Empagliflozin and its effect on endothelial dysfunction through inflammation and endoplasmic reticulum stress regulation

Marc-Antoine Campeau

Department of Chemical Engineering

McGill University, Montreal, Quebec, Canada

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“I'm roaming through the hills
All alone
I'm trying to find my direction home
A question of space
A matter of time
I follow the stars until the first light”

Dallas Green
a.k.a. City and Colour

Abstract

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) were developed to treat type 2 diabetes by the inhibition of renal glucose reabsorption. However, clinical trials have demonstrated that they additionally provide considerable cardioprotective benefits well beyond glycemic control. Treatment with SGLT2i (and in particular empagliflozin (EMPA; Jardiance®)) has shown to cause significant reductions in the rate of major adverse cardiovascular events such as heart failure. Endothelial cell (EC) function has been shown to change with EMPA treatment *in vitro* and *in vivo* through improved regulation of vasorelaxation and inflammatory response. The EC glycocalyx (GCX) is known to be disrupted in type 2 diabetes, leading to EC dysfunction which is an underlying cause of many cardiovascular diseases. Previous studies have shown that EMPA can overcome damage to the EC GCX, restoring the anti-inflammatory and mechanotransduction function of the cells *in vitro*.

This thesis investigates the mechanisms of EMPA that help overcome EC dysfunction. In the first study, sustained disruption of the GCX and exposure to shear was used to model pathological EC dysfunction. Sustained GCX disruption resulted in enhanced leukocyte adhesion while having no significant effect on endothelial nitric oxide synthase (eNOS) activation and intercellular adhesion molecule 1 (ICAM-1) expression. Transcriptome analysis revealed the induction of endoplasmic reticulum (ER) stress by the significant upregulation of genes belonging to the unfolded protein response (UPR) signaling. Treatment with EMPA normalized the leukocyte adhesion and attenuated the increased ER stress transcriptional expression under sustained GCX disruption, suggesting a redox mechanism alleviating EC inflammation independent of the GCX integrity.

The specific effect of EMPA on ER stress signaling was further explored with tunicamycin-stimulated ECs to model ER stress dysfunction. The apoptotic response and the oxidative regulated inflammation of ER stress were induced in ECs as observed by the unregulated protein expression of the downstream mediators C/EBP homologous protein (CHOP) and thioredoxin interacting protein (TXNIP)/NLR-family pyrin domain-containing protein 3 (NLRP3), respectively. EMPA effectively reduced CHOP and TXNIP/NLRP3 expression while having no effect on phospho-eIF2α, indicative of ER stress induction. Similarly, the reduced nuclear factor erythroid 2-related

factor 2 (nfr2) nuclear translocation by EMPA suggested the mitigation of oxidative stress, possibly through the metabolic regulation of ECs by the inhibition of the sodium-glucose cotransporter 1 (SGLT1).

EMPA partially resolved EC dysfunction under sustained GCX disruption and caused by tunicamycin-induced ER stress, demonstrating its effect in a pathologically relevant model of vascular dysfunction and its targeted impact on ER stress downstream signaling. As GCX shedding is becoming a clinical surrogate for deteriorating vascular health such as in the case of type 2 diabetes, the contribution of the improved EC function by SGLT2i treatment to vascular health and in turn better clinical outcome is of great interest. The therapeutic potential of SGLT2i to overcome cardiovascular complications relies on our understanding of the molecular mechanisms at the cellular level which is possible through *in vitro* studies.

Résumé

Les inhibiteurs du cotransporteur sodium-glucose 2 (SGLT2i) ont été développés pour traiter le diabète de type 2 en inhibant la réabsorption rénale du glucose. Cependant, des essais cliniques ont démontré qu'ils procurent également des avantages cardioprotecteurs considérables bien au-delà du contrôle glycémique. En effet, le traitement par SGLT2i (et en particulier l'empagliflozine (EMPA ; Jardiance®)) entraîne des réductions significatives du taux d'événements cardiovasculaires majeurs tel que l'insuffisance cardiaque. Il a été démontré que la fonction des cellules endothéliales (CEs) change avec l'EMPA *in vitro* et *in vivo* grâce à une meilleure régulation de la vasorelaxation et de la réponse inflammatoire. Le glycocalyx (GCX) des CEs est connu pour être dégradé dû au diabète de type 2, entraînant la dysfonction endothéliale qui est une cause sous-jacente de nombreuses maladies cardiovasculaires. Des études antérieures ont montré que l'EMPA peut surmonter les dommages causés au GCX, en restaurant la fonction anti-inflammatoire et de mécanotransduction des CEs *in vitro*.

Cette thèse étudie les mécanismes de l'EMPA qui aident à contrer la dysfonction endothéliale. Dans la première étude, la perturbation soutenue du GCX et l'exposition au cisaillement ont été utilisées pour modéliser un dysfonctionnement pathologique des CEs. La perturbation soutenue du GCX a entraîné une adhésion accrue des leucocytes tout en n'ayant aucun effet significatif sur l'activation de l'oxyde nitrique synthase endothéliale (eNOS) et l'expression de la molécule d'adhésion intercellulaire 1 (ICAM-1). L'analyse du transcriptome a révélé l'induction du stress du réticulum endoplasmique (RE) par la régulation positive significative de gènes appartenant à la signalisation de la réponse protéique dépliée (UPR). Le traitement avec l'EMPA a normalisé l'adhérence des leucocytes et a atténué l'expression transcriptionnelle accrue du stress du RE sous la perturbation soutenue du GCX, suggérant un mécanisme d'oxydoréduction atténuant l'inflammation des CEs, indépendant de l'intégrité du GCX.

L'effet spécifique de l'EMPA sur la signalisation du stress du RE a été exploré plus en détails avec des CEs stimulées par la tunicamycine pour modéliser le dysfonctionnement du stress du RE. La réponse apoptotique et l'inflammation régulée par le stress oxydatif lié au stress du RE ont été induites dans les CEs, tel qu'observé par l'augmentation de l'expression protéique des

médiateurs protéine homologue C/EBP (CHOP) et protéine interagissant avec thiorédoxine (TXNIP)/protéine 3 contenant domaine pyrine de la famille NLR (NLRP3), respectivement. L'EMPA a efficacement réduit l'expression de CHOP et de TXNIP/NLRP3 tout en n'ayant aucun effet sur le phospho-eIF2α, indiquant l'induction du stress du RE. De plus, la translocation nucléaire réduite du facteur nucléaire érythroïde 2 lié au facteur 2 (nfr2) par l'EMPA suggère l'atténuation du stress oxydatif, possiblement due à la régulation métabolique des CEs par l'inhibition du cotransporteur sodium-glucose 1 (SGLT1).

L'EMPA a partiellement résolu la dysfonction endothéliale sous la perturbation soutenue du GCX et causé par le stress du RE induit par la tunicamycine, démontrant son effet dans un modèle pathologique pertinent de dysfonctionnement vasculaire et son impact ciblé sur la signalisation du stress du RE. Alors que la perte du GCX devient un critère d'évaluation clinique pour la détérioration de la santé vasculaire, comme dans le cas du diabète de type 2, la contribution de la fonction endothéliale améliorée grâce au traitement par SGLT2i à la santé vasculaire et, par conséquent, aux meilleurs résultats cliniques est d'un grand intérêt. Le potentiel thérapeutique des SGLT2i pour surmonter les complications cardiovasculaires repose sur notre compréhension des mécanismes moléculaires au niveau cellulaire, ce qui est possible grâce aux études *in vitro*.

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AGE	Advanced glycosylation end products
AMPK	AMP-activated protein kinase
ATF3	Activating transcription factor 3
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATRA	All-trans retinoic acid
CANA	Canagliflozin
CCL-5	Chemokine (C-C motif) ligand 5
CVD	Cardiovascular diseases
CHOP	C/EBP homologous protein
DAPA	Dapagliflozin
EC	Endothelial cell
eIF2α	Eukaryotic translation initiation factor 2 subunit 1
EMPA	Empagliflozin
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
GCX	Glycocalyx
HAAEC	Human abdominal aortic endothelial cell
HAEC	Human aortic endothelial cell
HbA1c	Hemoglobin A1c

HCAEC	Human coronary artery endothelial cell
HDL	High-density lipoprotein
HFrEF	Heart failure with reduced ejection fraction
HS	Heparan sulphate
hsCRP	High-sensitivity C reactive protein
HUVEC	Human umbilical vascular endothelial cell
ICAM-1	Intracellular adhesion molecule 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
iNOS	inducible nitric oxide synthase
IRE1	inositol requiring enzyme 1
LDL	Low-density lipoprotein
MCP-1	monocyte chemoattractant protein 1
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9
NAC	N-Acetyl-L-cysteine
NHE	Na+/H+ exchanger
NF-κB	Nuclear factor κB
NLRP3	NLR-family pyrin domain-containing protein 3
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
PECAM-1	Platelet endothelial cell adhesion molecule 1

PERK	PKR-like endoplasmic reticulum kinase
ROS	Reactive oxygen species
SGLT1	Sodium-glucose cotransporter 1
SGLT2	Sodium-glucose cotransporter 2
SGLT2i	Sodium-glucose cotransporter 2 inhibitor
STZ	Streptozotocin
T2D	Type 2 diabetes
TLR4	Toll-like receptor 4
Tm	Tunicamycin
TNF-α	Tumor necrosis factor α
TXNIP	Thioredoxin interacting protein
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule 1

CHAPTER 1 INTRODUCTION

1.1 SGLT2 inhibitors: from type 2 diabetes treatment to vascular pleotropic effects

Type 2 diabetes (T2D) is characterized by the impairment of the physiological glucose regulation. Driven by the omnipresence of its risk factors including obesity and sedentary lifestyles, T2D incidence has increased consistently over the past decades with an estimated projection to 642 million people affected globally by 2040 [1]. Cardiovascular diseases represent the main burden on T2D patients' health, affecting approximatively 32.2% of T2D patients and is the leading cause of mortality [2]. Proper glycemic control with appropriate diet and medication can limit diabetes adverse effects due to their association to hyperglycemia [3], but significantly higher risk of cardiovascular complications remains.

Sodium-glucose cotransporter 2 inhibitor (SGLT2i) is a relatively new class of glucose-lowering drugs that have shown promising efficacy against T2D hyperglycemia through the limited reabsorption of glucose in the kidney microtubule [4, 5]. Following the recent issued guidance for newly developed drugs, the first SGLT2i long-term cardiovascular outcome trial, the EMPA-REG OUTCOME trial not only demonstrated the safe usage of empagliflozin (EMPA; Jardiance®) but revealed striking cardioprotective benefits with reduction in heart failure and cardiac death [6].

Based on preclinical and recent studies, the potential treatment indications of SGLT2i have rapidly expanded with cardiovascular benefits that go beyond the intended glycemic control [7]. Indeed, SGLT2i have been recommended for the treatment of T2D patients with established cardiovascular diseases [8] and very recently, SGLT2i dapagliflozin and empagliflozin have now been approved by the Food and Drug Administration as a heart failure treatment regardless of the diabetic status of the patient [9]. Although the use of SGLT2i for the treatment of cardiovascular complications has expanded, it remains nonetheless important to identify the mechanisms of action providing the cardiovascular protection.

In vitro studies have provided evidence of the direct effects of SGLT2i on vascular cell homeostasis that supports results from animal studies and the clinical literature. Endothelial cells (ECs) are fundamental to vascular and cardiac health and the restoration of their function potentially contributes to the positive clinical outcomes associated to SGLT2i treatment [10, 11]. Therefore, a deeper understanding of the effects of SGLT2i on ECs and the cellular mechanisms involved is needed to better define their cardioprotective benefits.

The overall objective of this thesis is to elucidate the mechanisms of EMPA that help overcome EC dysfunction. The glycocalyx (GCX), a surface layer composed of transmembrane core proteins and glycosaminoglycans/proteoglycan chains, is a critical mediator of EC mechanobiology [12-15] and inflammatory response [16-18]. Disruption of the GCX and exposure to laminar shear stress was used as a model of EC dysfunction, representative of the detrimental environment found in the vicinity of atherosclerotic plaques or under chronic hyperglycemic conditions [19-21]. Using this model, the anti-inflammatory effect of EMPA was further studied to unveil the possible implication of the GCX and the associated EC functions as these overlap with the reported beneficial effects of SGLT2i on ECs.

1.2 Hypotheses and objectives

Previous studies have demonstrated anti-inflammatory effects of EMPA in ECs and its ability to overcome the detrimental effects of GCX degradation. The overall objective of this thesis is to build upon previous findings **1) to study how EMPA overcomes EC dysfunction associated with GCX disruption and the dependency of the anti-inflammatory effect on GCX integrity, 2) to identify the inflammatory pathway(s) involved and demonstrate the direct effect of EMPA on the pathway functional targets.**

The first study (Chapter 4) was designed to test the hypothesis that **i) EMPA promotes an anti-inflammatory EC response independent of EC GCX integrity**. Our previous study has demonstrated that EMPA can reduce the inflammatory cell adhesion to ECs upon acute GCX disruption. Under sustained GCX disruption, EMPA was able to reduce EC inflammation but the mechanism remained elusive. Transcriptome analysis identified endoplasmic reticulum (ER) stress as the most relevant pathway underlying EC dysfunction in our model. The second study

(Chapter 5) was therefore designed to test the hypothesis that **ii) EMPA can attenuate ER stress signaling and the expression of downstream markers through oxidative stress mitigation**, relevant to the reduced inflammatory response observed in the first study.

By addressing these hypotheses, this thesis contributes to defining the pleotropic effect of EMPA on EC function. The findings described in this work will help to elucidate the cellular mechanisms by which EMPA provides cardiovascular protective effects in pathological settings and in turn, help to expand its therapeutic potential in the treatment of cardiovascular diseases implicating EC dysfunction.

1.3 Thesis organization

This thesis dissertation is manuscript-based and includes the related objectives, hypotheses and background of the doctoral research. This thesis consists of 7 chapters ordered as described below.

Chapter 1 Introduction, hypotheses and original contribution.

Chapter 2 Background and literature review

Chapter 3 Review article (drafted for submission) presenting the recent findings of the effects of SGLT2i treatment on vascular and cardiac health with a focus on endothelial functions and the possible mechanisms and signaling pathways involved. This chapter completes the literature review presented in Chapter 2.

Chapter 4 The first original manuscript (published in *Scientific Reports*) investigates the anti-inflammatory effect of EMPA on ECs based on our previous study [22]. The effect of EMPA on EC dysfunction caused by sustained GCX disruption was studied. EC dysfunction was also characterized through transcriptome analysis to identify relevant pathways of the inflammatory response (Objective 1).

Chapter 5 The second original manuscript (submitted) further explores the effects of EMPA on ER stress signaling downstream markers based on the transcriptional data from the first manuscript (Objective 2).

Chapter 6 Discussion of the main findings of the first and second manuscripts in relation to the initial hypothesis and how these findings relate to the current knowledge of the cardiovascular protective effects of SGLT2i.

Chapter 7 Conclusion and future perspective.

1.4 Contribution to original knowledge

This thesis contributes to the body of literature studying the pleotropic effects of SGLT2i on vascular cells, taking part in the ongoing research to identify the underlying cellular mechanisms in support to clinical findings. The detailed contributions are summarized below.

- Documentation of the current knowledge of the effects of SGLT2i on endothelial functions and the principal cellular mechanisms that potentially contribute to the cardio and vascular protective effects observed *in vivo* and clinically (Chapter 3).
- Further defining how EMPA has an anti-inflammatory effect on ECs, preventing leukocyte cell adhesion under sustained heparan sulfate (HS) degradation, suggesting the mitigation of the EC dysfunction caused by GCX disruption and shear exposure through a HS-independent mechanism (Chapter 4).
- Demonstrating that EMPA prevents the upregulation of mRNA and protein expression of thioredoxin interacting protein (TXNIP). Mitigation of oxidative stress under sustained HS degradation and shear exposure results in similar leukocyte adhesion reductions compared to EMPA, suggesting the implication of the redox homeostasis in EMPA-treated ECs (Chapter 4).
- Showing that EMPA impacts endoplasmic reticulum (ER) stress signaling by the transcriptional downregulation of markers from the unfolded protein response (UPR) under sustained HS degradation and shear exposure (Chapter 4).
- Documenting how EMPA limits the upregulation of ER stress downstream signaling markers and nuclear factor erythroid 2-related factor 2 (nrf2) translocation caused by tunicamycin-induced ER stress, further supporting the attenuating effect of EMPA on ER stress through a redox mechanism (Chapter 5).
- Clarifying that although human abdominal aortic ECs were not found to express sodium-glucose cotransporter 2 (SGLT2), they can still be metabolically repressed by EMPA at the concentrations tested, suggesting a protective mechanism through the mitigation of excessive oxidative stress mediated by the sodium-glucose cotransporter 1 (SGLT1) inhibition (Chapter 5).

1.5 Contribution of authors

The contribution of the authors for each chapter are detailed below.

Chapter 3 Marc-Antoine Campeau: Review and compilation of literature, conceptualization of the review framework, original draft writing, editing and reviewing final manuscript.

Richard Leask: conceptualization of the review framework, editing and reviewing final manuscript.

Chapter 4 Marc-Antoine Campeau: Experimental design and outline of the article, data collection, research and writing original draft, editing and reviewing final manuscript.

Richard Leask: funding acquisition, supervision, experimental design, editing and reviewing final manuscript.

Chapter 5 Marc-Antoine Campeau: Experimental design and outline of the article, data collection, research and writing original draft, editing and reviewing final manuscript.

Richard Leask: funding acquisition, supervision, editing and reviewed the final manuscript.

CHAPTER 2 BACKGROUND

2.1 Endothelial cells and vascular homeostasis

The cardiovascular system consists of the heart, the vasculature and the circulating blood. The vasculature is composed of a series of arteries and veins varying in size and organization. These vessels are structured as concentric cell layers of different types and functions. The innermost layer, the endothelium, is composed of endothelial cells (ECs) which line the entirety of the cardiovascular system including the inner wall of the heart and the aortic valve leaflets [23].

Due to their unique proximity to blood flow, ECs contribute to vascular homeostasis through the regulation of vasorelaxation, coagulation, inflammation as well as endocrine, paracrine and autocrine signaling [23, 24]. These mechanisms are, in part, governed by the effect of blood flow on ECs. Shear stress created by blood flow at the arterial wall impacts ECs because of their ability to sense and adapt to hemodynamic forces. Through mechanosensors, ECs can transform shear stress into biochemical signals to regulate cell mechanisms such as cytoskeletal adaptation, vasoregulation and inflammation, a process termed mechanotransduction [25].

Arterial blood is a heterogeneous and complex hemodynamic environment with shear stress magnitude up to 70 dyn/cm^2 and non-laminar pulsative flow patterns. The responses from ECs vary and depend on the localized forces. Mean positive shear found in straight segments of arteries provide atheroprotective benefits to the endothelium [26, 27]. *In vitro*, this effect can be modeled with levels of steady laminar shear stress of 5 to 30 dyn/cm^2 which have been shown to promote EC homeostasis. In a pivotal study, Dewey *et al.* demonstrated the effect of laminar shear stress on cultured ECs which changed shape to elongate and orient in the flow direction [28]. In addition, this type of flow positively regulates the endothelial nitric oxide synthase (eNOS) activation and induced anti-inflammatory phenotypic changes [27].

Consequently, vascular diseases develop more prominently in the curving or branching regions of the vasculature where flow separation occurs, creating susceptible areas of the endothelium exposed to flow reversal and oscillatory shear [26, 29]. When coupled to risk factors or non-uniform temporal and spatial gradients of shear, these flow patterns generate localized disturbed

hemodynamic forces and cause EC dysfunction [30, 31]. As initially demonstrated *in vitro* by DePaola *et al.*, large spatial gradients of shear caused significant changes in shape, density and proliferation among the observed EC population, indicative of localized EC dysfunction [32]. In addition, through localized or systemic vascular insults such as inflammation [19, 33], hyperglycemia [20] and disturbed hemodynamic forces [17, 34], cell mechanosensors and thus the associated mechanotransduction can be impaired, resulting in similar dysfunctional changes.

2.2 The endothelial glycocalyx

The glycocalyx (GCX) is a functional layer on the apical surface of ECs, extending into the lumen of blood vessels. The GCX is composed of glycosaminoglycan chains linked to transmembrane proteoglycans. This mesh structure is embedded with ions, signaling molecules and growth factors [35]. Through steric hindrance, the GCX regulates the proximal contact with circulating red blood cells and inflammatory cells, preventing adverse adhesion.

Once thought to be an inert structure, the GCX is now known to actively participate in EC homeostasis, primarily through mechanotransduction [14]. The GCX participate in the shear-regulated cellular alignment, nitric oxide (NO) production, cellular permeability and inflammatory cell adhesion. As a result, disruption of the GCX has profound effects on EC hemostasis (Fig. 2-1). Enzymatic degradation of GCX components has been used to study the effect of disruption on shear-regulated functions. Degradation of heparan sulfate (HS), the main component of EC GCX, results in impaired shear-regulated NO production and cellular alignment [15, 36-39]. Moreover, HS degradation generates a pro-inflammatory environment due to the loss of steric hindrance and the impairment of beneficial laminar stress mechanotransduction, resulting in increased cell permeability and leukocyte adhesion [16-18, 40].

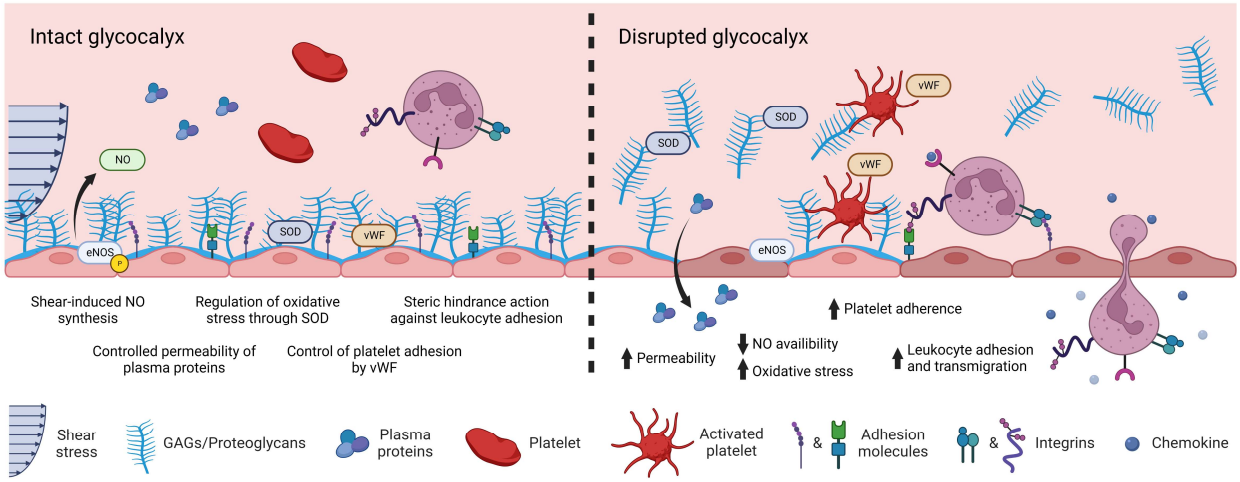

Figure 2-1. ***Glycocalyx functions in endothelial homeostasis****. An intact glycocalyx promotes the homeostasis of the endothelium through mechanotransduction, molecule harboring and steric hindrance. Shear-induced NO synthesis and cytoskeletal organization of ECs limit oxidative stress and permeability, respectively. Oxidative stress is also reduced by the presence of enzyme such as superoxide dismutase (SOD). Embedded von Willebrand factor (vWF) prevents thrombotic response. Upon disruption, shear-regulated and barrier functions are impaired leading the ECs to a pro-inflammatory state characterized by increased plasma protein permeability and reduced NO. Released SOD and vWF cause the increase in oxidative stress and the activation of platelet, respectively. The loss of steric hindrance also promotes the adhesion and transmigration of leukocytes. GAGs: glycosaminoglycans. This figure was created with BioRender.com.*

2.3 The inflammatory cascade of leukocyte adhesion

The recruitment of inflammatory cells from the blood stream to the endothelium surface is a cascade mechanism progressing from transient cell-cell interactions to firm adhesion. The common sequence of steps is tethering, rolling, adhesion, crawling and ultimately transmigration through the endothelium [41, 42]. The recruitment begins when endothelial cells undergo pro-inflammatory phenotypic changes induced by inflammatory mediators such as cytokines, triggering the recruitment of inflammatory cells. Activated ECs express E-selectin and P-selectin on their surface leading to the interaction and capture of leukocytes through their glycosylated ligands (tethering). Shear stress and transient low-affinity between the selectin ligands cause the captured leukocyte to start rolling, enhancing the contact of the leukocyte with the endothelium. A gradient in chemokine release activates and attracts the leukocyte towards sites of inflammation. Sustained interactions with chemokines induce conformational changes of the constitutive surface-expressed integrins of leukocytes which promote firm adhesion to the endothelium. Integrins LFA1 and MAC1 bind to immunoglobulin-like cell adhesion molecules (e.g. intercellular adhesion molecule 1; ICAM-1) expressed by activated ECs. Similarly, integrin VLA-4

binds to vascular cell adhesion molecule 1 (VCAM-1) [42, 43]. These high-affinity interactions cause the firm adhesion and arrest of the leukocyte on the surface of the endothelium in preparation for transmigration. The leukocyte gradually migrates to cell-cell junctions, the site of paracellular transmigration. This process is achieved through interactions of cell adhesion molecules and integrins as well as dissociation of junctional bonds between endothelial cells mediated by VE-cadherin and the platelet endothelial cell adhesion molecule 1 [44].

2.4 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an essential component of the cell protein folding and assembly. As a primary function, protein folding and quality control are regulated in the ER lumen prior to the protein transit to cell surface where they are secreted or displayed, ensuring normal cellular functions [45].

In certain physiological or pathological conditions where the folding capacity of the ER is exceeded due to an increase in protein folding demand, misfolded proteins accumulate in ER lumen, causing ER stress. In response, a series of adaptive signaling pathways termed the unfolded protein response (UPR) aim at restoring ER homeostasis through a balance of protective and proapoptotic signals [45]. First triggered by the release of chaperone molecules (binding immunoglobulin protein (BiP; Grp78)) binding to misfolded proteins, the membrane mediators of the UPR, namely PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and the activating transcription factor 6 (ATF6) regulate the activation of their respective signaling pathway. While each branch involves different mechanisms and intermediate effectors, they share similar outcomes by controlling cell faith. Transitional attenuation is first induced to progressively improve folding capacity but prolonged ER stress inevitably promotes apoptosis [45, 46].

Prolonged induction of ER stress is inherently coupled to cellular dysfunction due to increased oxidative stress through reactive oxygen species (ROS) generated during increased selective protein synthesis and the associated apoptosis/inflammatory downstream signaling [47-49]. In ECs, induction of ER stress has been shown to activate the PERK signaling pathway, causing the

upregulation of C/EBP homologous protein (CHOP), with increased NADPH oxidase activity and inflammatory cytokine expression [50, 51].

Additionally, oxidative stress and inflammation can be exacerbated through the upregulation of thioredoxin-interacting protein (TXNIP), impairing the redox regulation from thioredoxin and activating the NLR-family pyrin domain-containing protein 3 (NLRP3) inflammasome [52-56]. Moreover, TXNIP expression under ER stress was shown to be regulated though PERK and IRE1 activation [57].

Given its detrimental effects on vascular homeostasis, ER stress has been identified as an interesting therapeutic target in the development of cardiovascular diseases [58] with the active contribution of EC dysfunction [48, 49]. Indeed, evidence suggests that inhibition of ER stress could prevent the impaired endothelium-dependent vasorelaxation and inflammation caused by hypertension [59] or diabetes [60] and prevent the progression of atherosclerotic plaques [61].

2.5 Type 2 diabetes, cardiovascular complications and medication

Coexisting conditions that commonly afflict diabetic patients such as obesity, hypertension and dyslipidemia are cardiovascular risk factors, placing diabetes patients at a higher risk of developing cardiovascular diseases. Other systemic factors, in particular vascular oxidative stress and endothelial dysfunction contribute to the development and progression of cardiovascular diseases [62, 63]. Hyperglycemia promotes the increase of oxidative stress through ROS derived from the formation of advanced glycosylation end products (AGE). This causes the disruption of NO bioavailability and impair NO-dependent vasorelaxation which further aggravate EC dysfunction [64, 65]. In conjuncture to oxidative stress, inflammation is central to diabetes and is involved in the pathogenesis of cardiovascular complications [63, 66]. Increased levels of cytokines and macrophage infiltration are indicative of vascular inflammation, which directly relates to the pro-inflammatory phenotype of ECs [67]. These effects on the vasculature converge to EC dysfunction and cause a marked increase in the risk of complications from coronary artery diseases.

The management and medication for diabetes have significantly evolved over the years [68]. Modern classes of drugs including biguanides (metformin), dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists and sodium-glucose cotransporter 2 inhibitors (SGLT2i) are used to control glycemia with the added benefit of reduced risk of cardiovascular events [69]. Among them, SGLT2i have received the most attention due to their potential to alleviate vascular dysfunction possibly through mechanisms independent from their glucose-lowering effect.

2.6 SGLT2i prevent hyperglycemia-associated vascular dysfunction

Preclinical studies have demonstrated the efficacy of SGLT2i to reduce hyperglycemia and to positively affect the associated vascular dysfunction. Indeed, impaired vascular functions caused by chronic oxidative stress and inflammation have been shown to be improved with SGLT2i treatment in diabetic rodent models.

2.6.1 Oxidative stress

Treatment of diabetic rodent models with SGLT2i was demonstrated to attenuate the hyperglycemia-induced vascular oxidative stress. Empagliflozin (EMPA) reduced the systemic oxidative burst, aortic ROS and NADPH oxidase (Nox2) activity. EMPA normalized the burst in oxidative stress with similar effects being induced by NAPDH inhibitor and intracellular calcium inhibitor suggesting their implication in the increase of oxidative stress [70]. The overall oxidative burden was characterized by increased cardiac mitochondrial aldehyde dehydrogenase activity and increased levels of 3-Nitrotyrosine and 4-Hydroxynonenal positive protein, which were normalized by EMPA. Accordingly, the increased aortic protein expression of Nox2, heme oxygenase 1 (HO-1) and receptor of AGEs were also normalized by EMPA [71]. Similarly, short-term (3 weeks) administration of ipragliflozin to streptozotocin (STZ)-induced diabetic mice normalized the level of systemic oxidative stress as measured by level of urine 8-OHdG [72]. Oxidative conditions in KK-Ay mice were also improved by EMPA treatment with significant decreases in the cardiac level of lipid hydroperoxide and malondialdehyde as well as increased levels of anti-oxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase. These changes were associated to the upregulation of the myocardial expression of total nuclear factor

erythroid 2-related factor 2 (nrf2), nuclear nrf2 and HO-1, suggesting the activation of the nrf2/ARE pathway and the associated attenuation of myocardial oxidative stress [73]. More closely related to EC dysfunction, treatment of ZDF-Lepr$^{fa/fa}$ rats with EMPA has shown to limit endothelial ROS levels as treatment with the eNOS inhibitor L-NAME improved endothelial dihydroethidium staining, indicating partial prevention of eNOS uncoupling [71]. In additions, cardiac microvascular ECs isolated from EMPA-treated diabetic mice showed decreased levels of mitochondrial and intracellular ROS, demonstrating the impact of EMPA on hyperglycemia-induced oxidative damage to the endothelium [74].

2.6.2 Impaired vasorelaxation

Along with the mitigation of oxidative damage to the vasculature, treatment with SGLT2i restored EC-dependent vasorelaxation in hyperglycemic conditions. Treatment with EMPA over 7 weeks improved the impaired EC-dependent vasorelaxation in diabetic rats with a dose-dependent increase in phospho-eNOS Ser1177/eNOS ratio, suggesting increased eNOS activation [70, 71]. This activation was supported by improved eNOS regulatory signaling with the phospho-vasodilator stimulated phosphoprotein Ser239/cGMP-dependent protein kinase 1 expression ratio which is indicative of effective vasodilation being reduced in diabetic rats and restored to control levels by EMPA. Moreover, dihydrofolate reductase which regulates the restoration of the cofactor tetrahydrobiopterin (BH4) and thus promotes the functional coupling state of eNOS was also significantly increased by EMPA [71]. The restoring effect of SGLT2i on eNOS activation is potentially mediated through Akt signaling as Salim *et al.* showed the improvement of EC-dependent vasorelaxation following the treatment of diabetic mice with ipragliflozin through the concomitant upregulation of phospho-eNOS Ser1177 and phospho-Akt Ser473 [72]. A study by Zhou *et al.* demonstrated how the impairment of EC-dependent vasorelaxation by hyperglycemic conditions in diabetic mice correlates to EC dysfunction. Isolated cardiac microvascular ECs showed disruption of their metabolic and structural integrity, including reduced phospho-eNOS Ser1177 expression and barrier function which were normalized in ECs from EMPA-treated mice [74].

Interestingly, the protective effect of SGLT2i on NO-mediated vasorelaxation does not appear to be solely attributable to the systemic reduction of glucotoxicity. Indeed, El-daly *et al.* demonstrated the improvement of PAR2-mediated EC-dependent vasorelaxation with EMPA using *ex vivo* aortic rings cultured in high glucose conditions. The addition of BH4, SOD, NADPH oxidase inhibitor and sulforaphane, a nrf2-sensitive antioxidant gene response stimulant, resulted in similar improvements of the vasorelaxation response, suggesting a redox mechanism in the protective effect of EMPA. This effect was also found to be dependent on the concentration as incubation with concentrations above 1 μM resulted in the loss of the PAR2-mediated vasorelaxation [75].

2.6.3 Inflammation

Treatment with SGTL2i has been shown to normalize the diabetic-induced inflammation in rodents. Aortic mRNA upregulation of inflammatory markers, chemokine (C-C motif) ligand 2 (CCL-2; MCP-1), CD68, interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and ICAM-1 were suppressed by EMPA suggesting an anti-inflammatory effect against low-grade inflammation [70]. Similarly, Salim *et al.* showed the normalization of the endothelial adhesion molecules ICAM-1 and VCAM-1 expression in aortic tissue of STZ-induced diabetic mice with ipragliflozin treatment. Chemoattractant marker MCP-1 and the macrophage marker F4/80 were also normalized by ipragliflozin. Improvement of EC inflammation was suggested to be mediated through the regulation of AGEs as treatment of HUVECs with methylglyoxal yield similar increases in the inflammatory marker expressions [72]. Treatment with EMPA was shown to improve microvascular integrity in cardiac tissue. Restored VE-cadherin expression and lower counts of TUNEL-positive cells along with the normalized expression of ICAM-1 and VCAM-1 in microvessels demonstrated the improved vascular integrity and inflammation in EMPA-treated mice [74].

2.6.4 Atherosclerotic lesions and plaque inflammatory markers

Evidence points towards the potential of SGLT2i to attenuate atherosclerosis development as multiple studies varying in SGLT2i used, treatment duration and animal model demonstrated the reduction in atherosclerotic lesions. Aortic atherosclerotic lesion area was reduced in ApoE-/- mice following treatment with dapagliflozin (DAPA) [76, 77], luseogliflozin [78], EMPA [79] and

canagliflozin (CANA) [80]. Similar observations were made in Ldlr deficient rodents treated with phlorizin [81] and CANA [82]. It remains unclear if SLGT2i impact atherosclerotic phenotypic changes through the attenuation of the hyperlipidemia burden. Although luseogliflozin treatment normalized lipid accumulation in atherosclerotic plaques, short-term treatment (1 week) had minimal effect on lipid metabolism-related genes and long-term treatment did not impact hyperlipidemia (elevated cholesterol, elevated low-density lipoprotein, lower high-density lipoprotein) [78]. Conversely, phlorizin treatment attenuated the diabetes-induced increase in plasma cholesterol and triglyceride levels of Ldlr-/- mice while having no effect in the treated non-diabetic group [81]. Ultimately, plaque composition with SGLT2i showed less lipid accumulation and inflammatory cell infiltration [77, 81]. Beyond prevention, EMPA accelerated the regression of atherosclerosis in STZ-induced-diabetic mice. After 3 weeks of induced regression, aortic root lesion development was attenuated. Lesion lipid accumulation and CD68+ macrophage infiltration were further decreased and plaque collagen content enhanced suggesting improved regression and stabilization of the atherosclerotic lesions with EMPA treatment [83].

Significant reductions in the inflammatory response of atherosclerotic lesions from gene expression to macrophage activation have been found with SGLT2i treatment. Treatment of diabetic ApoE-/- mice with CANA or EMPA attenuated the upregulated expression of inflammatory and oxidative stress genes such as *MCP-1*, *ICAM-1*, *VCAM-1*, *NOX2*, *NOX4*, *p22phox* and *p47phox* and normalized the expression of MCP-1, ICAM-1 and VCAM-1 in the aortic root lesions [79, 80]. Similarly, treatment with DAPA resulted in decreased aortic ROS levels and reduced aortic NLRP3, ASC, Caspase-1, interleukin-1β (IL-1β) and interleukin-18 expression [76]. Short term treatment with luseogliflozin normalized the upregulation of cytokine F4/80, TNF-α, IL-1β and IL-6 mRNA in aortic tissue of NA/STZ-ApoE-/- mice [78].

Increased circulating monocytes, neutrophils and Ly6-C^{hi} cells and their activation for adhesion through increased CD11b expression caused by diabetes were normalized by phlorizin treatment, suggesting an anti-inflammatory action on systemic inflammation which in turn decreases atherosclerosis lesion development [81]. Indeed, multiple studies demonstrated the reduction of the infiltration of macrophages in atherosclerotic lesions with SGLT2i treatment [76-80, 83]. In

addition, the increased degree of foam cell formation as measured by the cholesterol ester accumulation in peritoneal cells of diabetic ApoE-/- and db/db mice was attenuated by DAPA and ipragliflozin treatment, respectively [77].

CHAPTER 3 BENEFICIAL EFFECTS OF SGLT2 INHIBITORS ON VASCULAR HOMEOSTASIS: A PERSPECTIVE FROM THE ENDOTHELIUM

3.1 Preface

In light of the growing evidence of the benefits of sodium-glucose cotransporter 2 inhibitors (SGLT2i) for the treatment of cardiovascular complications, studies of their pleotropic mechanisms are needed to understand the effects of the drugs on the cardiovascular system. While systemic effects have been documented, questions remain regarding the possible effects directly impacting vascular health. The impact of SGLT2i treatment on cardiomyocytes and cardiac functions have been prioritized due to their intimate role in the cardiomyopathies monitored during clinical trials. Consequently, the effects on endothelial cell (EC) dysfunction have not been sufficiently studied despite recent evidence suggesting that restoration of endothelial functions actively contributes to the SGLT2i cardioprotective benefits. This motivates the study of the specific signaling pathways involved and how they relate to the clinical outcomes. As studies on the subject were at first limited but are now rapidly evolving, a review of the literature was undertaken to orient the experimental design of the work presented in this thesis.

This review article summarizes the recent findings regarding the vascular effects of SGLT2i implicating endothelial functions. Integrating the findings of *in vitro* studies to our current knowledge of the cardiovascular protection observed clinically is crucial to fully comprehend the benefits of SGLT2i treatment.

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Beneficial effects of SGLT2 inhibitors on vascular homeostasis: A perspective from the endothelium

Marc-Antoine Campeau[1], Richard L. Leask[1,2]*

[1]Department of Chemical Engineering, McGill University, Montreal, QC, Canada

[2]McGill University Health Centre, Montreal, QC, Canada

*Corresponding Author

richard.leask@mcgill.ca

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3.2 Abstract

Since their introduction as a new class of drugs in the treatment of type 2 diabetes, sodium-glucose cotransporter 2 inhibitors (SGLT2i) have demonstrated their efficacity in the management of the cardiovascular complications. From animal studies, their cardiovascular protective benefits beyond their glucose-lowering actions are now recognized and suggest pleotropic effects on vascular cells. The mechanisms mediating these effects and their impact on the pathological outcome remains however elusive. SGLT2i treatment in non-diabetic rodents has demonstrated to improve cardiac and vascular functions, in part through restored endothelial cell (EC)-dependent vasorelaxation and reduced inflammation. Hence, recent *in vitro* studies have investigated the effects of SGTL2i on EC functions and the signaling pathways involved. Although evidence points toward the potential of SGLT2i to mitigate EC dysfunction, exploration of the pleotropic effects on ECs is necessary to adequately evaluate their contribution to the cardiovascular protective benefits. Molecular and cellular mechanisms including AMP-activated protein kinase (AMPK) activation, reduced endoplasmic reticulum (ER) stress induction and Na+/H+ exchanger (NHE) inhibition has been identified as potential mediators in the effects of SGLT2i. This review summarizes the recent *in vivo* and *in vitro* findings regarding the pleotropic effects of SGLT2i on vascular functions related to ECs. Integrating these findings to the animal and clinical data is of interest as improved EC function is expected to participate in the cardiovascular protective effects provided by SGLT2i treatment.

3.3 Introduction

Over the last decade, sodium-glucose cotransporter 2 inhibitors (SGLT2i) have emerged as a powerful tool in the treatment of type 2 diabetes (T2D). Designed to selectively inhibit the glucose reabsorption mediated by SGLT2 in the kidney proximal tubule, these competitive inhibitors provide an effective glycemic control independent of insulin. Preclinical studies demonstrated how treatment with SGLT2i diminish vascular complications associated with hyperglycemia such as increased oxidative stress and impaired vasodilation [1, 2]. The EMPA-REG OUTCOME trial was the first large clinical trial to show cardiovascular health benefits from SGLT2i treatment as it demonstrated a significant reduction in the morbidity, hospitalization and fatal complications rates in T2D patients treated with empagliflozin (EMPA) [3]. Following this, DAPA-HF [4] and EMPEROR-Reduced trial [5] showed that treatment with dapagliflozin (DAPA) and EMPA respectively lower the risk of cardiovascular death or hospitalization for heart failure independently of the presence or absence of diabetes.

Once thought to have their benefits tied to their antihyperglycemic effects, SGLT2i are becoming an emerging option as therapeutic agents in the treatment of cardiomyopathies independently of diabetes. Thus, ongoing studies and clinical trials are now aiming to better define the potential of SGLT2i to improve cardiovascular complications from atherosclerosis, ischemia and heart failure in non-diabetic conditions. Although their glucose-lowering action has been associated with beneficial systemic effects such as lower blood pressure and natriuresis [6], a consensus regarding other significant pleotropic effects is now being established. As such, the use of DAPA (Farxiga®; AstraZeneca) has been recently approved to mitigate the complications associated with heart failure (NYHA class II-IV) with reduced ejection fraction (HFrEF, LVEF ≤ 40%) in patients with and without T2D. Similar approval for EMPA (Jardiance®; Eli Lilly and Boehringer Ingelheim) shortly followed based on the results of the EMPEROR-Reduced trial [7].

Given its rapid onset, the cardiovascular benefits of SGLT2i may stem from improved vascular cell homeostasis. Based on this hypothesis, we and others have investigated the effects of SGLT2i on endothelial dysfunction and the underlying cellular mechanisms involved. Endothelial dysfunction, characterised by impaired nitric oxide (NO) synthesis, chronic inflammation and

prothrombotic state, is the pivotal point in the development of many cardiovascular diseases, often associated with hypertension, disturbed hemodynamic forces and metabolic disorders [8]. Interestingly, impaired vasomotion, inflammation and oxidative stress have been shown to be mitigated by SGLT2i treatment *in vivo* and *in vitro*, suggesting that improved endothelial cell homeostasis could contribute to the improved cardiovascular outcomes.

This review summarizes the recent findings regarding the direct vascular effects of SGLT2i, focusing primarily on EC functions related to cardiovascular pathologies. The impact of systemic effects (e.g. metabolic and hemodynamic effects) on other vascular cells and cardiac function will not be covered in depth in this work and the reader should be directed to these recent publications [6, 9]. A better understanding of the cellular mechanisms underlying the SGLT2i benefits is key to unlocking its full potential in the treatment of cardiovascular complications of diabetic and non-diabetic patients.

3.4 SLGT2 inhibitors

Over the years, the members of the SGLT2i family have continuously expanded. Derived from the naturally occurring SGLT1/SGLT2 competitive inhibitor phlorizin, iterations in the development of modern SGLT2i aimed at improving water solubility, oral bioavailability and hydrolysis resistance, greatly improving their half-life. Designed as C-glycosylated diarylmethane pharmacophore, variations in their chain modulates the selectivity toward SGLT2 vs SGLT1 [10, 11].

There are currently four SGLT2i approved by the Food and Drug Administration and the European Medicines Agency (Fig. 3-1). Canagliflozin (CANA; Invokana®; Janssen Pharmaceuticals), DAPA (Farxiga®; AstraZeneca), EMPA (Jardiance®; Eli Lilly and Boehringer Ingelheim) were first approved in 2013-2014 followed by Ertugliflozin (Steglatro®; Merck and Pfizer) in 2017. All had their efficacy demonstrated by efficiently controlling blood glucose level and reducing HbA1c formation in T2D [12-16]. Their selectivity toward SGLT2 vs SGLT1 ranges from approximatively 250:1, 1200:1, 2500:1 and 2000:1, respectively [11, 17]. Other SGLT2i including ipragliflozin (Suglat®) and luseogliflozin (Lusefi®) have been approved in Japan. In addition, sotagliflozin, developed as a dual SGLT1/SGLT2 inhibitor has recently shown promising results similar to the

other approved SGLT2i for the treatment of diabetes [18] and the prevention of fatal cardiovascular complications [19].

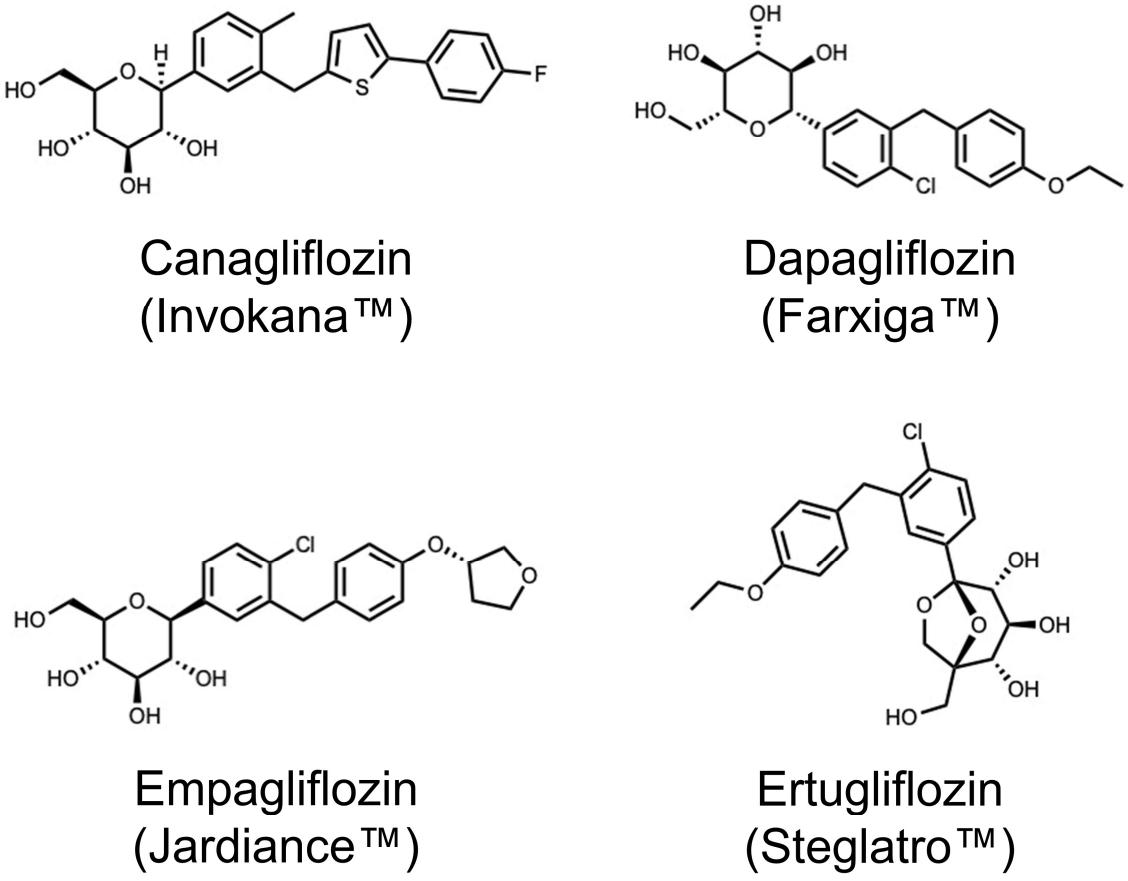

Figure 3-1. ***SGLT2i approved by the Food and Drug Administration and the European Medicines Agency for the treatment of T2D.***

3.5 Endothelial SGLT2 expression

Although more evidence of beneficial effects of SGLT2i on vascular cells has emerged, it remains unclear if theses effects are mediated through SGLT2, raising the question of expression and functionality. Pharmacokinetic studies revealed that plasma concentrations of SLGT2i is significantly lower compared to kidney and liver concentrations [20]. Moreover, the expression of SGLT2 at the transcriptional and protein levels in ECs is still debated although it has been previously established that SGLT1 is expressed in cardiac tissue and could potentially be inhibited by high SGLT2i concentrations [21, 22].

Recent studies reported basal or inducible expression of SGLT1 and SGLT2 at the transcriptional and protein levels in different conditions. Protein and mRNA expression of SGLT1 and/or SGLT2 were detected in porcine ECs [23, 24], human umbilical vascular ECs (HUVECs) [25-27], human aortic ECs (HAECs) [28] and human coronary ECs (HCAECs) [27]. Expression of SGLT2 was also showed in mouse aortic tissue [29, 30] while colocalization with CD31 suggested specific expression in endothelial cells [30]. Pro-inflammatory and oxidative stress treatment such as palmitic acid [26], angiotensin II (Ang II) [24], tumor necrosis factor α (TNF-α) [27], H_2O_2 and high glucose [23] have been shown to cause upregulation of SGLT1/SLGT2 expressions in cultured ECs. However, validation of the expression of SGLT2 can be difficult as the mRNA levels have been reported to be undetectable in some cases [27, 28] and that siRNA silencing of SGLT2 was without effect on protein expression [27].

In parallel to expression, glucose uptake assays with glucose analogues such as 2-NBDG or 2-[3H]-deoxyglucose to assess the functionality of SGLT2 have shown mixed results. El-daly *et al.* showed the inhibition of 2-NBDG uptake by mouse aortic ECs treated with 100 nm EMPA [29]. Similarly, Mancini *et al.* showed the inhibition of 2-[3H]-deoxyglucose uptake in ECs treated with 10 µM CANA or 10 µM DAPA [28]. Khemais *et al.* did not find changes in the basal glucose uptake of ECs following treatment with 100 nM of EMPA but increased uptake induced by oxidative stress and high glucose were normalized [23]. Interestingly, exposure of ECs to methyl α-D-glucopyranoside, a non-metabolizable glucose analogue transported through SGLTs, resulted in a similar pro-oxidant response to glucose, suggesting an effect independent of glucose metabolism [24].

As more evidence support the expression and functionality of SGLT2 in ECs, progress is being made in understanding how the impact of SGLT2 inhibition on glucose/ions homeostasis or other pathways could benefit cellular mechanisms which in turn contribute to better cardiovascular health.

3.6 SGLT2i treatment prevents hyperglycemia-associated vascular dysfunction

Pre-clinical and clinical studies have demonstrated the efficacy of SGLT2i to reduce the cardiovascular complications associated with hyperglycemic conditions. Persistent glycemic control through glucosuria translates to systemic effects including weight loss, natriuresis and reduced blood pressure [31] but also to the prevention of long-term vascular dysfunction. Indeed, treatment of diabetic rodents with SGLT2i was shown to alleviate excessive oxidative stress and restore NO vasorelaxation homeostasis which in turn lessen the inflammatory burden of the vasculature (Appendix A - Table 1). These changes directly related to the improvement of EC functions in atheroprone rodent treated with SGLT2i, resulting in the reduction of atherosclerosis plaque development, adhesion molecule expression and macrophage infiltration (Appendix A - Table 2).

Treatment with SGLT2i has shown to prevent diabetic-driven vascular dysfunction and atherosclerosis development, through their intended glucose secretion effect, leading to lower glucotoxicity, improved EC functions and in turn restored vascular homeostasis. The attenuation of vascular tissue inflammation by SGLT2i might help prevent the worsening of atherosclerotic plaques. This effect has often been correlated to the lower blood glucose [32, 33] or reduced markers of hyperglycemia (e.g. HbA1c) [34], implying that the observed improvements were induced through the reduction of glycemia. However, a few studies have also demonstrated that the anti-inflammatory effects of SGLT2i also exist in non-diabetic mice. DAPA treatment reduced aortic interleukin-1β (IL-1β), interleukin-18 and ASC protein expression in non-diabetic ApoE-/- mice [35]. Moreover, Nakatsu *et al.* showed that while NA/STZ induced diabetes did not exacerbate aortic root macrophage infiltration and oxidative stress, long-term treatment with luseogliflozin decreased CD68 and 4-Hydroxynonenal (4-HNE) positive area below levels of ApoE-/- mice suggesting the prevention of excessive macrophage infiltration and oxidative stress caused by atherosclerosis, independent of glycemic control [36]. Lim *et al.* also demonstrated how the protective mechanism of CANA against ischemia/reperfusion injury may rely on phenotypic changes of vascular cells rather than a continuous glucose-lowering effect from

CANA. As *ex vivo* ischemia/reperfusion injuries resulted in reduced infarct sizes in CANA-treated non-diabetic rats but acute exposure with CANA failed to induce a similar effect, it would suggest that the protective mechanism stems from significant changes in cell phenotype over time [37].

While most studies focused on the glucose-lowering actions of SGLT2i and largely attributed the observed benefits to the glycemic control, recent evidence now suggests that SGLT2i can impact vascular homeostasis independently of the hyperglycemic condition. As described further below, diabetes studies consisted only in the first step in the field of the cardioprotective benefits of SGLT2i as new pleotropic effects on vascular health are unveiled.

3.7 Effects of SGLT2i in non-diabetic animal models and the implication of EC dysfunction

In recent years, an increasing number of studies have explored the therapeutic potential of SGLT2i in non-diabetic conditions. The rapid onset of the vascular protective effects suggests a mechanism that goes beyond simple glycemic control [45]. A consensus regarding the cardioprotective effect of SGLT2i regardless of the diabetic status is now established. Recently, SGLT2i have been recommended to be prescribed to T2D patients with existing cardiovascular complication [46] and approved for heart failure treatment, regardless of the diabetic status of the patient. Although, these approvals benefit patient care, the exact mechanisms of the cardioprotective effects remain unclear.

Research now aims to clarify the mechanisms by which SGLT2i improve vascular and cardiac functions. In this regard, although the evidence on the efficacy of SGLT2i to alleviate the atherosclerotic burden or cardiac dysfunction are still fragmental, improved cardiovascular functions have been reported, demonstrating the direct implication of vascular cell homeostasis (Appendix A - Table 3). As the endothelium lines the entire cardiovascular system and regulates crucial vascular functions, the impact on EC functions represents an important aspect of the cardiovascular protection of SGLT2i.

3.7.1 Lipid metabolism

Changes in the metabolic handling of lipids can drastically impact vascular homeostasis and consists in a risk factor for the development of coronary artery disease. Excess lipids in the circulation or in tissue cause lipotoxicity and impair endothelial functions through oxidative and inflammatory mechanisms ultimately leading to the development of atherosclerosis [47, 48].

A pivotal study by Han *et al.* showed the reduction in the aortic arch and valve atheroma burden of ApoE-/- mice treated with EMPA for 8 weeks [49]. EMPA reduced the plaque composition in lipid and histocytes as well as reduced the coverage of CD68+ cells indicative of reduced macrophage infiltration. Treatment with EMPA induced a decrease in HOMA-IR and high-sensitivity C-reactive protein (hsCRP), indicative of improved insulin resistance and lower low-density lipoprotein (LDL) transcytosis, respectively. Hepatic enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) were also decreased while kidney function as measured by the creatinine level was unchanged between groups. Triacylglycerol levels were decreased by EMPA whereas high-density lipoprotein (HDL) and LDL were unchanged except at high doses of EMPA where HDL slightly increased [49]. In a fatty rat model (ZSF1), increased levels of glycemia, ALP, total cholesterol and triglycerides were shown to be attenuated by EMPA after 6 weeks [50]. Conversely, 5 week treatment of ApoE-/- mice with CANA only marginally improved the detrimental effects associated with atherogenesis but reduced total blood cholesterol, serum triglycerides and serum LDL [51]. Mean arterial pressure and lipid peroxidation (MDA) along with body weight and blood glucose were reduced in ApoE-/- mice treated with EMPA for 6 weeks but cholesterol and triglyceride levels did not differ between untreated and EMPA treated groups [52].

3.7.2 Cardiac and vascular impact

In addition to the possible metabolic impact on lipidemia, SGLT2i treatment has shown to positively affect the cardiovascular outcome in induced pathologies. Short-term treatment with EMPA prevented the worsening of cardiac function following induced heart failure by preventing a decrease in ejection fraction without affecting the indices of structural remodeling. Interestingly, improved cardiac output and work by EMPA in *ex vivo* functional assessment

suggested an effect independent of hemodynamic or systemic changes [53]. Conversely, Park *et al.* showed that EMPA significantly reduced the increase in blood pressure of ZSF1 rats and limited cardiac remodeling. Heart weight, volume and left ventricle wall thickness were normalized without further changes in functional markers [50]. Furthermore, EMPA treatment against hypertension was able to attenuate cardiovascular indications of heart failure with preserved ejection fraction. EMPA reduced left ventricle mass and thickness and in turn wall stress which were associated with reduced cardiomyocyte hypertrophy, resulting in improved diastolic function [54]. Hypertensive Dahl salt-sensitive rats treated with DAPA showed improvement of diastolic parameters with improved cardiomyocyte [Ca^{2+}] and [Na^{+}] homeostasis. However, *in vitro* treatment of cardiomyocytes isolated from non-treated hypertensive rats with DAPA showed no impact on the intracellular concentration of Na^{+} and Ca^{2+} [55]. Treatment with EMPA also improved vascular homeostasis in a model of Ang II-induced abdominal aortic aneurysms. EMPA prevented vascular remodeling and attenuated aneurysm progression by reducing macrophage infiltration and neovascularization which also coincide with a reduction in monocyte chemoattractant protein 1 (MCP-1; CCL-2), chemokine (C-C motif) ligand 5 (CCL-5), matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) expression in aortic tissue. Interestingly, MMP-2 and MMP-9 were detected principally in CD31+ and Mac3+ cells, indicating that the reduction in expression and vascular remodeling may be mediated by endothelial cells and macrophages [30].

Similar to the vascular effects observed in diabetic animal models, treatment with SGLT2i has been shown to attenuate vascular damage in the absence of diabetes. Administration of EMPA prior to ischemia/reperfusion injury resulted in reduced myocardial infarct size and expression of inflammatory markers interleukin 6 (IL-6) and inducible nitric oxide synthase (iNOS) in myocardial tissue, suggesting reduced potential of oxidative stress as iNOS reacts with anion superoxide to form peroxynitrite causing lipid peroxidation [52]. Similarly, myocardial oxidative state was significantly increased in infarct tissue and normalized by DAPA along with decreased levels of iNOS/CD68+ macrophages (M1) and reduced expression of IL-6, IL-1β and iNOS in infarct tissue, therefore promoting an increased M2/M1 ratio indicative of an attenuated inflammatory response [56]. Sayour et al demonstrated the acute effect of CANA on ischemia with a single

bolus IV injection of CANA resulting in reduced infarct size (-30%) without affecting the glycemic levels [57]. The single treatment induced the phosphorylation of AMP-activated protein kinase (AMPK) Thr172 in sham rats and restored the level of pACC Ser79 after ischemia. Similarly, although non-significant, phospho-endothelial NO synthase (eNOS) Ser1177 showed signs of restoration, improving the decreased level of phosphorylation. Levels of pAkt Ser473 were increased by CANA but had no effect in sham rats. Akt is a well-known cardioprotective mediator in the setting of acute myocardial ischemia/reperfusion injury. In addition, CANA reduced mRNA expression of p47phox and catalase as well as 4-HNE+ area in myocardial tissue, suggesting an anti-oxidant effect [57].

3.7.3 Endothelial dysfunction

Without significant effect on body weight, glucose secretion or atherosclerotic lesion development, DAPA for 4 weeks improved EC-dependent vasorelaxation in HFD-fed ApoE-/- adult mice and decreased inflammatory marker expression of P-IκBα, intercellular adhesion molecule 1 (ICAM-1) and F4/80 in aortic tissue, suggesting decreased nuclear factor-κB (NF-κB) activation and macrophage infiltration [58]. Similarly, EMPA treatment restored EC-dependent vasorelaxation and contractile response of mesenteric artery rings from ZSF1 rats [50]. *Ex vivo* exposure to CANA resulted in increased sensitivity of aortic rings to Ach-stimulated vasorelaxation and maximal vasorelaxation while having no effect on EC-independent vasodilation [57]. Similarly, *ex vivo* incubation of rat aortic rings with CANA significantly improved EC-dependent vasorelaxation with reduced gene expression of *IL1A* and *IL6*. Additionally, CANA prevented the upregulation of *Cd40* and caused a downregulation of *NoxO1*. Immunohistochemical analysis of the aortic tissue showed that CANA normalized ICAM-1 and platelet endothelial cell adhesion molecule expression and reduced nitro-oxidation at the endothelial layer [59].

Cardiac tissue of Dahl rats on a high salt diet showed induced EC dysfunction with increased expression of vascular cell adhesion molecule (VCAM-1) and E-selectin and decreased expression of eNOS. Moreover, increased expression of NF-κB, MCP-1 and IL-6 were also found. DAPA

treatment normalized the expression and blunted EC dysfunction. Interestingly, NF-κB activation (pNF-κB Ser536) was predominately located in ECs [55].

Increased expression of senescence markers, p53, p21 and p16, in the aorta inner curvature, was blunted by EMPA. In addition, EMPA improved eNOS expression and attenuated the increased VCAM-1 and tissue factor (TF) expression in lean control rats but not ZSF1 rats. This could suggest that EMPA protects atherogenesis-prone arterial sites by preventing pro-senescence and pro-atherothrombotic responses of low shear stress which seems absent in ZSF1 rats [50].

3.8 Protective effects on the endothelium: from macrovascular observations to the study of EC mechanisms *in vitro*

As more animal studies unveil the protective effects of SGLT2i against cardiovascular complication involving endothelial dysfunction, questions remain regarding the underlying cellular mechanisms. Several recent *in vitro* studies aimed at understanding the effects of SGLT2i on ECs independently of the systemic factors inherent to animal models (Appendix A - Table 4). As SGLT2i might impact EC homeostasis through multiple cellular mechanisms, integrating the recent *in vitro* findings will improve our interpretation of the role of the endothelium in the clinical benefits of SGLT2i.

3.8.1 Metabolic functions

Evidence points toward a possible restrictive effect of SGLT2i on the metabolic activity of ECs. These effects can either become detrimental at high concentrations or restraint cellular dysfunction through metabolic restriction.

Treatment of HUVECs with SGLT2i has been shown to inhibit DNA synthesis and thus cell proliferation to different extent without affecting cell viability. CANA (5 to 50 µM) induced a gradual decrease in DNA synthesis whereas DAPA and EMPA were better tolerated, only affecting synthesis at higher concentration (30-50 µM and 50 µM respectively) [25]. In addition, cell cycle progression appeared to be impacted due to the higher proportion of cells in the G0/G1 cycle compared to the S cycle. Drastic decreases in cyclin A and retinoblastoma protein phosphorylation expression were associated to reduced cell proliferation [25]. Cardiac

microvascular ECs isolated from diabetic mice showed increase senescence with higher proportion in G0/G1 cycle. Treatment with EMPA normalized metabolic activity and prevented mitochondrial fission resulting in improved endothelial functions in term of eNOS activity and paracellular permeability [39]

In line with the animal studies, treatment of ECs with SGLT2i *in vitro* can effectively resolve dysfunction induced by hyperglycemic condition. EMPA (10 µM) improved HUVECs viability in high glucose culture over 6 days, normalizing cell density which correlated with restored eNOS activity [2]. EMPA (100 nM) prevented the high glucose induced senescence of porcine coronary ECs. The redox-mediated senescence was mitigated by EMPA through the downregulation of NADPH oxidase and cyclooxygenase 2 (COX-2). Similarly, EMPA (100 nM) reduced the senescence of Ang II treated porcine coronary ECs similar to angiotensin type 1 receptor (AT1R)/NADPH oxidase inhibition, suggesting a redox sensitive mechanism. In addition, EMPA diminished the expression of senescence markers p53, p21 and p16 [23, 24]. EMPA (100-500 nM) was also found to limit hypoxic damage with advanced glycosylation end products (AGE) by improving cell viability and ATP content [52].

Together, this subset of results demonstrates the substantial effect of SGLT2i and its involvement in EC metabolic activity potentially impacting crucial cellular mechanisms.

3.8.2 eNOS activity and NO bioavailability

Further insight into the restoration of impaired vasodilation was found through *in vitro* studies. In multiple conditions inducing EC dysfunction, treatment with SGLT2i restored normal NO bioavailability but the exact impact on eNOS activity remains unclear. Phlorizin partially restored Akt and eNOS activation in response to palmitic acid induced dysfunction in HUVECs. The effect was demonstrated to be mediated through the activation of the PI3K/Akt/eNOS signaling pathway. Interestingly, SGLT1/SGLT2 gene silencing attenuated the Akt/eNOS impairment suggesting that direct inhibition of SGLT1/SGLT2 transport and downregulation of their expression might be involved in the protective effects observed [26]. However, decreased NO bioavailability in HCAECs and HUVECs caused by TNF-α was restored by DAPA and EMPA but the effect could not be related to improved eNOS activity. No significant changes in Cav-1 expression,

eNOS Ser1177 phosphorylation and Cav-1/eNOS complex in EMPA treated cells were found, indicating an effect independent of eNOS subcellular localization and posttranslational modifications [27]. In addition, EMPA restored normal NO availability of cardiac microvascular ECs (CMECs) [60] and HUVECs [2] treated with TNF-α and high glucose, respectively, but was without effect on the transcription, protein expression and phosphorylation levels of eNOS, suggesting improved NO availability through the mitigation of reactive oxygen species (ROS) level [60]. Together, these studies suggest that the restoration of NO bioavailability from SGLT2i treatment is mainly mediated through attenuation of oxidative stress rather than direct changes in eNOS expression and activity.

3.8.3 ROS and oxidative stress

Redox regulation is fundamental to cellular homeostasis as metabolic activity is inherently tied to production of ROS. Conditions promoting excessive oxidative stress, exceeding the redox regulation capacity of ECs, lead to dysfunction and exacerbated oxidative stress in a vicious circle. Observation of reduced oxidative markers in animal studies is supported by *in vitro* findings.

The induction of cytoplasmic and mitochondrial ROS by TNF-α in CMECs were prevented by EMPA [60]. Similarly, treatment with EMPA and DAPA respectively normalized and attenuated the ROS production induced by TNF-α [27]. However, the reduction could not be attributed to a direct antioxidant effect as tests with DPPH used as a free radical did not show similar results to BHT and ascorbic acid. Alternatively, TNF-α can induce ROS via NADPH oxidase 4 but EMPA did not alter *NOX4* nor *SOD1* and *SOD2* (radical scavenging enzymes) mRNA expression, suggesting a different TNF-α-altered intracellular mechanisms [60]. In comparison, Khemais-Benkhiat *et al.* showed that the reduction in the high glucose induced oxidative stress by EMPA was accompanied with the normalization of the NADPH oxidase subunits p22phox, p47phox and COX-2 expression [23]. In addition, sotagliflozin and EMPA also decreased Ang II induced oxidative stress. Interestingly, the increase in oxidative stress was found to be dependent on the active glucose transport via SGLT1/2 as decreases in extracellular glucose and sodium concentrations abolished the Ang II-induced increase in oxidative stress [24].

3.8.4 Inflammation

SGLT2i have shown to reduce systemic and localized inflammatory responses *in vivo* and recent studies emphasize the implication of ECs in the anti-inflammatory effects of SGLT2i. Discrepancies in the results exist regarding the capacity of different SGLT2i to alter cytokine levels and adhesion molecule expression.

Pretreatment of cells with CANA but not DAPA and EMPA inhibits the IL-1β-stimulated secretion of MCP-1 and IL-6. CANA attenuated mRNA expression of MCP-1 and IL-6 underlying the attenuated secretion of IL-1β-stimulated MCP-1/IL-6 [28]. Similarly, CANA but no DAPA or EMPA reduced lipopolysaccharides (LPS)-induced IL-6 release in HCAECs [63]. Inhibition of TLR4 resulted in similar results, suggesting the implication of the receptor through NF-κB activation [66]. CANA also reduced the high glucose redox-induced production of cytokines in TeloHAECs including interleukin 8 (IL-8), TNF-α, MCP-1 and IL-6 [61]. Increased secreted levels of CCL-2 and CCL-5 by Ang II stimulated HUVECs were normalized by EMPA (3μM) with reduced expression of adhesion molecules (ICAM-1 and VCAM-1). This is possibly mediated through NF-κB as EMPA also blunted the phosphorylation of p65 NF-κB triggered by short-term Ang II stimulation [30].

HUVECs treated with DAPA (1-2 nM) attenuated the TNF-α-induced increases in soluble ICAM-1 and VCAM-1 levels. Similarly, hyperglycemic conditions induced an increase expression of ICAM-1 and low concentrations of DAPA (1-10nM) attenuated the increase whereas higher concentrations (100 nM and 1 μM) did not have the same protective effect. Again, upregulation of NF-κB mRNA expression by TNF-α was attenuated by DAPA (1-2 nM) suggesting that changes in adhesion molecule expressions could rely on the transcriptional regulation of NF-κB signaling cascade [58].

However, other studies reported no significant effect of SGLT2i on adhesion molecules expression. Uthman *et al.* showed that treatment of HUVECs and HCAECs with EMPA (1 μM) did not resolve the TNF-α induced upregulation of ICAM-1 and VCAM-1 [27]. Similarly, EMPA did not change the transcription level nor the protein expression of VCAM-1 and E-selectin in CMECs normally induced by TNF-α via the NF-κB [60].

Regardless of the changes in adhesion molecules expressions, studies showed the reduction in inflammatory cell adhesion to ECs with SGLT2i. Pretreatment with EMPA (0.1-3 μM) significantly reduced the Ang II-induced leukocyte adhesion to HAECs in a dose-dependent manner [30]. Without altering the IL-1β-stimulated cell surface expression of E-selectin, VCAM-1 or ICAM-1, CANA inhibited the increased TNF-α-stimulated U937 promonocytic cell adhesion to HUVECs [28]. Similarly, EMPA (50 μM) prevented the increased leucocyte (NB4) adhesion to human abdominal aortic ECs caused by enzymatic glycocalyx degradation while having no impact on ICAM-1 expression [62].

3.9 Insights into EC mechanisms related to SGLT2i vascular benefits

Multiple potential pathways and mediators in the response of improved EC function by SGLT2i have now been studied. It is therefore reasonable to assume that SGLT2i impacts ECs through more than one mechanism although identifying signaling molecules as mediators or product of the effect of SGLT2i remains difficult with only a few studies including inhibitor experiments.

3.9.1 AMPK activation

Like other glucose-lowering agents, SGLT2i have been shown to impact AMPK, a major regulator of energy homeostasis promoting cardiovascular protective effects in part through improved EC function with improved NO synthesis, inhibition of ROS formation and NF-κB activation suppression [68]. However, discrepancies exist in the reported capacity of different SGLT2i to induce AMPK activation and in turn to improve vascular health. Increased or restored AMPK activation in rodent cardiac tissue, cardiomyocytes and ECs was shown following treatment with CANA [57] and EMPA [39, 69]. Diabetic induced reduction in AMPK activity and AMP:ATP ratio were restored by EMPA in CMECs [39]. Similarly, Koyani *et al.* showed the protective effects of EMPA on murine cardiomyocytes and macrophages with restored AMPK activation levels, resulting in improved cardiovascular functions with partially restored EC-dependent vasorelaxation [69]. In opposition, a study by Andreadou *et al.* which demonstrated the reduction in infarct sizes with EMPA treatment did not find increased AMPK activation levels in myocardial tissue [52]. *In vitro*, CANA but not DAPA or EMPA attenuated the LPS- or IL-1β stimulated IL-6 increase expression and induced increased AMPK activity [28, 63].

3.9.2 TLR4 and NF-κB activation

Through ligands binding, Toll-like receptor 4 (TLR4) activates NF-κB and associated inflammatory signaling cascade. Treatment of HUVECs with DAPA resulted in the downregulation of TLR4 and NF-κB p65 protein expression and reduced production of IL-6 and IL-8. Moreover, miRNAs involved in the regulation of inflammatory responses, i.e. miRNA-146a and miRNA-155 and were respectively upregulated and normalized by EMPA which support the anti-inflammatory effect of DAPA in LPS-stimulated HUVECs [66]. Similarly, the NF-κB p65 protein expression in EMPA-treated HAECs was normalized along with CCL-2, CCL-5, ICAM-1 and VCAM-1 expression, promoting the reduction of leukocyte adhesion [30].

3.9.3 Endoplasmic reticulum stress

Upon disruption of the protein-folding capacity of the endoplasmic reticulum (ER), cells can experience ER stress, which through the unfolded protein response (UPR) will determine cell apoptotic or survival response. Development of cardiomyopathy in rodents have shown improvement with SGLT2i treatment thought inhibition of ER stress signaling. EMPA downregulated the transcriptional and protein expression of ER stress markers, apoptotic response, and the NLRP3-mediated inflammation in cardiac tissue [70, 71]. Similar results were found in diabetic ob/ob mice treated with DAPA and supported by the observation of the reduction of ER stress inflammatory markers in a dose-dependent manner on isolated cardiofibroblast [72]. Moreover, treatment of human coronary artery ECs with DAPA, resulted in the normalization of the UPR regulatory protein activation, suggesting the direct attenuation of ER stress signaling by SGLT2i [64]. More work is however needed to relate the protective effects of SGLT2i on ER stress at the cardiac and endothelial cell levels (Fig. 3-2). Interestingly, other natural occurring compounds have shown to have protective effects against EC ER stress in comparable ways to SGLT2i. Quercetin [73], Mangiferin [74], Ilexgenin A [75] as well as Astragaloside IV [76] were shown to revoke the induction of ER stress by palmitate or high glucose in HUVECs through the reduction of ROS, leading to reduced TXNIP/NLRP3 activation, IL-1β/IL-6 secretion and apoptosis.

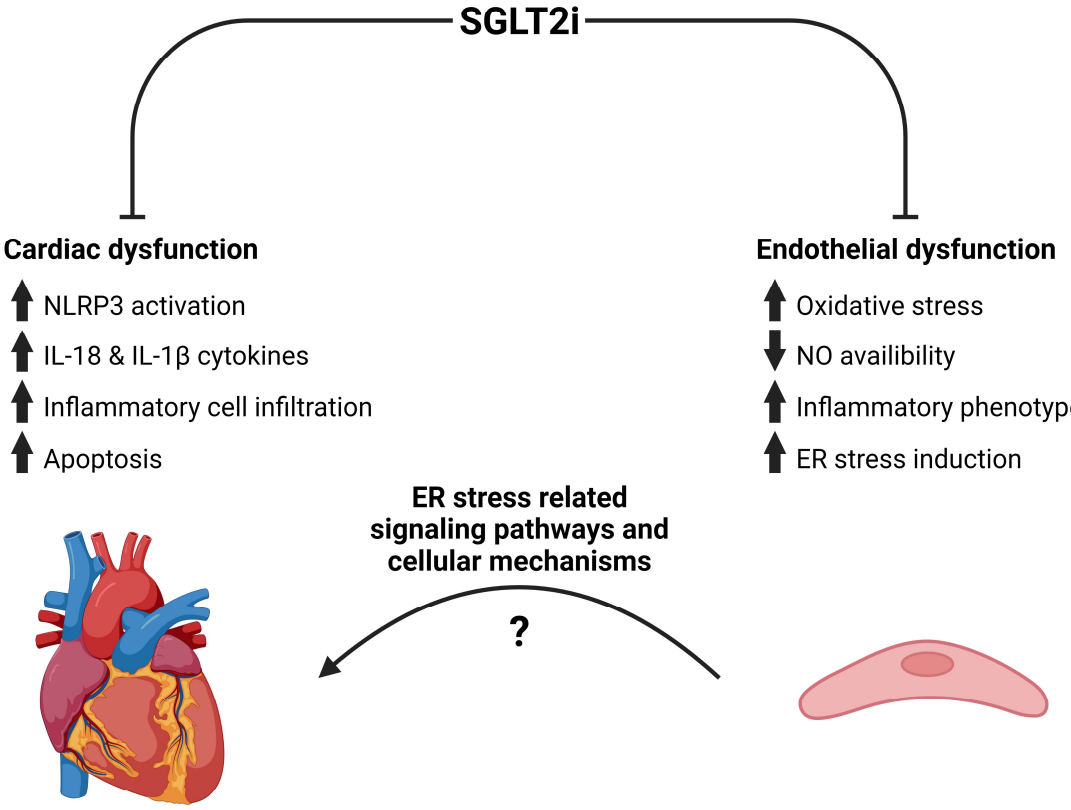

Figure 3-2. ***Cardiovascular benefits of SGLT2 inhibitors in relation to ER stress****. Treatment of rodents with SGLT2i has been shown to mitigate the complications of cardiomyopathies through the reduction in expression of ER stress markers. The improvement of cardiac functions was concomitant with reduction in NLRP3 activation, IL-18 & IL-1β secretion, infiltration of macrophages and the expression of apoptotic markers in myocardial tissue. Similarly, treatment of ECs with SGLT2i has been shown to mitigate the dysfunction associated to ER stress such as increased oxidative stress, impaired NO availability, inflammatory phenotype and the upregulation of the UPR regulatory proteins. How the improved EC functions by SGLT2i translate to better cardiac functions in the context of ER stress remains to be clarified. This figure was created with BioRender.com.*

3.9.4 NHE inhibition

Off-target inhibition of Na+/H+ exchanger (NHE) by SGLT2i has been demonstrated and proposed as a mechanism by which lower myocardial cytosolic Na+ concentration and NHE activity promote cardiac protection [77]. For instance, NHE1 was upregulated in rats fed with a high-salt diet and downregulated with DAPA treatment. Increased expression was primarily observed in the coronary endothelium, suggesting the direct involvement of endothelial NHE1 regulation in the protective effects of DAPA. This relation was confirmed with HUVECs, showing impaired pH recovery, similar to cariporide, a specific NHE inhibitor [55]. Treatment of HCAECs and HUVECs with EMPA resulted in reduced cytoplasmic Na+ concentration and NHE1 activity similar to

cariporide. Also, mitigation of TNF-α generated ROS by EMPA or cariporide resulted in similar reductions [67]. However, despite NHE inhibition with cariporide inducing similar anti-oxidant response to EMPA in porcine CAECs, it was not equally implicated in the prevention of cellular senescence by EMPA [24].

3.9.5 STAT3

The signal transducer and activator of transcription 3 (STAT3) is a major regulator of cellular mechanisms fundamental to cardiovascular homeostasis. STAT3 modulates myocardial inflammation through cytokine production which is in part mediated by paracrine signaling between ECs and cardiomyocytes [78]. DAPA, and to a lesser extent phlorizin treatment, induced a sustained activation of STAT3 as measure by the ratio of pSTAT3/STAT3 in rat myocardial infarct tissue. Immunohistochemistry further supported activation of STAT3 by the translocation of pSTAT3 (Thr705) into the nucleus. Treatment with S3I-201, a STAT3 inhibitor, abolished the beneficial changes in inflammatory marker expressions, suggesting that STAT3 activation act as a mediator in the anti-inflammatory effects of DAPA and phlorizin [56]. Similarly, while treatment of EMPA against ischemic injury had no significant effect on the activation of Akt, eNOS, GSK-3β, ERK1/2 and AMPK, it increased the expression and activation of STAT3 (Thr705) and reduced the associated IL-6 and iNOS myocardial expression [52]. A follow-up study further associated the improved recovery from ischemia to the activation of STAT3 by the chronic EMPA treatment. Notably, EMPA was shown to improve viability of ECs subjected to hypoxia through a STAT3 dependent mechanism and to reduce ROS production. In myocardial tissue, pSTAT3 (Thr705) colocalized with CD31+ and VEGFR2+ cells, suggesting the direct implication of the endothelium in the effect mediated by EMPA in response to hypoxia [79].

3.10 Concluding remarks

In conclusion, the recent evidence on the SGLT2i pleotropic effects point toward mechanisms at the cellular level, regulating crucial cell functions and signaling cascades that ultimately contribute to the cardiovascular benefits. It is becoming clear that these effects rely on the improvement of the homeostasis of vascular cells including ECs. Thus, to fully characterize the effect of SGLT2i on the vasculature and help predict the clinical outcome, the effect of SGLT2i on

EC signaling and the interplay between ECs and other vascular cells needs to be further studied. To this end, future clinical trials and studies should be encouraged to include the assessment of endothelial functions. Monitoring vasomotion and surrogate markers of endothelial cell activation such as hsCRP and Ox-LDL receptor 1 (LOX-1) in patients treated with SGLT2i would be of interest. From a mechanistic point of view, whether the effects of SGLT2i on vascular cells is mediated through the inhibition of SGLT1/2, the inhibition of unspecific targets or a combination of both remains to be clarified. The use of knock down animal models should be explored to refine our understanding of the impact of SGLT2i on the aforementioned signaling pathway participating in the cardiovascular protective effects.

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CHAPTER 4 EMPAGLIFLOZIN MITIGATES ENDOTHELIAL INFLAMMATION AND ATTENUATES ENDOPLASMIC RETICULUM STRESS SIGNALING CAUSED BY SUSTAINED GLYCOCALYX DISRUPTION

4.1 Preface

In continuation of our previous published study [22], the effects of empagliflozin (EMPA) on endothelial cells (ECs) were further investigated. Treatment of ECs with EMPA has been shown to reduce leukocyte adhesion following acute glycocalyx (GCX) degradation, suggesting an anti-inflammatory effect. However, the direct implication of the GCX remained unclear as the GCX regrowth and functionality after the culture period of 24h was not fully addressed. Thus, whether the effect of GCX mechanotransduction is directly implicated or if EMPA can independently mitigate the cellular dysfunction induced by GCX disruption remain to be answered. Therefore, this study aimed to clarify the role of the GCX in the EMPA anti-inflammatory effect by studying the potential mechanisms by which EMPA overcomes EC dysfunction associated to GCX disruption. Sustained GCX disruption was used to abolish regrowth and mechanotransduction. This condition served as a model for EC dysfunction in hyperglycemic or inflamed conditions where the integrity of the GCX is chronically compromised. Given the fact that the primarily endothelial functions related to the GCX and leukocyte adhesion such as endothelial nitric oxide synthase (eNOS) activation and intercellular adhesion molecule 1 (ICAM-1) expression were not significantly impacted by sustained heparan sulfate (HS) degradation and EMPA, a genome-wide transcriptome analysis was conducted to better characterize the EC dysfunction. This allowed us to screen, target and better report the impacted signaling pathways by GCX disruption and EMPA. Identifying the role of the GCX in relation to EMPA and the identified pathways contributes to our understanding of the effects of EMPA on EC dysfunction in pathologically relevant setting.

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Empagliflozin mitigates endothelial inflammation and attenuates endoplasmic reticulum stress signaling caused by sustained glycocalyx disruption

Marc-Antoine Campeau[1], Richard L. Leask[1,2]*

[1]Department of Chemical Engineering, McGill University, Montreal, QC, Canada

[2]McGill University Health Centre, Montreal, QC, Canada

*Corresponding Author

richard.leask@mcgill.ca

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4.2 Abstract

The disruption of the endothelial cell (EC) glycocalyx (GCX) leads to cellular dysfunction promoting inflammation and cardiovascular disease progression. Recent studies have shown that empagliflozin (EMPA; Jardiance), a sodium-glucose cotransporter 2 inhibitor used in the treatment of type 2 diabetes, can improve EC functions impacted by GCX disruption although the exact cellular mechanisms remain to be elucidated. In this study, the effect of EMPA on EC inflammatory response induced by sustained GCX disruption was investigated. Human aortic ECs were cultured under shear (10 dyne/cm^2) for 24h with or without sustained degradation of heparan sulfate (HS). HS degradation increased inflammatory cell adhesion to ECs. EMPA (50 µM) normalized adhesion levels under sustained HS degradation. Protein expressions of eNOS, phospho-eNOS Ser1177 and ICAM-1 remained unchanged between conditions. Transcriptome analysis revealed the induction of the unfolded protein response (UPR) through the increased expression of *ATF3*, *ATF4*, *DDIT3* (CHOP), *EIF2AK3* (PERK), *HSPA5* (Grp78), *PPP1R15A* (GADD34) and *TRIB3* which was in part downregulated by EMPA. mRNA and protein expression of thioredoxin interacting protein (TXNIP) was also downregulated by EMPA. Mitigation of oxidative stress with N-Acetyl-L-cysteine resulted in similar reduction in inflammatory cell adhesion compared to EMPA which could indicate a potential mechanism by which EMPA normalized the inflammatory response. In conclusion, this study demonstrated the potential of EMPA to resolve the inflammatory response of ECs caused by sustained GCX disruption while altering UPR signaling under endoplasmic reticulum stress.

4.3 Introduction

The endothelial glycocalyx (GCX) is a glycosaminoglycan and proteoglycan-rich functional layer lining the endothelium which promotes homeostasis through its role as a mechanosensor and as a barrier to inflammatory cell attachment. Disruption of the GCX in pathological conditions such as type 2 diabetes (T2D) and atherosclerosis [1, 2] causes endothelial cell (EC) dysfunction and thus, leads to cardiovascular disease progression. In particular, *in vitro* and *in vivo* degradation of heparan sulfate (HS), the main component of EC GCX, has been shown to impair shear-regulated nitric oxide (NO) production and promote adhesion of inflammatory cells [3-5]. Given its central role in EC homeostasis, the GCX is considered a potential therapeutic target to prevent or alleviate EC dysfunction and therefore benefit clinical outcomes [6, 7].

Empagliflozin (EMPA; Jardiance®) belongs to the sodium-glucose cotransporter 2 inhibitor (SGLT2i) class of drugs used in the treatment of T2D and more recently heart failure. This class has emerged as a promising new therapy for cardiovascular diseases as treatment with a SGLT2i has shown impressive beneficial outcomes in recent clinical trials (e.g. EMPA-REG OUTCOME (NCT01131676) [8], DAPA-HF (NCT03036124) and EMPEROR-Reduced (NCT03057977) [9, 10]). Among these, the EMPA-REG OUTCOME trial demonstrated significant reductions in the rate of hospitalization for heart failure and cardiovascular death in T2D patients treated with EMPA when compared with placebo [8]. Subsequent animal studies have suggested pleiotropy and extended protective effects by EMPA to atherosclerosis [11], heart failure [12, 13] and ischemia-reperfusion injury [14] in non-diabetic conditions, suggesting that the cardiovascular protection is not achieved solely through glycosuria.

As endothelial dysfunction is a central step in the development of cardiovascular complications, it has been hypothesized that SGLT2i can prevent or overcome EC dysfunction. Treatment with EMPA has been shown to improve EC-dependent vasorelaxation in diabetic rodents [15-17]. El-Daly *et al.* showed similar results with *ex vivo* mice aortic tissue in response to hyperglycemic conditions [18]. Moreover, EMPA reduced the inflammatory response and the adverse vascular remodeling in Ang II-induced AAA of ApoE(−/−) mice. Adhesion of inflammatory cells and adhesion molecule expressions in isolated mouse ECs were also reduced [19]. Recent *in vitro*

studies suggested that EMPA restored NO bioavailability and reduced oxidative stress in TNF-α treated ECs [20, 21]. Although cardiac and vascular protective mechanisms have been proposed [22], the exact mechanism by which EMPA promotes EC homeostasis and in turn contributes to better vascular health remains to be clarified.

Considering the importance of GCX health in EC function and the impressive ability of EMPA to improve cardiovascular outcomes, we hypothesized that EMPA can overcome EC dysfunction caused by GCX disruption which could, in part, explain the improvement in vascular health. We previously showed that EMPA can normalize the inflammatory response of ECs possibly through shear mechanotransduction of a restored GCX after acute degradation [23]. As the GCX is a dynamic surface layer with potential of regrowth, this work aims at exploring more closely the signaling pathways involved under sustained GCX disruption to identify other anti-inflammatory mechanisms of EMPA.

4.4 Materials and Methods

4.4.1 Cell culture and 3D culture model

Human abdominal aortic endothelial cells (HAAECs, ATCC, Coriell, CRL-2472) were cultured in endothelial cell growth medium (Promocell, C-22020), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) in culture flasks coated with 0.1% pig gelatin at 37°C and 5% CO2. Cells were grown to confluence and harvested from the flask with TrypLE (ThermoFisher, 12605028). Experiments were performed with cells at passage 5 and 6.

Tubular cell culture models were produced as previously described [59, 60]. Briefly, the silicon elastomer Sylgard™ 184 (Paisley, AVDC0184-39) was prepared by mixing the base and curing agent in a 10:1 ratio. Sylgard™ 184 was cured around a polished stainless steel rod in plexiglass molds. Following complete curing, the rod was removed, creating a straight hollow tube where cell can be seeded and grown. In preparation to cell culture, models were sterilized by boiling and coated with a 40 µg/mL human fibronectin (Akron Biotech, AK9715-0005) solution overnight. HAAECs were seeded at a concentration of 1x106 cells/mL and grown for 48h. Models were mounted on a rotor to ensure complete and even coverage of the inner surface. Cells were then

cultured for 24h in static or shear condition (laminar shear stress of 10 dyne/cm2) with or without 50 µM EMPA.

4.4.2 Heparan sulfate enzymatic degradation

GCX disruption was performed by enzymatic degradation of HS. HAAECs were treated with 0.5 U/mL of heparinase III (Sigma unit, enzyme 4.2.2.8, IBEX Pharmaceuticals, 50-012) in FBS-free media for 2 hours followed by the addition of 0.1 U/mL in the medium during the 24h of static or shear culture to achieve sustained degradation (sDEG) and to prevent HS regrowth. The initial condition of degradation (DEG T0) was assessed immediately after the initial 2h exposure to heparinase III.

4.4.3 Heparan sulfate immunostaining and confocal microscopy

Cells were fixed in situ with a 2%/0.1% paraformaldehyde/glutaraldehyde solution in PBS for 30 min without prior washes to prevent HS disruption. Sections of the models were cut and samples were blocked with a 2% normal goat serum (NGS) in PBS. Primary antibody for HS (1:100, mouse, 10E4, Amsbio, 370255) was diluted in 1% NGS/PBS and incubated overnight at 4°C. Cells were rinsed with PBS and incubated for 1h with the secondary antibody (ThermoFisher, goat anti-Mouse IgG, IgM (H+L) Alexa Fluor 488, A-10680) and DAPI (1:1000) diluted in 1% NGS/PBS. Cells were rinsed with PBS and the model section were cut, mounted using 0.2% Dabco/Glycerol (1:5, Sigma) and imaged via laser scanning confocal microscopy (Zeiss Exciter 800). Z-stack images were acquired at 10X magnification and maximum intensity projections were created to quantify the mean gray value of each image using ImageJ.

4.4.4 Cell morphology and shape index

Following the static or shear culture with or without EMPA, cells were fixed in situ with 1% paraformaldehyde in PBS for 20 min, rinsed with PBS and stained with a 4% crystal violet (BD Biosciences) solution for 10 min. Cell morphology was assessed through light microscopy (Leica DMIL microscope and Leica DC300 camera) images and quantified by computing the shape index of individual cells using Matlab scripts [59]. The shape index (SI) is used as a metric of cell

elongation where a perfectly round cell has a SI close to 1 and a straight elongated cell, a SI closer to 0.

4.4.5 Adhesion assay

Adhesion assays were performed as previously described [24]. Acute promyelocytic leukemia (NB4) cells were cultured in suspension and maintained at a concentration of $2x10^5$ to $1x10^6$ cells/mL in RPMI 1640 medium with 2 mM L-glutamine supplemented with 10% FBS and 1% penicillin-streptomycin. 48h prior to the adhesion assay, NB4 cells were stimulated with 10-6 M all-trans-retinoic acid (ATRA, Sigma, R2625) to induce differentiation into granulocytes. Adhesion assays were conducted following 24h of shear exposure and tumor necrosis factor-alpha (TNF-α; PeproTech, 300-01A, 10 ng/mL) stimulation of HAAECs to induce NB4 cell adhesion. As previously shown, TNF-α stimulation is necessary to reach significant numbers of NB4 cell adhesions [24]. Also, to assess the effect of oxidative stress on adhesion, HAAECs were treated with 1 mM of N-Acetyl-L-cysteine (NAC, Sigma, A9165) for 24h prior to the adhesion assay. A NB4 cell suspension at $5x10^5$ cells/mL was perfused through the models at a shear stress of 1.25 dyne/cm^2 for 1h. PFA 1% was then perfused to fix the adhered NB4 cells to the endothelial monolayer while removing non-adherent NB4 cells. The number of adhered NB4 cells was determined by manually counting cells from light microscope images (3-5/sample) at a magnification of 4X.

4.4.6 Protein collection and western blot

HAAECs were washed with cold PBS and lysed in situ in cold RIPA lysis buffer (ThermoFisher, 89900) with 2% Halt™ protease and phosphatase inhibitor cocktail (ThermoFisher, 78440). Samples were vortexed and kept on ice before centrifuging at 13000 RPM for 10 min. Protein quantification was performed through colorimetric assays and protein quantities were normalized between samples. Loading samples were prepared with Bolt LDS sample buffer and reducing agent (ThermoFisher, B0007, B0009). Gel electrophoresis were performed using the Mini Blot Gel tank and pre-cast Bolt 4-12% Bis-Tris Plus gels loaded with 20 µg of protein. Protein transfer was carried out using the Mini Blot module and 0.45 µm nitrocellulose membranes following manufacturer instructions. Following the transfer, membranes were blocked with 5% bovine serum albumin (BSA, BioShop, ALB001) in 0.1% Tween-20 TBS (TBST) for 1h. Primary

antibodies were diluted in blocking buffer and incubated at 4°C overnight on a shaking plate. Antibodies consisted of eNOS (1:500, BD Biosciences, 610297), phospho-eNOS Ser1177 (1:500, Abcam, ab184154), ICAM-1 (1:5000, Abcam, ab109361), GAPDH (1:10000, ThermoFisher, AM4300) and TXNIP (1:1000, Abcam, ab188865). After 3 washes in TBST, the membranes were incubated with horseradish peroxidase secondary antibodies (1:40000, ThermoFisher) diluted in blocking buffer for 1 hour at room temperature. Membranes were washed 3 times in TBST and signal detection was achieved with SuperSignal™ West Pico PLUS Chemiluminescent Substrate and UVP Biospectrum 810 MultiSpectral Imaging System. Proteins were quantified by densitometry using ImageJ and normalized to the loading control (GAPDH).

4.4.7 Transcriptome analysis

The transcriptome profiles of HAAECs under two sets of conditions were studied. Cells were statically cultured with or without EMPA for 6h or cultured under similar conditions to the adhesion assay (24h shear culture, TNF-α stimulation, CTL (Static), CTL, sDEG and sDEG-EMPA). Following treatment, cells were lysed in situ and RNA isolation was performed using the RNeasy Plus Micro kit (QIAGEN) according to manufacturer instructions. Sample RNA integrity was confirmed with the RIN using a BioAnalyzer (Agilent). The genomic transcriptome profile of each sample was determined with a Human Clariom S Array and analyzed using the Transcriptome Analysis Console software (SST-RMA method, Applied Biosystems, v4.0.2). Gene lists were generated by filtering minimal threshold expression and fold changes (≤ -1.5 or ≥ 1.5). The open-source pathway database REACTOME was used to analyze the gene lists and to identify potential pathways based on the number of matched entities [61].

4.4.8 Reactive oxygen species measurement

Reactive oxygen species (ROS) production was measured using the 2',7'-dichlorofluorescin diacetate (DCFDA) assay kit (Abcam, ab113851) according to the manufacturer instructions. Briefly, ECs were cultured in 96-well plates at a density of 30 000 cells/cm2 and stained with 10 µM H2DCFDA for 30 min prior to treatments. Tert-Butyl hydroperoxide (TBHP) at 50 µM was used as positive control, with or without 50 µM EMPA for 6h. Pretreatment with EMPA (preEMPA + TBHP) was also tested by incubating with EMPA for 18h prior to the assay. TNF-α (10 ng/mL) and

sDEG over 24h were also tested. After incubation, ROS production was measured by fluorescence unit on a Berthold fluorescence microplate reader with the FITC filter set. Measurements were normalized to the control sample i.e. H2DCFDA stained cells with no TBHP or EMPA treatment.

4.4.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism™ software. Results are presented as the mean values ± standard deviation of 3 independent experiments. Each experiment included 2-6 replicates for each condition and the mean was used in the analysis. When applicable, the normality of the data was confirmed by D'Agostino and Pearson test. Otherwise, the data was assumed to be normally distributed. Mean values were compared using t-tests, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-test with a 95% confidence interval. Statistical analysis of the transcriptome data was carried out through the Transcriptome Analysis Console software using default settings with eBayes analysis. p-values less than 0.05 were considered significant.

4.5 Results

4.5.1 EMPA induces an anti-inflammatory response independent of HS.

Sustained HS degradation was used to evaluate the inflammatory response of ECs to EMPA independent of HS. Inflammatory cell adhesion assays were performed on TNF-α treated cells similar to previous studies [3, 23, 24]. EMPA significantly reduced NB4 cell adhesions compared to control (Fig. 4-1, EMPA vs CTL * $p<0.001$). While sustained degradation caused significant increases in NB4 cell adhesion (sDEG vs CTL * $p<0.001$) showing an enhanced inflammatory response, EMPA reduced the adhesions to the same level as non-degraded samples (sDEG-EMPA vs sDEG # $p<0.001$) and below control (sDEG-EMPA vs CTL * $p<0.001$). This suggests that EMPA prevents the inflammatory response of ECs to TNF-α stimulation and HS degradation. As it persists under sustained degradation, it can be assumed that EMPA can reduce inflammation independently of HS.

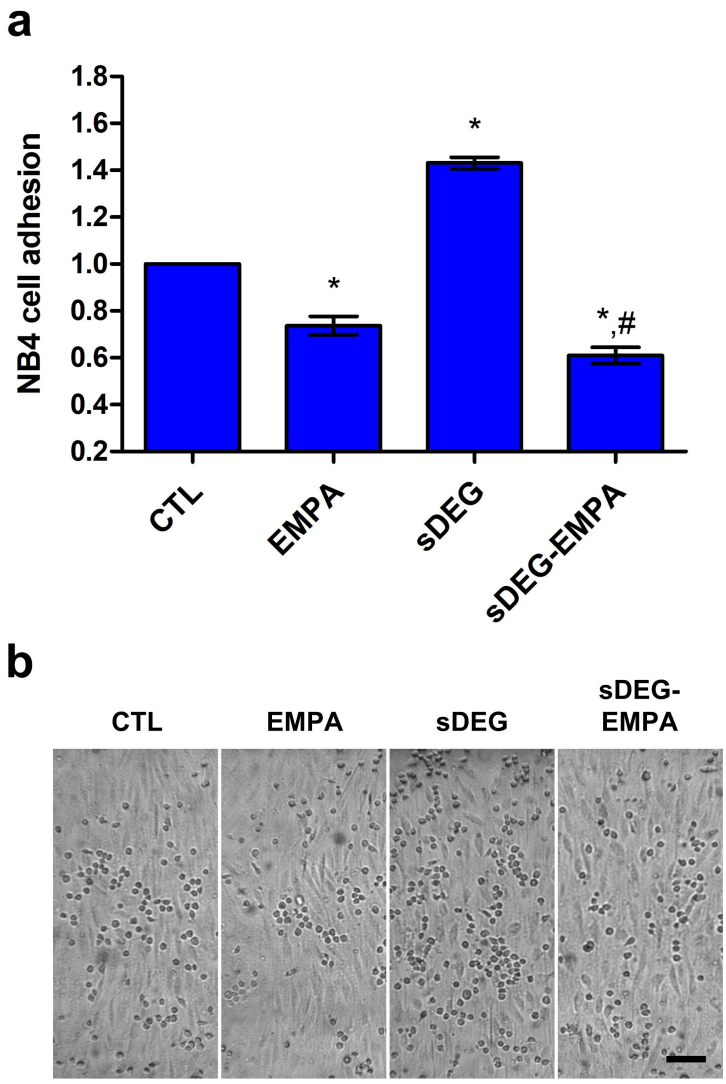

Figure 4-1. ***NB4 cell adhesion assay following shear culture for 24h.*** *(a) Relative numbers of NB4 cell adhesions to TNF-α stimulated HAAECs under sustained HS degradation with or without EMPA treatment. Degradation caused increases in adhesion compared to control (sDEG vs CTL * p<0.001). EMPA reduced the adhesion compared to control (EMPA vs CTL * p<0.001) and prevented the increase caused by degradation (sDEG-EMPA vs sDEG # p<0.001) resulting in adhesion levels below control (sDEG-EMPA vs CTL * p<0.001). (b) Representative images of each condition (scale bar = 100 µm).*

4.5.2 ICAM-1 and eNOS are not implicated in the HS independent EMPA anti-inflammatory response

The expression of eNOS and ICAM-1 were assessed to characterize the inflammatory response induced by sustained HS degradation and their potential role in the recovery effect provided by EMPA. After 24h of culture, eNOS expression in all static and shear TNF-α + conditions were reduced compared to TNF-α – controls (Fig. 4-2a, * p<0.001). There was no observed effect of EMPA nor

degradation on eNOS expression. Shear significantly increased the phospho-eNOS Ser1177 levels compared to the static control in absence of TNF-α (Fig. 4-2b, CTL TNF-α – shear vs CTL TNF-α – static # $p<0.001$). TNF-α stimulation impaired the shear-induced increase of phospho-eNOS Ser1177 to similar levels in all shear conditions suggesting reduced eNOS activation (* $p<0.001$). Again, no significant impact on phospho-eNOS Ser1177 caused by EMPA or degradation were found. Similarly, TNF-α induced the expression of ICAM-1 to similar levels in all conditions (Fig. 4-2c, * $p<0.001$) with no significant effect of shear, EMPA or degradation. Interestingly, the phospho-eNOS Ser1177/total eNOS ratio remained unchanged with or without TNF-α in shear condition while significantly higher ratios were found in static culture (Fig. 4-2d, * $p<0.01$). Taken together, these results suggest that the activation of eNOS and the expression of ICAM-1 are not directly implicated in the HS independent recovery of EC dysfunction by EMPA.

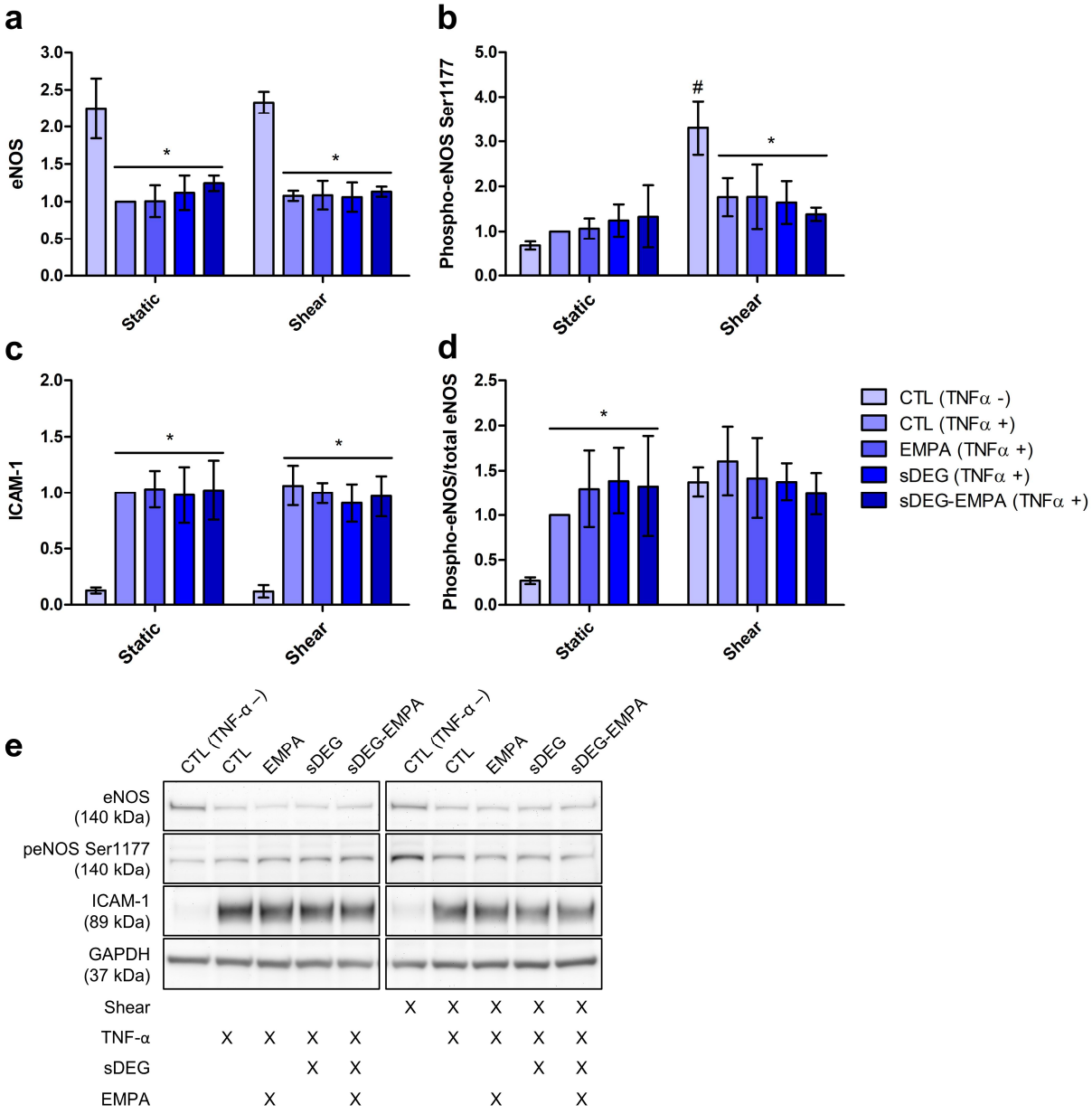

Figure 4-2. ***Protein expression quantification through western blot densitometry measurements after 24h of culture.*** *Expression of (a) eNOS, (b) phospho-eNOS Ser1177, (c) ICAM-1 and (d) the ratio phospho-eNOS/total eNOS were assessed. Shear increased the expression of phospho-eNOS Ser1177 compared to the static control (CTL TNF-α – shear vs CTL TNF-α – static # p<0.001). TNF-α stimulation reduced expression levels of eNOS in static and shear culture and reduced phospho-eNOS Ser1177 in shear culture compared to TNF-α – controls (* p<0.001). ICAM-1 expression was upregulated by TNF-α in static and shear culture compared to TNF-α – controls (* p<0.001). Phospho-eNOS/total eNOS ratios were increased in static condition compared to the TNF-α – control (* p<0.01) while no significant differences were observed in shear condition. No significant effects of degradation and EMPA were observed on the protein expression levels within TNF-α groups. (e) Representative bands and culture condition details. Full-length blots can be found in the Supplementary Fig. S4-4.*

4.5.3 The induction of the unfolded protein response caused by sustained HS degradation is partially restored by EMPA

To explore the possible signaling pathways impacted by the sustained degradation of HS and EMPA beyond the traditional mechanobiology and anti-inflammatory role of the GCX, a genome-wide transcriptome analysis was performed. We sought to better understand the mechanisms by which EMPA prevents the increased inflammatory response triggered by the sustained HS degradation under shear culture. EC dysfunction associated genes were screened and grouped to identify the signaling pathways involved based on their fold changes.

Shear exposure for 24h showed regulation of hallmark genes such as eNOS (*NOS3*), *KLF2* and COX-2 (*PTGS2*) while downregulating inflammatory signaling genes (*IL1B*, *IL6*) compared to the static control (CTL vs CTL (Static)). mRNA expression of ICAM-1 was also slightly downregulated by shear (Suppl. Table S4-1).

Sustained HS degradation under shear caused the upregulation of genes related to the unfolded protein response (UPR) and the C/EBP homologous protein (CHOP) transcriptional factors suggesting that ECs were subjected to endoplasmic reticulum (ER) stress. As such, activating transcription factor 3 (*ATF3*), DNA-damage-inducible transcript 3 (*DDIT3*; CHOP) and tribbles homolog 3 (*TRIB3*) were significantly upregulated under HS degradation compared to the control (Fig. 4-3a, sDEG vs CTL * $p<0.001$). The activation of the UPR was also apparent by the upregulation of the activating transcription factor 4 (*ATF4*), the eukaryotic translation initiation factor 2 α kinase 3 (*EIF2AK3*; PERK), the ER chaperone protein Grp78 (*HSPA5*) and the growth arrest and DNA damage-inducible protein GADD34 (*PPP1R15A*) (Fig. 4-3b, sDEG vs CTL * $p<0.001$). EMPA significantly reduced the expression of *ATF3*, *DDIT3*, *HSPA5*, *PPP1R15A* and *TRIB3* under HS degradation (sDEG-EMPA vs sDEG # $p<0.001$, \$ $p<0.01$, % $p<0.05$). The transcriptional regulation induced by sustained HS degradation and EMPA appeared to be mediated mainly through the PKR-like ER kinase (PERK, *EIF2AK3*)/eukaryotic initiation factor 2 (eIF2)/ATF4 branch of the UPR (Fig. 4-3c). Taken together, these results suggest that HS degradation under shear promotes EC dysfunction through the activation of the UPR and in turn, CHOP-mediated apoptosis, features of unresolved ER stress. Treatment with EMPA showed attenuation of ER stress through the transcriptional downregulation of key UPR genes.

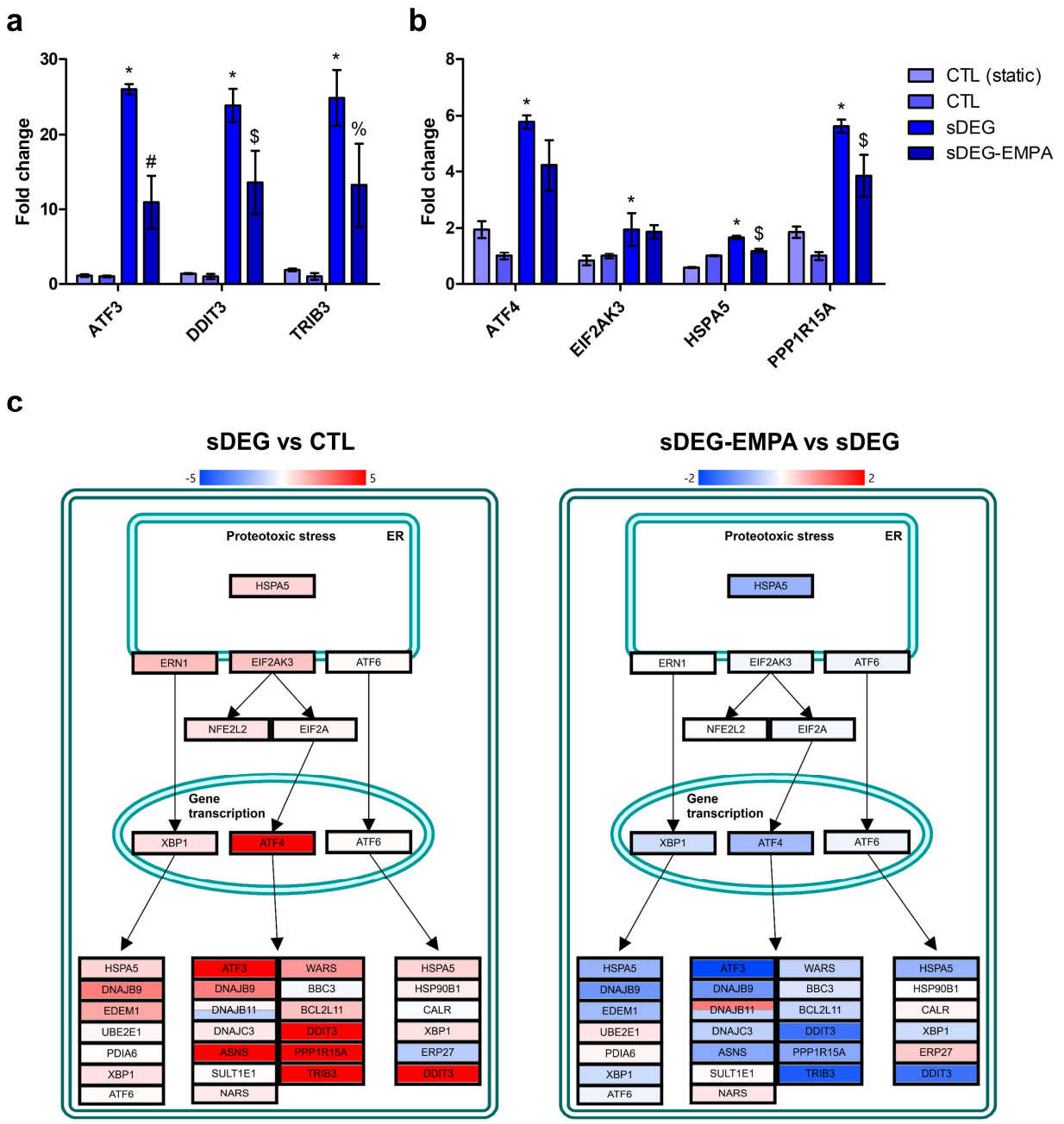

Figure 4-3. ***Impact of sustained HS degradation and EMPA treatment on ER stress gene transcription.*** *(a) Fold changes of ATF3, DDIT3 (CHOP), TRIB3 and (b) ATF4, EIF2AK3 (PERK), HSPA5 (GRP78) and PPP1R15A (GADD34). Sustained degradation caused significant fold increases in ATF3 (26.01), ATF4 (5.78), DDIT3 (23.85), EIF2AK3 (1.95), HSPA5 (1.67), TRIB3 (24.86) and PPP1R15A (5.63) expression (sDEG vs CTL * p<0.001). EMPA significantly reduced the expression of ATF3 (-2.37), DDIT3 (-1.76), HSPA5 (-1.40), PPP1R15A (-1.46) and TRIB3 (-1.88) (sDEG-EMPA vs sDEG # p<0.001, $ p<0.01, % p<0.05). (c) Simplified ER stress signaling pathway generated from the Transcriptome Analysis Console software showing regulation through the PERK/eIF2/ATF4 branch of the UPR. The original version of the pathway is made available through WikiPathways [25].*

4.5.4 EMPA potentially attenuates inflammation through the regulation of TXNIP

In parallel to the UPR, EMPA was found to reduce the expression of the thioredoxin interacting protein (TXNIP) which impacts oxidative stress regulation and has been related to ER stress. Treatment with EMPA for 6h in static culture was found to downregulate the mRNA expression of TXNIP (Fig. 4-4a, EMPA vs CTL * $p<0.05$). TXNIP mRNA levels were also significantly reduced by shear after 24h (Fig. 4-4b, CTL vs CTL (Static) * $p<0.001$). Sustained HS degradation partially abolished the shear-induced regulation (sDEG vs CTL # $p<0.01$, sDEG vs CTL (Static) $ $p<0.01$) and treatment with EMPA normalized the expression by significantly reducing the increased levels caused by degradation (sDEG-EMPA vs sDEG % $p<0.05$, sDEG-EMPA vs CTL (Static) * $p<0.001$). Sustained HS degradation also caused a significant increase in TXNIP protein expression (Fig. 4-4c, sDEG vs CTL * $p<0.001$) and similar to the mRNA levels, EMPA normalized the expression compared to the control (sDEG-EMPA vs sDEG # $p<0.01$). This suggests that TXNIP could be a potential mediator in the ER stress response as its mRNA and protein expression was modulated by shear, sustained HS degradation and EMPA.

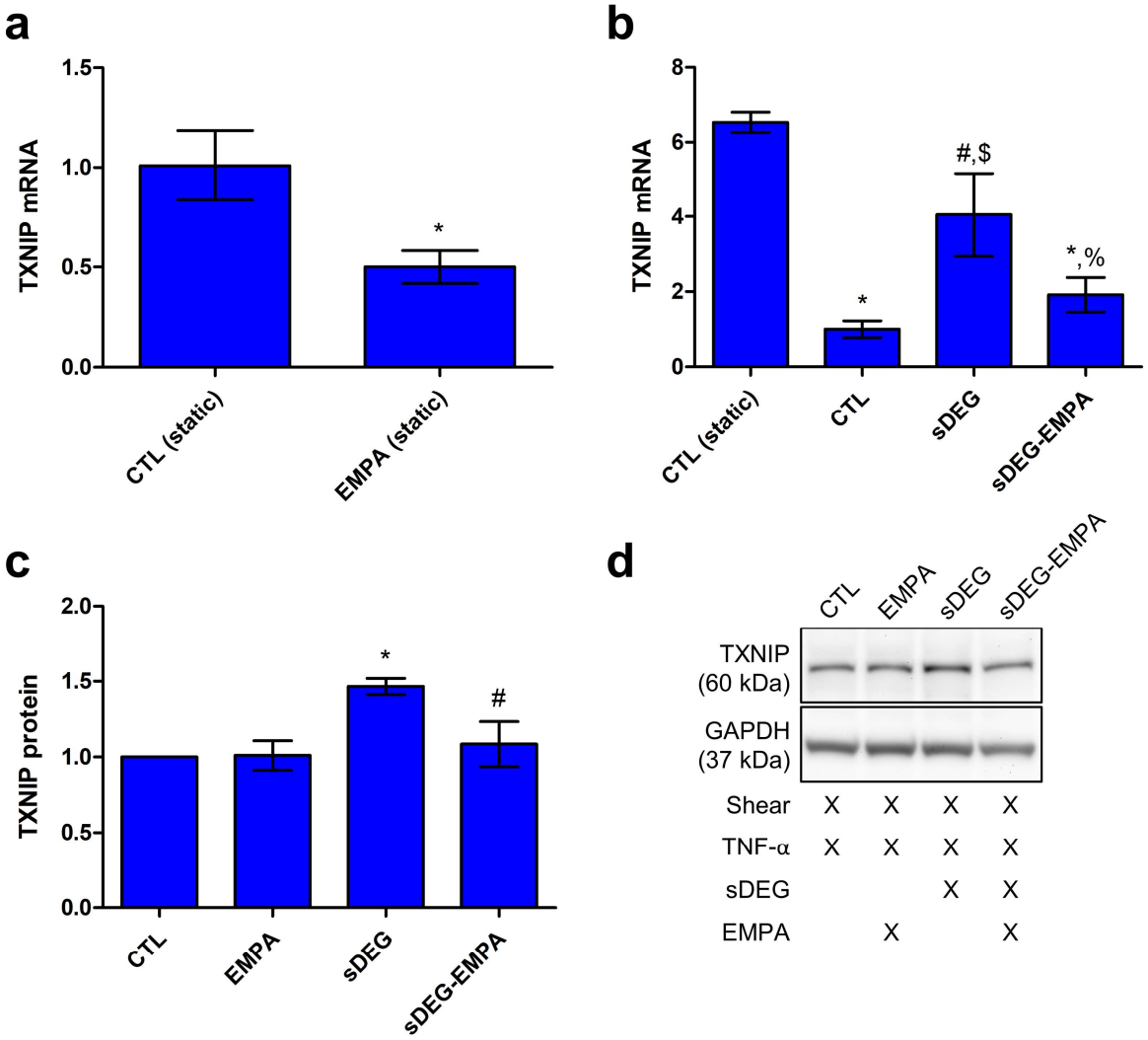

Figure 4-4. ***TXNIP mRNA expression from transcriptome analysis and protein expression in static and shear culture.*** *(a) EMPA significantly reduced TXNIP mRNA levels after 6h of static culture (EMPA vs CTL * p<0.05) (conditions in triplicates). (b) TXNIP mRNA level is differently regulated by shear, sustained HS degradation and EMPA after 24h. Shear downregulated TXNIP mRNA compared to the static control (CTL vs CTL (Static) * p<0.001) while sustained degradation partly abolished shear-induced downregulation (sDEG vs CTL # p<0.01, sDEG vs CTL (Static) $ p<0.01) in TNF-α stimulated ECs. EMPA treatment restored the shear-induced downregulation compared to sDEG (sDEG-EMPA vs sDEG % p<0.05, sDEG-EMPA vs CTL (Static) * p<0.001) (conditions in triplicates). (c) TXNIP protein expression levels in shear culture are increased under degradation compared to the control (sDEG vs CTL * p<0.01) in TNF-α stimulated ECs. EMPA reduced the expression under degradation (sDEG-EMPA vs sDEG # p<0.01), normalizing the expression compared to the control. (d) Representative bands and culture condition details. Full-length blots can be found in the Supplementary Fig. S4-4.*

4.5.5 N-Acetyl-L-cysteine treatment reduces NB4 cell adhesion similar to EMPA.

To further study the reduction in NB4 cell adhesion caused by EMPA, we sought to determine if the effect was mediated through the regulation of oxidative stress under shear and sustained HS

degradation. Prior to the adhesion assay, HAAECs were treated with NAC during shear exposure for 24h. Unlike EMPA, NAC did not significantly reduce the number of adhered NB4 cells compared to the control (Fig. 4-5, NAC vs CTL). However, under sustained degradation, treatment with NAC resulted in a significant reduction in adhesions (sDEG-NAC vs sDEG $ $p<0.001$), comparable to the effect provided by EMPA (sDEG-EMPA vs sDEG $ $p<0.001$). This could suggest that the mitigation of oxidative stress during shear exposure is a potential mechanism by which EMPA-treated ECs reduce their inflammatory response to sustained HS degradation.

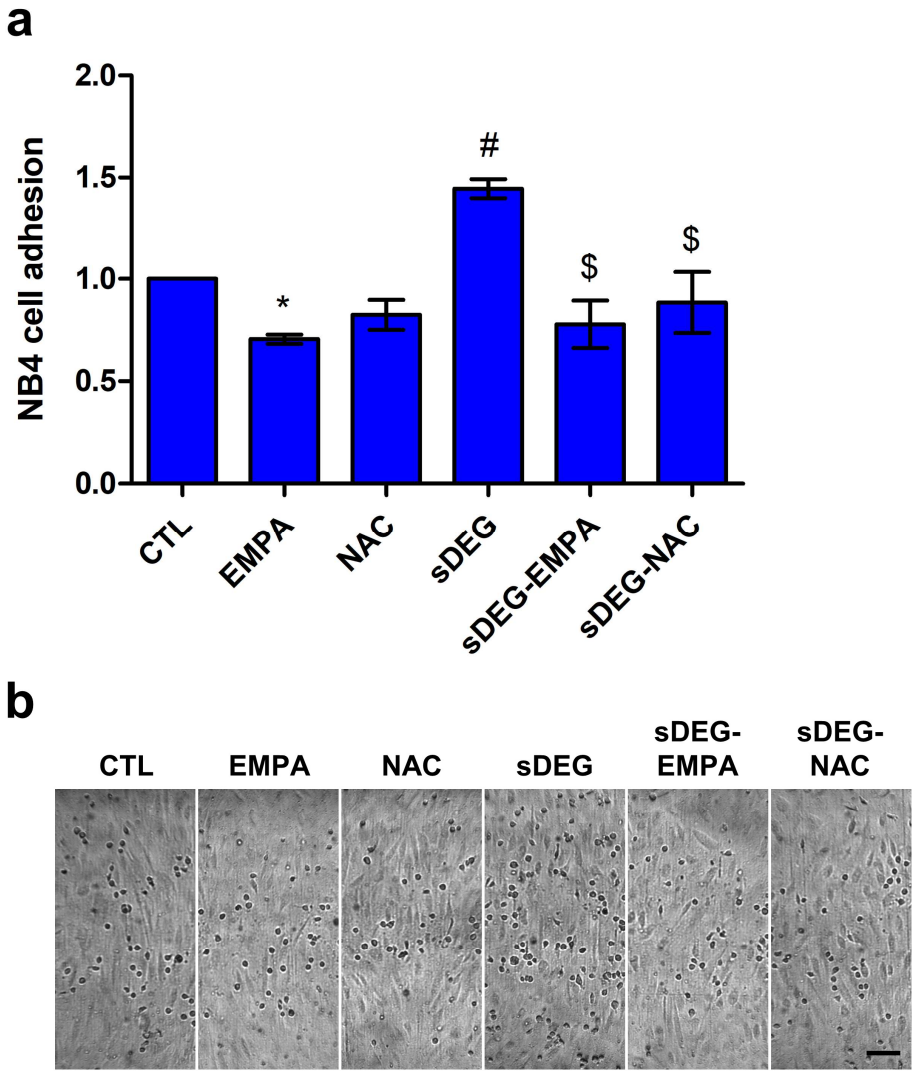

Figure 4-5. ***NB4 cell adhesion assay with NAC to mitigate oxidative stress.*** *(a) Relative numbers of NB4 cell adhesions to TNF-α stimulated HAAECs following treatments and sustained HS degradation under shear culture. EMPA reduced adhesion compared to control (EMPA vs CTL * $p<0.05$). Degradation caused increases in adhesion compared to control (sDEG vs CTL # $p<0.001$). Treatment with EMPA or NAC reduced the adhesion under degradation (sDEG-EMPA & sDEG-NAC vs sDEG $ $p<0.001$) and restored normal adhesion level. (b) Representative images of each condition (scale bar = 100 µm).*

4.6 Discussion

The disruption of the endothelial GCX has been associated with pathological conditions of the vasculature and impaired endothelial function making the role of a healthy GCX of interest in multiple diseases and treatment options. Normal GCX mechanotransduction helps maintain EC homeostasis through shear-regulated functions such as NO-driven vasodilation, cell barrier function and inflammatory cell transmigration. In this regard, treatment with SGLT2i has been demonstrated to improve vascular functions *in vivo* and *in vitro* in non-hyperglycemic conditions through improvement of EC homeostasis [13, 17, 19-21, 26, 27]. Recent studies have demonstrated that EMPA can improve EC function but the mechanisms involved remain elusive.

Given the potential of EMPA to remediate EC dysfunction, we sought to study the effects of EMPA in a pro-inflammatory chronic GCX disruption model by using sustained HS degradation. In this study, we were able to show the impact of sustained GCX disruption on the induction of ER stress and a possible mechanistic effect of EMPA to attenuate the associated inflammatory response which could contribute to the improved vascular health observed clinically [8-10].

Under normal conditions, the GCX at the apical surface of ECs allows the transduction of shear stress through the cytoplasmic membrane, transforming sensing forces into biochemical signals [28]. Thus, laminar shear stress induces the adaptive remodeling of the cytoskeleton i.e. cell elongation and the production of NO through the activation (phosphorylation Ser1177) of eNOS [29, 30]. Upon disruption of the surface GCX, these transduced effects are impaired, leading ECs to a pro-inflammatory state, promoting inflammatory cell adhesion [3]. Hyperglycemic conditions have been shown to cause vascular GCX disruption *in vivo* [1, 31] and *in vitro* [32] thus treatment with EMPA was studied for its potential benefits on HS integrity, which could prevent EC dysfunction.

The sustained HS degradation in our study reduced the HS mean intensity at EC surface by approximatively 85% and prevented regrowth during the 24h culture period (Suppl. Fig. S4-1) which is in line with previous in vitro studies [33, 34]. EMPA did not improve HS intensity in control condition or under sustained degradation. Similarly, treatment with EMPA did not overcome the shear-induced elongation loss under sustained HS degradation (Suppl. Fig. S4-2) but resulted in significantly less NB4 cells adhered to the EC surface under degraded condition (Fig. 4-1). Taken together, these results

define the mechanosensory and anti-inflammatory role of the GCX as HS-dependent and indicate that EMPA can reduce inflammation independently of HS-mediated EC functions.

The impact on inflammation downstream of known GCX mechanotransduction was assessed through eNOS homeostasis and ICAM-1 expression to characterize the inflammatory response of treated ECs. Degradation of HS has been linked to reduced shear-induced NO production in ECs [5, 35]. Ebong *et al.* and others further demonstrated that the impairment of NO production is mediated through reduced phospho-eNOS Ser1177 levels and is concomitant with loss of EC remodeling [30, 36]. In our case, stimulation with TNF-α resulted in significant reductions in eNOS and phospho-eNOS Ser1177 levels with no further decrease due to HS degradation, indicating that TNF-α may have eclipsed the impact of degradation on NO production (Fig. 4-2). Nonetheless, no improvement of phospho-eNOS/eNOS levels were found with EMPA, indicating that eNOS activation might not contribute directly to the anti-inflammatory effect of EMPA. Although we did not find significant changes in the expression level of phospho-eNOS/eNOS induced by EMPA, recent studies have shown improved NO production and EC-dependent vasodilation in response to EMPA. Treatment with 1 μM EMPA restored NO bioavailibility in TNF-α stimulated cardiac microvascular ECs (CMECs) [21] and human coronary arterial ECs (HCAECs) while eNOS phosphorylation levels remained unchanged under static conditions [20]. Conflicting results exist regarding the effects of EMPA on inflammation and adhesion molecule expressions. Ortega *et al.* showed the reduction in ICAM-1 and VCAM-1 expression in aortic sections of Ang-II stimulated mice [19]. Moreover, reduction of inflammatory cell adhesion in a dose-dependent manner was demonstrated with Ang-II stimulated HUVECs. Expression of ICAM-1 and VCAM-1 was also found to be reduced in CMECs isolated from EMPA-treated mice [17]. Conversely, expression of ICAM-1 and VCAM-1 remained unchanged in TNF-α stimulated CMECs [21] and HCAECs [20] treated with 1 μM of EMPA. We previously showed that ICAM-1 expression correlates with NB4 cell adhesion [3], however, the protein expression of ICAM-1 (Fig. 4-2) were unaffected by sustained HS degradation or EMPA suggesting that regulation of the adhesion molecule ICAM-1 may not contribute to the variations in the number of NB4 cell adhesions.

From the transcriptome analysis, genes form the PERK/eIF2/ATF4 branch of the UPR were identified as being significantly regulated by sustained HS degradation and EMPA, indicative of ER stress induction (Fig. 4-3). Sustained degradation caused the concomitant upregulation of ATF3 and CHOP (*DDIT3*), likely through the signaling cascade of eIF2α phosphorylation and ATF4 transcriptional

regulation [37]. Downstream of CHOP, upregulation of GADD34 (*PPP1R15A*) and TRIB3 are related to the inhibitory regulation of eIF2α phosphorylation and ER stress-mediated cell apoptosis [38], respectively. Together, these changes evoke the progressive induction of UPR due to unresolved ER stress leading to increase reactive oxygen species (ROS) production and the activation of apoptotic signaling through CHOP [39-41]. To the best of our knowledge, very few studies have established a direct link between GCX degradation and ER stress. Recently, Dhounchak *et al.* demonstrated the correlation between the loss HS proteoglycans and the upregulation of ER stress gene expression (*DDIT3* (CHOP), *HSPA5* (Grp78) and *ATF3*) in beta cells of diabetic murine models [42]. Similar observations were made with MIN6 cells where the induction of ER stress with thapsigargin caused the degradation of intracellular HSPG and HS, suggesting a degradation mechanism at the cellular level. Moreover, shear is known to have a regulatory effect on ER stress. While laminar shear stress limits ER stress [43], low shear or disturbed flow promote ER stress signaling with inflammation and apoptosis [44, 45]. This could, in part, explain how sustained HS degradation triggers ER stress through the loss of normal laminar shear stress response.

Treatment with EMPA caused small but significant reductions in the transcriptional expression of *ATF3*, *DDIT3* (CHOP), *HSPA5* (Grp78), *PPP1R15A*, and *TRIB3* under HS degradation suggesting a dampened apoptotic response (Fig. 4-3). Other studies have also found similar dampening effects of EMPA on ER stress. Treatment of ApoE-/- and C57Bl/6 mice with EMPA for 5 weeks resulted in the reduction of hepatic ER stress as shown by the downregulation of ER stress signaling genes including *HSPA5* (Grp78), *ERN1* (IRE1), *XBP1*, *DDIT3* (CHOP), *ATF4*, *ATF6* and *GADD45A* and the reduced ratio of phospho-eIF2α/eIF2α [46, 47]. A study by Zhou and Wu demonstrated the potency of EMPA on ER stress related cardiomyopathy of diabetic rats [48]. Treatment with EMPA for 8 weeks resulted in improved cardiac functions along with reduced cardiomyocyte apoptosis and protein expression of ER stress markers (Grp78, CHOP and cleaved caspase-12) suggesting a lesser impact of ER stress on the cardiomyocytes.

ER stress is inherently tied to oxidative stress as the adaptive protein folding is coupled to ROS production [49]. Additionally, oxidative stress can be exacerbated through the action of TXNIP which is related to ER stress by its concomitant activation. TXNIP is a critical regulator of ROS signaling implicated in diverse vascular pathologies due to its role in downstream inflammation and apoptosis. Through its inhibitory effect, TXNIP can impair the thiol-redox system thioredoxin (TRX), promoting

oxidative stress. Lerner *et al.* demonstrated how activation of the UPR sensors, IRE1α and PERK, resulted in increased TXNIP mRNA levels and increased ROS levels in mouse embryonic fibroblasts [50]. Deletion of IRE1α, PERK or TXNIP abolished the detrimental effects associated with unresolved ER stress. Moreover, disturbed flow has been shown to upregulate TXNIP protein expression which caused increased leukocyte-EC adhesion through increased ICAM-1 and VCAM-1 expression [51]. TXNIP is also known to interact with and activate the NLRP3 inflammasome resulting in enhanced inflammation as well as promoting apoptosis through the dissociation of TRX from the apoptosis signal-regulating kinase 1 (ASK1) [52, 53].

Given the detrimental effects associated to the expression of TXNIP and its implication in ER stress, we hypothesize that it plays a regulatory role in the effects provided by EMPA. Indeed, TXNIP mRNA levels were downregulated by EMPA after 6h in static condition (Fig. 4-4). Sustained HS degradation caused the loss of the shear-induced downregulation of TXNIP mRNA and an increase in TXNIP protein. EMPA respectively reduced and normalized the mRNA and protein expression, which correlates with the trend observed in NB4 cell adhesion. These results are in accordance with previous studies showing the role of shear-regulated TXNIP in the inflammatory response of HUVECs [52] and the correlation between TXNIP expression and leukocyte-endothelium adhesiveness [51].

The transcriptomic profile of degraded samples (sDEG & sDEG-EMPA vs CTL) was also characterized by the differential regulation of redox genes (Suppl. Table S4-1) suggesting an adaptive response to oxidative stress. While we were unable to show the direct reduction of ROS by EMPA in static culture (Suppl. Fig. S4-3) or under shear (data not shown), a few studies have shown that SGLT2i can effectively reduce oxidative stress in different conditions. Pre-clinical studies demonstrated the potential of EMPA to reduce whole blood oxidative burst, cardiac NAPDH oxidase activity as well as aortic and endothelial ROS levels in diabetic rats [15, 16]. Zhou *et al.* also showed reduction in intracellular and mitochondrial ROS by EMPA in CMECs isolated from diabetic rats [17]. *In vitro* treatment of CMECs/HCAECs with 1 µM EMPA resulted in the reduction of TNF-α induced oxidative stress as measured by intracellular and mitochondrial ROS suggesting that EMPA has a protective effect against ROS production in normoglycemic conditions while a direct anti-oxidant effect was excluded by the authors.

By virtue of its role in EC homeostasis, the disruption of the GCX also coincide with increased ROS production, promoting a vicious circle of degradation as ROS will further promote GCX disruption [54, 55]. In this context, unresolved ER stress with increased TXNIP expression can cause ROS production and may result in increased NB4 cell adhesion. To test this, NAC was used to promote ROS scavenger and mitigate oxidative stress during shear exposure. Perfusion of NAC has been previously shown to prevent hyperglycemic damage of the glycocalyx [1] and to reduce the level of hyaluronan shedding during renal fluid resuscitation [56]. In our case, incubation with NAC resulted in similar reductions of adhesion compared to EMPA suggesting an oxidative stress mediated mechanism by EMPA. However, the exact impact of EMPA on the interplay between ER stress and TXNIP, resulting in reduced oxidative stress and in turn reduced inflammation, remains to be clarified. TXNIP knockout ECs could represent an interesting model to test the EMPA effect dependency on TXNIP expression.

The study of the glycocalyx *in vitro* as well as conclusions drawn from transcriptional data comes with inherent limitations. First, the glycocalyx of ECs in culture is known to lack the thickness of its *in vivo* counterpart which may lessen the impact of degradation on inflammatory cell adhesion [57]. Although different from chronic GCX disruption, sustained enzymatic degradation of HS was used as an inflammatory stimulus to mimic EC GCX shedding as found in diabetic or curved/branching vasculature. Furthermore, while the effect of HS degradation was the focus of this study, degradation of other components of the GCX can also impact EC homeostasis which could induce different signaling cascades and therefore change the role of EMPA in the restoration of GCX functions. Although the use of TNF-α under sustained HS degradation does not allow to fully dissociate their respective impact on EC inflammation in all experiments, TNF-α addition was required to perform adhesion assays and was therefore use in subsequent experiments to properly interpret the results. The single time-point studied does not reflect transient response or feedback regulation between ER stress and inflammation. Moreover, a suprapharmacological concentration of EMPA was used compared to the reported serological concentrations of treated patients [58] in order to study the reduction in inflammation previously reported and to highlight the potential cellular mechanisms that may occur under lower serological concentrations over longer periods of time.

In conclusion, the present study suggests a possible mechanism by which EMPA may contribute to better endothelial health. Our results point toward the potential of EMPA to attenuate EC inflammation independently of HS-mediated functions, as shown by the reduction of inflammatory

cell adhesion under sustained HS degradation. Downregulation of pro-apoptotic signaling and TXNIP expression by EMPA may contribute to oxidative stress mitigation therefore limiting oxidative damage under unresolved ER stress and resulting in reduced inflammatory cell adhesion. Future work should aim at studying the regulatory mechanisms associated with the expression of TXNIP on the inflammatory response through the NLRP3 inflammasome and apoptotic signaling. A better understanding of the effects of SGLT2i on GCX-mediated EC functions could benefit new strategies to limit EC dysfunction in pathological conditions where chronic GCX damage is found.

4.7 Acknowledgement

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4.8 Author contribution

MAC and RL conceived and designed the study. MAC performed the experiments. MAC interpreted the data. MAC wrote the manuscript. MAC and RL reviewed and edited the manuscript. All authors have read and approved the final manuscript.

4.9 Competing interests

The authors declare no competing interests.

4.10 Data availability

The datasets generated for this study are available on request to the corresponding author.

4.11 Supplementary information

Empagliflozin mitigates endothelial inflammation and attenuates endoplasmic reticulum stress signaling caused by sustained glycocalyx disruption

Marc-Antoine Campeau[1], Richard L. Leask[1,2]*

[1]Department of Chemical Engineering, McGill University, Montreal, QC, Canada

[2]McGill University Health Centre, Montreal, QC, Canada

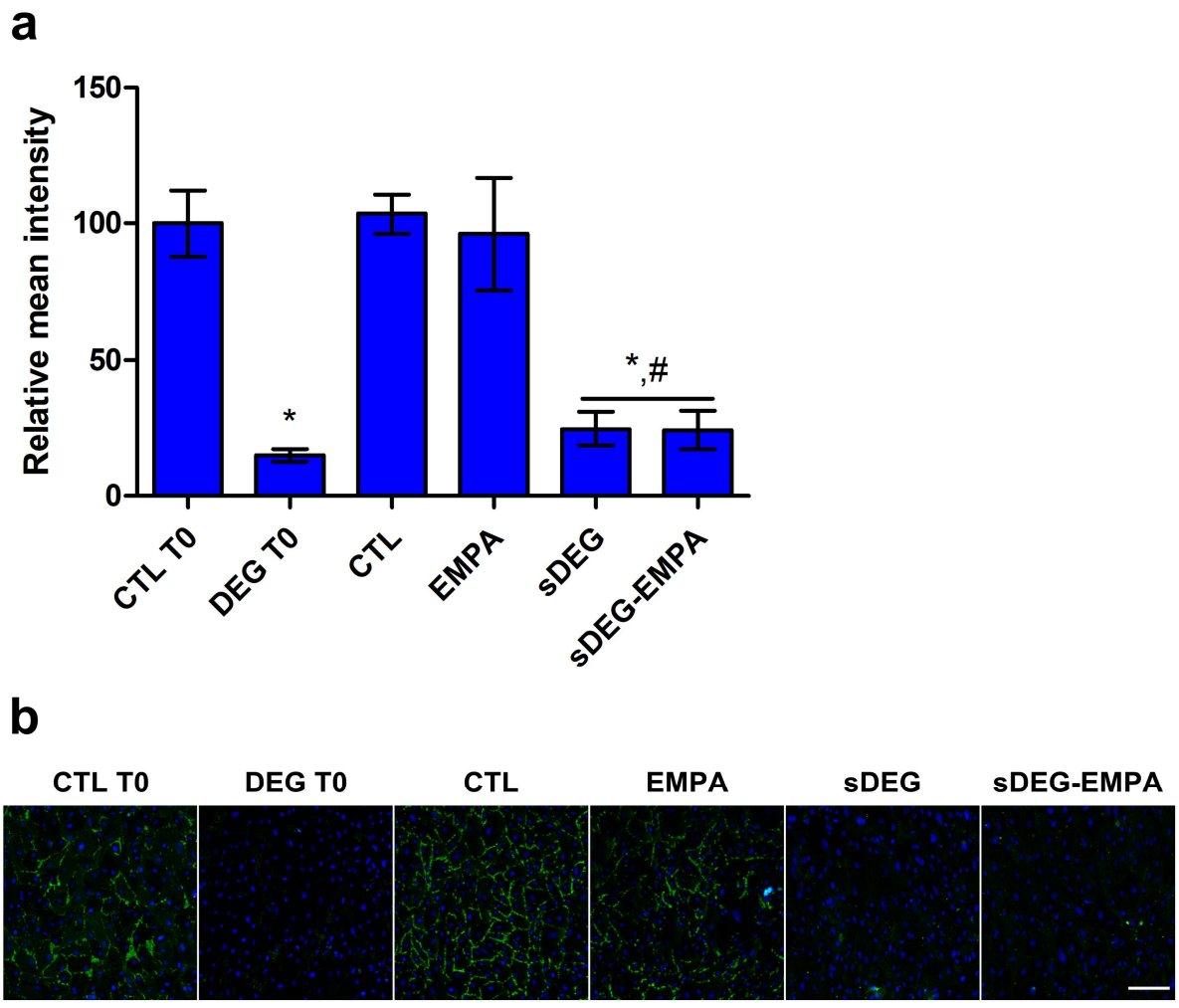
*Corresponding Author

richard.leask@mcgill.ca

Keywords: Sodium/glucose cotransporter 2 (SGLT2) inhibitor, empagliflozin (EMPA), endothelial cell dysfunction, type 2 diabetes, endoplasmic reticulum stress (ER stress), glycocalyx, inflammation, oxidative stress

EMPA does not restore HS integrity under sustained degradation

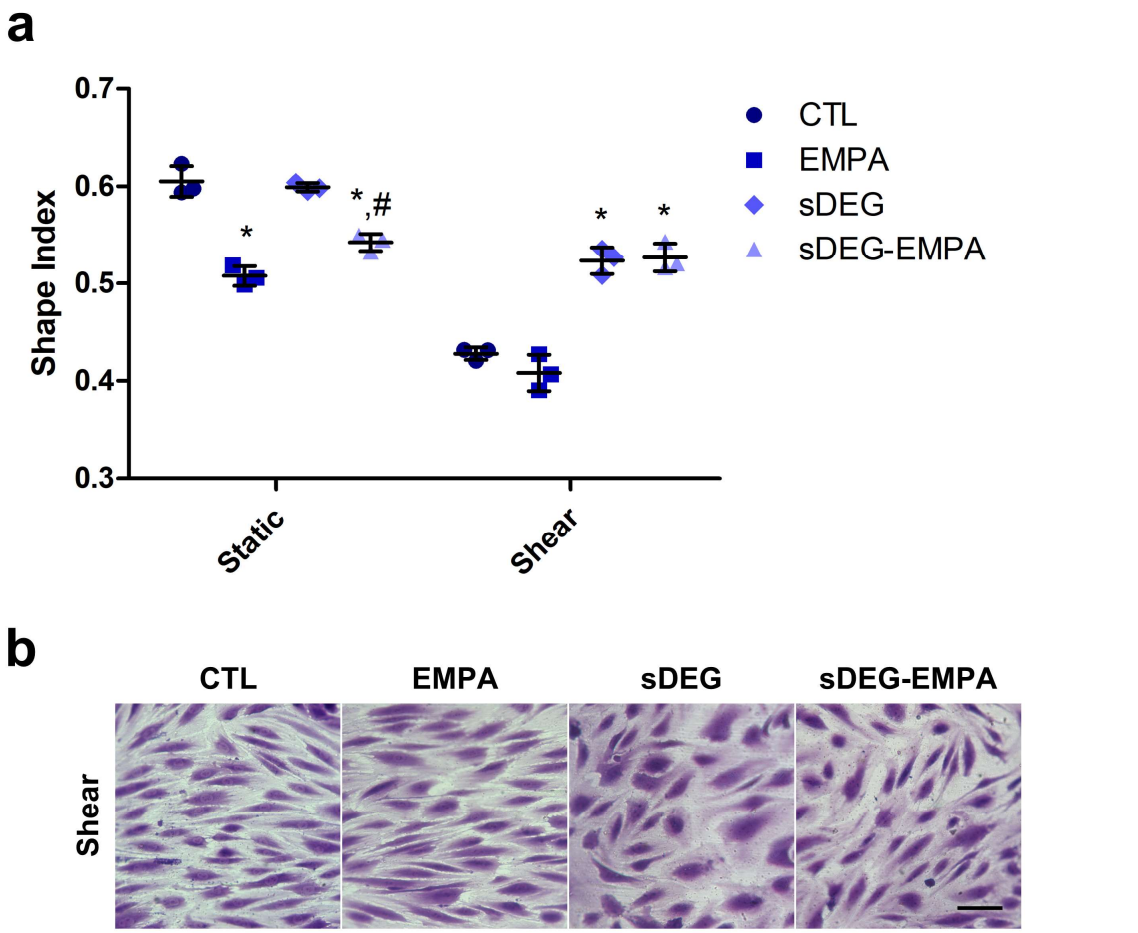
HS integrity and the impact of EMPA under shear conditions were studied through HS immunostaining. The initial condition of degradation has shown to reduce the mean HS intensity by approximately 85% compared to control (Suppl. Fig. S4-1, DEG T0 vs CTL T0 * $p<0.001$). The mean HS intensities were similar between the EMPA-treated cells and the control after 24h (EMPA vs CTL). Cells under sustained degradation showed minimal regain in intensity indicating continued degradation of HS over the 24h period of culture (sDEG & sDEG-EMPA vs CTL # $p<0.001$; vs CTL T0 * $p<0.001$) with no effect of EMPA. Taken together, these results suggest that treatment with EMPA for 24h does not enhance HS on EC surface or prevent the effect of sustained degradation.



Supplementary Figure S4-1. ***Relative mean intensity of HS immunostaining images to assess HS integrity.*** *(a) Relative intensity of HS at initial conditions (T0) and 24h of shear culture. Initial condition following degradation (DEG T0) reduced HS intensity by approximatively 85% compared to control (DEG T0 vs CTL T0 * $p<0.001$). Sustained degradation maintained HS degraded over 24h compared to control (sDEG & sDEG-EMPA vs CTL # $p<0.001$; vs CTL T0 * $p<0.001$). (b) Representative HS immunostaining images (scale bar = 200 µm).*

EMPA does not restore cell elongation in response to shear under sustained HS degradation

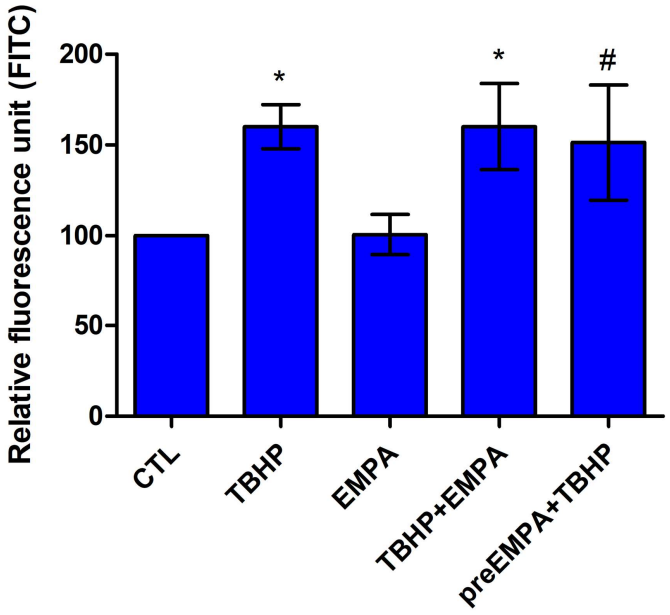
In accordance with previous results, EMPA caused cell elongation (i.e. reduction in shape index (SI)) in static culture (Suppl. Fig. S4-2, EMPA vs CTL * p<0.001). The EMPA-induced elongation also persists under sustained degradation in static culture (sDEG-EMPA vs sDEG # p<0.001; vs CTL * p<0.001). Conversely, the effect of EMPA on shear-induced elongation was absent under degradation with impaired cell elongation as shown by the increased SI (sDEG & sDEG-EMPA vs CTL * p<0.001), suggesting that EMPA does not alleviate EC dysfunction caused by the loss of normal HS mechanotransduction.



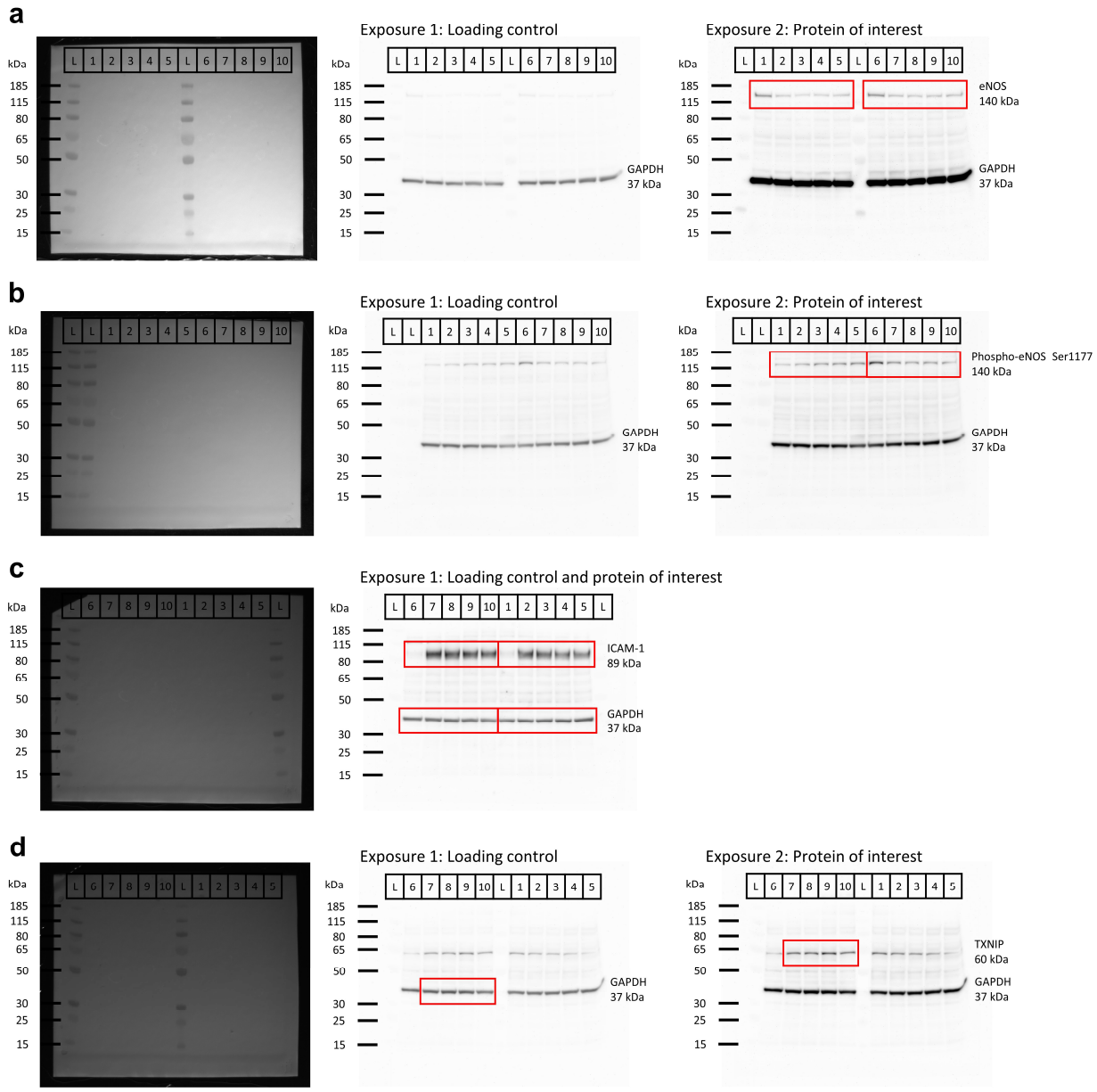
Supplementary Figure S4-2. ***HAAEC cell elongation under sustained HS degradation with or without EMPA treatment in static and shear conditions for 24h.*** *(a) SI measurements. EMPA induced reduction in SI in static culture (EMPA vs CTL * p<0.001, sDEG-EMPA vs sDEG # p<0.001; vs CTL * p<0.001). Sustained degradation under shear caused an increase in SI compared to control vs CTL (sDEG & sDEG-EMPA vs CTL * p<0.001) with no effect of EMPA. (b) Representative images of shear culture conditions (scale bar = 200 µm).*

EMPA does not directly reduce ROS production caused by TBHP

We sought to determine if EMPA has a direct protective effect against oxidative stress. Treatment with EMPA for 6h did not reduce the baseline ROS production level (Suppl. Fig. S4-3, EMPA vs CTL). While incubation with TBHP resulted in a significant increase in measured ROS after 6h (TBHP vs CTL * $p<0.01$), simultaneous treatment with EMPA for 6h or pretreatment with EMPA for 18h (preEMPA+TBHP) failed to limit the increase (TBHP+EMPA vs CTL * $p<0.01$, preEMPA+TBHP vs CTL # $p<0.05$). Adhesion assay conditions (TNF-α stimulation and sustained HS degradation) did not show significant ROS production compared to control in static culture (data not shown).



Supplementary Figure S4-3. ***Relative ROS production measurements using the H2DCFDA ROS assay kit.*** *TBHP used as a positive control resulted in increased ROS production after 6h compared to CTL (TBHP vs CTL * $p<0.01$). Simultaneous incubation (TBHP+EMPA) or preincubation (preEMPA+TBHP) with EMPA did not reduce the TBHP-induced increase in ROS production (TBHP+EMPA vs CTL * $p<0.01$, preEMPA+TBHP vs CTL # $p<0.05$).*



Supplementary Figure S4-4. ***Whole Western blots from Figure 2e and 4d.*** *Full-length blots of (**a**) eNOS, (**b**) phospho-eNOS Ser1177, (**c**) ICAM-1, (**d**) TXNIP and the loading control GAPDH. Blots are presented as unprocessed images obtained from the UVP Biospectrum 810 MultiSpectral Imaging System. Bright field images and multiple exposures of the same blot, when applicable, are presented in columns. The cropped areas used in Figure 2e and 4d are indicated by boxes. Samples included (1) CTL (TNF-α –) Static, (2) CTL (TNF-α +) Static, (3) EMPA (TNF-α +) Static, (4) sDEG (TNF-α +) Static, (5) sDEG-EMPA (TNF-α +) Static, (6) CTL (TNF-α –) Shear, (7) CTL (TNF-α +) Shear, (8) EMPA (TNF-α +) Shear, (9) sDEG (TNF-α +) Shear, (10) sDEG-EMPA (TNF-α +) Shear, (L) Protein ladder.*

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CHAPTER 5 EMPAGLIFLOZIN REDUCES ENDOPLASMIC RETICULUM STRESS ASSOCIATED TXNIP/NLRP3 ACTIVATION IN TUNICAMYCIN STIMULATED AORTIC ENDOTHELIAL CELLS

5.1 Preface

From the transcriptome analysis of the previous study [84], we identified endoplasmic reticulum (ER) stress as a candidate pathway in the effect of empagliflozin (EMPA) to overcome endothelial cell (EC) dysfunction. Under sustained glycocalyx (GCX) disruption, EMPA downregulated the transcription of unfolded protein response (UPR) markers, mainly from the PKR-like endoplasmic reticulum kinase (PERK)/Activating transcription factor 4 (ATF4)/C/EBP homologous protein (CHOP) branch as well as the thioredoxin interacting protein (TXNIP), suggesting attenuated ER stress through oxidative stress mitigation. Despite the relevance of GCX disruption and shear mechanotransduction to EC dysfunction, the model used in the first study was changed to further study the effect of EMPA on ER stress signaling in controlled conditions with higher throughput.

Hence, ECs were statically cultured and treated with tunicamycin, a potent inhibitor of N-glycosylation, resulting in misfolded protein accumulation in the ER and thus, ER stress. As the cellular dysfunction under ER stress is multifaceted, induction and downstream markers were targeted to study the different regulatory paths of cell fate under ER stress. Downstream of PERK, phosphorylation of eIF2α mediate ATF4 transcriptional regulation of CHOP, involved in the ER stress apoptosis response. In parallel, oxidative stress promotes TXNIP expression and the activation of NLR-family pyrin domain-containing protein 3 (NLRP3), exacerbating inflammation. Consequently, protein expression of phospho-eIF2α, CHOP, TXNIP/NLRP3 were studied to determine how EMPA can impact ER stress induction, apoptosis and oxidative-driven inflammation, respectively.

This manuscript builds on the previous study to demonstrate the direct effect of EMPA on the signaling pathway of ER stress. Of interest, ER stress has been related to the development of

cardiovascular diseases and its attenuation by EMPA possibly represents a relevant part of the cardioprotective effect of SGLT2i.

This manuscript has been submitted for publication.

Empagliflozin reduces endoplasmic reticulum stress associated TXNIP/NLRP3 activation in tunicamycin stimulated aortic endothelial cells

Marc-Antoine Campeau[1], Richard L. Leask[1,2]*

[1]Department of Chemical Engineering, McGill University, Montreal, QC, Canada

[2]McGill University Health Centre, Montreal, QC, Canada

*Corresponding Author

richard.leask@mcgill.ca

Keywords: sodium/glucose cotransporter 2 (SGLT2) inhibitor, empagliflozin (EMPA), endoplasmic reticulum stress (ER stress), thioredoxin interacting protein (TXNIP), NLR-family pyrin domain containing 3 (NLRP3), C/EBP homologous protein (CHOP), nuclear factor erythroid 2-related factor 2 (nrf2), interleukin-6 (IL-6).

5.2 Abstract

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) have proven to be of therapeutic significance for cardiovascular diseases beyond the treatment of type 2 diabetes. Recent studies have demonstrated the beneficial effects of SGLT2i on endothelial cell (EC) dysfunction but the underlying cellular mechanisms remain to be clarified. In this study, we sought to understand the effect of empagliflozin (EMPA; Jardiance®) on cell homeostasis and endoplasmic reticulum (ER) stress signaling. ER stress was induced by tunicamycin (Tm) in human abdominal aortic ECs treated with EMPA over 24h. Tm-induced ER stress caused increases in the protein expression of thioredoxin interacting protein (TXNIP), NLR-family pyrin domain-containing protein 3 (NLRP3), C/EBP homologous protein (CHOP) and in the ratio of phospho-eIF2α/eIF2α. EMPA (50-100 μM) resulted in a dampened downstream activation of ER stress as seen by the reduced expression of CHOP and TXNIP/NLRP3 in a dose-dependent manner. Nuclear factor erythroid 2-related factor 2 (nrf2) translocation was also attenuated in EMPA-treated ECs. These results suggest that EMPA improves redox signaling under ER stress which in turn attenuates the activation of TXNIP/NLRP3.

5.3 Introduction

Sodium glucose cotransporter 2 inhibitors (SGLT2i) have attracted significant attention due to their therapeutic potential that exceeds their intended glycemic control effect. Results from multiple clinical trials and studies have shown that treatment with SGLT2i is associated with beneficial effects against heart failure [1-3], myocardial infarction [4] and atherosclerosis [5] independently of diabetes. While part of the clinical benefits observed can be attributed to systemic effects [6], accumulating evidence points towards direct pleiotropic effects on endothelial cell (EC) function [7]. Therefore, understanding the underlying cellular mechanisms that contribute to improved EC function by SGLT2i treatment is necessary to reveal the full clinical potential of SGLT2i.

Treatment with SGLT2i has been shown to improved EC-dependent vasorelaxation [8, 9] and the inflammatory response of vascular cells through mediation of redox state [10, 11]. One possible mechanism by which SGLT2i impact cellular homeostasis is through the mediation of endoplasmic reticulum (ER) stress. Accumulation of unfolded and misfolded proteins in the ER initiates a stress response which impacts redox homeostasis, inflammation and apoptosis [12]. The unfolded protein response (UPR) signaling pathway aims to restore ER homeostasis through increased protein folding capacity which is inherently coupled to reactive oxygen species (ROS) production [13].

Treatment with the SGLT2i empagliflozin (EMPA) has been previously shown to downregulate the transcriptional and protein expression of ER stress markers in rodent cardiac tissue [3, 14]. Moreover, treatment of human coronary artery ECs with dapagliflozin, a different SGLT2i, prevented the induction of ER stress through the normalization of the UPR regulatory protein activation [15]. We previously found that EMPA can mitigate the transcriptional upregulation of ER stress downstream signaling markers as well as the thioredoxin interacting protein (TXNIP) mRNA and protein expressions in response to EC dysfunction caused by glycocalyx degradation [16]. Thus, attenuation of ER stress activation and the associated downstream oxidative stress and inflammation could represent one of the underlying cellular mechanisms contributing to the clinical benefits of SGLT2i treatment. In this study, prolonged ER stress was induced in ECs with

tunicamycin (Tm) and the impact of EMPA treatment on UPR activation and downstream signaling was identified.

5.4 Materials and Methods

5.4.1 Cell culture

Human abdominal aortic endothelial cells (HAAECs, ATCC, Coriell, CRL-2472) were cultured in endothelial cell growth medium (Promocell, C-22020), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen) in culture flasks coated with 0.1% pig gelatin at 37°C and 5% CO2. Cells were grown to confluence and harvested from the flask with TrypLE (ThermoFisher, 12605028). Experiments were performed with cells at passage 5 and 6.

Culture plates were coated with a 10 µg/mL human fibronectin (Akron Biotech, AK9715-0005) solution for 1h. HAAECs were seeded at a density of 25 000 cells/cm^2 and grown overnight to reach near confluency. EMPA treatment consisted in pretreating the cells for 18h prior to ER stress induction. Tm was used to pharmacologically induce ER stress through the inhibition of N-linked glycosylation [17]. Cells were treated with 1 µg/mL Tm with or without EMPA (50 µM) in FBS-free media for up to 24h. The effect of the concentration of EMPA was also tested by treating the cells with Tm and EMPA (1, 10 and 100 µM) for 24h.

5.4.2 MTS assay

The effect of EMPA on HAAEC metabolic activity was evaluated through cell proliferation with MTS assays (Abcam, ab197010) following the manufacturer's instructions. ECs were seeded at 50% confluency (1.2 x10^4 cells/cm2) in 96-well plates and treated with vehicle (DMSO) or EMPA at various concentrations for 24h. Concentrations of EMPA ranging from 10 to 500 µM were tested. Vehicle (DMSO) control of 0.05% and 0.50%, corresponding to EMPA concentrations of 50 and 500 µM respectively, were tested. 3h prior to the end of the treatment, the MTS reagent was added to each well. Absorbance was measured on a Benchmark Plus Microplate Spectrophotometer (Bio-Rad) at 490 nm and normalized to the control.

5.4.3 Protein collection and western blot

Protein expressions were studied as previously described [16]. Briefly, HAAECs were washed with cold PBS and lysed in situ in cold RIPA lysis buffer (ThermoFisher, 89900) with 2% Halt™ protease and phosphatase inhibitor cocktail (ThermoFisher, 78440) before being centrifuged. Protein quantification was performed through colorimetric assays to normalize sample concentrations. Samples were prepared with Bolt LDS sample buffer and reducing agent (ThermoFisher, B0007, B0009). Gel electrophoresis was performed using Bolt 4-12% Bis-Tris Plus gels loaded with 20 µg of protein. Following transfer, membranes were blocked with 5% bovine serum albumin (BSA, BioShop, ALB001) or 4% BSA/1% milk in 0.1% Tween-20 TBS (TBST) for 1h. Primary antibodies were diluted in blocking buffer and incubated at 4°C overnight. Antibodies used included NLR-family pyrin domain-containing protein 3 (NLRP3) (1:500, Proteintech, 19771-1-AP), C/EBP homologous protein (CHOP) (1:500, Proteintech, 15204-1-AP), TXNIP (1:1000, CST, 14715), phospho-eIF2α (1:1000, CST, 3398), eIF2α (1:5000, CST, 5324), SGLT1 (1:500, abcam, ab14686) and SGLT2 (1:500, abcam, ab37296) as well as GAPDH (1:20000, ThermoFisher, AM4300) and Vinculin (1:5000, Sigma-Aldrich, V9131) as loading controls. Membranes were then incubated with horseradish peroxidase secondary antibodies (1:40000, ThermoFisher) diluted in blocking buffer for 1 hour at room temperature and signal detection was achieved with SuperSignal West Pico PLUS Chemiluminescent Substrate and Bio-Rad ChemiDoc Imaging System. Proteins were quantified by densitometry using ImageJ (1.52p) and normalized to the loading control.

5.4.4 IL-6 ELISA

Production of interleukin 6 (IL-6) by HAAECs was measured using a commercial ELISA kit (ThermoFisher, KHC0061). Following treatment for 24h, the supernatant was collected and centrifuged to remove cell debris. Samples were diluted to match the concentration range of the standard curve and the ELISA was performed according to the manufacturer's instruction. Absorbance was measured on a Benchmark Plus Microplate Spectrophotometer (Bio-Rad) at 450 nm and the concentrations of IL-6 were determined through the calibration curve.

5.4.5 nrf2 immunostaining and translocation

Cells were fixed with 2% paraformaldehyde in PBS for 15 min. Cells were blocked and permeabilized with a 2% normal donkey serum (NDS)/0.2% Triton X-100 solution in PBS. Primary antibody for the nuclear factor erythroid 2-related factor 2 (nrf2) (1:200, rabbit, Proteintech, 16396-1-AP) was diluted in 1% NDS/PBS and incubated overnight at 4°C. Cells were rinsed with PBS and incubated for 1h with the secondary antibody (donkey anti-rabbit IgG, Alexa Fluor 555, ThermoFisher, A-31572) and DAPI (1:1000) diluted in 1% NDS/PBS. Cells were rinsed with PBS and imaged at 10X magnification via laser scanning confocal microscopy (Zeiss LSM 800). Using imageJ, the nuclear and cytoplasmic regions were isolated from the DAPI and Alexa Fluor 555 channel signal, respectively. The nrf2 translocation was define as the ratio between the mean gray value of the nucleus and the cytoplasm of individual cells. At least 3 images and 15 cells per image were analyzed for each condition.

5.4.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism™ software. Results are presented as the mean values ± standard deviation of 3 independent experiments. Mean values were compared using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-test with a 95% confidence interval. p-values less than 0.05 were considered significant.

5.5 Results

5.5.1 Time-dependent attenuation of Tm-induced ER stress by EMPA

To assess the impact of EMPA on the induction and downstream signaling of ER stress, the phospho-eIF2α/eIF2α ratio and the protein expression of TXNIP, NLRP3 and CHOP were studied over 24h (Fig. 5-1). Treatment of ECs with Tm showed constant increased levels of the eIF2α phosphorylation ratio after 2h, 8h, and 24h (Fig. 5-1A, Tm & Tm+EMPA vs CTL * $p<0.001$) indicating ER stress induction with no significant difference caused by EMPA treatment. TXNIP expression showed gradual increases in expression over time with a significant increase after 8h and a maximum expression after 24h of treatment with Tm (Fig. 5-1B, Tm vs CTL 8h $ $p<0.05$, 24h * $p<0.001$). EMPA treatment limited the Tm-induced increase with a significantly reduced

expression of TXNIP (Fig. 5-1B, Tm+EMPA vs Tm 24h # $p<0.001$). While NLRP3 expression remained at basal level after 2h and 8h, it was significantly upregulated after 24h in Tm-treated cells compared to control (Fig. 5-1C, Tm vs CTL 24h * $p<0.001$). EMPA significantly reduced the increase in expression, indicating a reduced inflammatory response (Fig. 5-1C, Tm+EMPA vs Tm 24h # $p<0.001$). CHOP expression peaked after 8h in Tm-treated cells and remained above the control level after 24h (Fig. 5-1D, Tm & Tm+EMPA vs CTL 8h & 24h * $p<0.001$). EMPA caused a small but significant reduction in CHOP expression at 8h (Fig. 5-1D, Tm+EMPA vs Tm 8h # $p<0.01$), suggesting a possible attenuation of the apoptotic response. Taken together, these results demonstrate the attenuation of EC dysfunction caused by prolonged ER stress induction. As the increase in phospho-eIF2α/eIF2α ratio remained constant over the time period analyzed but EMPA caused significant changes in ER stress downstream signaling, EMPA may not directly impact the induction of ER stress by Tm but rather mitigate the downstream effects in term of inflammation, apoptosis and redox control.

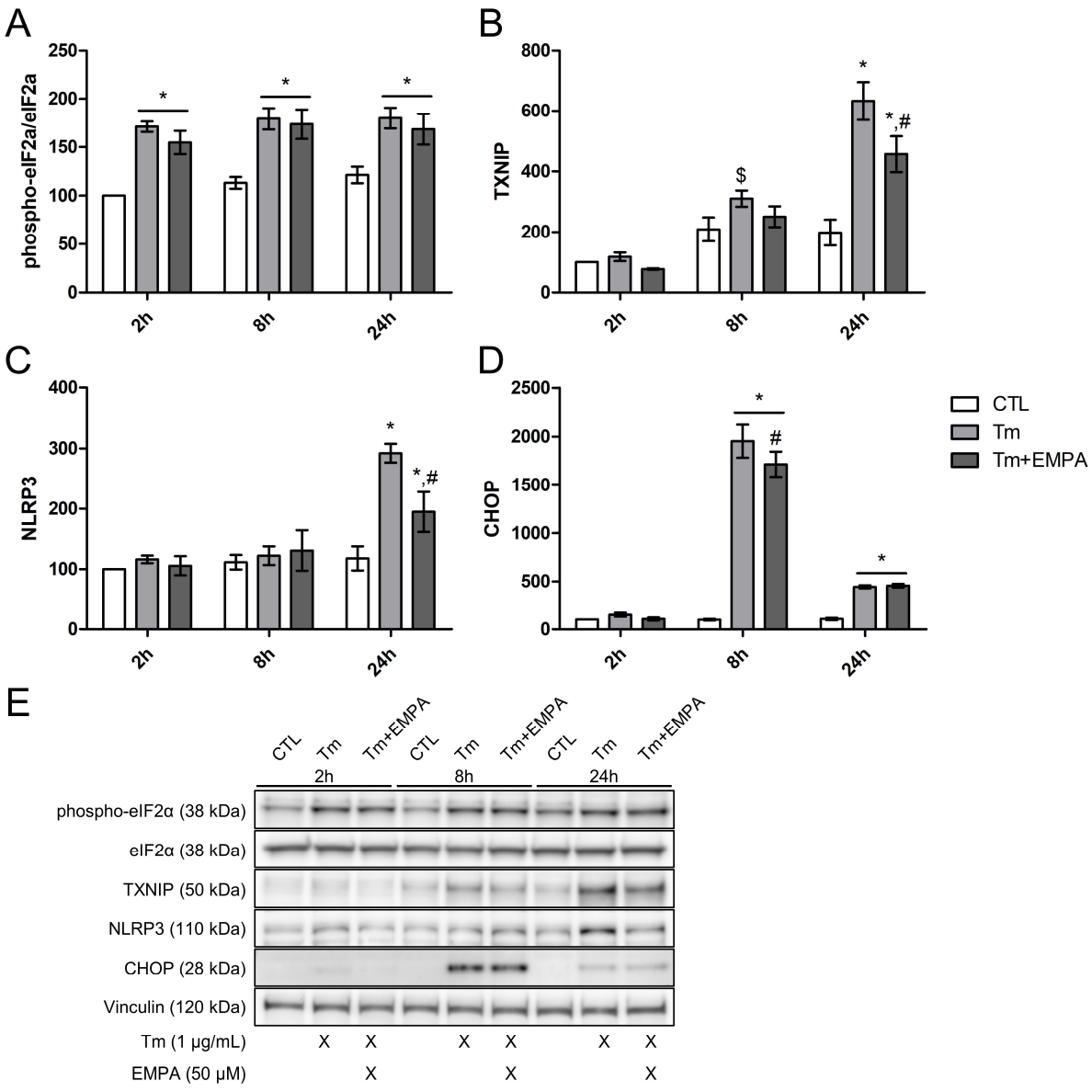


Figure 5-1. ***Protein expression of ER stress signaling markers over time.*** *(A) The phospho-eIF2α/eIF2α ratio and the expression of (B) TXNIP, (C) NLRP3, (D) CHOP were quantified after 2h, 8h and 24h of treatment. The phospho-eIF2α/eIF2α ratio was significantly increased by Tm (1 µg/mL) compared to CTL (* p<0.001) and remained elevated over 24h. TXNIP expression was significantly increased by Tm after 8h ($ p<0.05) and 24h (* p<0.001) compared to their respective CTL. EMPA (50 µM) reduced the increased TXNIP expression caused by Tm at 24h (# p<0.001) but expression remained elevated compared to CTL (* p<0.001). NLRP3 expression was significantly increased by Tm after 24h (* p<0.001) with EMPA limiting the increase (vs Tm # p<0.001; vs CTL * p<0.001). CHOP expression induced by Tm peaked at 8h and remained elevated after 24h compared to CTL (* p<0.001). EMPA significantly reduced the expression of CHOP at 8h (# p<0.01) (n=3). (E) Representative bands and culture condition details.*

5.5.2 Concentration-dependent effect of EMPA on TXNIP and NLRP3 expression

Given the reduced ER stress signaling by EMPA after 24h, we sought to determine the concentrations at which EMPA can effectively reduce the expression of TXNIP and NLRP3 (Fig. 5-

2). EMPA alone did not significantly change the basal expression of TXNIP or NLRP3 compared to control (Fig. 5-2A&B, EMPA vs CTL). Low concentrations (1 & 10 µM) of EMPA were not able to reduce the significant increases in expression of TXNIP or NLRP3 caused by Tm (Fig. 5-2A&B, Tm, Tm+EMPA 1 & Tm+EMPA 10 vs CTL * $p<0.001$), However, a concentration of 100 µM EMPA resulted in a significantly reduced expression for both TXNIP and NLRP3 (Fig. 5-2A&B, Tm+EMPA 100 vs Tm # $p<0.001$). These results suggest that the concentration threshold for EMPA to be effective against ER stress induced TXNIP and NLRP3 increased expression in ECs in vitro is high and likely to be in the range of 10-100 µM at 24h.

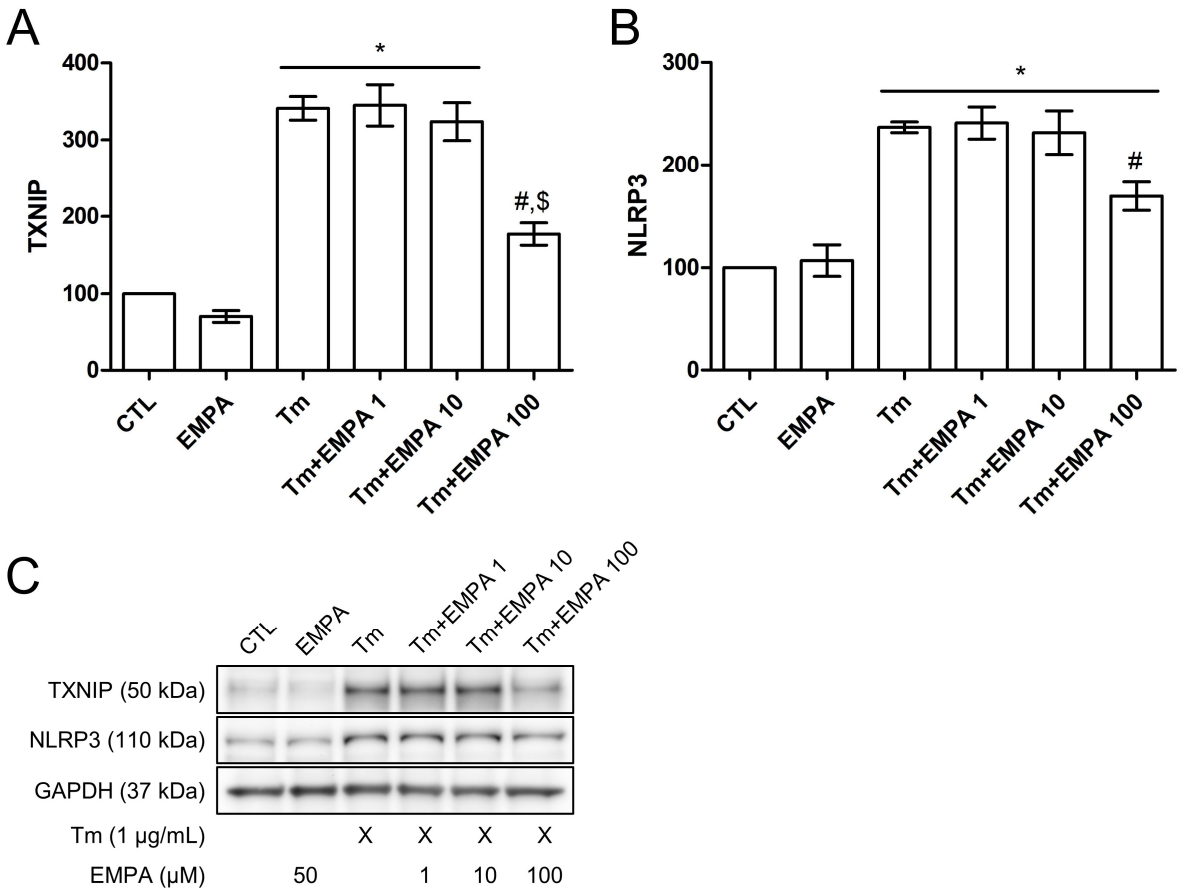


Figure 5-2. ***Effect of EMPA concentration on TXNIP and NLRP3 protein expression.*** *Expression of (A) TXNIP and (B) NLRP3 following treatment with EMPA (1, 10 or 100 µM) for 24h. TXNIP and NLRP3 expression were significantly increased by Tm (1 µg/mL) after 24h compared to CTL (* $p<0.001$). Low concentrations of EMPA (1 and 10 µM) had no effect on the increased expressions. The concentration of 100 µM caused significant decreases in the expression of TXNIP and NLRP3 compared to Tm (# $p<0.001$) while remaining elevated compared to CTL (TXNIP $ $p<0.01$; NLRP3 * $p<0.001$) (n=3). (C) Representative bands and culture condition details.*

5.5.3 EMPA reduces nrf2 nuclear translocation

The nuclear translocation of nrf2 was investigated as it is a transcriptional regulator of inflammatory and redox signaling genes (Fig. 5-3). Tm caused a significant increase in nrf2 nuclear intensity (Fig. 5-3A, Nucleus Tm vs CTL # $p<0.001$) which translates to increased nrf2 translocation (Fig. 5-3B, Tm vs CTL * $p<0.001$). Treatment with EMPA caused a slight decrease in nuclear intensity compared to control (Fig. 5-3A, EMPA vs CTL * $p<0.05$) and normalized the increased nuclear intensity caused by Tm (Fig. 5-3A, Tm+EMPA vs Tm $ $p<0.001$), resulting in a reduced translocation level (Fig. 5-3B, Tm+EMPA vs Tm # $p<0.01$, vs CTL $ $p<0.01$). In addition, combined treatment of Tm and EMPA caused a slight decrease in cytoplasmic intensity compared to the control (Fig. 5-3A, Tm+EMPA vs CTL * $p<0.05$). The changes in nrf2 translocation caused by EMPA suggests a reduced nrf2 transcriptional regulation and an attenuated anti-oxidant response from Tm-induced ER stress.

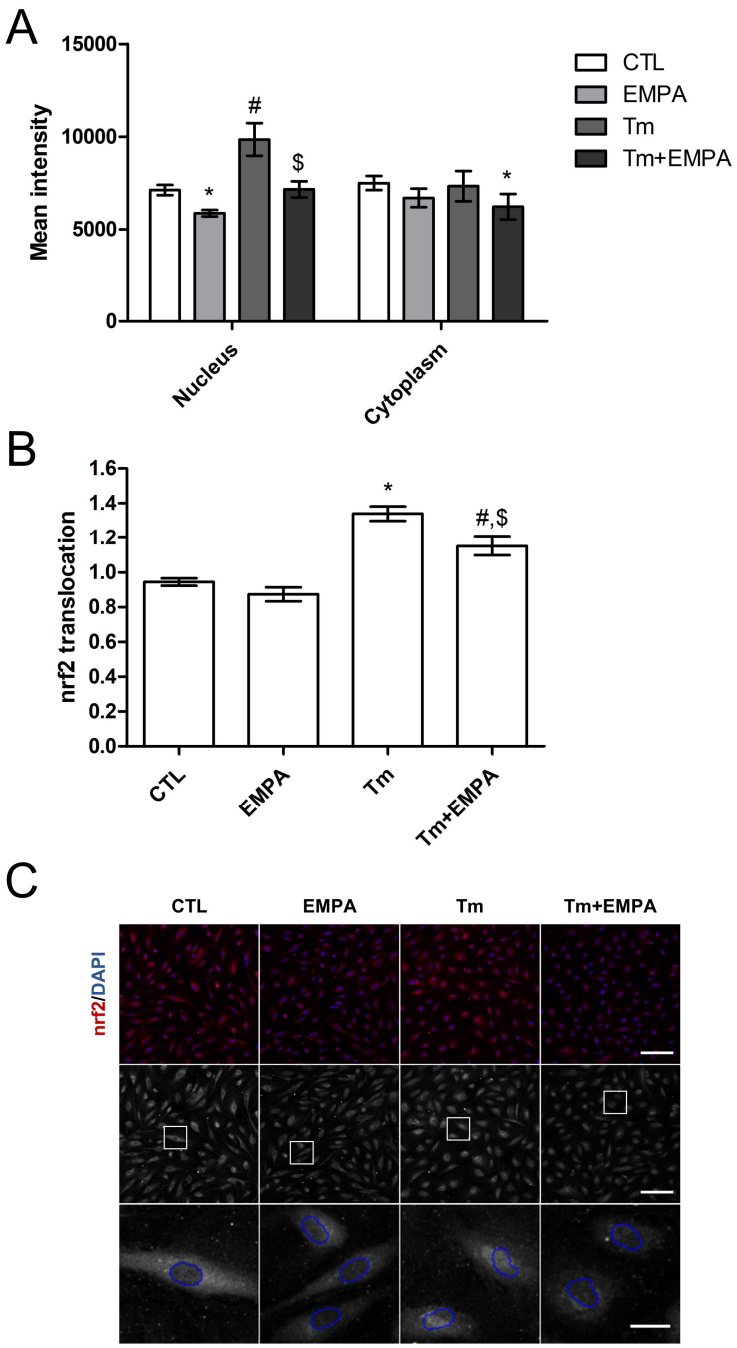


Figure 5-3. ***nrf2 nuclear translocation.*** *(A) Mean gray value (MGV) measurements of the nuclear and cytoplasmic nfr2 signal. Tm (1 µg/mL) induced a significant increase in nuclear nrf2 intensity compared to CTL (# p<0.001). EMPA (50 µM) reduced the nuclear intensity compared to CTL (* p<0.05) and normalized the increase caused by Tm ($ p<0.001). Treatment with Tm+EMPA caused a slight decrease in cytoplasmic intensity compared to CTL (* p<0.05). (B) Nrf2 translocation was assessed with the ratio between the nuclear and cytoplasmic MGV. Tm induced a significant increase in translocation compare to CTL (* p<0.001). Treatment with EMPA limited the increase induced by Tm (# p<0.01) with levels remaining above CTL ($ p<0.01) (n=3). (C) Representative images of nrf2/DAPI immunostaining. Top panel: nrf2 (red; Alexa Fluor 555), nucleus (blue; DAPI), scale bar=150 µm. Middle panel: gray scale images for analysis. Bottom panel: Enlarged area with examples of outlined nucleus, scale bar=25 µm.*

5.5.4 EMPA does not impact the Tm-induced IL-6 secretion

As the UPR has been shown to elicit an inflammatory response through the nuclear factor κB (NF-κB) signaling, the potential effect of EMPA on IL-6 production was examined. Tm caused a significant increase in IL-6 supernatant concentration after 24h compared to control (Fig. 5-4, Tm vs CTL * $p<0.001$). However, no changes in IL-6 concentration measurements compared to Tm-treated cells were observed with the EMPA treatment (Tm+EMPA vs Tm), indicating that EMPA had no impact on the IL-6 production induced by ER stress.

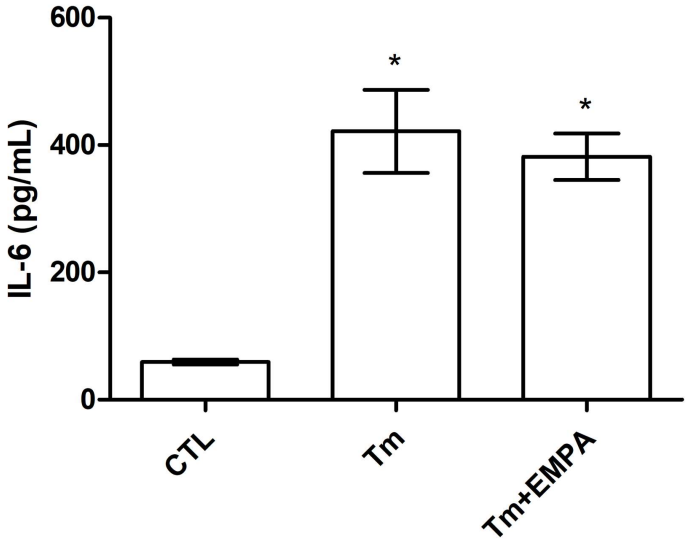


Figure 5-4. ***IL-6 production quantification.*** *IL-6 concentration in culture supernatant was measured through ELISA assays following 24h of treatment. Tm (1 µg/mL) significantly increased the production of IL-6 from cells after 24h compared to CTL (* $p<0.001$). Treatment with EMPA (50 µM) had no effect on the Tm-induced IL-6 concentration increase (n=3).*

5.6 Discussion

ER stress arises from the accumulation of unfolded or misfolded proteins due to a demand exceeding ER capacity. In response, the UPR aims to restore ER homeostasis but prolonged UPR activation has been shown to contribute to cardiovascular disease development and progression [18] through increased oxidative stress and inflammation leading to EC dysfunction [19, 20]. In this study, we report the capacity of EMPA to reduce TXNIP/NLRP3 downstream signaling and to attenuate nrf2 nuclear translocation, indicative of a moderation of ER stress induced EC dysfunction. This follows our previous findings suggesting that EMPA has an anti-inflammatory effect through the regulation of ER stress markers, attenuating leukocyte adhesion caused by sustained glycocalyx degradation [16].

UPR activation in ECs has been identified as a marker for atherosclerosis plaque development [21] and is related to impaired endothelium-dependent vasorelaxation and the upregulation of EC inflammatory markers [22, 23]. Previous studies have also found reduction of ER stress markers in SGLT2i-treated rodents resulting in improved cardiac and vascular functions [3, 14, 24, 25]. Thus, the attenuation of ER stress by EMPA represents an interesting therapeutic approach to mitigate EC dysfunction associated with cardiovascular diseases.

In line with our previous results [16], induction of ER stress in ECs has been related to the activation of the PKR-like ER kinase (PERK) and the eukaryotic translation initiation factor 2 subunit 1 (eIF2α) with increased CHOP expression and oxidative stress [26]. Accordingly, exposure to oxidized phospholipids has been shown to cause the upregulation of the UPR target genes including *ATF4* and *DDIT3* (CHOP) as well as the inflammatory genes *IL6* and *IL8* [27].

Parallel to UPR activation, redox homeostasis can be impaired by the upregulation of TXNIP, an inhibitor of thioredoxin (TRX), which has been shown to promote oxidative stress and inflammation in ECs [28, 29]. Moreover, TXNIP has been identified as a direct activator of the NLRP3 inflammasome [30], exacerbating subsequent inflammatory responses [31-34]. Thus, as prolonged activation of the UPR promotes EC dysfunction through oxidative stress and inflammation, we sought to determine the effect of EMPA on the phosphorylation of eIF2α and the downstream targets TXNIP, NLRP3 and CHOP.

Treatment of HAAECs with Tm caused a sustained ER stress induction as shown by the constant increase of the phospho-eIF2α/eIF2α ratio over 24h (Fig. 5-1). In contrast to a recent study by Kapadia *et al.* which showed the normalization of PERK phosphorylation by dapagliflozin (1 µM) in Tm-treated HCAECs [15], no significant changes in the eIF2α phosphorylation, directly downstream to PERK, were found with EMPA in our case. Despite no indication of reduced ER stress activation, EMPA attenuated the peak expression of CHOP, TXNIP and NLRP3. The increased expressions of TXNIP and NLRP3 and the attenuating effect of EMPA were concomitant, supporting a direct activation of NLRP3 by TXNIP in oxidized conditions as found by others [31-33, 35-37].

Interestingly, the peak expression of CHOP precedes that of TXNIP/NLRP3. CHOP signaling is complex and can promote both protective and proapoptotic signals depending on the stress stimuli [38, 39]. Prolonged activation of the UPR through the PERK-eIF2α-ATF4 axis triggers an increase in CHOP expression leading to cell apoptosis through the increase of protein synthesis and oxidative stress [40-42]. Thus, oxidative stress caused by CHOP signaling may contribute to the dissociation of thioredoxin from TXNIP, further affecting the redox regulation and promoting the interaction between TXNIP and NLRP3 (Fig. 5-5). A concentration-dependent effect was also observed on the Tm-induced increased protein expressions of TXNIP and NLRP3 as the efficacy of EMPA only appears at high concentrations (Fig. 5-2).

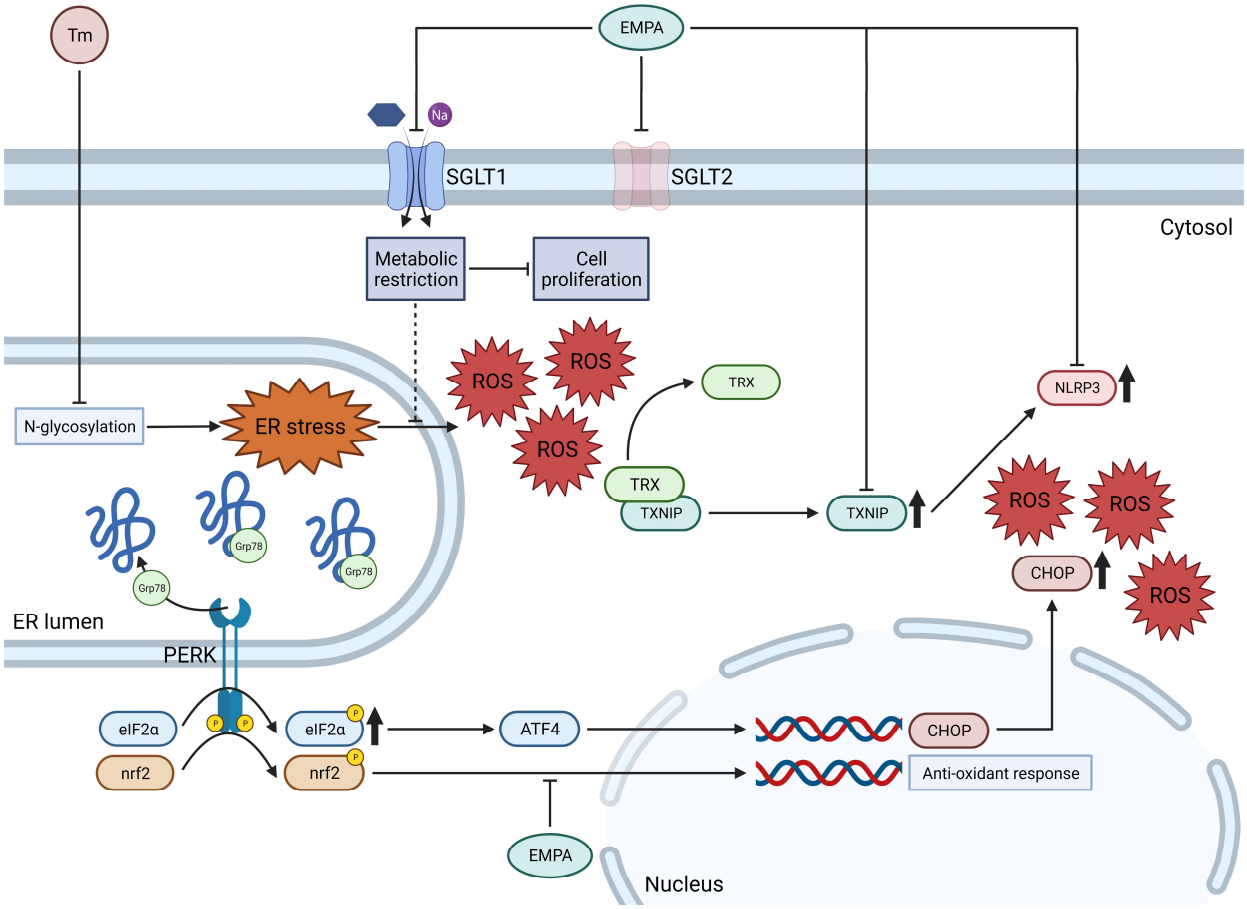


Figure 5-5. ***Impact of EMPA on the ER signaling pathway and downstream regulation.*** *Treatment of HAAECs with Tm induces ER stress through the inhibition of N-glycosylation. This causes the activation of the UPR transmembrane regulator PERK as the chaperone molecules Grp78 bind the accumulating misfolded proteins. eIF2α and nrf2 are two direct substrates of PERK. Increased phosphorylation of eIF2α inhibits general translation and induces the transcriptional regulation of CHOP through ATF4, promoting oxidative stress and apoptosis. PERK activates nrf2 causing its nuclear translocation and the transcription of redox regulatory enzymes as an anti-oxidant response. The inherent increase in ROS under ER stress promotes the dissociation of TRX from TXNIP, leading to NLRP3 activation. EMPA potentially mitigate ER stress downstream signaling through the inhibition of SGLT1. The metabolic restriction imposed on HAAECs could limit the oxidative damage of excessive ROS which would in turn attenuates TXNIP/NLRP3 activation and nrf2 translocation. This figure was created with BioRender.com.*

Previous animal studies showed that treatment with SGLT2i can attenuate ER stress signaling and contribute to improved vascular and cardiac health. Treatment of diabetic rodents with EMPA or dapagliflozin reduced the myocardial expression of ATF4/CHOP [14] and NLRP3 [25], respectively. Leng *et al.* showed the reduction in NLRP3 serum level and inflammatory signaling in dapagliflozin-treated diabetic ApoE(−/−) mice resulting in attenuated atherosclerotic lesion area [24]. A recent animal study conducted by Byrne *et al.* showed that EMPA prevents cardiac dysfunction associated to NLRP3 in rodents with heart failure. Short-term treatment with EMPA

resulted in lower expression of inflammasome activation markers including TXNIP in intact mouse heart and following ischemia/reperfusion injury [3]. Based on our results and the observations from animal studies, treatment with SGLT2i appears to attenuate downstream signaling related to ER stress and this effect is not limited to cardiac tissue but extents to ECs, which could contribute to prevent the early development of cardiovascular complications.

Nrf2 is an important PERK-mediated regulator of redox homeostasis under ER stress [43, 44]. Nuclear translocation of nrf2 promotes redox protection against EC dysfunction in oxidative-driven cardiovascular diseases [45, 46]. Nrf2 signaling has been related to TXNIP as nrf2 KO mice exhibited increased oxidative stress and TXNIP cardiac expression [47]. Moreover, negative regulation of NLRP3 by nrf2 through the interaction of the TRX/TXNIP complex has been demonstrated in rat cerebral arteries following infarction [48]. Treatment with Tm resulted in increased nrf2 translocation (Fig. 5-3), indicative of a more pronounced anti-oxidant response under ER stress which was normalized by EMPA. However, EMPA and canagliflozin have been shown to induce the activation of nrf2 with increased expression of nuclear nrf2 in rodent cardiac tissue under oxidative conditions [49, 50]. This discrepancy with our results suggests that nrf2 regulation in cultured ECs may differ from animal tissue.

In addition to oxidative stress, prolonged activation of UPR can profoundly affect the inflammatory signaling through the activation of NF-κB and the subsequent release of cytokines. Following the PERK-mediated eIF2α phosphorylation, the translation attenuation of the specific inhibitor IκB promotes NF-κB activation, nucleus translocation and inflammatory gene transcription [51, 52]. Indeed, treatment with Tm caused an increased production of IL-6 suggesting NF-κB activation (Fig. 5-4). However, addition of EMPA failed to reduce IL-6 concentration levels. Although the anti-inflammatory effect of EMPA has been previously shown in mice with the reduction of IL-6 myocardial expression [4] and IL-6 serum concentration [5], the induction of NF-κB activation under ER stress may differ from systemic pathological inflammation. Interestingly, *in vitro* studies showed how SGLT2i differ in their capacity to reduce IL-6 with canagliflozin being the only one compared to dapagliflozin and EMPA to effectively reduce IL-6 levels in IL-1β-stimulated HUVECs [53] and LPS-stimulated HCAECs [54].

To further examine the mechanistic effect of EMPA on ECs, the expression of SGLT1 and SGLT2 was studied. Protein and mRNA expression of SGLT1 and/or SGLT2 has been previously detected in multiple EC lines [11, 55-58]. The expressions of SGLTs were also shown to be upregulated in culture conditions promoting inflammation or oxidative stress. However, cases with low levels of mRNA and protein expression impede the certainty of the expression in ECs [53, 59]. In our case, as SGLT1 expression was confirmed but SGLT2 could not be detected (Suppl. Fig. S5-1), it is reasonable to assume that the effect of EMPA on HAAECs are likely to be mediated through SGLT1 and not SGLT2 as the high concentrations tested can theoretically inhibit SGLT1 [60].

Despite other transmembrane transporters of glucose such as GLUT1/3 which are expressed in ECs with overall glucose transport contribution superior to SGLT1 [59], a dose-dependent effect of EMPA on metabolic activity was demonstrated by the gradual decrease of HAAEC proliferation with concentrations exceeding 50 µM (Suppl. Fig. S5-2). These results are in accordance with Behnammanesh *et al.* who showed that EMPA is well tolerated by HUVECs to concentrations up to 50 µM, with decreases in DNA synthesis (24h) and proliferation (3 days) but no impact on cell viability [57].

Therefore, it is possible that the observed effects of EMPA on ER stress signaling is related to metabolic restriction. Through SGLT1 inhibition, EMPA could limit oxidative damage under prolonged ER stress and in turn attenuate TXNIP/NLRP3 and nrf2 activation (Fig. 5-5). Further work on the metabolic impact of SGLT1 inhibition by EMPA is required to clarify its beneficial impact on EC functions.

In this study, Tm was used to block N-glycosylation and induce ER stress. Other pharmaceutical and physiological inducers of ER stress could potentially change the observed effects of EMPA, however, preliminary tests showed that the use of thapsigargin, a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, resulted in similar reductions in NLRP3 expression (data not shown), suggesting that the beneficial effects of EMPA is independent on the mechanism of ER stress induction. The PERK-eIF2α-ATF4 axis of the UPR was primally targeted based on our previous results but other signaling pathways were likely to be activated

and be implicated in the cellular response. Also, pleotropic effects of EMPA different from the targeted inhibition of SGLT1/2 cannot be ruled out.

In conclusion, our results suggest that EMPA attenuates ER stress downstream apoptotic and inflammatory signaling as shown by the reduced TXNIP/NLRP3 activation. This effect is possibly mediated through SGLT1 inhibition, promoting metabolic restriction and in turn limiting excessive oxidative stress caused by prolonged UPR activation. The mitigation of oxidative stress by EMPA is supported by the reduced nrf2 translocation indicative of a lesser anti-oxidant response. As more studies explore the pleotropic effects of EMPA on vascular cell mechanisms, its therapeutic potential for oxidative-driven cardiovascular diseases will be greatly enhanced.

5.7 Supplementary information

Empagliflozin reduces endoplasmic reticulum stress associated TXNIP/NLRP3 activation in tunicamycin stimulated aortic endothelial cells

Marc-Antoine Campeau[1], Richard L. Leask[1,2]*

[1]Department of Chemical Engineering, McGill University, Montreal, QC, Canada

[2]McGill University Health Centre, Montreal, QC, Canada

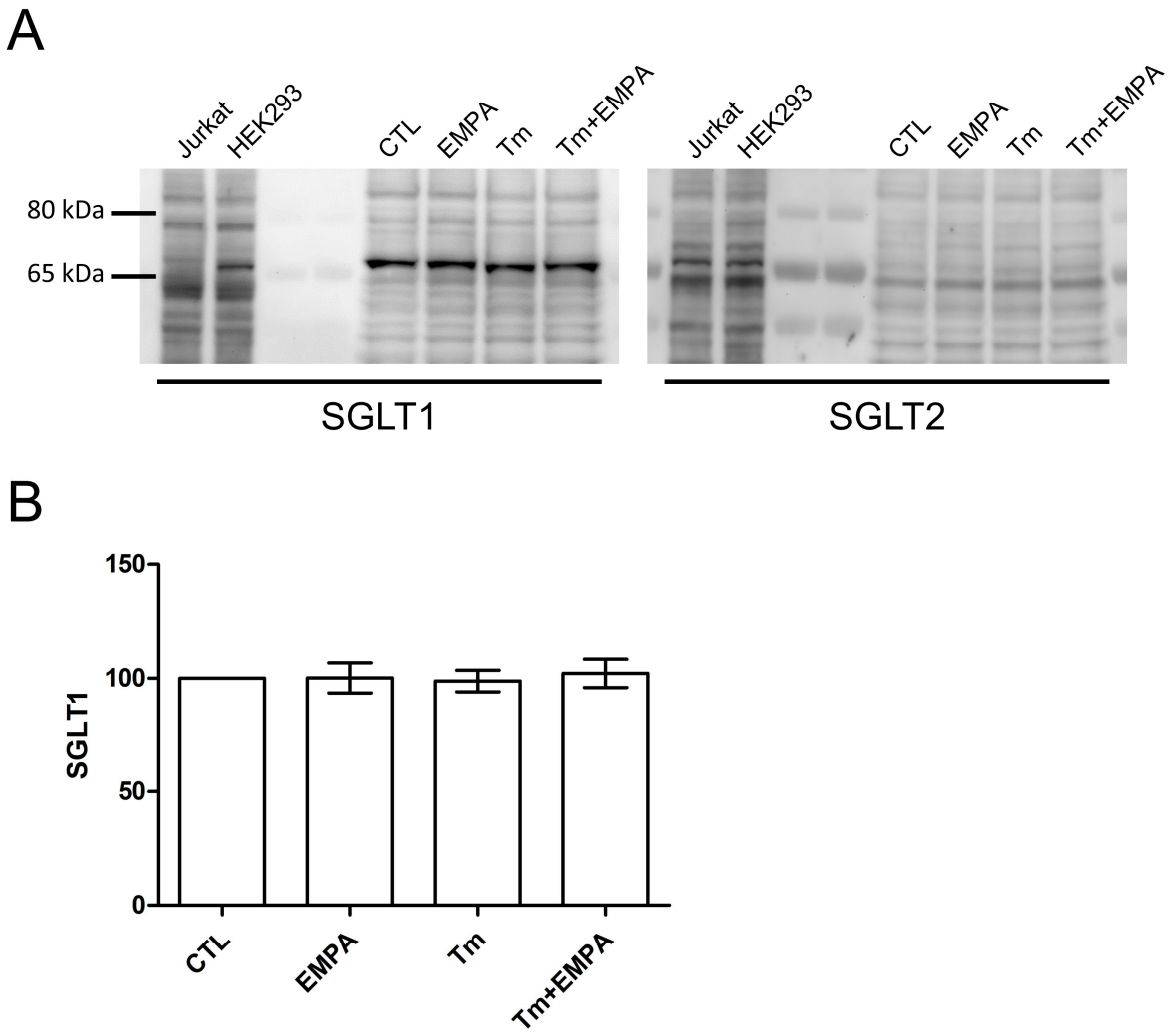
*Corresponding Author

richard.leask@mcgill.ca

Keywords: sodium/glucose cotransporter 2 (SGLT2) inhibitor, empagliflozin (EMPA), endoplasmic reticulum stress (ER stress), thioredoxin interacting protein (TXNIP), NLR family pyrin domain containing 3 (NLRP3), C/EBP homologous protein (CHOP), nuclear factor erythroid 2-related factor 2 (nrf2), interleukin-6 (IL-6).

SGLT1 but not SGLT2 is expressed in HAAECs

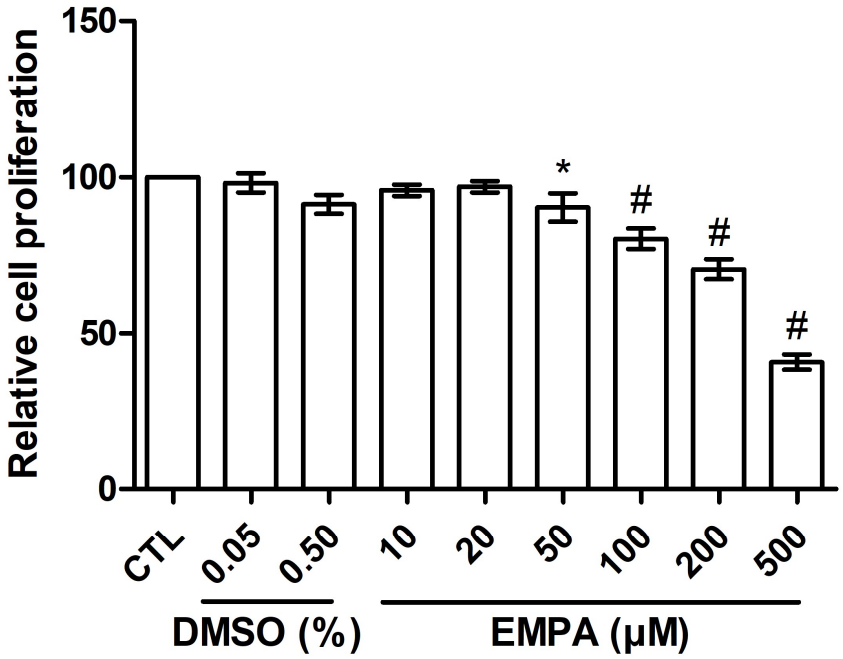
Protein expression of SGLT1 but not SGLT2 was detected through western blot (Suppl. Fig. S5-1A). Bands at approximatively 70 kDa were observed for SGLT1 and confirmed with the HEK293 cell lysate. Conversely, while immunoblotting of SGLT2 was confirmed by the Jurkat cell and HEK293 cell lysates, no detectable bands were found for HAAEC samples, which suggests that SGLT2 is not expressed in the HAAEC line tested. Additionally, SGLT1 expression remained constant with no impact of Tm or EMPA (Suppl. Fig. S5-1B).



Supplementary Figure S5-1. ***SGLT1 and SGLT2 expression in HAAECs.*** *(A) The protein expression of SGLT1 and SGLT2 in HAAECs was determined through western blot. HAAECs showed expression of SGLT1 with bands detected at approximatively 70 kDa. No bands were detected for SGLT2 with HAAEC samples. Positive controls, Jurkat cell and HEK293 lysate, both showed expression of SGLT2 whereas SLGT1 was detected in HEK293 lysate only. (B) SGLT1 protein expression. Treatment of HAAECs with EMPA (50 µM) or Tm did not impact SGLT1 protein expression in the conditions tested.*

EMPA reduces HAAEC proliferation at high concentrations

Treatment of HAAECs with EMPA for 24h showed a concentration-dependent effect on metabolic activity as measured by the cell proliferation, proportional to the MTS reagent absorbance. While low concentrations (10-20 µM) did not significantly impact proliferation, higher concentrations (≥50 µM) resulted in significant reductions compared to the control (Suppl. Fig. S5-2, EMPA 50 µM vs CTL * $p<0.05$; EMPA 100-500 µM vs CTL # $p<0.001$). Vehicle controls (DMSO) confirmed that the reductions are attributable to the effect of EMPA as 50 µM and 500 µM were equivalent to 0.05 and 0.50% DMSO, respectively.



Supplementary Figure S5-2. ***Effect of EMPA on HAAEC metabolic activity.*** *Treatment of HAAECs with EMPA for 24h caused reductions in cell proliferation as a measure of metabolic activity in a dose-dependent manner. Cell proliferation was significantly reduced compared to CTL with EMPA 50 µM (* $p<0.05$) and EMPA 100-500 µM (# $p<0.001$). Concentration of EMPA 50 µM and 500 µM correspond to vehicle control of DMSO 0.05% and DMSO 0.50% respectively.*

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CHAPTER 6 DISCUSSION

As research progresses to define the therapeutic potential of sodium-glucose cotransporter 2 inhibitors (SGLT2i), *in vitro* studies offer a unique perspective to explore their direct effects on vascular cells, independent of the benefits related to the systemic control of glycemia. So far, SGLT2i have been found to improve endothelial cell (EC) functions in a number of conditions both *in vivo* and *in vitro*. The findings presented in this thesis demonstrates a potential mechanism by which empagliflozin (EMPA) attenuates EC dysfunction associated with the signaling of ER stress in the pathologically relevant condition of glycocalyx (GCX) disruption. Ultimately, through improved intracellular functions and paracrine signaling, ECs can contribute to the restoration of vascular homeostasis and in turn to the cardiovascular protective effect of SGLT2i.

The main objectives of this thesis are **1) to study how EMPA overcomes EC dysfunction associated with GCX disruption and the dependency of the anti-inflammatory effect on GCX integrity** and **2) to identify the inflammatory pathway(s) involved and demonstrate the direct effect of EMPA on the pathway functional targets.** Chapter 3 contributes to the overall elucidation of these objectives. Chapter 4 addresses the first objective and part of the second objective while Chapter 5 realises the second objective.

The GCX is a dynamic structure involved in shear-regulated cellular mechanisms through heparan sulfate (HS) shear stress mechanotransduction. HS-mediated mechanotransduction has been related to NO production [37, 38], cell alignment and elongation [15, 16, 36] and cellular permeability [85]. Previous work from our lab and others has implicated HS in the regulation of the inflammatory phenotype and leukocyte adhesion in response to shear [16, 18, 86]. Disruption of the GCX through HS degradation resulted in reduced endothelial nitric oxide synthase (eNOS) expression, increased intercellular adhesion molecule 1 (ICAM-1) expression and leukocyte adhesion. This pro-inflammatory phenotype was the result of the loss of HS shear mechanotransduction triggering nuclear factor κB (NF-κB) activation [16]. In addition, the study from Cooper *et al.* which served as a starting point for the work of this thesis, demonstrated an anti-inflammatory effect of EMPA by attenuating the increased leukocyte adhesion caused by acute HS degradation and shear exposure [22]. Although the reduction in adhesion was

concomitant with the recovery of shear-induced cell elongation and HS staining intensity, the direct role of HS mechanotransduction remained in question.

Exposure to shear has been shown to regulate the EC GCX layer integrity and synthesis with laminar flow enhancing HS coverage and disturbed flow promoting shedding [87-89]. Following acute degradation, GCX components can regrowth over the course of 16-24h *in vitro* and potentially regain the associated mechanotransduction regulation [87, 90]. Building on the previous studies, to address the first objective which was **1) to study how EMPA overcomes EC dysfunction associated with GCX disruption and the dependency of the anti-inflammatory effect on GCX integrity**, experiments were designed to account for the potential regrowth of HS and to study the impact of EMPA on GCX-related EC functions (Chapter 4). In accordance with others, preliminary results showed significant regrowth of HS after 6 and 24h of culture following acute degradation. The inclusion of heparinase III for the experiment duration, condition termed as sustained HS degradation, silenced the contribution of HS mechanotransduction to EC homeostasis. This condition of degradation is similar to the chronic GCX disruption *in vivo* with atherosclerotic plaques and the shedding of GCX components under hyperglycemic conditions. Sustained HS degradation resulted in almost complete abolishment of HS intensity at cell surface and impairment of shear-induced cell elongation. Surprisingly, the reduced adhesion of leukocyte induced by EMPA was also observed under sustained HS degradation, indicative of a HS-independent mechanism. In addition, no changes in markers of EC function related to HS mechanotransduction (i.e. eNOS activation and ICAM-1 expression) were observed. Therefore, the results of **Chapter 4 support the initial hypothesis that i) EMPA promotes an anti-inflammatory EC response independent of EC GCX integrity.**

In parallel to the work of Chapter 4, a systemic review of the current literature on the effects of SGLT2i on vascular homeostasis and specifically on EC function was conducted to orient the study of the underlying mechanisms (Chapter 3). While evidence of SGLT2i beneficial effects on ECs from *in vitro* studies were scattered and fractional at first, the consensus regarding the existence of direct pleotropic effects on vascular cells has slowly gained ground in the literature over the past few years. As described in Chapter 3, anti-inflammatory effects of SGLT2i through restored vasoregulation and reduced adhesion molecule expression and cytokine production have been

previously reported but could not be validated (Chapter 4) or were technically challenging (e.g. cytokine release quantification in flow loop media) with our model. Other GCX-related functions such as paracellular permeability and migration were explored but no significant effects of EMPA were found. Consequently, a different approach was required to study how EMPA mitigated the inflammatory response of ECs caused by sustained HS degradation.

To address the second objective, **2) to identify the inflammatory pathway(s) involved and demonstrate the direct effect of EMPA on the pathway functional targets**, the genome-wide transcriptome analysis allowed for a broad overview of the dysfunction caused by sustained HS degradation and the impact of EMPA. Endoplasmic reticulum (ER) stress was identified as a significant pathway in the dysfunction induced by sustained HS degradation due to the significant upregulation of the unfolded protein response (UPR) regulatory genes. EMPA attenuated the ER stress response with lower transcriptional expression of these genes, suggested an attenuated or delayed ER stress induction. Downregulation of the thioredoxin interacting protein (TXNIP) transcription and protein expression by EMPA pointed toward a redox-sensitive mechanism contributing to improved EC inflammation under sustained HS degradation and shear.

In Chapter 5, the effect of EMPA on ER stress signaling was further studied by inducing stress with tunicamycin. This allowed to assess the direct effect of EMPA on downstream pathway targets in a more precise and targeted manner. EC protein expression showed the attenuation of downstream TXNIP/NLR-family pyrin domain-containing protein 3 (NLRP3) but no changes in the phosphorylation of eIF2α, suggesting the mitigation of the redox-sensitive TXNIP/NLRP3 activation independent of changes in ER stress induction. Despite our inability to demonstrate direct attenuation of oxidative stress using fluorescent probes with our experimental setup, Chapter 4 and 5 converged toward the mitigation of oxidative stress in the protective effect of EMPA. Indeed, reduction of oxidative stress by treatment with N-Acetyl-L-cysteine (NAC) resulted in similar leukocytes adhesion under sustained degradation compared to EMPA (Chapter 4). The reduction in nuclear factor erythroid 2-related factor 2 (nrf2) nuclear translocation by EMPA suggests an impact on the anti-oxidant response from ER stress (Chapter 5). In relation to nrf2 transcriptional regulation, genes of redox enzymes including heme oxygenase 1 (*HMOX1*), NADPH oxidase 4 (*NOX4*), NAD(P)H dehydrogenase quinone 1 (*NQO1*) and superoxide dismutase

2 (*SOD2*) were found to be differently regulated under sustained degradation (Chapter 4). Finally, the increased expression of TXNIP which contributes to oxidative stress and inflammation through thioredoxin inhibition and NLRP3 activation respectively was downregulated by EMPA. While Chapter 4 demonstrates the attenuation of EC inflammatory response by EMPA in an unspecific yet pathologically relevant setting of chronic GCX disruption, Chapter 5 supports that EMPA is able to reduce oxidative driven ER stress signaling which was part of the dysfunction observed under sustained HS degradation (Figure 6-1). Overall, results from **Chapter 4 and 5 support the hypothesis that ii) EMPA can attenuate ER stress signaling and the expression of downstream targets through oxidative stress mitigation.**

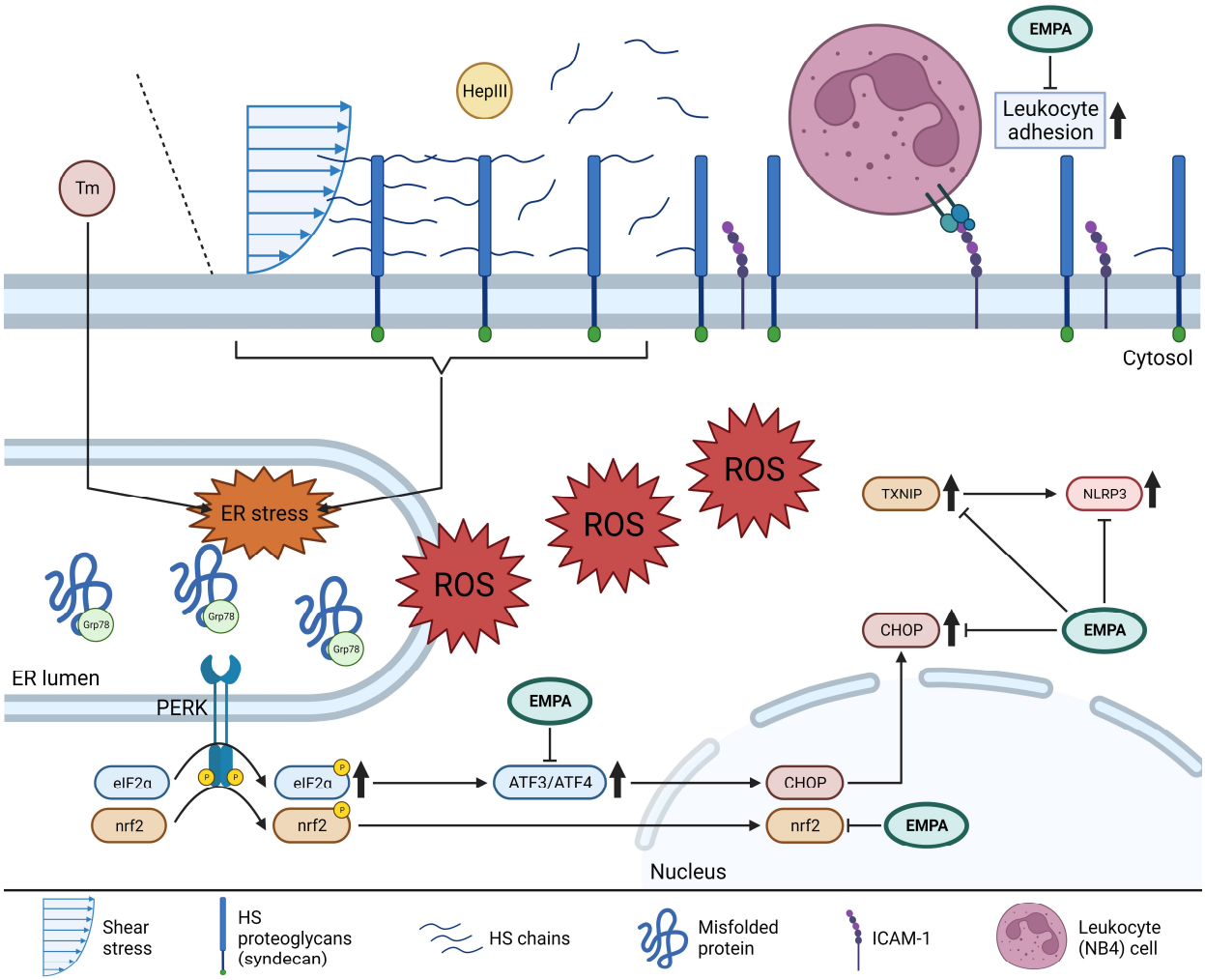

Figure 6-1. ***EMPA mitigates EC dysfunction associated to ER stress under sustained GCX disruption or pharmacological induction of ER stress with tunicamycin.*** *Sustained degradation of surface HS under shear or tunicamycin treatment resulted in ER stress induction in HAAECs. Through the activation of PERK-eIF2α-ATF3/4-CHOP signaling pathway, protein expression of CHOP, TXNIP and NLRP3 were upregulated. Oxidized condition due to prolonged ER stress induces the activation of NLRP3 inflammasome by TXNIP, exacerbating the inflammatory response and promoting enhanced NB4 cell adhesion. EMPA attenuates the PERK signaling cascade and reduced oxidative stress mediated EC dysfunction as shown by the reduced expression of ATF3/4, CHOP, TXNIP and NLRP3 as well as the attenuation of nrf2 translocation. This potentially contributes to the anti-inflammatory effect of EMPA limiting NB4 cell adhesion under sustained HS degradation. This figure was created with BioRender.com.*

The interplay between EC GCX disruption, ER stress signaling and the cardioprotective effects of SGLT2i remains to be clarified. Evidence suggests that disruption of the GCX and exposure to shear results in cellular responses similar to exposure to disturbed flow [16, 34] which has been shown to trigger ER stress signaling in ECs. Indeed, while laminar shear stress can limit ER stress [91], low shear or disturbed flow promote ER stress signaling with inflammation and apoptosis [92, 93]. ER stress has been found to be a marker of atheroprone endothelium [61]. Moreover,

products derived from the activation of the NLRP3 inflammasome, including IL-1β, IL-18 and caspase-1, have been related to atherosclerosis and cardiomyopathies [94]. For instance, exposure to atheroprone flow caused the activation of the NLRP3 inflammasome through the activation of sterol regulatory element binding protein 2 (SREBP2) and the increase in oxidative stress in ECs, which was demonstrated to lead to atherogenesis in mice [95]. Of note, disturbed flow found in susceptible area such as atherosclerotic plaques cause the shedding of the glycocalyx [19]. Together, these observations support that shedding of the GCX triggers EC dysfunction in part through ER stress and NLRP3 inflammation activation which participate in the development or progression of atherosclerotic plaques.

The elevation in the serum concentration of endothelial glycocalyx components is under study for the prognostic of cardiovascular outcomes. Increased concentration of syndecan-1 has been proposed as a marker for the worsening of cardiac function in patients with ischemic heart disease or heart failure (HF) [96]. A recent clinical trial looked at the perfused boundary region in microvessels to assess the glycocalyx thickness of type 2 diabetes (T2D) patient treated with glucagon-like peptide-1 receptor, SGLT2i or their combination. Interestingly, 12-month treatment with both drugs and their combination resulted in a significant improvement of the glycocalyx thickness which was not observed in insulin-treated patients, suggesting improved endothelial health [97].

In light of the direct benefits of SGLT2i on endothelial functions in clinically relevant pathological conditions such as GCX disruption, the contribution of improved EC homeostasis toward the overall vascular and cardiac outcome required further investigation. Despite being identified as an early event in cardiovascular disease development, EC dysfunction is often reported as an outcome of systemic inflammation/oxidation in animal studies of cardiac and vascular complications such as HF. Naturally, since clear benefits for HF patients have been reported, research has been focusing on the effect of SGLT2i on cardiomyocyte/cardiac functions [11]. A few recent studies, however, demonstrated that the positive effects of SGLT2i on ECs can benefit cardiomyocyte/myocardial functions. Juni *et al.* showed that the *in vitro* treatment of cardiac microvascular ECs with EMPA restored normal NO-mediated cardiomyocyte contraction and relaxation through the reduction of reactive oxygen species (ROS) [98]. In addition, Cappetta *et*

al. showed how the improvement of rat cardiac functions following dapagliflozin treatment could be related to ECs. Through the reduction of endothelial NF-κB and Na+/H+ exchanger (NHE1) activation by dapagliflozin, improved endothelial functions likely contributed to lower inflammation and fibrosis to ultimately result in restored cardiac function [99]. Moving forward, both *in vitro* and animal studies will be required to fully unveil the complete interactions taking place in the cardiovascular system. As illustrated herein, *in vitro* experiments provide a controlled but often less applicable testing of the drug effects on vascular cells. Signaling pathways and cellular mechanisms have been highlighted by using suprapharmacological concentrations of EMPA over a shorter period in opposition to the intended long-term exposure of the oral medication. Therefore, and as it is often the case, the conclusions from the *in vitro* experiments will need to be further validated *in vivo*.

In clinical settings, numerous studies have demonstrated the existence of endothelial dysfunction in the form of impaired vasodilation in the pathophysiology of HF. As such, the peripheral endothelial dysfunction assessed via venous occlusion plethysmography or flow-mediated dilation was identified as a significant predictor of HF worsening. Collectively, reduced nitric oxide synthesis and bioavailability due to increased oxidative stress appears to be the main underlying cause [100, 101] which has been shown to be positively impacted by SGLT2i treatment in *in vitro* and animal studies. However, the EMBLEM trial investigated the effects of EMPA on endothelial function in T2D patients with established cardiovascular diseases (HF and coronary artery diseases) and found no differences in the reactive hyperemia peripheral arterial tonometry index after 24 weeks of treatment [102]. With the recent approval of SGLT2i for HF treatment, further investigation into the EC function of diabetic and non-diabetic SGLT2i-treated patients will be possible. Clarifying the relation between improved EC function and the cardioprotective effects of SGLT2i in patients is necessary to translate the recent *in vitro* and animal findings into clinical significance.

CHAPTER 7 CONCLUSION AND FUTURE PERSPECTIVE

7.1 Conclusion

Endothelial function is fundamental to vascular homeostasis and as studies start to unveil the direct effects of sodium-glucose cotransporter 2 inhibitors (SGLT2i) on endothelial cells (ECs), it is becoming crucial to understating the cellular mechanisms potentially contributing to the clinical outcomes. Restoration of cellular dysfunction by SGLT2i can however take many forms and collective effort will be required to fully elucidate SGLT2i beneficial effects.

The work presented in this thesis dissertation aimed **1) to study how empagliflozin (EMPA) overcomes EC dysfunction associated with glycocalyx (GCX) disruption and the dependency of the anti-inflammatory effect on GCX integrity** and **2) to identify the inflammatory pathway(s) involved and demonstrate the direct effect of EMPA on the pathway functional targets.**

Based on previous observation that EMPA could limit increased leukocyte adhesion to ECs caused by acute heparan sulfate (HS) degradation, we sought to determine whether EMPA anti-inflammatory effect relied on GCX integrity and mechanotransduction. As the reduction in adhesion persisted under sustained HS degradation, the effect was demonstrated to be independent of HS integrity. The transcriptome analysis revealed the significant induction of endoplasmic reticulum (ER) stress signaling under sustained HS degradation. EMPA attenuated the upregulation of key mediators from the unfolded protein response as well as thioredoxin interacting protein (TXNIP). In support to these observations, specific induction of ER stress with tunicamycin strengthen the hypothesis regarding the attenuation of ER stress signaling by EMPA. Protein of the apoptotic and oxidative-driven inflammatory response were downregulated, indicative of attenuated EC dysfunction. Mechanistically, EMPA possibly attenuates oxidative damage under HS degradation or prolonged ER stress by metabolic restriction through sodium-glucose cotransporter 1 (SGLT1) inhibition. Together, the findings presented support the hypotheses that **i) EMPA promotes an anti-inflammatory EC response independent of EC GCX integrity** and **ii) EMPA can attenuate ER stress signaling and the expression of downstream markers through oxidative stress mitigation.**

In conclusion, the results showed the potential of EMPA to alleviate EC inflammatory response in conditions of sustained GCX disruption. This effect was further related to the capacity of EMPA to reduce ER stress downstream signaling, demonstrating its positive impact in a clinically relevant model and toward the underlying cellular signaling. These observations contribute to the body of evidence suggesting that through direct cellular mechanisms, SGLT2i promote endothelial cell homeostasis which is relevant to the improved cardiovascular outcomes reported from clinical trials.

7.2 Future perspective

As discussed, more work is needed to unveil the true benefit of SGLT2i in cardiovascular complication treatment. The clinical perspective often relies on the macro observation of the cardiovascular condition and the direct effects on vascular cells are not fully integrated to the medical practice as question remains unanswered.

Further exploration of the interplay between ECs and cardiomyocytes in the context of SGLT2i treatment is necessary. Using a direct or indirect co-culture system could clarify the action and contribution of each cell type on vascular homeostasis. In addition, refining our analysis of the signaling through knockdown and knockout models could help identify the critical mediators in the effects of SGLT2i and define the interplay mechanisms between ECs and cardiomyocytes.

Based on the findings of the present thesis, given the relation between EMPA treatment and TXNIP expression, TXNIP knockout or overexpression is likely to change the capacity of EMPA to mitigate EC dysfunction. It would be interesting to see if EMPA preserves its attenuating effect on ER stress in TXNIP knockout ECs. However, given the reported effects of EMPA on multiple signaling pathways in ECs (Chapter 3), it is unlikely that TXNIP consists in the sole mediator of EMPA. Overexpression of TXNIP and the associated increase in oxidative stress could also be a model to study the potential of EMPA to reduced oxidative damage. Likewise, as our conclusion implies the mitigation of oxidative stress by EMPA, further assessment of this effect would be beneficial. Redox enzyme expression and the nuclear protein fraction of the nuclear factor erythroid 2-related factor 2 (nrf2) could be used to expand on our results. More technical

approaches such as the use of an ARE Luciferase Reporter Lentivirus could be used to assess the progression of oxidative stress in live culture.

Whether the effects of SGLT2i are in part or fully mediated through the inhibition of SGLT1/2 remained puzzling. The discrepancy in the reported expression of SGLT2 in ECs complicates the study of the direct mode of action of SGLT2i through SGLT1 and SGLT2. Silencing of these transporters has been previously shown to change the effects of phlorizin on HUVECs [103] but undetectable SGLT2 mRNA in ECs [104, 105] render the study of the role of SGLT2 challenging. Murine SGLT2 knockout models have been previously used to assess the role of SGLT2 in hyperglycemia, kidney injury and pancreatic β-cell death [106-108]. Similar models could be used to determine if the observed cardiovascular benefits of SGLT2i treatment are in part mediated through SGLT1/2 inhibition.

The possible implication of SGLT1/2 inhibition on vascular cells metabolic activity and how changes in that metabolic activity and cell cycle progression relate to metabolic disorders (and the implication of ER stress) need further investigation. To this end, SGLT2i, and to some extent other metabolic acting drugs, could benefit from more advanced transcriptome analysis to map their regulation on cell metabolism and to predict their impact in tissues and organs. This could be performed in conjuncture with metabolic assays in an attempt to relate the possible shift in metabolic activity to the protective effect of SGLT2i.

A few natural occurring compounds have been shown to have protective effects against EC ER stress in comparable ways to SGLT2i. Quercetin [109], Mangiferin [53], Ilexgenin A [110] as well as Astragaloside IV [56] were shown to revoke the induction of ER stress by palmitate or high glucose in HUVECs through the reduction of reactive oxygen species, leading to reduced TXNIP/NLR-family pyrin domain-containing protein 3 (NLRP3) activation, IL-1β/IL-6 secretion and apoptosis. As these compounds enhance or restore AMP-activated protein kinase (AMPK) activation, impacting cellular energy homeostasis, the suppression of ER stress-associated oxidative stress by AMPK was proposed as a potential protective mechanism. However, activation of AMPK through SGLT2i treatment remains uncertain and the protective potential appears to be dependent on the vascular cell type and the SGLT2i used. Mancini et al.

demonstrated that Canagliflozin but not EMPA up to a concentration of 100 µM can increase AMPK activity in HUVECs [104]. Given that few studies comparing SGLT2i reported differences in their capacity to alleviate inflammation or oxidative stress, it would be of interest to study if these differences stem from their different specificity toward SGLT1/2. In other words, could the presence or absence of a class effect inform us about the mechanisms of action of SGLT2i on vascular functions? For instance, canagliflozin, dapagliflozin and EMPA, all were shown to inhibit cardiac Na+/H+ exchanger activity and cytoplasmic sodium concentration in mouse cardiomyocytes but only canagliflozin and EMPA resulted in cardiac vasodilation [111].

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Appendix A

Table 1. Effects of SGLT2i treatment on hyperglycemia-related cardiovascular dysfunction in diabetic rodent models

Animals	Model/Vascular intervention	SGLT2i treatment	SGLT2i-related effects	Comments	Reference
Wistar rats	STZ-induced T1D	EMPA 10 and 30 mg/kg/day 7 weeks	↑ EC-dependent vasorelaxation Ø EC-independent vasorelaxation ↓ Whole blood oxidative burst ↓ Cardiac Nox2 activity ↓ Aortic ROS Ø Endothelial ROS ↓ eNOS expr., ↑ peNOS/eNOS ratio ↓ Nox1, Nox2 and HO-1 epxr. ↓ MCP-1 expr. ↓ MCP-1, CD68, IL-6, TNF-α and ICAM-1 mRNA ↓ Aortic wall thickness and collagen content	eNOS inhibitor (L-NAME) decreased endothelial ROS signal, suggesting eNOS uncoupling which is not completely prevented by EMPA treatment.	[1]
C57BL/6J mice	STZ-induced T1D	Ipragliflozin 3 mg/kg/day 3 weeks	↑ EC-dependent vasorelaxation Ø EC-independent vasorelaxation ↓ Systemic oxidative stress (urine 8-OHdG) ↑ aortic pAkt/tAkt expr. ↑ aortic peNOS/teNOS expr. ↓ aortic ICAM-1, VCAM-1, MCP-1 and F4/80 mRNA		[38]
Zucker Diabetic Fatty rats (ZDF-Lepr$^{fa/fa}$) Lean controls rats (ZDF-Lepr$^{+/+}$)	T2D	EMPA 10 and 30 mg/kg/day 6 weeks	↑ EC-dependent vasorelaxation ↓ cGKI, ↑ pVASP expr. ↑ DHFR, ↓ peNOS (Thr495) ↓ Whole blood oxidative burst ↓ Cardiac NADPH oxidase activity ↓ Aortic ROS ↓ Endothelial ROS ↓ Cardiac 4-HNE-positive proteins ↓ aortic Nox2, HO-1, RAGE expr. ↓ aortic IFN-γ, Cox-2, iNOS, SELP mRNA	Inhibitory effect of L-NAME on ROS staining of the endothelium is indicative of eNOS uncoupling. EMPA partly restore normal eNOS activity and regulation.	[2]
C57BL/6J mice	STZ-induced diabetes	EMPA 10 mg/kg/day	↑ LVEF, ↑ E/A, ↓ systolic LV volume ↓ cardiac collagen fiber deposition	Variations in cardiac function attributable to changes in cardiac structure	[39]

		20 weeks	↑ myocardial microperfusion ↑ myocardial CD31-positive microvessels ↑ peNOS level in microvessels ↑ EC-dependent vasorelaxation ∅ EC-independent vasorelaxation ↑ VE-Cadherin expression ↓ ICAM-1 and VCAM-1 expr.	Beneficial effect of EMPA on the vasodilatation, microvessel integrity and barrier function.	
KK-Ay mice C57BL/6J mice	T2D HFD	EMPA 10 mg/kg/day 10 weeks	↑ Cardiac function (EF%, FS%, FAC%, E/A, ECG) ↓ Cardiac lipid hydroperoxide, NOX4 and MDA expr. ↑ Cardiac SOD, GSH-Px expr. ↓ Myocardial fibrosis, ↓ TGF-β, collagen I and III ↑ nfr2 expr.	EMPA promotes reduction in oxidative stress by nuclear translocation of nfr2. EMPA normalizes TGF-β/Smad pathway in diabetic mice heart.	[40]
Male C57BL/6 mice Ex vivo (aorta rings)	Hyperglycemic conditions, 10 and 25 mM glucose	CANA, DAPA, EMPA 25 nM to 10 µM	↑ EC-dependent vasorelaxation ↓ SGLT2 mRNA ↑ Oxygen consumption rates (25 mM Glu) ↓ Proton leak (25 mM Glu)	Biphasic effect of [EMPA] on EC-dependent vasorelaxation Comparable effect between the different SGLT2i	[29]
Male C57BL/6 J-lepob mice ($ob/ob^{-/-}$)	Prediabetes (Obese, insulin resistance)	EMPA 1.5 mg/kg/day 10 weeks	↑ Coronary flow velocity reserve ↑ Fractional area change ↑ Plasma L-Arginine	EMPA improves cardiac functions Supported by NO regulation improvement	[41]
Male Wistar rats	STZ-induced diabetes CCA clamping cerebral I/R injury	EMPA 10 mg/kg 1 and 24h after I/R injury	↓ MDA (lipid peroxides) ↑ GSH, Catalase ↓ cerebral TNF-α level ↓ Caspase-3+ (apoptotic) cells ↓ brain infarct volume ↓ degenerative neurons		[42]

Akt: Protein kinase B (PKB), CCA: Common carotid artery, CD31: Platelet endothelial cell adhesion molecule, cGKI: cGMP-dependent protein kinase I, Cox-2: cyclooxygenase-2, DHFR: dihydrofolate reductase, E/A: early (E) and late (A) ventricular filling velocity ratio, eNOS: endothelial nitric oxide synthase, GSH: glutathione, GSH-Px: glutathione peroxidase, HO-1: Heme oxygenase 1, HFD: High fat diet, 4-HNE: 4-hydroxynonenal, ICAM-1: intercellular adhesion molecule 1, IFN-γ: interferon gamma, IL-6: Interleukin-6, iNOS: inducible nitric oxide synthase, I/R: Ischemia/reperfusion, LV: left ventricle, LVEF: left ventricle ejection fraction, MCP-1: Monocyte Chemoattractant Protein-1, MDA: malondialdehyde, Nox1: NADPH oxidase 1, Nox2: NADPH oxidase 2, Nox4: NADPH oxidase 4, RAGE: receptor for advanced glycation end products, ROS: Reactive oxygen species, SELP: P-selectin, SOD: superoxide dismutase, STZ: Streptozotocin, T1D: Type 1 diabetes, T2D: Type 2 diabetes, TGF-β: Transforming growth factor β, TNF-α: Tumor necrosis factor, VASP: vasodilator stimulated phosphoprotein, VCAM-1: Vascular cell adhesion protein 1

Table 2. Effects of SGLT2i treatment on atherosclerosis-related vascular dysfunction in diabetic rodent models

Animals	Model/Vascular intervention	SGLT2i treatment	SGLT2i-related effects	Comments	Reference
Male C57BL/6J ApoE$^{-/-}$ mice	WD STZ-induced diabetes	EMPA 20 mg/kg/day 8 or 12 weeks	↓ atherosclerotic lesions ↓ aortic MCP-1, VCAM-1, NOX2, p22phox mRNA ↓ lipid deposition ↓ MCP-1, VCAM-1 positive area ↓ macrophage accumulation ↑ EC-dependent vasorelaxation Ø EC-independent vasorelaxation ↓ PVAT MCP-1, ICAM-1, VCAM-1, CD68, p22phox, p47phox mRNA	EMPA reduced atherosclerotic markers of inflammation, oxidative stress and lipid accumulation	[43]
Zucker Diabetic Fatty rats (Lepr^{fa}/Crl)	T2D	CANA Fortified chow, normalized to 10 µM in plasma 4 weeks	↓ infarct size/area at risk	Effect independent of diabetic phenotype	[37]
C57BL/6J ApoE$^{-/-}$ mice	NA/STZ-induced diabetes	Luseogliflozin 0.1% Mixed in diet 1 week or 6 months	↓ aortic F4/80, TNF-α, IL-1, IL-6 mRNA ↓ aortic ICAM-1, PECAM-1, MMP-2 mRNA ↓ Aortic atheroma area ↓ Aortic lipid accumulation ↓ Aortic root CD68+ area ↓ Aortic root 4-HNE+ area	↓ CD68 and 4-HNE positive area below ApoE$^{-/-}$, suggesting a protective effect independent of glycemic control.	[36]
Male C57BL/6J mice	High cholesterol diet Atherosclerosis progression/ regression STZ-induced diabetes	EMPA 35 mg/kg/day 3 weeks	↓ Aortic root lesion area ↓ Aortic lipid accumulation ↓ Aortic root CD68 positive area ↑ collagen plaque content (Sirius Red) Ø circulating leukocytes Ø Ly6C^{lo} and Ly6C^{hi} monocytes ↓ Ki67+ macrophage proliferation ↓ leukocyte adhesion density		[33]
Male C57BL6 Ldlr$^{-/-}$ mice	Cholesterol rich diet STZ-induced diabetes	Phlorizin 400 mg/kg/0.5 day 4 weeks	↓ Total plasma cholesterol and triglycerides ↓ Plasma cholesterol VLDL and LDL and TG LDL ↓ Atherosclerotic lesions ↓ Aortic root CD68+ area ↓ Aortic root lipid accumulation ↓ circulating monocytes, neutrophils and Ly6-C^{hi}		[32]

			↓ CD11b monocytes/neutrophils expr.		
Male ApoE$^{-/-}$ mice	HFD STZ-induced diabetes Male db/db mice	DAPA 1.0 mg/kg/day 4 weeks Ipragliflozin 1.0 mg/kg/day 4 weeks	↓ Atherosclerotic lesion ↓ Aortic atheroma area ↓ Aortic lipid accumulation ↓ Plaque macrophage infiltration ↓ Peritoneal cholesterol ester accumulation ↓ Macrophage pro-inflammatory markers (Lox-1, CD36, ACAT1)		[34]
Male ApoE$^{-/-}$ mice	HFD STZ-induced diabetes	DAPA 1.0 mg/kg/day 12 weeks	↓ NLRP3, IL-1β, IL-18 serum conc. ↓ aortic lesion area ↓ Lesion macrophage and SMC infiltration ↓ aortic ROS ↓ NLRP3, ASC, Casp-1, IL-1β, IL-18 aortic expr.	DAPA inhibits the release of IL-1β/IL-18 through the ROS-NLRP3-caspase-1 pathway.	[35]
Male ApoE$^{-/-}$ mice	WD STZ-induced diabetes	CANA 30 mg/kg/day 8 and 12 weeks	↓ Atherosclerotic lesion area ↑ EC-dependent vasorelaxation Ø EC-independent vasorelaxation ↓ ICAM-1, VCAM-1, MCP-1, F4/80, IL-6 aortic gene expr. ↓ iNOS, NOX2, NOX4, p22phox, p47phox aortic gene expr. ↓ Oxidative stress (urinary 8-OHdG) ↓ lipid deposition ↓ Macrophage infiltration ↓ ICAM-1 & VCAM-1 in aortic lesions		[44]

ApoE: Apolipoprotein E, ASC: PYCARD, Casp-1: Caspase-1, HFD: High fat diet, 4-HNE: 4-hydroxynonenal, ICAM-1: intercellular adhesion molecule 1, IL-1: Interleukin 1, IL-6: Interleukin-6, IL-18: Interleukin 18, iNOS: inducible nitric oxide synthase, LDL: low density lipoprotein, MCP-1: Monocyte Chemoattractant Protein-1, MMP-2: Metalloproteinase 2, NA: nicotinamide, NLRP3: NLR family pyrin domain containing 3, Nox2: NADPH oxidase 2, Nox4: NADPH oxidase 4, PECAM-1: Platelet endothelial cell adhesion molecule, PVAT: Perivascular adipose tissue, ROS: Reactive oxygen species, SMC: Smooth muscle cells, STZ: Streptozotocin, TG: Triglycerides, TNF-α: Tumor necrosis factor, VCAM-1: Vascular cell adhesion protein 1, VLDL: very low density lipoprotein, WD: Western diet

Table 3. Effects of SGLT2i treatment on vascular dysfunction in non-diabetic rodent models

Animals	Model/Vascular intervention	SGLT2i treatment	SGLT2i-related effects	Comments	Reference
Male C57BL/6J mice	WD I/R injury	EMPA 10 mg/kg/day 6 weeks pretreatment	↑ cardiac function ↓ infarct size Ø myocardial pAkt/tAkt, peNOS/teNOS, pGSK3β/tGSK3β, pERK1/2/tERK1/2, pAMPK/tAMPK expr. ↑ myocardial pSTAT3/tSTAT3 expr. Ø pNF-κB/tNF-κB expr. (WB) ↓ myocardial IL-6 and iNOS expr. (WB)	No effect of EMPA (50 nM - 50 μM) on calcium retention capacity of isolated heart mitochondria. EMPA limited infarct size by regulating the inflammatory response of the myocardium through STAT3 activation.	[52]
Male ApoE$^{-/-}$ mice	WD Atherosclerosis	EMPA 1 and 3 mg/kg/day 8 weeks	↓ aortic arch/valve atheroma burden ↓ Histiocyte and CD68+ cells infiltration ↓ weight, mass and fat % ↓ circulating AST/ALT ↓ circulating TNF-α, IL-6 and MCP-1, hsCRP	Anti-atherosclerotic properties of EMPA were attributable to the improvement of the inflammatory response of the vasculature, liver and fat tissues.	[49]
Male C57BL/6J mice	TAC (%EF <45%) HF	EMPA 10 mg/kg/day 2 weeks	↑ % EF ↑ cardiac output and work Ø structural remodeling indices	EMPA prevented progressive decline of cardiac functions caused by induced HF in non-diabetic mice.	[53]
Male C57BL/6J mice Male ApoE$^{-/-}$ adult mice aged mice	HFD Atherosclerosis	DAPA 1.0 mg/kg/day 4 weeks	↑ EC-independent vasorelaxation (C57BL/6J) ↑ EC-dependent vasorelaxation (adult and aged ApoE$^{-/-}$) Ø EC-independent vasorelaxation (adult ApoE$^{-/-}$) Ø % plaque area (adult ApoE$^{-/-}$) ↓ p-Iκβα, ICAM-1 and macrophage infiltration (aged ApoE$^{-/-}$)	DAPA effect in non-diabetic (ApoE$^{-/-}$) mice; independent on glucose regulation Anti-inflammatory effect of DAPA could contribute to the EC dysfunction attenuation/atherosclerosis progression.	[58]
Male C57BL/6J ApoE$^{-/-}$ mice	Ang II–induced AAA	EMPA 1 and 3 mg/kg/day 28 days	↑ survival rate ↓ maximal suprarenal aorta external diameter ↓ elastic laminae degradation ↑ smooth muscle α-actin cells ↓ macrophage infiltration ↓ MCP-1 and CCL-5 expr. ↓ MCP-1, CCL-5 and VEGF mRNA ↓ MMP-2 and MMP-9 expr., ↑ TIMP-1 expr. ↓ p38 MAPK and p65 NF-κB	Dose-dependent effect of EMPA SGLT2 detected in aortic tissue, located in CD31+ cells (ECs).	[30]
ZDF	Hypertension	EMPA 30 mg/kg/day 6 weeks	↓ systolic blood pressure ↓ cardiac volumes, wall thickness Ø cardiac output and LVEF	EMPA promoted cardioprotection in hypertensive ZDF rats while maintaining EC homeostasis/preventing low shear	[50]

hypertensive heart failure F1 hybrid (ZSF1)-HFpEF rats Lean control (ZL)			↑ EC-dependent vasorelaxation Ø EC-independent vasorelaxation ↓ senescence markers (p53, p21 and p16) (lean) ↑ eNOS expr. (lean) ↓ VCAM-1 expr. (lean) ↓ SGLT1/SGLT2 expr. (lean)	induced EC dysfunction in lean Zucker rats.	
Male Sprague-Dawley rats *Ex vivo* aortic rings	Left anterior descending coronary artery occlusion (LAD) I/R injury	CANA 3 ug/kg Single intravenous injection Ex vivo 10 µM	↓ myocardial infarct size ↑ cardiac function ↑ pAMPK/AMPK (sham) ↑ pACC/ACC, peNOS/eNOS ↑ pAkt/Akt (I/R injury) ↓ p47phox mRNA ↓ 4-HNE positive area ↑ EC-dependent vasorelaxation Ø EC-independent vasorelaxation	Bolus injection following I/R injury preserved cardiac function and modulated phosphorylation of cardioprotective signaling mediators, independent of metabolic changes.	[57]
Male Wistar rats	Anterior descending artery ligation MI	DAPA 0.1 mg/kg/day Phlorizin 0.4 g/kg/day 3days or 4 weeks	↓ LV superoxide ↓ LV nitrotyrosine ↑ pSTAT3/STAT3, nucleus translocation ↓ iNOS/CD68+ macrophages (M1) ↑ IL-10/CD68+ macrophages (M2) ↑ M2/M1 macrophage ratio ↓ IL-6, IL-1β, iNOS mRNA ↑ CD206, IL-10 mRNA ↓ α-SMA fibroblast ↓ collagen deposition	Protective impact of DAPA/Phlorizin through STAT3 signaling pathways Considering specificity and concentration used, DAPA should not inhibit SGLT1 effectively	[56]
Male C57BL/6 ApoE$^{-/-}$ mice	HFD Atherosclerosis	CANA 10 mg/kg/day 5 weeks	↓ blood cholesterol, serum TG, serum LDL ↓ aortic atherosclerotic plaque area Ø plaque collagen content ↓ aortic VCAM-1 (mRNA) and MCP-1		[51]
Male Sprague-Dawley rats	Deoxycorticosterone acetate (DOCA) hypertensive salt model	EMPA Mixed in chow 0.35mg/g 4 weeks	Ø systolic blood pressure ↓ LV weight/TL ↑ hematocrit and hemoglobin ↓ LV wall thickness ↓ wall stress ↓ cardiomyocyte size Ø LV fibrosis	No noticeable cardiovascular effect on non-hypertensive animals suggesting that effects induced by SGLT2i treatment might only become evident when intravascular volume is increased.	[54]

Male Wistar rats *Ex vivo* aortic rings	Thoracic aorta I/R injury	50 µM CANA	↑ EC-dependent vasorelaxation ↓ maximal contractility ↓ IL1a, IL6, CD40 mRNA ↓ NOXO1 mRNA ↓ ICAM-1 expr. ↑ PECAM-1 expr. ↓ nitro-oxidative stress (nitrotyrosine)		[59]
Male Dahl salt-sensitive rats	8% NaCl chow 5 weeks Hypertension	DAPA 0.1 mg/kg/day 6 weeks	↑ E/A ratio ↓ end-diastolic pression, IVRT ↑ Ca2+ transient amplitude ↓ cardiomyocyte diastolic [Ca2+] ↓ cardiomyocyte diastolic [Na+] ↓ CAMKII, $Na_v1.5$, NCX1 ↓ VCAM-1, E-selectin, NF-κB, MCP-1, IL-6 cardiac expr. ↑ eNOS cardiac expr. ↓ SGLT1 cardiac expr. ↓ NHE1 cardiac expr.	DAPA chronic treatment improved Ca2+ and Na+ homeostasis but no acute effect was found. pNF-κB Ser536 and NHE1 expression were principally located at the endothelium layer, suggesting EC implication in DAPA effect. DAPA might interfere with NHE1 activity in ECs.	[55]

α-SMA: Alpha-Smooth Muscle Actin, AAA: Abdominal aortic aneurysm, ACC: acetyl-CoA carboxylase, Akt: Protein kinase B (PKB), ALT: Alanine aminotransferase, AMPK: AMP-activated protein kinase, Ang II: Angiotensin II, AST: Aspartate aminotransferase, CAMKII: Ca2+/calmodulin-dependent protein kinase II, CCL-5: chemokine (C-C motif) ligand 5, E/A: early (E) and late (A) ventricular filling velocity ratio, eNOS: endothelial nitric oxide synthase, HFD: High fat diet, 4-HNE: 4-hydroxynonenal, ICAM-1: intercellular adhesion molecule 1, IL-1: Interleukin 1, IL-6: Interleukin-6, IL-10: interleukin-10, iNOS: inducible nitric oxide synthase, I/R: Ischemia/reperfusion, LDL: low density lipoprotein, IVRT: Isovolumetric relaxation time, LV: left ventricle, LVEF: left ventricle ejection fraction, MAPK: Mitogen-activated protein kinase, MCP-1: Monocyte Chemoattractant Protein-1, MMP-2: Metalloproteinase 2, MMP-9: Metalloproteinase 9, NCX1: Sodium-calcium exchanger 1, NF-κB: Nuclear factor-kappa B, NHE1: Sodium–hydrogen antiporter 1, NOXO1: NADPH oxidase organizer 1, PECAM-1: Platelet endothelial cell adhesion molecule, STAT3: Signal transducer and activator of transcription 3, TAC: Transverse aortic constriction, TIMP-1: TIMP metallopeptidase inhibitor 1, TG: Triglycerides, VCAM-1: Vascular cell adhesion protein 1, WD: Western diet

Table 4. Effects of SGLT2i on EC functions in cell culture experiments

Cells	EC dysfunction treatment	SGLT2i treatment	SGLT2i-related effects	Comments	Reference
ECs	AGE 1 mg/mL	EMPA 500 nM (cotreatment)	↑ Viability ↑ ATP content		[52]
HUVECs	VEGF-stimulation 10 ng/mL	EMPA 0.1-100 µM (cotreatment)	Ø Proliferation Ø Migration		[49]
HUVECs	Hyperglycemic 30 mM glucose	EMPA 1 and 10 µM (cotreatment, day 4-6)	↑ Viability/Density ↑ eNOS activity		[2]
HUVECs	Palmatic acid 300 µM	Phlorizin 50 nM Post treatment 30 min	↓ SGLT1/SGLT2 expr. ↑ Glucose uptake ↑ NO release ↑ pAKT, peNOS expr.	Conc/time effect on AKT activation by phlorizin PI3K inhibitor abolished pAKT and peNOS restoration siRNA SGLT1/SGLT2 attenuated PA dysfunction, indicating functional receptors	[26]
HUVECs HAECs	IL-1β stimulation 5-10 ng/mL	CANA DAPA EMPA 0.3-30 µM Pretreatment 15-30 min	↑ AMPK activation (CANA 10-30 µM) ↓ MCP-1 and IL-6 secretion (CANA 10) Ø Endothelin-1 secretion Ø ICAM-1, VCAM-1 and E-selectin expr. (CANA 10) ↓ U937 cell adhesion Ø Proliferation/Viability	SGLT2 protein expr. detected but not mRNA CANA and DAPA limited glucose uptake CANA limited inflammatory response through AMPK-dependent and transcriptional (independent) mechanisms	[28]
HUVECs	TNF-α stimulation 10 ng/mL Hyperglycemic (20-30 mM)	DAPA 1.0-5.0 nM (cotreatment)	↓ ICAM-1/VCAM-1 expr. ↓ NF-κB mRNA	DAPA-mediated transcriptional regulation of adhesion molecule expression	[58]
MAECs		EMPA 100 nM SOTA 500 nM 1h	↓ Glucose uptake (2-NBDG)	SGLT2 expr. confirmed by immunostaining EMPA limited glucose uptake	[29]
CMECs Isolated from diabetic/EMPA-treated animals	FCCP 5 µM 2h		↓ mitochondrial fission ↓ intracellular ROS ↓ mitochondrial ROS ↑ peNOS expr. ↓ ICAM-1 and VCAM-1 expr. ↓ Paracellular permeability ↑ AMPK activation	AMPK/Drp1-dependent EMPA beneficial effect through inhibition of mitochondrial fission Restore F-actin homeostasis and migration capacity	[39]
HUVECs HAECs		CANA 5-50 µM DAPA 30-50 µM	↓ DNA synthesis, ↓ cell proliferation	CANA disrupted cell cycle progression SGLT1/SGLT2 protein expr. detected	[25]

MAECs		EMPA 50 µM 24h	↓ Cyclin A expr.		
HAAECs	TNF-α stimulation 10 ng/mL Acute HepIII treatment Shear (10 dyn/cm^2)	EMPA 50 µM 24h (cotreatment)	↓ leukocyte (NB4) adhesion ↑ shear-induced elongation ↑ Heparan sulfate surface coverage		[62]
CMECs	TNF-α stimulation 10 ng/mL 6h	EMPA 1 µM (cotreatment)	↑ NO bioavailability Ø VCAM-1 and E-selectin expr. Ø VCAM-1, E-selectin and SOD2 mRNA Ø eNOS, NOX4 and SOD1 mRNA ↓ intracellular ROS ↓ mitochondrial ROS Ø pJNK expr.	EMPA restore normal EC-mediated (NO) CM contractility ROS level reduction by EMPA not through anti-oxidant effect	[60]
Porcine CAECs	High glucose 25 mM	EMPA 100 nM (cotreatment)	↓ senescence SA-β-gal activity ↓ senescence p21, p16 markers ↓ ROS ↓ p22phox, p47phox, Cox-2 ↑ eNOS expr. ↓ VCAM-1, TF, ACE, AT1R expr. (WB, 96h) ↓ Glucose uptake (2-NBD)	Comparison with H_2O_2 treated cells suggests that the effect of EMPA is mediated through regulation of oxidative stress	[23]
HUVECs	Ang II 1 µM	EMPA 0.1-3 µM pretreatment	↓ Leukocyte adhesion ↓ CCL-2 (MCP-1) and CCL-5 secretion ↓ ICAM-1 and VCAM-1 expr. ↓ p38 MAPK and p65 NF-κB	SGLT2 protein expr. detected EMPA limited/normalized Ang II mediated inflammatory response Concentration-dependent effect	[30]
HUVECs HCAECs	TNF-α stimulation 10 ng/mL	EMPA/DAPA 1 µM (cotreatment)	↓ ROS ↑ NO bioavailability Ø peNOS, eNOS expr. Ø Permeability Ø ICAM-1/VCAM-1 expr. Ø SGLT2 expr.	NO bioavailability restored through reduction of ROS, independent of peNOS SGLT2 protein expr. detected but not mRNA	[27]
HCAECs	LPS 1 µg/mL	CANA 3-10 µM DAPA 0.5-10 µM EMPA 1-10 µM (pretreatment)	↓ IL-6 release (CANA 10) ↓ HKII expr (Cana 10) Ø HK activity (Cana 10) ↓ pERK/ERK (Cana 10) ↑ pAMPK/AMPK (Cana 10)	Anti-inflammatory effect of CANA mediated through reduced HKII expression, ERK 1/2 phosphorylation and AMPK activation HKII not implicated in AMPK activation by CANA No effects for DAPA and EMPA	[63]
HCAECs		DAPA 1 µM	↓ pIRE1α	DAPA normalizes ER stress markers	[64]

	Tunicamycin 1 µM	(cotreatment)	↓ pPERK ↓ ATF6 ↓ GRP78		
HCAECs	10% stretch 24h	CANA 3 µM DAPA 1 µM EMPA 1 µM	↓ cell permeability ↓ intracellular ROS ↑ VE-Cadherin expr. (IF & WB) Ø IL-6 and IL-8 secretion	Improvement of barrier function by EMPA mediated through ROS mitigation (similar effects to NHE1 and NOX inhibitors)	[65]
TeloHAECs	High glucose 25 mM	CANA 5 µM pretreatment	↑ SIRT6 expr. ↓ SGLT2 expr. ↓ NF-κB expr. ↓ MMP-9 expr. ↓ ROS (intra/extracellular) ↓ IL-8, TNF-a, MCP-1, IL-6	SIRT6 silencing blocks the beneficial effects of SGLT2i and may act as an SGLT2 upstream regulator	[61]
Porcine CAECs	Ang II 100 nM	EMPA 100 nM (cotreatment)	Ø ROS 6h ↓ ROS 24h ↓ senescence p53 & p21 ↑ NO formation ↑ eNOS expr. ↓ VCAM-1, MCP-1, TF, ACE, AT1R expr.	Oxidative stress inhibitors normalized SGLT1/2 expression similar to EMPA Results suggest that SGLT1/2 have a role in the Ang II/AT1R/NADPH oxidase pro-oxidant response	[24]
HUVECs	LPS 20 ng/mL Glucose 5.5 or 25mM 24h	DAPA 0.005-100 µM	↓ cell viability (DAPA ≥ 25 µM) ↓ IL-6, IL-8 secretion (DAPA 0.005-0.05 µM) ↓ TLR-4, p65 NF-κB (DAPA 0.5 µM) ↑ miR-146a (DAPA 0.05-0.5 µM) ↓ miR-155 (DAPA 0.05-0.5 µM) ↓ M1/M2 macrophage ratio (DAPA 0.05 µM)	Most effects observed were independent of glucose conditions Similar effects of DAPA were observed on macrophages	[66]
HCAECs HUVECs	TNF-α 10 ng/mL	EMPA 1 µM	↓ NHE activation ↓ $[Na^+]_C$ ↓ intracellular ROS	EMPA reduces TNF-a induced ROS through NHE inhibition	[67]

ACE: angiotensin-converting enzyme, AGE: Advanced glycolysation end-product, Akt: Protein kinase B (PKB), AMPK: AMP-activated protein kinase, AT1R: anti-angiotensin type 1 receptor, ATF6: Activating transcription factor 6, ATP: adenosine triphosphate, Cox-2: cyclooxygenase-2, CMECs: Cardiac microvascular endothelial cells, Drp1: Dynamin-related protein 1, eNOS: endothelial nitric oxide synthase, Grp78: Binding immunoglobulin protein (BiP), HAECs: Human aortic endothelial cells, HCAECs: Human coronary artery endothelial cells, HKII: Hexokinase II, HUVECs: Human umbilical vein endothelial cells, ICAM-1: Intercellular adhesion molecule 1, IL-1β: Interleukin-1β, IL-6: IL-6: Interleukin-6, IRE1α: inositol-requiring enzyme 1 α, MAECs: mouse aortic endothelial cells, MAPK: Mitogen-activated protein kinase, MCP-1: Monocyte Chemoattractant Protein-1, MMP-9: Metalloproteinase 9, NF-κB: Tumor necrosis factor, NHE: Na+/H+ exchanger, NOX4: NADPH oxidase 4, PERK: Eukaryotic translation initiation factor 2-alpha kinase 3, pJNK: c-Jun

N-terminal kinase, ROS: Reactive oxygen species, SIRT6: Sirtuin 6, SOD: superoxide dismutase, TF: tissue factor, TLR4: Toll-like receptor 4, TNF-α: Tumor necrosis factor, VCAM-1: Vascular cell adhesion protein 1**,** VEGF: Vascular endothelial growth factor