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THÈSE

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Evaluation du rôle des co-transporteurs sodium-glucose SGLT1 et 2 dans l'induction de la sénescence et de la dysfonction des cellules endothéliales à l'aide d'une approche *in vitro* et *in vivo*

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ANIMUM FORTUNA SEQUITUR

Everyone has talent. What is rare is the courage to follow the talent to the dark place where it leads.

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LIST OF PUBLICATIONS

Publications

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2. Sonia Khemais-Benkhiat, Eugenia Belcastro, Noureddine Idris-Khodja, **Sin-Hee Park**, Lamia Amoura, Malak Abbas, Cyril Auger, Laurence Kessler, Eric Mayoux, Florence Toti, Valérie B. Schini-Kerth, "Angiotensin II-induced redox-sensitive SGLT2 expression promotes high glucose-induced endothelial cell senescence", Journal of Cellular and Molecular Medicine, 109, 461 (2019).
3. Kushal Sharma, Hyun-Ho Lee, Dal-Seong Gong, **Sin-Hee Park**, EunyoungYi, Valérie Schini-Kerth, Min-Ho Oak, "Fine air pollution particles induce endothelial senescence via redox-sensitive activation of local angiotensin system", Environmental Pollution, 252, 317-329 (2019)
4. Hira Hasan, **Sin-Hee Park**, Cyril Auger, Eugenia Belcastro, Kensuke Matsushita, Benjamin Marchandot, Hyun-Ho Lee, Abdul Wahid Qureshi, Gilles Kauffenstein, Patrick Ohlmann, Valérie B Schini-Kerth, Laurence Jesel, Olivier Morel, "Thrombin induces angiotensin II-mediated senescence in atrial endothelial cells: Impact on pro-remodeling patterns", Journal of clinical medicine, 8(10), 1570 (2019)
5. **Sin-Hee Park** et al., "The AT1R/NADPH oxidase pro-oxidant pathway induces expression of SGLT1 and 2 to sustain glucose and Na^+-dependent oxidative stress promoting endothelial dysfunction in response to Ang II and circulating microparticles of coronary artery disease patients", in preparation

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7. HH. Lee, **SH. Park** et al., “An anthocyanin-rich blackcurrant extract induced NO-mediated relaxation in coronary artery rings and eNOS phosphorylation in cultured endothelial cells: Role of sodium-glucose cotransporter 1 and 2.”, in preparation
8. MA. Farooq, L. Amoura, S. Gaertner, Z. Niazi, **SH. Park** et al., “Oral intake of EPA:DHA 6:1 improves ageing-related blunted endothelium-dependent relaxations and increased contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases”, in preparation
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6. S. Gaertner, MA. Farooq, B. Pollet, L. Amoura, S. Khemais-Benkhiat, **SH. Park** et al., “Ageing-related endothelial dysfunction in the femoral vein is mediated by cyclooxygenases: Role of thromboxane prostanoid receptors”, European Society of Cardiology Congress, August 2018, Munich(Germany)
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8. MA. Farooq, L. Amoura, S. Gaertner, Z. Niazi, **SH. Park** et al., “Oral intake of EPA:DHA 6:1 improves ageing-related blunted endothelium-dependent relaxations and increased contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases”, European Society of Cardiology Congress, August 2017, Barcelona(Spain) & International Conference on the Mechanism of Action of Nutraceuticals and the International Union of Basic and Clinical Pharmacology; Natural Products Section, September 2017, Aberdeen(Scotland) & Doctoral School Days, March 2018, Strasbourg

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ABBREVIATIONS

AA: arachidonic acid

ACE: angiotensin-converting enzyme

ACh: acetylcholine

ADP: adenosine di-phosphate

AGEs: advanced glycation end-products

ARB: angiotensin receptor blocker

ASC: apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain

AT_1R: angiotensin II type 1 receptor

AT_2R: angiotensin II type 2 receptor

ATP: adenosine tri-phosphate

Ang II: angiotensin II

BH2: dihydrobiopterin

BH4: tetrahydrobiopterin

BK: bradykinin

BK_{Ca}: calcium-sensitive potassium channels of large conductance

COX: cyclooxygenase

CVD: cardiovascular disease

DM: diabetes mellitus

ECEs: endothelin converting enzymes

ECs: endothelial cells

ED: endothelial dysfunction

EDCFs: endothelium-dependent contractile factors

EDH: endothelium-derived hyperpolarization

EDRF: endothelium-derived relaxing factor

EETs: epoxyeicosatrienoic acids

EMA: European Medicines Agency

EPCR: endothelial protein C receptor

ET-1: endothelin-1

FAK: focal adhesion kinase

FDA: Food and Drug Administration

GTP: guanosine triphosphate

H_2O_2: hydrogen peroxide

HAECs: human aortic endothelial cells

HF: heart failure

HFpEF: heart failure with preserved ejection fraction

HNF: hepatocyte nuclear factor

HR: hazard ratio

HUVECs: human umbilical vein endothelial cells

Hsp90: heat shock protein

ICAM-1: intercellular adhesion molecule

IFG: impaired fasting glucose

IRS-1: insulin receptor substrate-1

JNK: jun C-terminal kinase

KLF2: krüppel-like factor 2

LDL: low density lipoprotein

LOX-1: lectin-type oxidized LDL receptor-1

MAPK: mitogen activated protein kinase

MCP-1: monocyte chemoattractant protein-1

MMP: matrix metalloproteinase

MPs: microparticles

MitoNCX: mitochondrial NCX

NCX: Na^+/Ca^{2+} exchanger

NF-κB: nuclear factor κB

NHE: Na^+/H^+ exchanger

NKA: Na^+/K^+-ATPase

NLRP3: nucleotide-binding domain leucin-rich repeat [LRR] and pyrin-containing receptor 3

NO: nitric oxide

O_2^-: superoxide anion

OH·: hydroxyl radical

$ONOO^-$: peroxynitrite

PA: palmitic acid

PAI-1: plasminogen activator inhibitor-1

PARs: protease activated receptors

PDGF: platelet derived growth factor

PECAM-1: platelet–endothelial cell adhesion molecule-1

PGG_2: prostaglandin G_2

PGH_2: prostaglandin H_2

PGI_2: prostacyclin

PKA: protein kinase A

PKC: protein kinase C

PYK2: proline-rich tyrosine kinase 2

RAS: renin-angiotensin system

ROS: reactive oxygen species

SA-β-gal: senescence-associated β-galactosidase

SASP: senescence-associated secretory phenotype

SGLTs: sodium-glucose cotransporters

SHRSP: stroke-prone spontaneously hypertensive rats

SMCs: smooth muscle cells

SOD: superoxide dismutase

STAT1: signal transducer and activator of transcription-1

STZ: streptozotocin

T2DM: type 2 diabetes mellitus

TCA: tricarboxylic acid

TGF-β : transforming growth factor

TNF-α: tumor necrosis factor-alpha

TXA_2: thromboxane A_2

VCAM-1: vascular cell adhesion molecule-1

ZSF1: zucker diabetic fatty/Spontaneously hypertensive heart failure F1 hybrid

cAMP: cyclic adenosine monophosphate

cGMP: cyclic 3′,5′-guanosine monophosphate

eNOS: endothelial nitric oxide synthase

ox-LDL: oxidized-low density lipoprotein

sGC: soluble guanylyl cyclase

t-PA: tissue-type plasminogen activator

vWF: von Willebrand factor

INTRODUCTION

Chapter I

The endothelium

I.1 Anatomy and physiology of the blood vessels

The blood vessels which supply the organs with nutrients, gases and other substances by circulating blood throughout the body are the key connection between the heart and the tissues. Various types of blood vessels that differ in some structures have the same general characteristics. Arteries and arterioles which carry blood away from the heart have thicker walls and smaller lumens than veins and venules which return blood to the heart to resist surge of blood pressure. Between arterioles and venules, the capillaries exchange nutrients and wastes surrounding tissues through the thin walls.

The vascular wall of arteries and veins consists of three distinct layers, called tunics: the tunica intima, the tunica media and the tunica externa.

The tunica intima (also called the tunica interna) which is the most interior layer is comprised of a single layer of endothelial cells (ECs) in contact with blood and connective tissues. In case of the artery, there is also internal elastic membrane (also called the internal elastic lamina) which is a thick layer of elastic fibers at the frontier with the tunica media. The endothelium is continuous with endocardium lining the inner heart chambers and not only plays a role in separating the blood from the vessel wall as a physical barrier but also has physiologically critical functions contributing to regulate vascular tone, inflammation, and coagulation in vascular biology.

The tunica media is the middle layer composed of circularly organized strands of smooth muscle cells (SMCs) sustained by elastic fiber and connective tissue. This smooth muscle layer is responsible for controlling contraction and relaxation by neuronal and chemical mechanisms to regulate blood flow and blood pressure.

The tunica externa (also called the tunica adventitia) which is the outermost layer mostly made up of collagenous connective fibers functions to protect and hold the vessel in relative circumstance (Marieb 2004) (Figure 1).

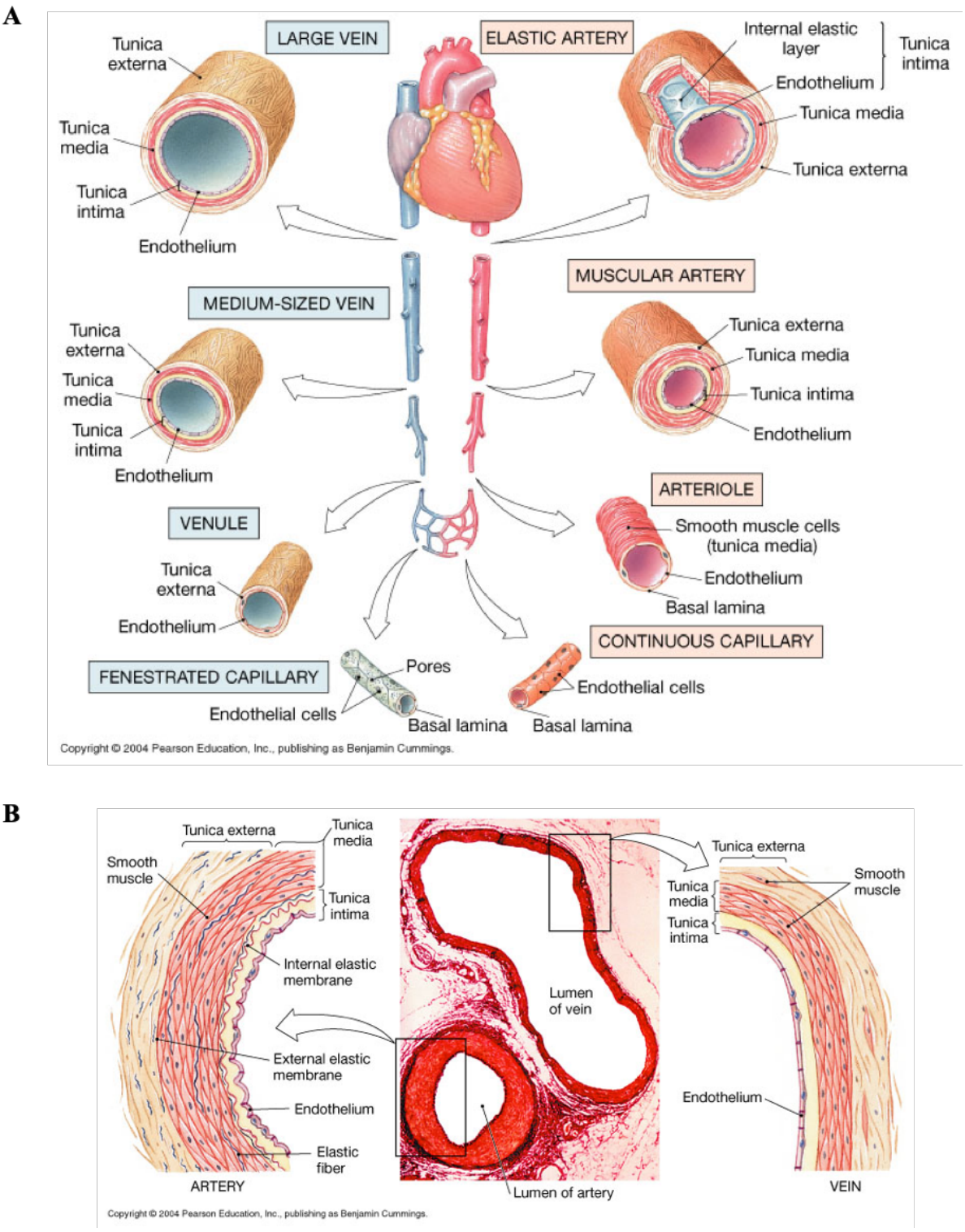

Figure 1. Structure of blood vessels. (A) Differential types of blood vessels. (B) Comparison of tunics in artery and vein (© Pearson Education, Inc. 2004).

I.2 Vascular endothelium

The surface of ECs which have various shape across the vascular tree, but typically thin and slightly elongated in the direction of blood flow, is constituted by approximately 1 to 6 X 10^{13} cells and weighs about 1 kg covering a surface area of more than 1,000 m^2 in an adult human body (Jaffe 1987). The ECs serve as a central regulator in sustaining vascular homeostasis interacting with numerous released autocrine and paracrine substances in response to biological, physical and chemical stimuli. In fact, because of their morphology and location in direct contact with circulating blood, ECs have a very special localization to modulate diverse vascular biological responses, including thrombosis, leukocyte trafficking, vascular tone, platelet aggregation, inflammation and angiogenesis (Gori et al. 2007).

Under physiological conditions, the blood stream is in contact with the endothelium that controls the appropriate hemostatic balance between procoagulant and anticoagulant systems and helps blood to remain fluid by providing anticoagulant and antiplatelet surface inhibiting clotting and platelet adhesion. ECs express anticoagulant factors such as endothelial protein C receptor (EPCR), thrombomodulin, tissue factor pathway inhibitor, heparan, ecto-ADPase, tissue-type plasminogen activator (t-PA), prostacyclin (PGI_2), and nitric oxide (NO). Whereas following vascular damage and under pathological conditions, the endothelium shifts the balance to a procoagulant/prothrombotic phenotype by synthesizing tissue factor, plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), thromboxane A_2 (TXA_2), and protease-activated receptors (PARs) (Bombeli et al. 1997; Rodgers 1988; Gross & Aird 2000).

The passage of leukocytes from the circulatory system to the surrounding sites of vascular injury through the endothelium is a critical event in the inflammatory process of atherogenesis involving a multistep molecular cascade that includes capture, rolling, initial attachment, arrest, and transmigration. The recruitment of leukocytes at sites of infection or

injury is mediated by preformed components expressed on the endothelial surface, containing Weibel-Palade bodies and their major components, vWF, and P-selectin which mediate both leukocyte and platelet adhesion. It is concluded by firm adhesion between leukocyte integrins and immunoglobulin family, such as platelet–endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) or junctional adhesion molecule and transmigration into inflamed site (Albelda & Buck 1990; Carlos & Harlan 1994).

Another crucial function of endothelium is to regulate the transport of solutes via a passively paracellular route and macromolecules using either transcellular or paracellular routes across the semi-permeable blood vessel endothelial barrier (Minshall & Malik 2006).

I.3 Key role of the endothelium in vascular homeostasis

The endothelium contributes to the regulation of vascular tone and the function of smooth muscle and circulating blood cells via well controlled release of various regulatory substances in response to both mechanical and humoral stimuli (Figure 2).

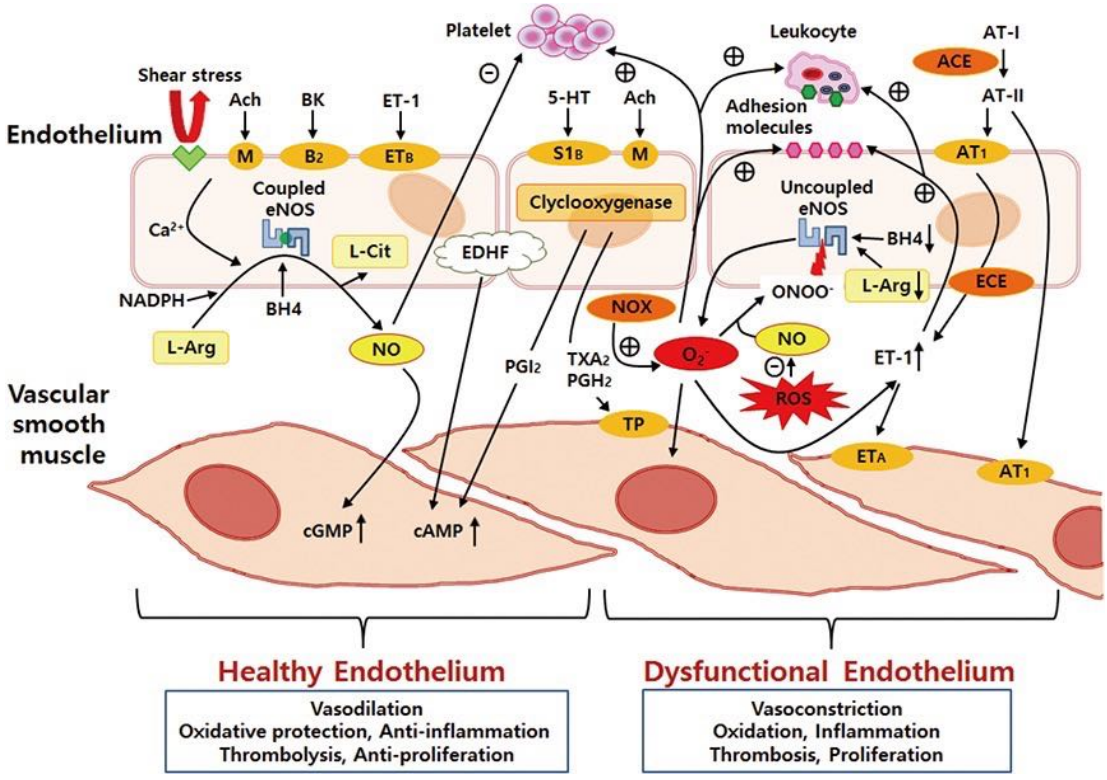

Figure 2. The release of various endothelium-derived regulatory substances and their effects on the smooth muscle and circulating blood cells (Park & Park 2015). ACE, angiotensin-converting enzyme; Ach, acetylcholine; AT-I, angiotensin I; AT-II, angiotensin II; AT_1, angiotensin 1 receptor; BH4, tetrahydrobiopterin; BK, bradykinin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ECE, endothelin converting enzyme; eNOS, endothelial nitric oxide synthase; EDHF, endothelium derived hyperpolarizing factor; ET_A and ET_B, endothelin A and B receptors; ET-1, endothelin-1; L-Arg, L-arginine; L-Cit, L-citrulline; M, muscarinic receptor; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric

oxide; NOX, nicotinamide adenine dinucleotide phosphate oxidase; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; ROS, reactive oxygen species; $S1_B$, serotonin receptor; TP, thromboxane prostanoid receptor; TXA_2, thromboxane; 5-HT, serotonin; Θ, inhibition; ⊕, stimulation.

I.3.1 Endothelium-derived relaxing factors

I.3.1.1 Nitric oxide

The presence of an endothelium-derived relaxing factor (EDRF) was recognized by Furchgott and Zawadzki in 1980. They observed that the relaxation of rabbit aortic rings by acetylcholine (ACh) is dependent on the intact endothelium (Furchgott & Zawadzki 1980). A few years later, it was revealed that EDRF was NO released from ECs (Palmer et al. 1987; Furchgott 1988). In the intact blood vessel, NO which is a free radical gas with a short half-life is produced by endothelial NO synthase (eNOS) in ECs through the oxidation of the amino acid L-arginine to L-citrulline (Palmer et al. 1988).

Under normal conditions, eNOS remains inactive by binding to the structural protein caveolin-1 located in caveolae, small invaginations of the cell membrane (Bucci et al. 2000). When agonists acting on membrane receptors such as ACh, bradykinin (BK), adenosine di-phosphate (ADP), adenosine tri-phosphate (ATP), thrombin and substance P increase the intracellular level of calcium, the displacement of eNOS from caveolin-1 to calcium/calmodulin occurs followed by the activation of eNOS (Topper et al. 1996). eNOS is the predominant enzyme in the regulation of vascular tone since eNOS knockout mice show increased systemic and pulmonary arterial pressures and endothelium-dependent relaxations in response to ACh of large arteries is markedly reduced (Huang et al. 1995; Chataigneau et al. 1999; Brandes et al. 2000). On the contrary, eNOS-overexpressing transgenic mice are hypotensive (Ohashi et al. 1998). NO generated from ECs diffuses into the adjacent SMCs,

where it binds to the enzyme soluble guanylyl cyclase (sGC) by targeting the ferrous heme prosthetic group. The activated sGC enzyme catalyzes the increased conversion rate of guanosine triphosphate (GTP) to cyclic 3′,5′-guanosine monophosphate (cGMP) and subsequent relaxation of the vascular SMCs (Ignarro 1991).

In addition, shear stress evokes NO production by activating specialized calcium-activated potassium channels on the ECs surface, causing calcium entry into the cell (Cooke et al. 1991). In the first phase, the prompt separation to caveolin-1 and the following interaction with heat shock protein (Hsp90), which is a chaperon protein, are observed. Then, the protein kinase A (PKA)-dependent phosphorylation of eNOS at Ser1177 is immediately stimulated. In the second phase, the proline-rich tyrosine kinase 2 (PYK2)-dependent phosphorylation of the eNOS inhibitor site Tyr657 and the PKA-dependent phosphorylation of the eNOS activator site Ser633, contribute to control NO production (Fleming 2010; Boo & Jo 2003; Balligand et al. 2009).

Apart from vasorelaxation, endothelium-derived NO inhibits the adhesion and aggregation of platelets (Nong et al. 1997), the activation of leukocytes (Kubes et al. 1991) as well as the migration (Marks et al. 1995) and proliferation (Garg & Hassid 1989) of SMCs. Furthermore, inhibition of eNOS promotes advanced atherosclerosis in mice and rabbits (Kauser et al. 2000; Cayatte et al. 1994). The apoE$^{-/-}$ mice with the additional deletion of eNOS also showed enhanced progression of atherosclerosis and abdominal aortic aneurysm generation and ischemic heart disease, indicating that endogenous eNOS-derived NO plays a major role in anti-atherosclerotic process (Kuhlencordt et al. 2001; Chen et al. 2001).

I.3.1.2 Prostacyclin

Prostaglandin G_2 (PGG_2), Prostaglandin H_2 (PGH_2), and PGI_2 derived from the arachidonic acid (AA) are major products of vascular cyclooxygenase (COX). There are two isoforms of COX encoded by two distinct genes. COX-1 is constitutively expressed in many tissues including ECs, and can also be overexpressed, for example, in ECs, by shear stress (Doroudi et al. 2000). Both ECs and to a lesser extent SMCs of healthy blood vessels express COX-1 being the prominent isoform under steady states or under chronic shear stress (Tang & Vanhoutte 2008; Potter et al. 2011; DeWitt et al. 1983). COX-2 is not constitutively expressed, but can be induced rapidly and transiently when the endothelium is disturbed or exposed to inflamed states. PGI_2 is produced from PGH_2 by prostacyclin synthase (Coleman et al. 1994). PGI_2 acting as a paracrine factor binds to the IP receptor which is present on both vascular SMCs and platelets to inhibit vasoconstriction and platelet aggregation, respectively, by activating adenylyl cyclase and the subsequent formation of the second messenger cyclic 3',5'-adenosine monophosphate (cAMP) (Nicosia et al. 1987). In fact, the effect of PGI_2 on endothelium-dependent responses is enhanced in eNOS knockout mice (Sun et al. 1999). In coronary arteries of obese Zucker rats and in the mesenteric vascular bed of streptozotocin (STZ)-induced diabetic mice, endothelial dysfunction is improved by a compensatory up-regulation of the expression and activity of COX-2 (Sánchez et al. 2010; Nacci et al. 2009). Likewise, in coronary arterioles and forearm blood flow of humans with diabetes and hypertension, COX-2-derived PGI_2 contributes as a compensatory factor for the diminished bioavailability of NO (Bulut et al. 2003; Meeking et al. 2000; Szerafin et al. 2006).

I.3.2 Endothelium-derived hyperpolarization

Endothelium-derived hyperpolarization (EDH), which causes hyperpolarization of the neighboring smooth muscle by inducing a more negative cell membrane potential, also plays significant role in the endothelium-dependent regulation of vascular tone (Feletou & Vanhoutte 1988). In general, EDH-mediated responses involve an increase in calcium levels resulting in potassium efflux via the stimulation of calcium-sensitive potassium channels of small and intermediate conductance in ECs. The SMCs respond to the transmission by electrical coupling through myo-endothelial gap junctions and/or discharge of potassium into the intercellular space, leading to hyperpolarizaion and relaxation (Edwards & Weston 2004; Sandow & Hill 2000) (Figure 3).

In addition, the endothelium releases substances that evoke hyperpolarization of the SMCs such as lipoxygenases derivatives (Faraci et al. 2001), epoxyeicosatrienoic acids (EET) (Campbell et al. 1996) and reactive oxygen species (Ellis & Triggle 2003). EETs, derived from cytochrome P450 epoxygenases, especially play a substantial role in endothelium-dependent hyperpolarization and relaxation of smooth muscle by opening of calcium-sensitive potassium channels of large conductance (BK_{Ca}) in several vascular beds (Quilley & McGiff 2000) such as large coronary arteries from dogs (Widmann et al. 1998), cows and pigs as well as human (Miura et al. 1999). In isolated porcine and bovine coronary arteries and in cultured ECs including those of human origin, receptor-dependent and -independent agonists such as ACh, BK, AA and pulsatile stretch prompt EETs release from ECs, an effect blocked by cytochrome P450 inhibitors (Popp et al. 1998; Rosolowsky & Campbell 1996; Popp et al. 1996; Gebremedhin et al. 1998; Fisslthaler et al. 1999; Campbell et al. 1996).

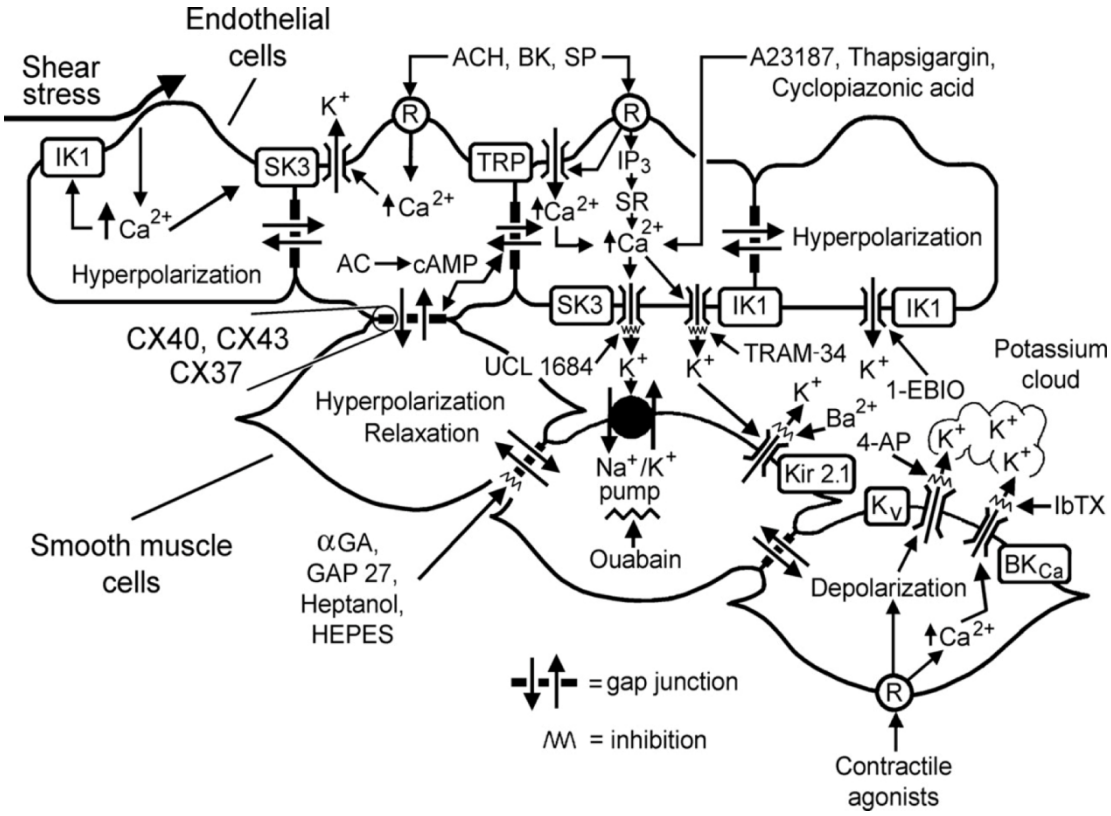

Figure 3. Endothelium-derived hyperpolarization (EDH)-mediated responses (Feletou & Vanhoutte 2006). R indicates receptor; Bk, bradykinin; SP, substance P; IP3, inositol trisphosphate; SR, sarcoplasmic reticulum; TRP, transient receptor potential channel; AC, adenylyl cyclase; cAMP, cyclic-AMP; αGA, glycyrrhetinic acid derivatives; CX, connexin; 4-AP, 4-aminopyridine; IbTX, iberiotoxin; SK3, small conductance calcium-activated potassium channel formed by SK3 α-subunits; IK1, intermediate conductance calcium-activated potassium channel formed by IK1 α-subunits; Kir2.1, inward rectifying potassium channel constituted of Kir2.1 α-subunits; K_V, voltage-gated potassium channels; BK_{Ca}, large conductance calcium-activated potassium channels.

I.3.3 Endothelium-derived contracting factors

I.3.3.1 Endothelin-1

Endothelin-1 (ET-1), which is generated from big ET-1 by endothelin converting enzyme (ECE), acts as a prominent endogenous vasoconstrictor peptide (Yanagisawa et al. 1988) and is also produced by vascular SMCs, macrophages and leukocytes (Ehrenreich et al. 1990; Sessa et al. 1991). The effects of ET-1 appear through the interaction with two G-protein-coupled receptors, ET_A which has a high affinity for ET-1 and is predominately expressed on vascular SMCs, and ET_B which is also localized on the ECs as well as SMCs (Arai et al. 1990; Sakurai et al. 1990). Binding to ET_A receptor contributes to increase the intracellular calcium concentration resulting in contraction of SMCs. By contrast, the activation of ET_B receptor on the ECs counteracts the vasoconstricting action of ET-1 on the SMCs by promoting the release of NO and PGI_2, two potent vasodilators (de Nucci et al. 1988). In fact, an *in vivo* study using ECs-specific ET-1 knockout mice has shown that endothelium-derived ET-1 sustains basal vascular tone and blood pressure (Kisanuki et al. 2010). In a human study with normotensive subjects, it was observed that both an inhibitor of ECE and a selective ET_A antagonist prevented forearm vasoconstriction by proET-1 (Haynes & Webb 1994).

I.3.3.2 Thromboxane A_2 & Prostaglandin H_2

The effects of PGI_2 counterbalance those of TxA_2 that is converted from AA into PGG_2/PGH_2 through COX-1 and the following synthesis by thromboxane synthase. TxA_2 leads to platelet aggregation and vasoconstriction by binding to the TP receptor to contribute to maintain cardiovascular homeostasis (Moncada & Vane 1979). $TP^{-/-}$ mice are

normotensive but show abnormal vascular responses to TxA_2 and have a higher sensitivity to bleeding (Thomas et al. 1998). The elimination of TP receptors alleviates vascular proliferation and activation of platelets in response to vascular injury in mice (Cheng et al. 2002), delays atherogenesis in $apoE^{-/-}$ mice (Kobayashi & Narumiya 2002), prevents angiotensin II (Ang II)- and L-NAME-induced hypertension and the subsequent cardiac hypertrophy (Francois et al. 2008; Francois et al. 2004). Moreover, in $apoE^{-/-}$ mice and in patients with atherosclerosis, thromboxane synthase mRNA is expressed within the vascular wall of mice atherosclerotic aorta and in the core of human atherosclerotic lesions, and is associated with increased generation of TxA_2, contributing to plaque instability and its thrombotic complications (Gabrielsen et al. 2010).

I.3.3.3 Angiotensin II

Ang II, the active peptide generated from angiotensinogen by the circulating renin-angiotensin system (RAS), plays an essential role in controlling vascular homeostasis, and also in the initiation and development of hypertension, heart failure and atherosclerosis (Kim & Iwao 2000; Schmidt-Ott et al. 2000; Dzau 1986) (Figure 4). The action of both circulating and local Ang II, which is generated from angiotensin I by angiotensin-converting enzyme (ACE) expressed on the ECs, are mediated by activating Ang II type 1 receptor (AT_1R) or type 2 receptor (AT_2R) (de Gasparo et al. 2000). AT_1R is mainly expressed in different tissues and cells associated with cardiovascular function such as vascular smooth muscle contraction and proliferation. The activation of AT_1R facilitates numerous physiological and pathophysiological activities of Ang II via complicated intracellular signaling pathways and the generation of ROS through the activation of NAD(P)H oxidase. Conversely, the AT_2R is highly expressed in the fetal development, and its expression is very low in the adult cardiovascular system. The role of the AT_2R and signaling pathways induced

by its stimulation remain under debate. However, it appears that the AT_2R plays a role in vasodilation, and may be increased as a counteracting mechanism in cardiac hypertrophy, hypertension and atherosclerosis (Lemarié & Schiffrin 2010).

The rise in oxidative stress stimulated by Ang II leads to diminished endothelial relaxation and endothelial dysfunction in experimental hypertensive rat model (Rajagopalan et al. 1996). In humans with elevated RAS activity, ROS-mediated endothelial dysfunction associated with vascular growth and inflammation has been involved in the formation of atheroma (Kolakovic et al. 2017). Ang II also acts as a proatherosclerotic factor causing vasoconstriction and stimulates the expression not only of cell adhesion molecules (CD31/PECAM-1, VCAM-1, ICAM-1) but also of growth factors, cytokines, and chemokines within the vascular wall (Touyz & Schiffrin 2000; Ushio-Fukai et al. 2002; Touyz & Schiffrin 1999).

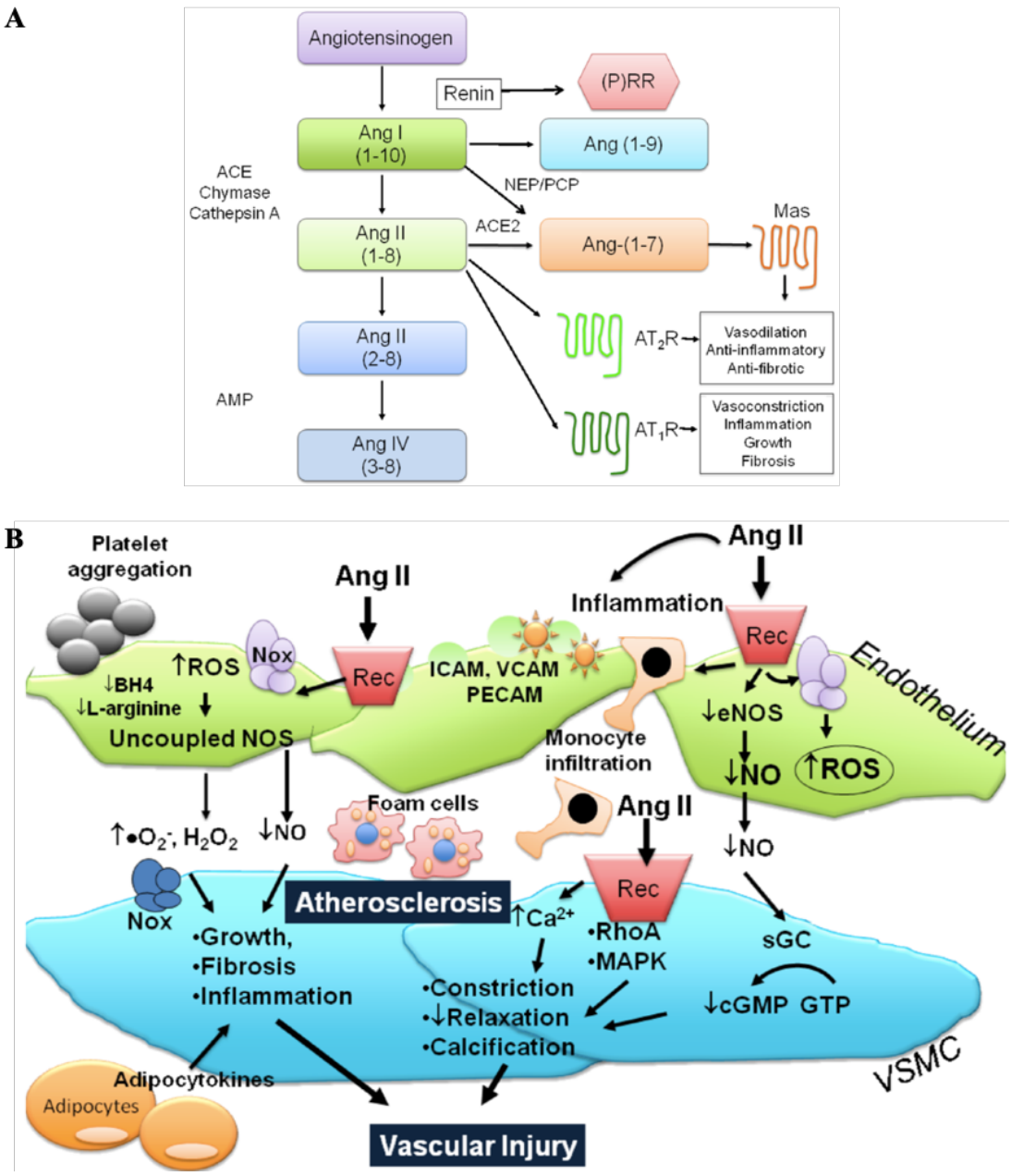

Figure 4. Schematic describing the circulating renin-angiotensin system (RAS) and the vascular effects induced by angiotensin II (Ang II). (A) The components of RAS and its receptors. (B) The role of Ang II on vascular cell functions (Montezano et al. 2014).

I.4 Endothelial dysfunction and cardiovascular diseases

Endothelial dysfunction (ED) is a well-established early step associated with the accumulation of cardiovascular risk factors and leads to the development of atherosclerosis (Anderson et al. 1995; Kinlay & Ganz 1997) (Figure 5). The deleterious changes of endothelial function are characterized by an imbalanced contribution between the bioavailability of vasodilators and endothelium-derived contracting factors, causing an impaired endothelium-dependent vasodilation (Lerman & Burnett 1992). Furthermore, specific states of endothelial activation, which are proinflammatory, proliferative, and procoagulatory responses, favor the progressive development of atherogenesis (Anderson 1999). Therefore, the status of endothelial function has been suggested to be an important prognostic marker of future cardiovascular events in patients with cardiovascular risk factors (Gokce et al. 2003).

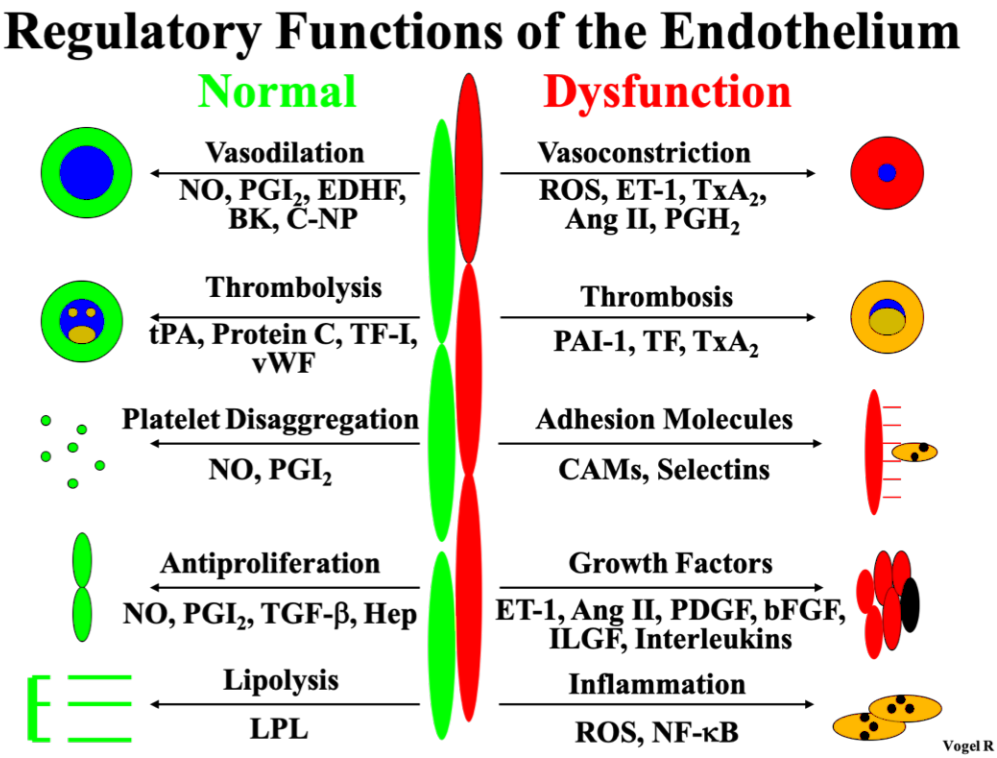

Figure 5. The differences between normal and dysfunctional endothelium (Vogel). NO, nitric oxide; PGI_2, prostacyclin; EDHF, endothelium derived hyperpolarizing factor; BK,

bradykinin; C-NP, C-type natriuretic peptide; ROS, reactive oxygen species; ET-1, endothelin-1; TXA_2, thromboxane; Ang II, angiotensin II; PGH_2, prostaglandin H_2; t-PA, tissue-type plasminogen activator; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; CAMs, cell adhesion molecules; TGF-β, transforming growth factor; PDGF, platelet derived growth factor; bFGF, basic fibroblast growth factor; ILGF, insulin-like growth factor; LPL, lipoprotein lipase; NF-κB: nuclear factor κB.

I.4.1 Atherosclerosis

ED is an early indicator of the development of atherosclerosis, which is observed before structural alterations of the vascular wall are detectable by ultrasound or angiography (Luscher & Barton 1997). The early stages of atherosclerosis are characterized by a reduced NO bioavailability due to an increased level of ROS in response to cardiovascular risk factors (Warnholtz et al. 1999). The antiatherogenic role of NO is described in several experimental models of atherosclerosis such as $apoE^{-/-}$ mice as well as in humans. In these studies, the reduced endothelial NO formation facilitates the development of atherosclerotic lesion formation in the coronary artery and the aorta, and treatment of L-arginine retains vessel morphology. The increase in superoxide anion production is involved in the reduced bioavailability of NO in arteries predisposed to atherosclerosis (Cai & Harrison 2000; Landmesser & Harrison 2001). The enhanced ROS generation involves NADPH oxidase (Spiekermann et al. 2003), xanthine oxidase (White et al. 1996; Berry et al. 2000) and leads to the degradation of NO by O_2^- and the oxidation of tetrahydrobiopterin (BH4) into dihydrobiopterin (BH2), which is not a cofactor of eNOS (Vásquez-Vivar et al. 2002; Tiefenbacher et al. 2000; Laursen et al. 2001; Landmesser et al. 2003). In addition, the pathological endothelial phenotype characterizing a dysfunctional state participates in the early and late stages of atherosclerotic lesion formation by increasing ECs permeability,

expression of adhesion molecules, cytokines and chemokines release, recruitment of leukocytes, enhanced low density lipoprotein (LDL) oxidation, activation of platelets, and VSMCs migration and proliferation. Furthermore, ED is not only the initial stage of the progression of atherosclerotic disease that occurs plaque formation, but it also can promote plaque growth leading to clinical cardiovascular complications, as one of the essential mechanisms in atherosclerotic diseases (Hegele 1996; Willerson 2002; Chiu & Chien 2011) (Figure 6).

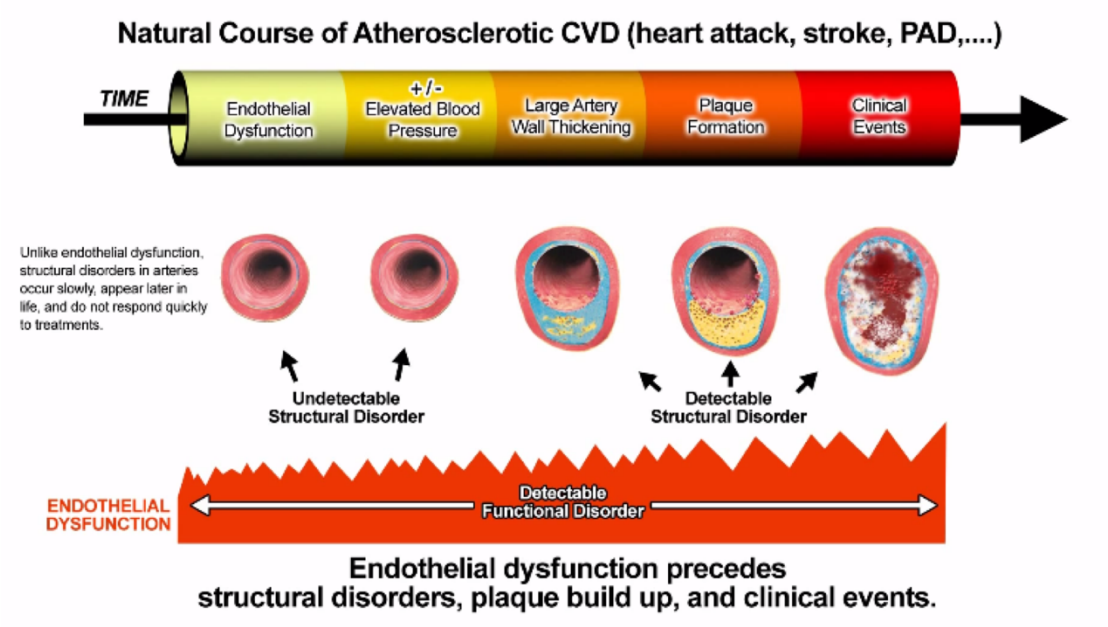

Figure 6. Endothelial dysfunction as an early indicator in the initiation and progression of atherosclerosis (© Cenegenics Medical Institute of Phoenix, AZ).

I.4.2 Aging

Aging is a physiological process causing a progressive worsening of structure and function of organs and is an independent cardiovascular risk factor for ED even in the absence of other risk factors such as diabetes, smoking, hypertension (Zeiher et al. 1993). Endothelial senescence, an irreversible phenomenon of cell cycle arrest, is known as both replicative senescence by aging over the time and premature senescence induced by pathological conditions with an increased expression of senescence-associated β-galactosidase (SA-β-gal) activity and p16INK4a (Dimri et al. 1995; Chen et al. 2006) (Figure 7). The senescent ECs are characterized by a prooxidative, and proinflammatory senescence-associated secretory phenotype (SASP) via the two major tumor suppressor pathways, known as the p53/p21 also involved in apoptosis and the p16/pRB pathways (Coppé et al. 2010). Several studies have shown that a variety of stimuli involved in cardiovascular disease promote endothelial senescence by increasing the intracellular level of oxidative stress and/or telomerase activity. Exposure of endothelial progenitor cells to Ang II promotes the onset of senescence through NADPH oxidase-dependent prooxidant response (Imanishi et al. 2005). Incubation of HUVECs with either high glucose or advanced glycation end-products (AGEs) increases SA-β-gal activity (Yokoi et al. 2006; Chen et al. 2002). Exposure of ECs to ox-LDL also promotes ECs senescence by impairing telomerase activity (Breitschopf et al. 2001). In addition, an increased SA-β-gal staining is observed in the thoracic aorta of Zucker diabetic rats (Chen et al. 2002) and mice model of oxidative stress (Ota et al. 2008). Furthermore, senescent ECs appear in human aortic arch and coronary arteries at sites overlapping atherosclerotic plaques (Vasile et al. 2001; Minamino et al. 2002).

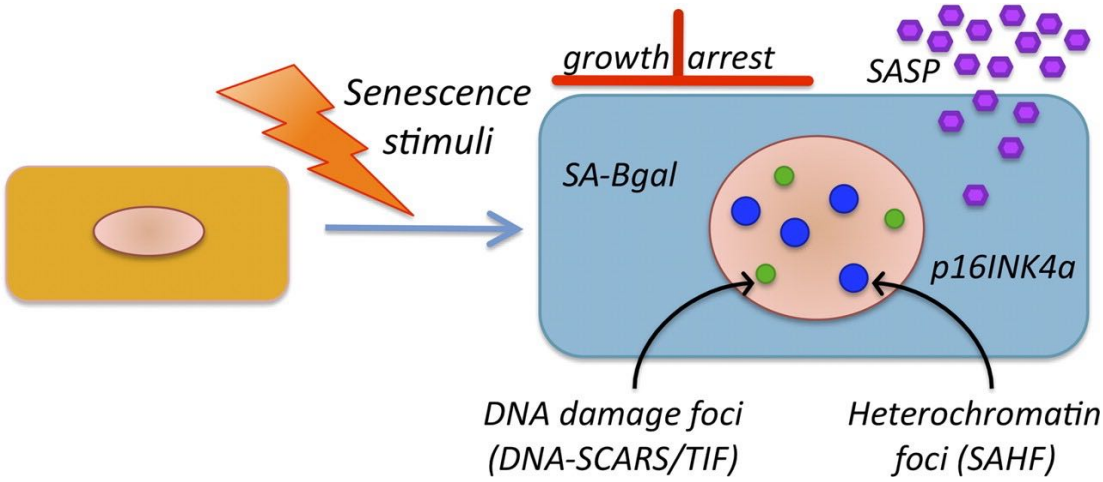

Figure 7. A principle features of senescence cells (Rodier & Campisi 2011).

I.4.3 Hypertension

Elevated blood pressure is a crucial risk factor associated with increasing prevalence of myocardial infarction, heart failure, and stroke, resulting in higher cardiovascular mortality (Levy et al. 1996; Stamler et al. 1989). The hypertensive-associated ED is a common state characterized by an imbalance between endothelium-derived vasodilators and vasoconstrictor factors and increased oxidative stress which has an important role in the decreased bioavailability of NO, and antioxidant capacity. In fact, an enhanced oxidative stress in patients with essential hypertension causes lower NO availability leading to ED (Taddei et al. 1998). Antioxidants such as vitamins C and E ameliorate endothelium-dependent relaxation of mesenteric artery rings in stroke-prone spontaneously hypertensive rats (SHRSP) by decreasing NADPH oxidase activation and by increasing superoxide dismutase (SOD) activity (Chen et al. 2001). In addition, an elevation in Ang II levels influences an increase in blood pressure and sustenance of hypertension through the activation of NADPH oxidase to produce ROS and the inhibition of the angiotensin system improved ED in hypertensive state (Vanhoutte & Tang 2010; Park et al. 2001; Schiffrin et al. 2000). Schiffrin et al reported an

upregulation of Ang II receptors in mesenteric arteries of DOCA-salt hypertensive rats (Schiffrin et al. 1983). Koh et al. identified that the treatment of hypertensive patients with the AT_1R antagonist candesartan resulted in improved endothelial function, decreased PAI-1 and reduced level of vascular oxidative stress as well as decreased level of inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-alpha (TNF-α) (Koh et al. 2003).

I.4.4 Diabetes

Diabetes mellitus is a major chronic health problem affecting about 422 million adults worldwide. Its prevalence rate is increasing rapidly with higher than optimal blood glucose levels causing about 2.2 million deaths by raising the risk of cardiovascular diseases over the past few decades (World Health Organization 2016). The direct and indirect effects of hyperglycemia on the vascular tree are contributing to the induction of both macrovascular complications including stroke, coronary artery diseases, and peripheral vascular diseases, and microvascular complications including diabetic retinopathy, nephropathy, and neuropathy (Fowler 2008). ED is an early key event facilitating the initiation and development of diabetic vascular complications through multiple mechanisms including NADPH oxidase-derived oxidative stress, and eNOS uncoupling with the subsequent formation of ROS instead of NO (Hink et al. 2001). Studies using coronary arteries from animal models of type II diabetes indicate prominent impaired endothelium-dependent relaxations as well as worsened responses to vasoconstrictors (Elmi et al. 2008; Khazaei et al. 2008; Koltai et al. 1997; Bagi et al. 2003). Furthermore, peripheral arterioles (mesenteric, femoral, skeletal muscle and adipose tissue) of obese Zucker rats and insulin-resistant mice have an elevated level of myogenic tone, which may impact blood flow and, hence, the susceptibility to ischemic injury (Lagaud et al. 2001; Frisbee et al. 2002). In addition,

hyperglycemia increases the formation of AGEs, which also contribute to ED by promoting oxidative stress as well as activation of inflammatory response including overexpression of VCAM-1 and tissue factor in HUVECs (Schmidt et al. 1995; Wautier et al. 2001).

I.4.5 Dyslipidemia

Hypercholesterolemia is one of the most well described risk factor triggering coronary artery disease (Multiple Risk Factor Intervention Trial Group 1982). Hypercholesterolemia as well as levels of LDL cholesterol lead to impaired endothelial function in both the coronary and the peripheral circulation in human subjects (Creager et al. 1990; Casino et al. 1993). Lowering cholesterol levels even within the normal range improved endothelium-dependent NO production and hence ameliorated endothelial function (Masumoto et al. 2001). It is worthy of note that reducing the average cholesterol level in patients with established coronary artery disease results in diminished myocardial infarction rates, and this protective effect may be partly because of improvement of the endothelial function (Gilligan et al. 1994; Casino et al. 1995).

I.4.6 Obesity

The progression of vascular disease in obesity may relate to the impact of the metabolic syndrome comprising insulin resistance, hypertension, dyslipidemia and excessive oxidative stress on the physiology of the endothelial function. Indeed, the effects of caloric restriction on endothelial function have been described to improve endothelium-dependent vasodilation in obese subjects with hypertension. Reduction of weight may improve endothelial function indirectly by ameliorating blood pressure and lipid profile (Sasaki et al. 2002). A lipase inhibitor, orlistat, prevented absorption of fat as well as weight loss in a double-blind,

placebo-controlled study involving 23 patients associated to an improved endothelial function (Bergholm et al. 2003). The aspect of aggravated oxidative stress in central obesity is based on an extension of the cytosolic triglyceride capacity in non-adipose tissue (Bakker et al. 2000). The deposit of long-chain fatty acyl-coenzyme A esters involved in fatty acids metabolism is postulated to prohibit mitochondrial adenosine translocation, with subsequent excess generation of oxygen radicals and accumulating evidence revealed that antioxidants may improve insulin resistance and ED (Paolisso & Giugliano 1996).

I.4.7 Underlying mechanisms of endothelial dysfunction

I.4.7.1 Disturbed shear stress

Even though the overall vascular tree is subjected to the cardiovascular risk factors of ED, phenotypic alterations to the endothelial function is expressed at specific regions of arteries such as bifurcations, branch points, and inner curvatures, where disturbed flow takes place and atherosclerosis is developing (Figure 8). Hemodynamic forces are not similar in the whole vascular system. While blood flow in the straight blood vessels of the arterial tree is commonly steady laminar with high shear stress, that in bifurcations is disturbed with low shear stress shifting endothelial function toward an atherogenic endothelial phenotype. Low shear stress promotes atherosclerosis by upregulating the expression of adhesion molecules, growth factors, cytokines, ET-1 and by impairing NO and PGI_2 production, and lipid uptake and catabolism, as well as inducing inflammation and prooxidative responses (VanderLaan et al. 2004; GimbroneJr 1999; Malek et al. 1999).

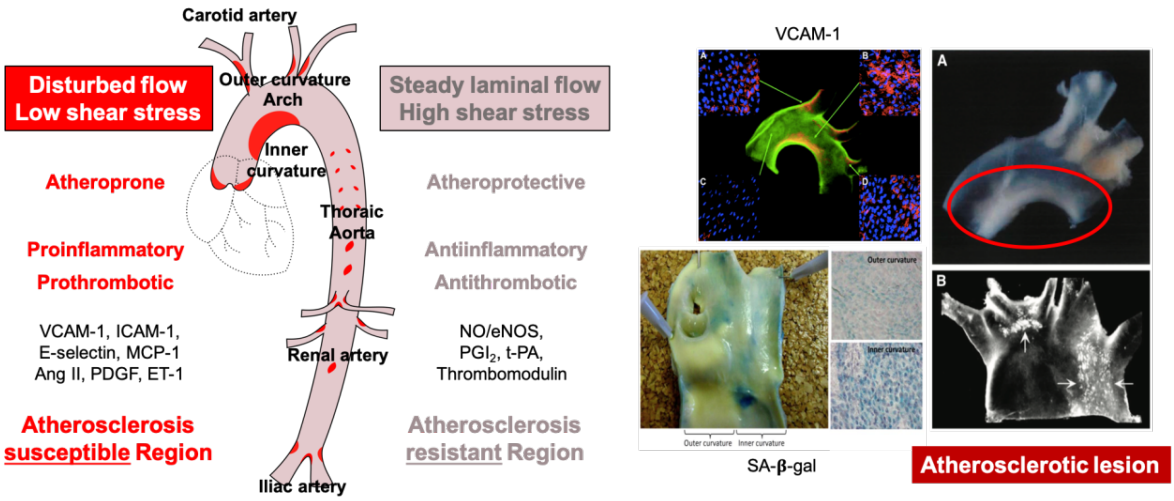

Figure 8. A schematic of the major arterial tree presenting hemodynamic forces responsible for atherosclerosis susceptible sites and various phenotypic modulations in the aortic arch (Nakashima et al. 1994; Nakashima et al. 1998; Suo et al. 2007; Warboys et al. 2014).

A number of *in vitro* studies have characterized responses of ECs to shear stress as a function of time. Early responses of ECs to shear stress imply activation of specific channels or proteins at the plasma membrane including the induction of transmembrane K^+ and Ca^{2+} channels opening (Naruse & Sokabe 1993; Olesen et al. 1988; Yoshikawa et al. 1997), stimulation of heterotrimeric G-proteins (Gudi et al. 1998), and PECAM-1 phosphorylation (Osawa et al. 1997). Moreover, within minutes, several intracellular signaling cascades are activated such as the calcium-dependent phosphorylation of eNOS at Ser1177 promoting its activation, and NO production (Gudi et al. 1998), PI3-kinase activation and signaling by integrins (Tzima et al. 2001). Within minutes to hours, shear stress causes activation of Rho family GTPases (Birukov et al. 2002; S. Li et al. 1999; Tzima et al. 2001; Tzima et al. 2002; Tzima et al. 2003; Tzima 2006; Wojciak-Stothard & Ridley 2003), as well as the adaptor protein Shc, c-Src, focal adhesion kinase (FAK) (Li et al. 1997), protein kinase C (PKC) (Traub et al. 1997), mitogen activated protein kinase (MAPK) (Tseng et al. 1995) and jun C-terminal kinase (JNK) (Li et al. 1997). Shear stress also initiates ROS generation in ECs

within minutes (Hsieh et al. 1998) and facilitates long-term cellular effects via activation of diverse shear stress-responsive transcription factors including c-fos, c-myc, c-jun, and nuclear factor κB (NF-κB) (Khachigian et al. 1995). Subsequent to persistent shear stress for hours to days, ECs adapt by elevating expression of krüppel-like factor 2 (KLF2) (Dekker et al. 2002; SenBanerjee et al. 2004), E-selectin, platelet-derived growth factor (PDGF), ICAM-1, transforming growth factor-β (TGF-β), tissue factor and MCP-1 (Khachigian et al. 1995; Nagel et al. 1994; Resnick et al. 1993; Sampath et al. 1995).

I.4.7.2 Local angiotensin system

Although the primary targets of Ang II are VSMCs, Ang II has numerous effects on ECs, such as eliciting ROS generation, promoting apoptotic signaling pathways, and promoting thrombosis. The intracellular formation of ROS induced by Ang II stimulates the activation of the transcription factor of NF-κB subsequent to the degradation of its cytoplasmic inhibitor, IκB, resulting in raised levels of VCAM-1 (Pueyo et al. 2000). A similar observation was reported by Arenas et al., who showed that Ang II upregulates the secretion of inflammatory cytokine such as TNF-α, which critically contributes to vascular inflammation and promotes vascular disorders, and matrix metalloproteinase (MMP)-2 from ECs (Arenas et al. 2004). Ang II through AT_1R triggers the production of TNF-α via a PKC-dependent pathway in adult heart (Kalra et al. 2002). Ang II-induced NF-κB-mediated inhibition of fibrinolysis and induction of cell adhesion molecules including VCAM-1 and ICAM-1 contributes to the initiation and progression of atherosclerosis. Ang II-induced MCP-1 and IL-6 expression are dependent on the NAD(P)H oxidase-derived oxidative stress in VSMCs from rats as well as humans (Marui et al. 1993; Kranzhöfer et al. 1999; Chen et al. 1998). In cultured human coronary artery ECs, Ang II induced the lectin-like receptor for ox-LDL (LOX-1), which is a crucial component in the formation of atherosclerotic lesions (Li et al. 1999). Moreover, in

ApoE$^{-/-}$ mice, infusion of Ang II resulted in accelerated development of atherosclerosis and aneurysms (Weiss et al. 2001).

I.4.7.3 Reactive oxygen species

An increased vascular generation of ROS in diabetes, hypertension, obesity, aging is the most common contributor promoting the development of ED, which ultimately leads to cardiovascular disease (Figure 9). ROS which are mostly free radicals possess unpaired electrons or have oxidizing potential including molecular oxygen, have several sources in vascular cells. The reactive intermediate superoxide anion (O_2^-) is responsible for the production of other reactive species under physiological responses, such as hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), and is generated by the mitochondrial electron chain, NADPH oxidase, COXs, uncoupled eNOS and xanthine oxidase (Förstermann & Münzel 2006; Dröge 2002).

Numerous evidences suggest that ROS participate in vascular signaling and proatherogenic responses by modulating redox-sensitive transcription and transduction pathways (Kunsch & Medford 1999). In the surroundings of an elevated level of oxidative stress, ECs lose their protective phenotype and express proinflammatory molecules such as VCAM-1, ICAM-1 and MCP-1, all of which promote interactions between leukocyte and endothelium and contribute to the early stage of atherosclerosis. Appearance of inflammatory signals is predominantly regulated by NF-κB, which is a redox-sensitive transcription factor that also contributes to promote VSMCs growth, vascular remodeling, and atherogenesis (Valen et al. 2001). Cultured cells overexpressing free radical-scavenging enzymes such as catalase reveal restrained NF-κB activation in response to TNF-α, while those overexpressing SOD show intracellular deposit of hydrogen peroxide and activation of NF-κB. Furthermore,

NF-κB activity is inhibited by antioxidants such as N-acetylcysteine and pyrrolidine dithiocarbamate (Kunsch & Medford 1999).

Oxidants also play a regulatory role in intracellular signaling. MAPK and tyrosine kinases are major regulatory proteins that modulate cellular responses to stress and growth stimuli (Wolin 2000; Kunsch & Medford 1999). In vascular cells, growth factors and Ang II are potent stimulators of extracellular signal-regulated kinase and p38 MAPK that stimulate proliferation and migration of SMCs fibroblasts via mechanisms involving hydrogen peroxide. These events cause neointimal growth contributing to atheroma progression and restenosis. Activation of VSMCs by the mitogen PDGF induces intracellular generation of hydrogen peroxide and phosphorylation of tyrosine (Sundaresan et al. 1995). This process is prohibited by increasing intracellular concentrations of catalase, and by N-acetylcysteine. ROS alter both Akt kinase and activity of caspase, which play an important role in proliferation of ECs and stimulation of apoptotic signals leading to loss of ECs, respectively (Irani 2000).

Moreover, ROS also modify metabolism of collagen matrix via activation of proteolytic MMPs that play a critical part in plaque characteristics and stability (Channon 2002). Expression of MMPs is raised in shoulder areas of atherosclerotic plaques where its enhanced activity may increase vulnerability possibly leading to plaque rupture (Galis et al. 1994). Atherectomy specimens from patients with unstable coronary syndromes show elevated levels of ROS compared with subjects with stable angina, suggesting a mechanistic role of ROS in composition of plaque and its activity (Azumi et al. 2002). N-acetylcysteine prevented the expression and stimulation of MMP-9 in hypercholesterolaemic rabbits, indicating a promising role for antioxidant treatment in the tuning of the plaque stability (Galis et al. 1998).

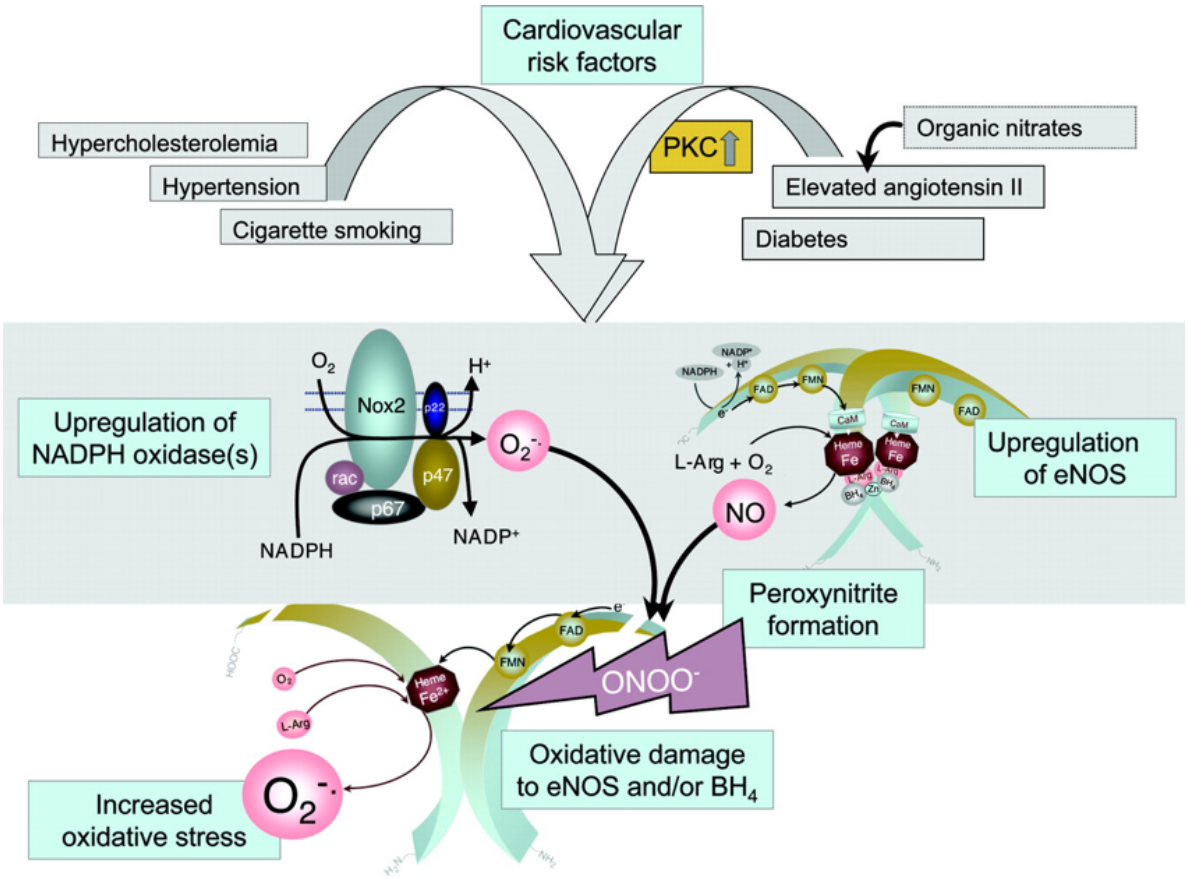

Figure 9. Various mechanisms of oxidative stress-mediated endothelial dysfunction by cardiovascular risk factors (Förstermann & Münzel 2006).

I.4.7.4 Microparticles

Circulating microparticles (MPs) are membrane vesicles with a diameter of 0.1 - 1 µm sheded from different cell types during cell activation by shear stress, cytokines, thrombin, and calcium ionophore A23187, apoptosis or senescence (Leeuwenberg et al. 1992; Sims et al. 1989; Miyazaki et al. 1996, Abbas 2017). The endothelium is one of the principal targets of circulating MPs acting as a biological transcellular signal delivery system by carrying membrane and cytoplasmic proteins and constituents, all of which represent their activated parent cells (Mause & Weber 2010). Under normal conditions, MPs which are mainly

released from platelets and to a lesser extent from ECs and leukocytes, participate in the regulation of ECs homeostasis (Tushuizen et al. 2011; Owens & Mackman 2011) (Figure 10).

Under stress conditions, on the other hand, increased concentrations of circulating MPs contribute to ED especially by modulating the balance between NO and ROS generation in addition to stimulating procoagulant and proinflammatory responses (Mostefai et al. 2008; Brodsky et al. 2004). Moreover, a recent study by our lab has shown that the incubation of ECs with circulating MPs from acute coronary syndrome patients induced premature endothelial senescence involving the Ang II-induced NADPH oxidase pathway (Abbas et al. 2017).

Therefore, MPs isolated from blood have been considered as potential diagnostic biomarkers of vascular damage and inflammation in cardiovascular diseases including diabetes, acute myocardial infarction and atherothrombosis (Diamant et al. 2004; Feng et al. 2010; Boulanger et al. 2001).

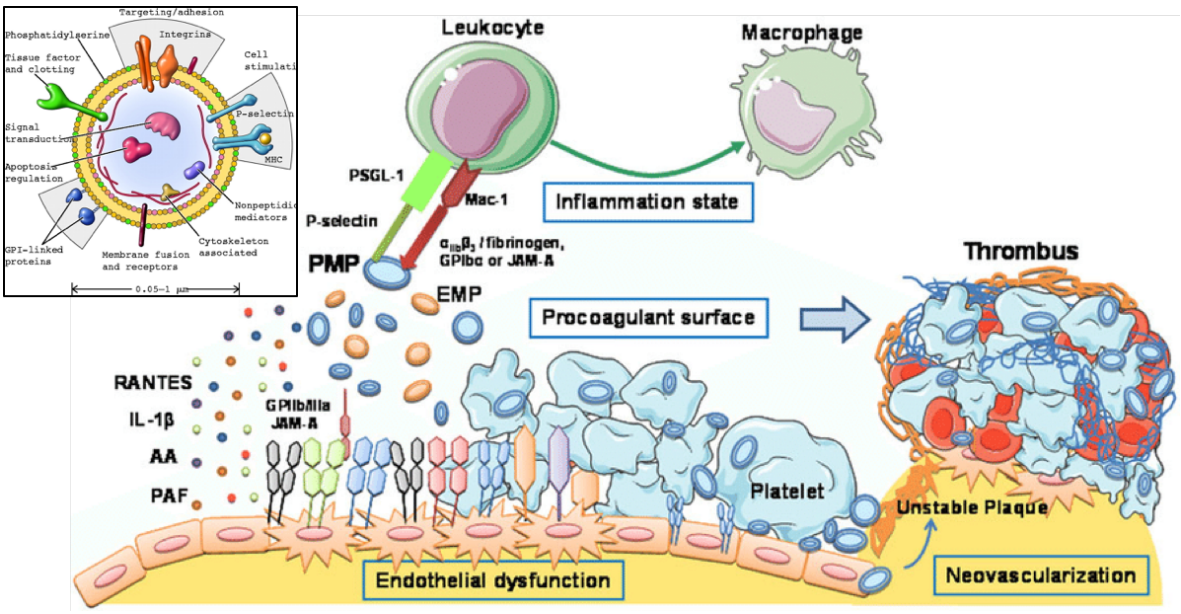

Figure 10. Composition and molecular mechanisms of circulating microparticles (MPs) on atherosclerosis plaque progression (Hugel et al. 2005; Badimon et al. 2016).

Chapter II

Gliflozins in cardiovascular diseases

II.1 Introduction

II.1.1 Gliflozins and sodium-glucose cotransporters

Gliflozins are a class of blood glucose-lowering medications used in the management of type 2 diabetes by inhibiting sodium-glucose cotransporter 2 (SGLT2) mainly responsible for the reabsorption of glucose in the kidney.

Glucose transporters play a crucial role in the regulation of glucose homeostasis by carrying glucose across the plasma membranes. Among the glucose transport proteins in different tissues, sodium-glucose cotransporters (SGLTs), which are integral membrane proteins, transport glucose via a symport mechanism with the concomitant transfer of sodium and are independent of the presence of insulin. The driving force is due to the active sodium removal of the cell by the basolateral sodium/potassium-ATPase, thus promoting a low cytosolic [Na^+] and hence, triggering glucose and sodium uptake. In human, six isoforms of SGLTs have been identified, but SGLT1 and SGLT2 are the best studied members of the SLC5A gene family (Table 1).

Human gene (locus)	Protein	Transport type	Human tissue distribution
SLC5A1 (22q12.3)	SGLT1	Cotransporter of glucose or galactose with Na^+, (H^+) Uniporter of urea and water	Small intestine, trachea, kidney, heart, brain, testis, prostate
SLC5A2 (16p11.2)	SGLT2	Cotransporter of glucose with Na^+	Kidney, brain, liver, thyroid, salivary glands
SLC5A4 (22q12.3)	SGLT3	Glucose activated Na^+ (H^+) channel	Small intestine (cholinergic enteric neurons), skeletal muscle, kidney, uterus, testis, lung, brain, thyroid
SLC5A9 (1p33)	SGLT4	Cotransport of mannose, fructose or glucose with Na^+	Kidney, small intestine, brain, liver, heart, lung, trachea, uterus, pancreas
SLC5A10 (17p11.2)	SGLT5	Cotransport of mannose, fructose or glucose with Na^+	Kidney cortex
SLC5A11 (16p12.1)	SGLT6	Cotransport of myoinositol, chiro-inositol with Na^+	Thyroid, brain, heart, muscle, spleen, liver, lung

Table 1. Human SLC5A gene family (Wright 2013).

The average molecular weight of these transmembrane glycoproteins with 14 transmembrane helices is approximately 60 to 80 kDa and they contain 580 to 718 residues.

In the kidney of healthy individuals, approximately 160 - 180 g glucose per day filtered by the glomeruli is completely reabsorbed by the proximal tubules until the glucose concentration in the glomerular filtrate does not exceed the maximum glucose transport capacity. Nearly all of glucose reabsorption in the proximal tubules (S1/S2) occurs through both SGLT2 and SGLT1 proteins. SGLT2, a low-affinity, high-capacity transporter, is responsible for approximately 90% of this process that is independent of insulin and the remaining 10% is removed in the S3 segment by SGLT1, a related high-affinity, low-capacity transporter. SGLT1 is also substantially expressed in the small intestine (Wright et al. 2007; Wright 2001; Wright & Turk 2004; Hediger & Rhoads 1994; Brown 2000) (Figure 11).

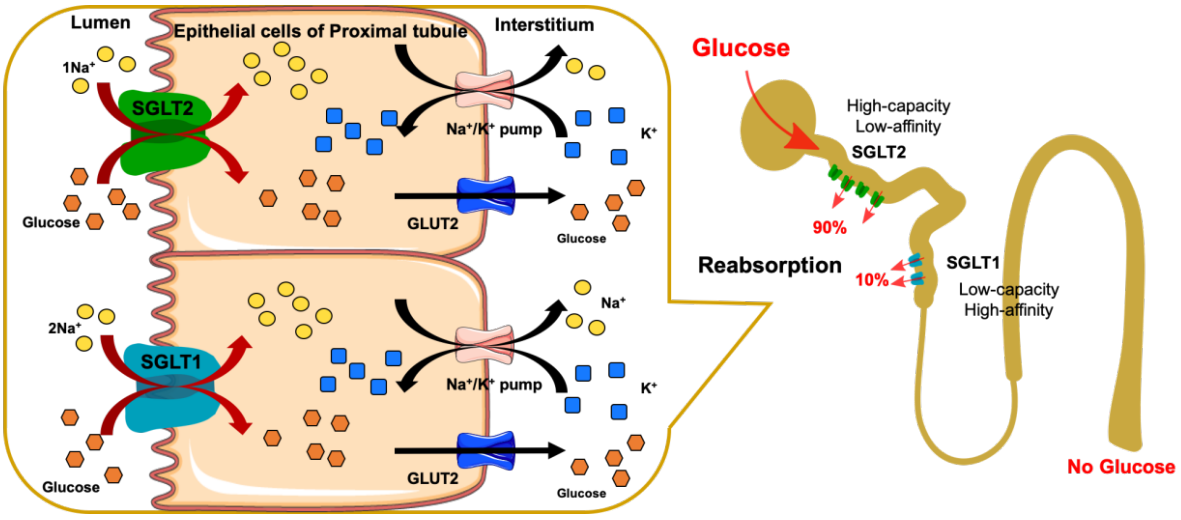

Figure 11. Reabsorption of glucose in the kidney of healthy individuals.

The expression and activity of SGLT2 are higher in T2DM patients, and consequently, glucose reabsorption is more pronounced, which contributes to promote hyperglycemia in the blood circulation, ultimately leading to development of cardiovascular complications (Figure 12). Lowering hyperglycemia by inhibiting SGLT2 represents an insulin-independent

strategy that minimizes blood glucose levels by preventing excessive glucose reabsorption in the kidney and hence diminishing the renal glucose threshold.

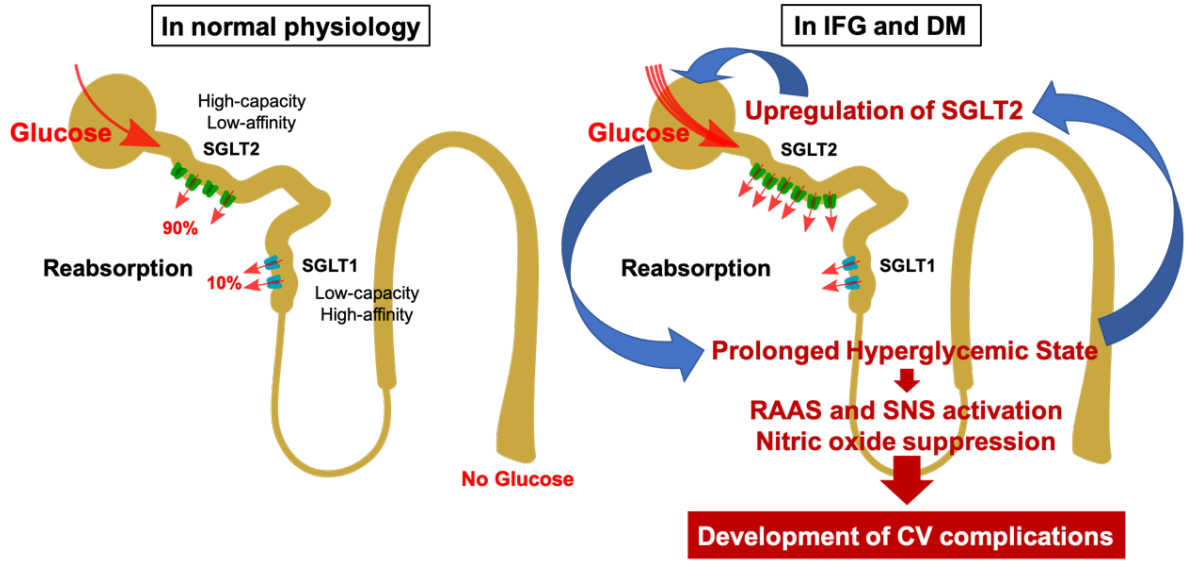

Figure 12. The maladaptive glucose homeostasis mechanism leading to upregulation of SGLT2 which eventually contributes to development of cardiovascular complications in impaired fasting glucose (IFG) and diabetes mellitus (DM).

Indeed, studies in primary cultures of human exfoliated proximal tubular epithelial cells from fresh urine of patients with T2DM showed that SGLT2 expression and renal glucose uptake were elevated compared with that from healthy individuals (Rahmoune et al. 2005). In addition, several studies in diabetic rodent models demonstrated increased renal SGLT2 and SGLT1 expression, including raised renal mRNA expression of SGLT1 and SGLT2 in diabetic obese Zucker rats compared with age-matched leans (Tabatabai et al. 2009) and also in alloxan-induced diabetic rats (Vestri et al. 2001). The mechanisms involved in upregulation of SGLT2 are still poorly understood. Recent studies suggested roles for insulin, PKC, and PKA activation and regulation of human SGLT2 activity (Ghezzi & Wright 2012). Ang II and AT_1R (Osorio et al. 2009), and hepatocyte nuclear factor (HNF)–1α, a

transcription factor (Freitas et al. 2008) have been implicated in the increased SGLT2 expression in diabetic rats.

II.1.2 Discovery and development of gliflozins

The first SGLT inhibitor discovered was phlorizin, a natural phenolic O-glucoside consisting of a dihydrochalcone moiety, isolated from the bark of the apple tree in 1835 (Petersen 1835) (Figure 13A). It was firstly used in the treatment of infectious diseases such as malaria, because its bitter taste was similar to that of other tree extracts used as antipyretics (De Koninck 1836). In 1886, von Mering revealed that ingestion of phlorizin causes glycosuria and, a century later, investigations of its mechanism of action led to the characterization of SGLTs (Mering 1886). In 1987, *in vivo* evidence demonstrated the efficacy of phlorizin treatment to reduce glycemic level and normalize insulin sensitivity in diabetic rats (Rossetti et al. 1987). However, phlorizin acts as a nonspecific SGLT inhibitor by targeting both SGLT1 and SGLT2. The dual inhibition of SGLT1 and 2 restricts its applicability for human as a drug. Indeed, owing to poor selectivity, this drug displays important gastrointestinal side effects linked to the high expression level of SGLT1 in the intestine. In addition, limited oral bioavailability due to the hydrolysis of the O-glycoside by intestinal glycosidases (Betz et al. 1975) represents an important concern. Such limitations triggered important efforts, to develop new SGLT2 inhibitors appropriate for oral administration that led to the discovery of C-aryl glucoside-derived gliflozins, presenting nonhydrolyzable C−C bond (Larson 2015) (Figure 13B).

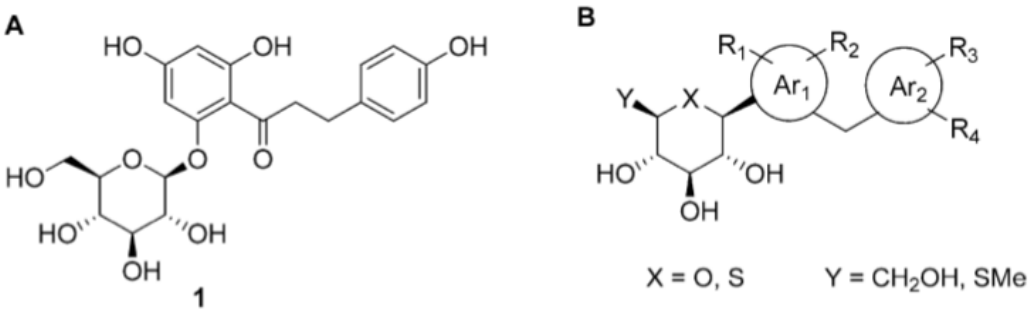

Figure 13. (A) Chemical structure of phlorizin and (B) Markush structure of gliflozins (Aguillón et al. 2018).

II.1.3 Classification and characteristics of gliflozins

Six gliflozins have now been approved as prescription medicines for the treatment of type 2 diabetes. Four of these agents are currently approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA): canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin. Other gliflozins, such as ipragliflozin, luseogliflozin, and tofogliflozin, are currently approved in Japan. Other gliflozins, including LX-4211 (sotagliflozin) and bexagliflozin, are in late development (Figure 14).

Because of the latent gastrointestinal side effects involved in SGLT1 inhibition, pharmaceutical companies have preferred medicines with greater selectivity for SGLT2 compared with SGLT1 for clinical development. As indicated in Table 2, the selectivity of main gliflozins under clinical development is > 100-fold greater for SGLT2 over SGLT1, except LX4211. LX4211 is a novel dual inhibitor of SGLT1 and SGLT2 that was designed to lessen glucose absorption in the gastrointestinal tract by inhibiting SGLT1 and renal glucose reabsorption by inhibiting SGLT2 for T1DM patients of which adequate glycemic control is not continuously achieved, in despite of insulin therapy.

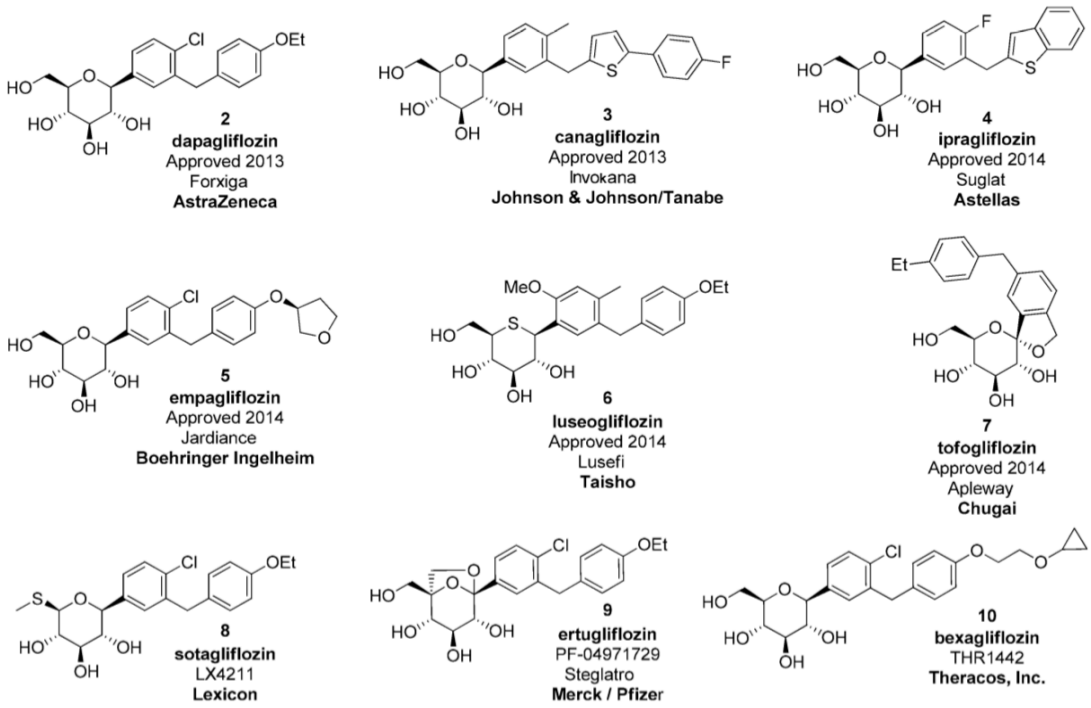

Figure 14. Chemical structures of SGLT2 inhibitors approved by the FDA and EMA (dapagliflozin, ertugliflozin, canagliflozin, and empagliflozin), approved by Japan's PMDA (tofogliflozin, ipragliflozin, and luseogliflozin), and currently in phase III clinical trials (sotagliflozin and bexagliflozin) (Aguillón et al. 2018).

Agent	IC_{50} for SGLT2	IC_{50} for SGLT1	SGLT2 selectivity (fold)
Phlorizin	34.6	210	6
Tofogliflozin	2.9	8,444	2,912
Empagliflozin	3.1	8,300	2,680
Luseogliflozin	2.26	3,990	1,770
Dapagliflozin	1.12	1,391	1,242
Ipragliflozin	7.38	1,876	254
Canagliflozin	4.4	683	155
LX4211 (Sotagliflozin)	1.8	36	20

Table 2. Selectivity of gliflozins for SGLT2 and SGLT1 (Abdul-Ghani et al. 2013).

II.2 Cardiovascular and metabolic protective effects of gliflozins

Cardiovascular risk factors include ageing, hypertension, hyperglycemia, dyslipidemia, obesity and are characterized with subclinical inflammation, and early ED. Intensive glycemic control with current antidiabetic agents may improve some risk factors such as dyslipidemia and blood pressure but negatively impact other risk factors such as body weight. Moreover, the cardiovascular mortality and all-cause mortality are commonly not reduced, indicating glycemic control alone is not enough to interfere the problem of cardiovascular risks in diabetic patients. Recent studies have suggested that gliflozins are able to improve cardiovascular outcomes independently of glycemic control. The impact of gliflozins on cardiovascular risk factors is described in Figure 16.

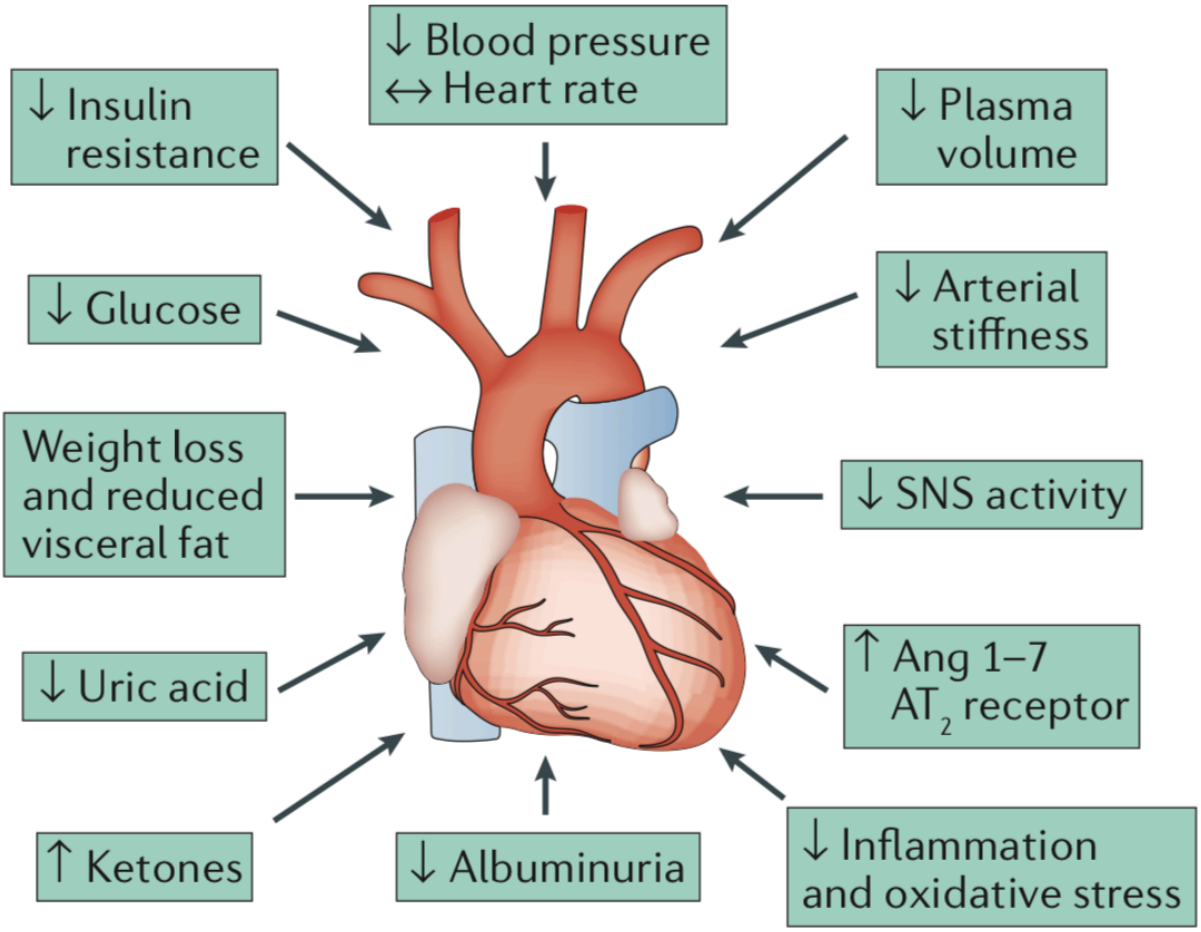

Figure 15. Schematic indicating effect of gliflozins on metabolic and cardiovascular outcome (DeFronzo et al. 2017).

II.2.1 *ex vivo* studies

Although several studies demonstrated the absence of SGLT2 expression in the heart (Van Steenbergen et al. 2017; Di Franco et al. 2017; Vrhovac et al. 2015), others have shown that the SGLT2 mRNA is present in most human tissue (Zhou et al. 2003) and that the SGLT2 protein can also be observed in ECs. In HUVECs, both SGLT1 and SGLT2 expressions were revealed at the protein and mRNA level and their expression levels as upregulated by palmitic acid (PA), which resulted in reduced phosphorylation of Akt, eNOS, and insulin receptor substrate-1 (IRS-1) and release of NO (Li et al. 2018). PA-induced ED was partially reversed with phlorizin, and knockdown of SGLT1 or SGLT2 (Li et al. 2018). In addition, empagliflozin decreased glucose uptake in cultured ECs like sotagliflozin, assessed by glucose analog 2-NBDG uptake. Pre-treatment of ECs with empagliflozin, dapagliflozin or canagliflozin for 24 h caused a potent vasorelaxation in hyperglycemic (25 mM glucose) mice aortic rings following addition of ACh or a PAR2 agonist. Additionally, empagliflozin prevented mitochondrial dysfunction by elevated oxygen consumption rates and lowered proton leakage in hyperglycemic mouse aortic rings (El-Daly et al. 2018).

In human umbilical vein endothelial cell (HUVECs) and human aortic endothelial cells (HAECs) stimulated by IL-1β, canagliflozin diminished secretion of MCP-1 and IL-6, which was at least partially associated with raised AMPK activity (Mancini et al. 2018). Dapagliflozin reduced TNF-α or hyperglycemia-induced ICAM-1 and VCAM-1 protein levels and expression of NFκB mRNA induced by TNF-α in HUVECs (Gaspari et al. 2018). While increased extracellular glucose level alone is insufficient to activate a pro-atherogenic state, the combination of glucotoxic and inflammatory stimuli might promote an environment prone to stimulate the development of atherosclerosis since inflammation is considered a critical aspect of cardiovascular disease in diabetes (Sharma et al. 2018). Current perceptions of the pathophysiology of heart failure (HF), especially HF with preserved ejection fraction

(HFpEF) suggests that the presence of cardiovascular risk factors cause microvascular inflammation that ultimately promotes the development of HF (Paulus & Tschöpe 2013). Vascular proinflammatory inducers may include interferon-γ and Ang II act via the signal transducer and activator of transcription-1 (STAT1) and by decreasing AMPK activity (He et al. 2015). In addition, high Na^+ loading causing an increase in fasting blood glucose levels, oxidative stress and insulin resistance are lilkely to contribute to inflammatory burden (Wan et al. 2018). Endothelial inflammation, in turn, leads to disturbed NO-cGMP-PKG signaling and elevated leukocyte trafficking, which subsequently stimulates cardiomyocyte hypertrophy and myofibroblast differentiation promoting cardiac remodeling. Ye et al. studied the effects of dapagliflozin in LPS-stimulated cardiac fibroblasts isolated from WT mice. Dapagliflozin prevented LPS-induced upregulation of nucleotide-binding domain leucin-rich repeat [LRR] and pyrin-containing receptor 3 (NLRP3), apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), IL-1β and caspase-1 mRNA levels. The effects of dapagliflozin on NLRP3, TNF-α and caspase-1 were similar to A769662, an AMPK activator, and these anti-inflammatory effects as well as phosphorylation of AMPKα at Thr172 were blocked by compound C, an AMPK inhibitor. Altogether, these data supports the view that dapagliflozin may induce anti-inflammatory responses in myofibroblasts, mediated by increased AMPK activation (Ye et al. 2017).

II.2.2 *in vivo* studies

One of the benefits of gliflozins unlike other antihyperglycemic drugs is that they do not promote hypoglycemia, since they can reinforce endogenous glucose production by raising plasma glucagon concentration. Indeed, dapagliflozin directly activated pancreatic alpha cells to stimulate glucagon secretion in healthy mice (Bonner et al. 2015). Glucagon is known as the crucial hormone for hepatic gluconeogenesis that promotes ketogenesis and improves

cardiac contractility, although such alterations are likely secondary effects to reduce glucose toxicity, which improves beta cell function and insulin sensitivity associated with atherosclerosis risk (Howard et al. 1996). Glycosuria also leads to weight and fat mass reduction due to enhanced lipid oxidation compensating for calorie loss. Ipragliflozin reduced body weight and fat mass by elevating fatty acid oxidation in high-fat diet-induced obese rats (Yokono et al. 2014). Osorio et al. showed that phlorizin prevented the development of hypertension in STZ-induced diabetic rats fed a normal diet or a high salt diet, indicating also a role of SGLT in the development of hypertension, possibly by lowering excessive sodium and water reabsorption (Osorio et al. 2010).

Reduction of oxidative stress involved in the pathophysiology of atherosclerosis and improvement of progressive heart failure have also been demonstrated in animal studies. Empagliflozin reduced atherosclerotic plaque formation by ameliorating inflammation and insulin resistance in $ApoE^{-/-}$ mice (Han et al. 2017). Canagliflozin alleviated obesity-induced inflammation in mice (Naznin et al. 2017), and dapagliflozin markedly reduced macrophage infiltration, inflammatory gene expression and oxidative stress in the kidney of *db/db* mice (Terami et al. 2014). Ipragliflozin ameliorated the reduced phosphorylation of Akt and eNOS, decreased ROS production, MCP-1, VCAM-1 and ICAM-1, and improved endothelial dysfunction in STZ-induced diabetic mice (Salim et al. 2016). Oelze et al. showed that empagliflozin improved endothelium-dependent relaxations in STZ-induced diabetic rats (Oelze et al. 2014). In 2016, Kusaka et al. tested the effect of empagliflozin in genetic prediabetic rats with metabolic syndrome. After 10 weeks of treatment, empagliflozin significantly decreased left ventricular weight, size of cardiomyocyte, cardiac interstitial fibrosis and macrophage infiltration (Kusaka et al. 2016). In 2017, Shi et al. studied the effect of empagliflozin in aristolochic acid induced heart failure in zebrafish embryos. Empagliflozin treatment improved heart failure morphology and attenuated heart failure

markers expression including atrial natriuretic peptide and brain natriuretic peptide (Shi et al. 2017)

II.2.3 Clinical trials in humans

The EMPA-REG OUTCOME trial (2010–2015) revealed a major cardioprotective effect of empagliflozin, which was significantly associated to lower rates of cardiovascular mortality (38% relative risk reduction), and lower rates of overall mortality (32% relative risk reduction), and lower rates of hospitalization for heart failure (35% relative risk reduction) in T2DM patients with established cardiovascular diseases (Zinman et al. 2015) (Figure 16). Such beneficial effects of empagliflozin were expected to be a class effect of gliflozins mediated via hemodynamic and metabolic actions. Several trials supported such a concept as summarized in Table 3. The CANVAS trial (2009–2017) reported that canagliflozin significantly decreased the composite of cardiovascular cause death, nonfatal myocardial infarction or nonfatal stroke [hazard ratio (HR) 0.86, 95% confidence interval (CI) 0.75–0.97] and hospitalization for heart failure (HR 0.67, 95% CI 0.52–0.87) (Neal et al. 2017). The CVD-REAL study evaluated the cardiovascular effect of gliflozins compared with other glucose-lowering drugs indicated that gliflozins significantly diminished the rate of hospitalization for heart failure (HR 0.49, 95% CI 0.41–0.57) and all-cause death (HR 0.61, 95% CI 0.51–0.73) (Kosiborod et al. 2017). In the DECLARE-TIMI 58 trial (2013–2019) including patients with T2DM who had or were at risk for atherosclerotic CVD, treatment with dapagliflozin resulted in a lower rate of cardiovascular death or hospitalization for HF, which reflected a lower rate of hospitalization for HF (27% relative risk reduction) (Wiviott et al. 2019). Ongoing cardiovascular outcome trials of gliflozins include VERTIS CV (ertugliflozin, 2013–2019) and RECEDE-CHF (empagliflozin, 2017–2019) may help to verify this anticipation (Cannon et al. 2018; Mordi et al. 2017; Raz et al. 2018).

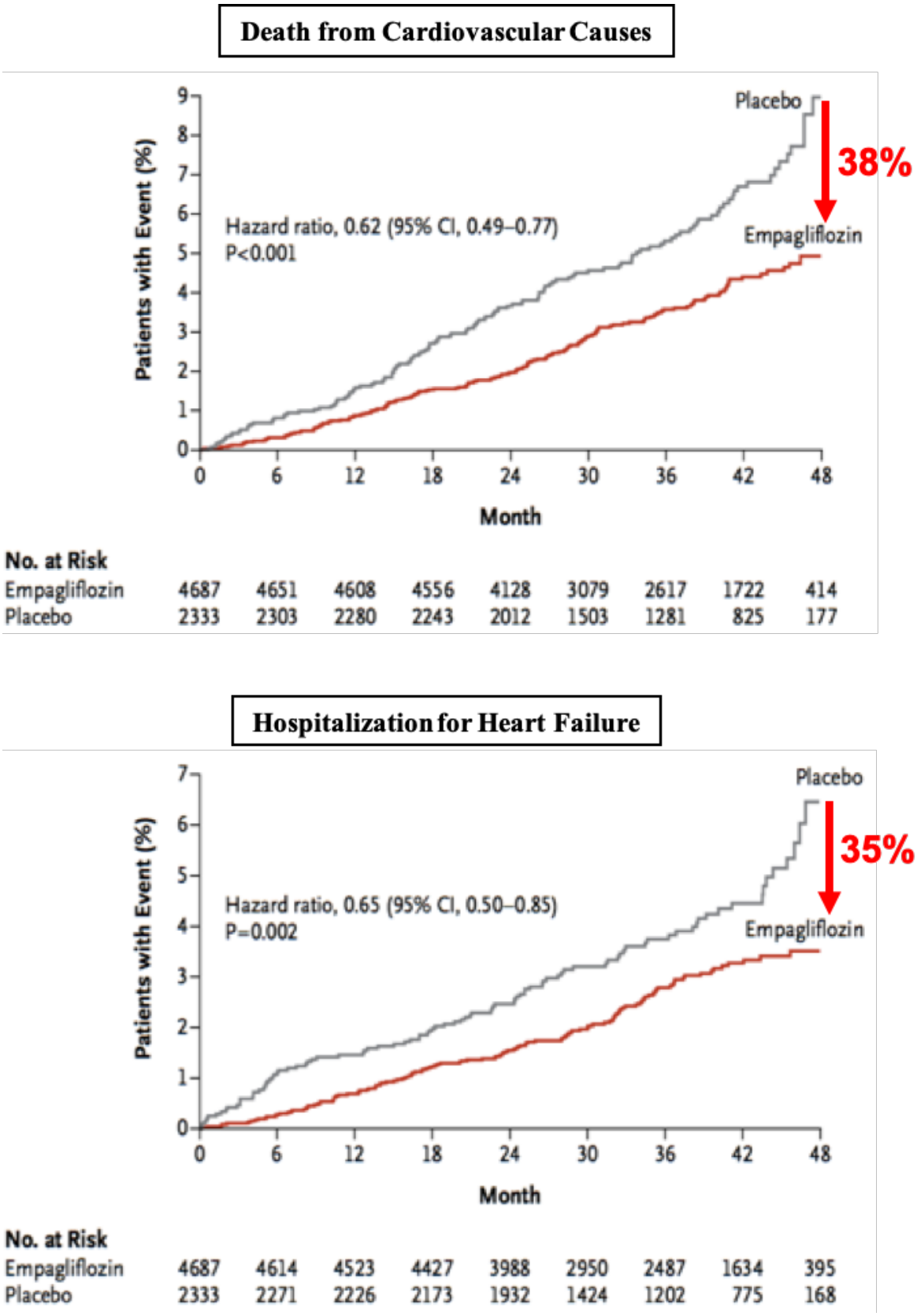

Figure 16. Cardiovascular Outcomes from EMPA-REG study (Zinman et al. 2015).

Trial name (publication year)	Major inclusion criteria	Number of patients randomized	Median follow-up, years	Intervention	Major findings
EMPA-REG OUTCOME (2015)	HbA1c 7.0%–9.0% (if drug naïve) or 7.0%–10.0% (if receiving stable glucose-lowering medication >12 weeks pre-randomization); established CVD	7,020	3.1	Empagliflozin 10 mg versus empagliflozin 25 mg versus placebo (analyzed as empagliflozin pooled vs placebo)	↓ Cardiovascular-cause death (38% RRR) ↓ All-cause death (32% RRR) ↓ HHF (35% RRR) ↔ MI, stroke
CANVAS Program (2017) (CANVAS + CANVAS-R)	HbA1c 7.0%–10.5%; ≥30 years history of CVD, or ≥50 years high risk of CVD	10,142 (CANVAS 4,330 + CANVAS-R 5,812)	2.4	Canagliflozin 100 mg versus canagliflozin 300 mg versus placebo	↓ Composite of cardiovascular-cause death, nonfatal MI or nonfatal stroke (HR 0.86, 95% CI 0.75–0.97) ↓ HHF (HR 0.67, 95% CI 0.52–0.87) ↔ All-cause death, cardiovascular-cause death, nonfatal MI, nonfatal stroke
CVD-REAL* (2017)	T2DM; new users of SGLT2 inhibitors or other GLD	(Not randomized; observational) 309,056	Retrospec-tive registries study	SGLT2 inhibitors versus other classes of GLD	↓ HHF (HR 0.49, 95% CI 0.41–0.57) ↓ All-cause death (HR 0.61, 95% CI 0.51–0.73)
DECLARE-TIMI 58 (2019)	HbA1c 6.5%–12%; ≥40 years established CVD	17,160	4.2	Dapagliflozin 10 mg versus placebo	↓ Cardiovascular death or HHF (HR, 0.83; 95% CI, 0.73-0.95) ↔ All-cause death, composite of cardiovascular death, MI or ischemic stroke

Ongoing trial	Patient population	Estimated enrollment, N[b]	Estimated end date	Intervention	Primary outcome
CREDENCE	HbA1c 6.5%–12.0%; ≥30 years; eGFR ≥30–<90 mL/min/1.73 m^2; stable dose ACE inhibitor or ARB; UACR >300–≤5,000 mg/g	4,200	June 2019	Canagliflozin 100 mg versus placebo	Composite: end-stage kidney disease, doubling of serum creatinine, renal or CV death
VERTIS CV	HbA1c 7.0%–10.5%; ≥40 years; history or evidence of atherosclerotic vascular disease	8,000	October 2019	Ertugliflozin 5 mg versus ertugliflozin 15 mg versus placebo	Composite: CV death, non-fatal MI, or non-fatal stroke

Table 3. Completed and ongoing cardiovascular outcome trials of gliflozins in patients with T2DM (Lahnwong et al. 2018; Cavaiola & Pettus 2018).

II.3 Potential targets for the gliflozin-induced cardiovascular mechanisms

Despite the pronounced cardiovascular benefits of gliflozins, the biological mechanisms underlying the cardioprotective effects mediated by interaction with diverse cardiac targets and/or by influences in extracardiac tissues are not fully understood.

II.3.1 Sodium-glucose cotransporters

Several studies have recently shown that gliflozins directly decreased glucose uptake by inhibiting SGLT2 in ECs. While SGLT2 has not been detected at all in the heart (van Deel et al. 2017), converging evidences report the presence of SGLT2 in non-cardiac ECs. Recent studies have disclosed SGLT2 in ECs at protein level and that its expression levels is modified by exposure to PA or SGLT2-specific siRNA (El-Daly et al. 2018; Li et al. 2018). Li et al. demonstrated that phlorizin improved the endothelial dysfunction and insulin resistance through activation of the PI3K/Akt/eNOS signaling pathway leading to the release of NO, partially by attenuating the upregulation of SGLT1 and SGLT2 in PA-induced HUVECs. Furthermore, empagliflozin prevented intracellular hyperglycemia by inhibiting SGLT2 expression and glucose uptake. Such mechanism is likely to induce, glucose-induced oxidative stress that suppresses PAR2-mediated endothelium-dependent vasodilation through NAPDH oxidase/ROS-triggered signaling pathway involving EGFR/Src/Rho-kinase and PKC in cultured ECs *in vitro* and mice *in vivo* (El-Daly et al. 2018; Li et al. 2018).

In contrast to SGLT2, SGLT1 is highly expressed in the heart (Zhou et al. 2003). Cardiac-specific SGLT1 overexpression promoted hypertrophy and left ventricular dysfunction and this deletion improved cardiomyopathy in mice (Ramratnam et al. 2014). An increased mRNA level of SGLT1 was observed in hearts from mice and humans with T2DM and ischemic cardiomyopathy (Banerjee et al. 2009) and also in obese humans in the absence

of T2DM (Lambert et al. 2015). Moreover, heart failure alone was associated with higher protein expression levels of cardiac SGLT1 in both lean and obese individuals. In good agreement with increased SGLT1 expression levels, the SGLT-mediated glucose uptake is significantly augmented in myocytes from diabetic compared to control rats. In addition, phlorizin and glucose-free solution considerably decreased Na^+ entry in myocytes from diabetic rats. The SGLT-mediated Na^+ influx was ≈7 times higher in diabetic compared to control myocytes. Altogether, these findings suggest that upregulation of SGLT1 in diabetic cardiomyocytes is mostly responsible for the excess Na^+ entry, which may contribute to oxidative stress.

II.3.2 Na^+/H^+ exchanger

All mammalian cells sustain a low level of intracellular Na^+ ($[Na^+]_i$) by actively expelling Na^+ through the energy consuming Na^+/K^+-ATPase (NKA). This energy-dependent mechanism will allow the establishment of the gradient of electrochemical Na^+ that permits the transmembrane transfer of other ions such as H^+ via the Na^+/H^+ exchanger (NHE) and Ca^{2+} through the Na^+/Ca^{2+} exchanger (NCX), uptake glucose for energy via SGLTs and amino acids through Na^+-amino acid cotransporter and, in excitable cells, initiation and propagation of action potentials via voltage-gated Na^+ channels. Alterations in $[Na^+]_i$ critically influence the function of these transporters, hence $[Na^+]_i$ homeostasis is vital for a variety of major cellular processes.

The disturbed balance between the passive Na^+ entry and active Na^+ pumping out of the cell is observed in both humans and animal models with heart failure and T2DM, resulting in raised $[Na^+]_i$ and $[Ca^{2+}]_i$ (Despa et al. 2002; Despa 2018; Lambert et al. 2015). Increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ are coupled to diminished mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) in cardiac myocytes, which leads to energetic deficit and redox malfunction of mitochondria (Murphy

& Eisner 2009; Bay et al. 2013). Elevated $[Ca^{2+}]_i$ is identified in LPS- and hyperglycemia-induced endothelial dysfunction (Wang et al. 2008; Cui et al. 2013), in cardiomyocytes during IR injury (Garcia-Dorado et al. 2012) and atrial fibrillation (Neef et al. 2010). Elevations in $[Na^+]_i$ and $[Ca^{2+}]_i$ are induced by altered activity of ion channels and/or several transporters, including the NCX, the NHE-1, the ryanodine receptor regulating SR-calcium release and NKA, and targeting these ion-controlling transporters has been suggested to improve cardiovascular function (Baartscheer et al. 2003; Despa et al. 2012; Karmazyn 2013; Sasahara et al. 2013; Luo et al. 2013) (Figure 17).

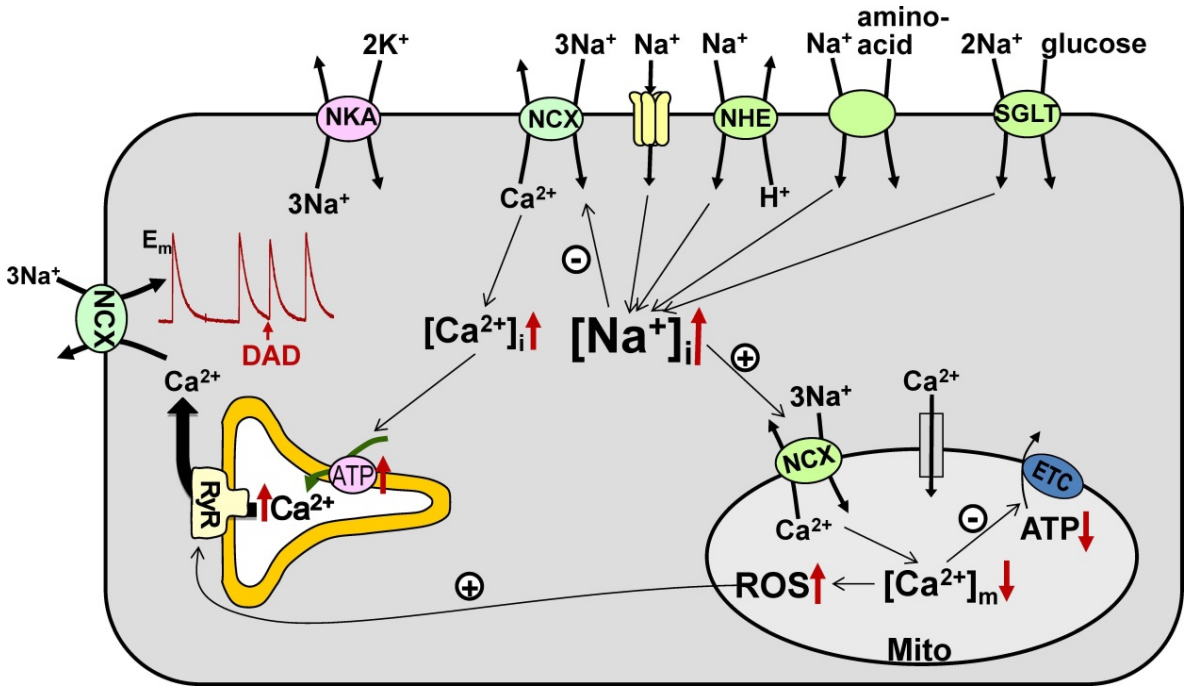

Figure 17. Potential pathways leading to elevated $[Na^+]_i$ via the involved of various transporters in cardiomyocytes (Despa 2018).

Elevated NHE activity emerges to play a key role in the pathophysiological process of HF and diabetes (Figure 18). The activity of cardiac NHE-1 is prominently augmented, and inhibition of NHE-1 by cariporide alleviated cardiac necrosis and infarct size, decreased the progression of cardiac remodeling, fibrosis, hypertrophy and systolic dysfunction, and diminished vascular abnormalities in experimental heart failure models (Kusumoto et al.

2001; Engelhardt et al. 2002; Aker et al. 2004; Kilić et al. 2014; Baartscheer et al. 2005; Baartscheer et al. 2003). Clinical studies showed that cariporide reduced the risk of myocardial damage and cardiovascular mortality in patients undergoing coronary artery bypass surgery (Boyce et al. 2003; Mentzer et al. 2008).

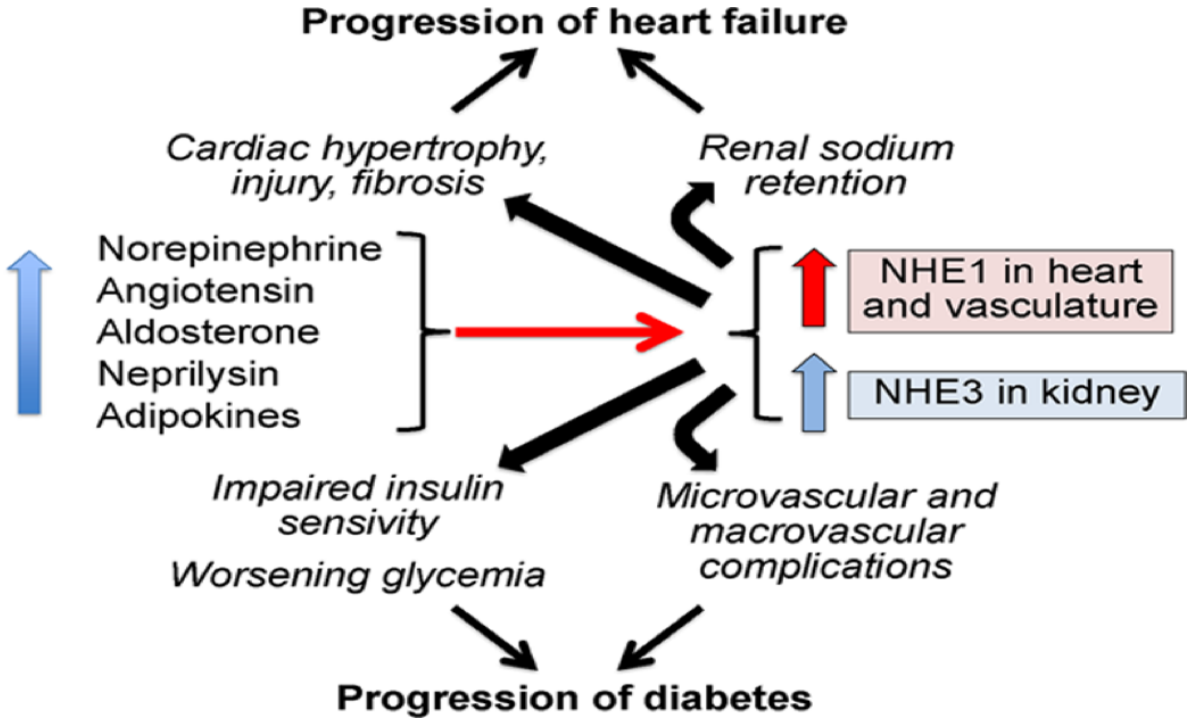

Figure 18. Mechanisms of Na^+/H^+ exchanger (NHE) activation contribute to progression of both heart failure and diabetes (Packer 2017).

Likewise, upregulation of NHE isoforms may promote organ dysfunction in glucose intolerance state. Raised NHE-1 expression has been suggested to contribute to vascular abnormalities of diabetes mediated by insulin resistance (Russell et al. 2005), hyperglycemia-induced coronary endothelial dysfunction (Vial et al. 2008), promotion of mesenteric vascular hypertrophy and AGE-induced neointimal proliferation (Wu et al. 2013), enhanced procoagulant activity (Telejko et al. 2003), and facilitation of the atherogenetic properties in human monocytes (Sarigianni et al. 2010). Furthermore, Ang II has been identified as one of the major stimuli of NHE-1 expression in both the heart and vasculature,

and of NHE-3 in the kidney (Cingolani et al. 2013; Liu et al. 2014; Xiao & Allen 2003; Lütken et al. 2009). The stimulatory effect of Ang II on NHE-1 has been linked to myocardial stiffness and this action is abolished by losartan, an AT_1R antagonist in the rabbit (Leite-Moreira et al. 2006). In addition, ramipril (ACE inhibitor) and valsartan (AT_1R antagonist) prevented the increase mRNA and protein of cardiac NHE-1 and NHE-1 activity in rats with post-MI (Sandmann et al. 2001).

Interestingly, two recent studies (Baartscheer et al. 2017; Uthman et al. 2018) revealed that empagliflozin, dapagliflozin, and canagliflozin block the NHE-1 activity and flux by directly binding to the extracellular Na^+-binding site of NHE-1, and thus declined myocardial $[Na^+]_i$ and $[Ca^{2+}]_i$ and reinforced $[Ca^{2+}]_m$ in rat, rabbit and mouse.

II.3.3 Na^+/Ca^{2+} exchanger

Subsequent to rise of $[Ca^{2+}]_i$ through the NCX resulting from increased $[Na^+]_i$, induces signaling cascades that lead to disturbance of mitochondrial homeostasis, including deteriorated ATP function and enhanced ROS generation, contributing to enhanced cardiac hypertrophy and remodeling (Bay et al. 2013).

In cardiomyocytes, NCX is the principal route for Ca^{2+} efflux from the cells, which exchanges three Na^+ for one Ca^{2+} ions (Bers 2001). Elevation of $[Na^+]_i$ by a few mM modifies Ca^{2+} fluxes via NCX, resulting in higher levels of $[Ca^{2+}]$ in the cytosol and sarcoplasmic reticulum, and subsequently exaggerated contractions. $[Na^+]_i$ also influences the $[Ca^{2+}]_m$ level through the mitochondrial NCX (MitoNCX), which is the central pathway for mitochondrial Ca^{2+} release in the heart (Griffiths 2009). A rise in $[Na^+]_i$ facilitates mitochondrial Ca^{2+} extrusion and thus lower $[Ca^{2+}]_m$ (Cox & Matlib 1993; Maack et al. 2006). Since $[Ca^{2+}]_m$ activates several dehydrogenases associated with the tricarboxylic acid (TCA)

cycle (McCormack et al. 1990), restoration of NADH and NADPH from their oxidized forms is delayed at lower $[Ca^{2+}]_m$. Delayed regeneration of the NADH/NADPH pool restricts the rate of electron transport and thus declines mitochondrial ATP synthesis driving cellular processes. NADPH is continuously used for neutralize the H_2O_2 generated by the electron transport chain in oxidative phosphorylation and lower levels of NADPH may result in oxidative stress (Bertero & Maack 2018).

II.3.4 Na^+/K^+ ATPase

Several studies in myocardium from human and animal models demonstrated a reduced protein expression level of NKA in HF. A decreased protein level and activity of NKA was observed with unaltered NCX protein expression level in failing human hearts (Schwinger et al. 1999), and in myocytes from rabbits with HF (Bossuyt et al. 2005). Despa et al. showed no changes in either the maximal transport rate of Na^+ or the affinity for $[Na^+]_i$ from failing rabbit hearts compared to that of control, by determining the rate of $[Na^+]_i$ fall as a function of $[Na^+]_i$ in ventricular myocytes (Despa et al. 2002). Reduced maximal Na^+ efflux rate mainly via NKA-α_2 isoform without changed $[Na^+]_i$-affinity were observed in myocytes from HF rats with myocardial infarction (Semb et al. 1998; Swift et al. 2008) as well as in mice with end-stage HF following knockout of $SERCA_2$ (Louch et al. 2010)

In addition, there are few studies of NKA function and expression in diabetic cardiomyopathy. NKA activity is decreased by 21% in the myocardium of STZ-induced diabetic rats (Kjeldsen et al. 1987), and a declined NKA current appeared in myocytes from alloxan-induced diabetic rabbits, which indicates a decline in the NKA affinity for $[Na^+]_i$ but no alteration in the maximal NKA activity (Hansen et al. 2007). Cardiac myocytes from HIP rats, which is a T2DM animal model, showed no modification in NKA-mediated Na^+ efflux

for $[Na^+]_i$ in the 0-20 mM of physiological range compared to their control WT rats (Lambert et al. 2015).

Aim of the study

Aim of the study

The vascular endothelium plays a critical role in the dynamic regulation of vascular functions in health and disease. Even though the overall vascular tree is subjected to the exposure of cardiovascular risk factors-related ED, phenotypic alterations to a dysfunctional endothelium is most markedly expressed at specific regions of arteries such as bifurcations, branch points, and inner curvatures, where disturbed flow and low shear stress take place. A dysfunctional state of the endothelium contributes to the initiation of the pathogenesis of cardiovascular diseases such as coronary artery disease (CAD), which is the major clinical manifestation of atherosclerosis. Senescent ECs are observed in human aortic arch and coronary arteries at sites overlapping atherosclerotic plaques. Moreover, since overexpression of the senescent marker p53 in ECs promoted ED and reduced formation of NO, cellular senescence pathway appears to act as a key upstream signaling event promoting ED. Endothelial senescence is characterized by the down-regulation of eNOS expression and the subsequent reduced formation of NO, a potent vasoprotective factor. The senescent-associated phenotype leads to the acquisition of pro-inflammatory and pro-thrombotic properties by ECs. A variety of findings support that ED is an independent predictor of deleterious events in CAD patients that can be evaluated by the circulating level of MPs, which act as a biological transcellular signal delivery system and consequently contribute to endothelial senescence and dysfunction. In fact, our previous study has indicated that incubation of ECs with circulating MPs from patients with acute coronary syndrome induces premature endothelial senescence and thrombogenicity via activation of the Ang II-induced NADPH oxidase pathway.

Gliflozins, the first class of oral antidiabetics that eliminate excess glucose from the body, have shown remarkable cardiovascular protective effect with reduced risk of cardiovascular mortality and hospitalization for heart failure in T2DM patients wtih a high cardiovascular

risk. Protective effects of empagliflozin have also been observed on β-cell function, oxidative stress, AGE/RAGE signaling and inflammation in Zucker fatty diabetic rats, a well-established rat model of T2DM. The findings of this *in vivo* study also indicated a protective effect on ED suggesting that glucose uptake across cell membranes involves glucose transporters including the SGLTs promoting oxidative stress and glucotoxicity. Recent findings have indicated that the cardioprotective effect of empagliflozin appears to be independent of glycemic control. Moreover, since high glucose causes the redox-sensitive upregulation of SGLT1 and 2 through local angiotensin system promoting endothelial senescence, it may be possible that SGLT2 inhibitors protect the endothelial function.

Therefore, we hypothesized that SGLT1 and 2 may contribute to the alteration of the cardiovascular system under a normal glucose concentration by promoting endothelial senescence and dysfunction. This hypothesis was investigated in a primary coronarary endothelial cell culture model, *ex vivo* in blood vessels and *in vivo* using an experimental model of metabolic syndrome as well as a translational approach.

In the first part of the scientific work, we have examined whether Ang II (a potent NADPH oxidase-dependent inducer of oxidative stress) stimulates SGLT1 and 2 expression in ECs, and assessed the underlying mechanism and their functional consequences.

In the second part of the scientific work, we determined the effect of empagliflozin on both the endothelial and vascular function, and the heart structure and function in an experimental model of metabolic syndrome. In the experimental model of metabolic syndrome with HFpEF, the obese ZSF1 rat, and its' lean control, we have evaluated 1) Oxidative stress-induced premature endothelial senescence and pro-atherosclerotic responses *in vivo* at arterial sites at risk, 2) Endothelial dysfunction in mesenteric artery rings, and 3) Characterized the underlying mechanism.

To determine the potential clinical relevance of the experimental findings, we have evaluated the effect of circulating MPs from CAD patients on SGLT1 and 2 expression in ECs, and determined their role on pro-atherosclerotic responses.

But de l'étude

L'endothélium vasculaire joue un rôle essentiel dans la régulation dynamique des fonctions vasculaires à la fois en conditions physiologiques et pathologiques. Bien que la totalité de l'arbre vasculaire soit exposée aux facteurs de risque cardiovasculaire, des altérations phénotypiques de l'endothélium s'expriment préférentiellement au niveau de régions spécifiques tels que les bifurcations, les points de ramification et les courbures artérielles, où se produisent un écoulement perturbé et une faible contrainte de cisaillement. Un endothélium dysfonctionnel contribue à l'initiation de la pathogenèse de maladies cardiovasculaires, telles que la maladie coronarienne, qui est la principale manifestation clinique de l'athérosclérose. Des CE sénescentes sont détectées dans les crosses aortiques et les artères coronaires humaines au niveau des plaques d'athérosclérose. Par ailleurs, considérant que la surexpression du marqueur de sénescence p53 dans les CE favorise la DE et la réduction de formation de NO, la voie de la sénescence cellulaire semble constituer une voie de signalisation clé impliquée dans la DE. La sénescence endothéliale est caractérisée par une diminution de l'expression de la eNOS et par une formation réduite de NO, un puissant facteur vasoprotecteur. Le phénotype associé à la sénescence conduit à l'acquisition de propriétés pro-inflammatoires et pro-thrombotiques par les CEs. De nombreux résultats montrent que la DE constitue un facteur prédictif indépendant d'événements cardiovasculaires chez les patients atteints de coronaropathie qui peut être évalué par le niveau de MP circulantes, qui agissent comme un système de transmission transcellulaire du signal biologique et contribuent par conséquent à la sénescence et à la DE.

Une étude récente menée au laboratoire a permis de montrer que l'incubation de CEs avec des MPs circulantes provenant de patients atteints d'un syndrome coronarien aigu induisait la sénescence endothéliale prématurée ainsi qu'un phénotype pro thrombotique via l'activation d'une voie NADPH/Ang II.

Les gliflozines, la première classe d'antidiabétiques oraux qui éliminent l'excès de glucose du corps, montrent un effet protecteur cardiovasculaire remarquable avec un risque réduit de mortalité cardiovasculaire et d'hospitalisation pour insuffisance cardiaque chez les patients atteints de DT2 présentant un risque cardiovasculaire élevé. Des effets protecteurs de l'empagliflozine ont également été observés sur la fonction des cellules β, le stress oxydant, la signalisation AGE / RAGE et l'inflammation chez le rat diabétique gras Zucker, un modèle de DT2 bien établi chez le rat. Les résultats de cette étude in vivo ont également mis en évidence un effet protecteur sur la DE suggérant que l'absorption cellulaire du glucose implique des transporteurs de glucose, y compris les SGLT favorisant le stress oxydatif et la glucotoxicité. Des découvertes récentes indiquent que les effets cardioprotecteurs de l'empagliflozine sont indépendants du contrôle de la glycémie. De plus, comme une glycémie élevée induit l'expression de SGLT1 et 2 par le système angiotensine locale et favorise la sénescence endothéliale, il est possible les inhibiteurs du SGLT2 exercent un effet protecteur de la fonction endothéliale.

Par conséquent, nous avons émis l'hypothèse que les SGLT1 et 2 pourraient contribuer à l'altération du système cardiovasculaire en condition de glycémie normale en favorisant la sénescence et la DE. Cette hypothèse a été testée in vitro dans un modèle de culture de CEs coronaires primaires ex vivo dans les vaisseaux sanguins et in vivo en utilisant un modèle expérimental de syndrome métabolique ainsi qu'une approche translationnelle.

Dans la première partie du travail scientifique, nous avons testée l'hypothèse selon laquelle l'Ang II (un puissant inducteur du stress oxydant dépendant de la NADPH oxydase) stimulait l'expression des SGLT1 et 2 dans les CEs et évalué les mécanisme sous-jacents ainsi que leurs conséquences fonctionnelles.

Dans la deuxième partie du travail scientifique, nous avons déterminé l'effet de l'empagliflozine sur les fonctions endothéliale et vasculaire, ainsi que sur la structure et la

fonction du cœur dans un modèle expérimental de syndrome métabolique. Dans un modèle expérimental de syndrome métabolique avec insuffisance cardiaque à fraction d'éjection préservée (HFpEF), le rat obèse ZSF1, nous avons évalué 1) la sénescence endothéliale prématurée induite par le stress oxydatif et les réponses pro-athérosclérotiques in vivo au niveau des sites artériels à risque, 2) la DE au niveau de l'artère mésentérique, et 3) caractérisé les mécanismes sous-jacents.

Pour déterminer la pertinence clinique potentielle des résultats expérimentaux, nous avons évalué l'effet des MP circulantes de patients atteints de coronaropathie sur l'expression des SGLT1 et 2 dans les CEs et déterminé leur rôle sur les réponses pro-athérosclérotiques.

RESULTS

Article I

Article I

Circulating microparticles from coronary artery disease patients up-regulate sodium-glucose cotransporters 1 and 2 expression to promote atherosclerotic responses in endothelial cells: Role of the Ang II/AT1R/NADPH oxidase pathway

The angiotensin system is a major determinant of NADPH oxidase-mediated oxidative stress promoting endothelial dysfunction in diabetes, hypertension and aging. SGLT2 inhibitors have been shown remarkable cardiovascular protective effect with reduced risk of cardiovascular mortality and hospitalization for heart failure in T2DM patients with a high cardiovascular risk, an effect that was independent of glucose control. Moreover, high glucose and H_2O_2 have been shown to cause a redox-sensitive up-regulation of SGLT1 and 2 in coronary artery ECs. Circulating microparticles (MPs) from patients with coronary artery diseases (CAD) have been shown to promote endothelial senescence and dysfunction involving the pro-oxidant local angiotensin system. In the present study, we aimed to investigate whether angiotensin II (Ang II, a potent NADPH oxidase-dependent inducer of oxidative stress) and CAD-MPs stimulate SGLT1 and 2 expression in ECs, and assessed their role in the induction of endothelial senescence and dysfunction.

Exposure of ECs to Ang II increased the SGLT1 and SGLT2 expression. Ang II increased the level of oxidative stress, SA-beta-gal activity, senescence markers (p53, p21 and p16), VCAM-1, MCP-1, tissue factor and the local angiotensin system (ACE, AT1R), and down-regulated that of eNOS. CAD-MPs from 7/10 patients decreased eNOS level and from 10/10 patients increased VCAM-1 level. The Ang II- and CAD MPs-induced expression of SGLT1 and 2, VCAM-1 and down-regulation of eNOS were prevented by the NADPH oxidase inhibitor VAS-2870, the AT1R antagonist losartan, and by the dual SGLT1/2 inhibitor, sotagliflozin and the selective SGLT2 inhibitor, empagliflozin.

In conclusion, the present findings indicate that Ang II and CAD-MPs via the activation of the local angiotensin system are potent inducers of SGLT1 and 2 expression to promote endothelial senescence and dysfunction, subsequent to the induction of glucose- and sodium-dependent oxidative stress leading to pro-atherosclerotic responses. They further suggest that inhibition of SGLT1 and/or SGLT2 might be an attractive therapeutic strategy to protect endothelial function, and the subsequent development of cardiovascular diseases.

Article I

Des microparticules circulantes provenant de patients atteints de coronaropathie régulent positivement l'expression des cotransporteurs sodium-glucose 1 et 2 et favorisent les réponses athérosclérotiques dans les cellules endothéliales: rôle de la voie Ang II/AT1R/NADPH oxydase

Le système angiotensine est un déterminant majeur du stress oxydatif induit par la NADPH oxydase et favorise la dysfonction endothélial associée au diabète, à l'hypertension et au vieillissement. Les inhibiteurs du SGLT2 ont démontré un effet protecteur cardiovasculaire remarquable avec un risque réduit de mortalité cardiovasculaire et d'hospitalisation pour insuffisance cardiaque chez les patients atteints de DT2 avec un risque cardiovasculaire élevé, un effet indépendant de leur contrôle de la glycémie. De plus, il a été montré que des taux de glucose élevés ou l'H_2O_2 entrainaient une surexpression des SGLT1 et 2 sensible au stress oxydant dans les CEs des artères coronaires. Il a été montré par ailleurs que les microparticules (MPs) circulantes de patients atteints de maladies coronariennes favorisaient la sénescence et la dysfonction endothéliale par induction du système angiotensine local pro-oxydant. Dans la présente étude, nous avons cherché à déterminer si l'angiotensine II (Ang II, un puissant inducteur du stress oxydant dépendant de la NADPH oxydase) et les MP de patients coronariens stimulaient l'expression des SGLT1 et 2 dans les CEs et nous avons évalué leur rôle dans l'induction de la sénescence et la dysfonction endothéliale.

L'Ang II favorise l'expression des SGLT1 et SGLT2 dans les CEs. L'Ang II induit un stress oxydatif, l'activité SA-bêta-gal, les marqueurs de sénescence (p53, p21 et p16), VCAM-1, MCP-1, le facteur tissulaire et le système angiotensine local (ACE, AT1R) et réprime l'expression de la eNOS. Les MPs de patients coronariens diminuent le niveau de eNOS (7/10) et augmentent le niveau de VCAM-1 (10/10). L'induction de l'expression des

SGLT1 et 2, de VCAM-1 et la régulation à la baisse de la eNOS induits par les MP de patients coronariens ou par l'Ang II sont prévenus par un inhibiteur de la NADPH oxydase (VAS-2870), par un antagoniste de AT1R (losartan) ainsi que par un inhibiteur mixte des SGLT1/2 (sotagliflozine) ou sélectif du SGLT2 (empagliflozine).

En conclusion, les résultats indiquent que l'Ang II et les MP de patients coronariens (via l'activation du système angiotensine local) sont de puissants inducteurs de l'expression des SGLT1 et 2 et favorisent la sénescence et les dysfonctions endothéliales consécutives à l'induction d'un stress oxydant dépendant du glucose et du sodium pour conduire à des réponses pro-athérosclérotiques. Ils suggèrent en outre que l'inhibition de SGLT1 et / ou SGLT2 pourrait être une stratégie thérapeutique attrayante pour protéger la fonction endothéliale et le développement de maladies cardiovasculaires.

The AT1R/NADPH oxidase pro-oxidant pathway induces expression of SGLT1 and 2 to sustain glucose and Na^+-dependent oxidative stress promoting endothelial dysfunction in response to Ang II and circulating microparticles of coronary artery disease patients

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Abstract

Background: Sodium-glucose cotransporter (SGLT)2 inhibitors have demonstrated cardiovascular (CV) protection in type 2 diabetes patients with established CV disease independently of glycemic control. Circulating microparticles (MPs), surrogate markers of CV disease, can promote endothelial dysfunction via the angiotensin system. Since H_2O_2 stimulated expression of SGLT1 and 2 in endothelial cells (ECs), the possibility that Ang II induces SGLT1 and 2 expression in ECs and the underlying mechanism and functional consequences were determined. Moreover, the role of such a pathway in MPs-induced ECs dysfunction was evaluated.

Methods: ECs were isolated from porcine coronary arteries, protein expression level by Western blot analysis, oxidative stress by dihydroethidium staining, and senescence by senescence-associated beta-galactosidase activity (SA-beta-gal activity). MPs were collected from blood of patients with coronary artery disease (CAD-MPs).

Results: Ang II increased the low level of SGLT1 and SGLT2 proteins in ECs in a time- and concentration-dependent manner starting at concentrations as low as 10 nM. Ang II induced a sustained pro-oxidant response that was dependent on extracellular glucose and Na^+, and inhibited by a NADPH oxidase inhibitor, an AT1R antagonist and by the dual SGLT1 and 2 inhibitor, sotagliflozin (Sota), and the selective SGLT2 inhibitor, empagliflozin (Empa). Ang II increased SA-beta-gal activity, senescence markers (p53, p21 and p16), VCAM-1, MCP-1, tissue factor (TF) and the local angiotensin system (ACE, AT1R), and down-regulated that of eNOS, which were all inhibited by Sota and Empa. CAD-MPs from 7/10 patients increased the expression of SGLT1, SGLT2 protein levels and from 10/10 patients the VCAM-1 level and from 7/10

decreased the eNOS level, all these responses were inhibited by a NADPH oxidase inhibitor, an AT1R antagonist, Sota and Empa.

Conclusions: These findings indicate that Ang II is a potent inducer of the expression of SGLT1 and SGLT2 in ECs to sustain the glucose- and Na^+-dependent oxidative stress, which, in turn, promotes endothelial senescence and dysfunction. Since the AT1R/NADPH oxidase/SGLT1 and 2 pathway mediates endothelial dysfunction to CAD-MPs, inhibition of SGLT1 and/or SGLT2 appears as an attractive strategy to restore the protective endothelial function and, hence, prevent the development of cardiovascular diseases.

Introduction

Cardiovascular diseases are the leading cause of mortality worldwide involving the heart and/or blood vessels.[1] The renin-angiotensin system is a vital constituent in controlling physiological and pathological regulation of the cardiovascular system.[2] Previous evidences have indicated an important role of both the circulating and the local angiotensin system in the induction of endothelial dysfunction in experimental models of hypertension, diabetes, aging and atherosclerosis, and in individuals with high cardiovascular risk factors.[3-8] ECs express high levels of angiotensin-converting enzyme (ACE) promoting formation of angiotensin II (Ang II).[9] Ang II is a potent inducer of endothelial senescence promoting endothelial dysfunction by inducing vascular oxidative stress involving NADPH oxidase.[10-12] The rise in oxidative stress stimulated by Ang II leads to diminished endothelial relaxation and endothelial dysfunction in the experimental hypertensive rat model.[13]

The vascular endothelium plays a critical role in the pathogenesis of coronary artery disease (CAD) which is the major clinical manifestation of atherosclerosis. During the past decades, although medical interventional procedures and secondary prevention medications, such as ACE inhibitors or statins, have substantially advanced and improved cardiovascular outcome, atherosclerosis still remains a principal contributor to cardiovascular mortality worldwide. Experimental and clinical studies indicate that endothelial dysfunction acts as an independent predictor of detrimental events in CAD patients.[14, 15] Moreover, plasma membrane-derived circulating MPs have emerged as a surrogate biomarker and effector of endothelial dysfunction and cardiovascular risk,[16, 17] and behave as a biological transcellular signal delivery system.[18] They consequently contribute to endothelial senescence and dysfunction especially by modulating the NO/ROS balance in favor of oxidative stress, which promotes procoagulant and proinflammatory responses.[19, 20] Indeed, a previous study has shown that the incubation of endothelial cells (ECs) with circulating MPs from acute

coronary syndrome patients induces premature endothelial senescence and thrombogenicity through activation of Ang II/AT_1R/NADPH oxidase pathway.[21]

Recent findings have emphasized the potential role of sodium-glucose cotransporters (SGLTs), which transport glucose across the plasma membrane via a symport mechanism with the concomitant transfer of sodium and independent of the presence of insulin, in the development of cardiovascular disease. Several clinical trials have demonstrated the cardiovascular effects of SGLT2 inhibitors by significantly lowering the mortality from cardiovascular causes and hospitalization for heart failure in T2DM patients with established cardiovascular diseases[22-24] and these effects appear to be independent of glycemic control.[25] Potential mechanisms contributing to the beneficial effects include a reduction in blood pressure, arterial stiffness and albuminuria, and natriuresis, diuresis, improvement in lipid profile, myocardial energetics by increasing oxidation of ketone bodies and visceral adiposity, and weight loss.[26] In addition, an effect on blood vessels may also contribute since high glucose caused the redox-sensitive upregulation of SGLT1 and 2 through the local angiotensin system promoting endothelial senescence.[27] Despite of the remarkable cardiovascular benefits, role of SGLT1 and SGLT2 on the endothelial function remains poorly described.

Therefore, the present study examined whether Ang II and MPs isolated from the plasma of patients with CAD induce SGLT1 and 2 expression to promote endothelial dysfunction characterized by premature senescence and prothrombotic responses. Moreover, since Ang II through NADPH oxidase-mediated oxidative stress is a strong inducer of endothelial senescence, the potential role of SGLT1 and 2 in the deleterious effect of both Ang II and CAD-MPs on endothelial dysfunction was determined.

Methods

Materials

Empagliflozin was provided by Boehringer Ingelheim Pharma GmbH & Co KG (Biberach an der Riss, Germany). All other chemicals were from Sigma-Aldrich (Sigma-Aldrich Chemie SARL, St Quentin Fallavier, France) unless otherwise specified.

Patients and isolation of circulating microparticles

The Institutional Review Board has approved the study and all participants gave informed assent. Sixteen patients with coronary artery diseases (CAD) (between 50 and 88 years old) were enrolled at the University Hospital of Strasbourg, France. The extent of CAD was characterized by coronary angiography. Patients with a history of chronic inflammatory disorders or atrial fibrillation were excluded.

Blood samples collected by venous puncture into tubes containing 129 mmol/L sodium citrate were processed within 1 h 30 min. Platelet-poor plasma (PPP) samples containing circulating microparticles (MPs) were acquired by double centrifugations at room temperature and immediately stored at -80 °C until use as previously described.[28, 29] For *ex vivo* experiments, PPP samples from individual CAD patients were thawed and centrifuged twice at 14,000 *g* for 1 h at 4 °C each. The MPs pellets after centrifugation were concentrated in calcium and magnesium-free Hank balanced salt solution (HBSS) before the addition to ECs. To determine the effects of modulators, washed MPs were produced from pooled PPP samples of 6 CAD patients. Briefly, 50 ml of PPP samples were subjected to a double centrifugation step (14,000 *g*, 1 h, 4 °C) and the final pellet was resuspended in 1.2 ml of HBSS. The concentration of MPs was measured by prothrombinase assay using a microplate reader set in kinetics software and referred to as nanomolar phosphatidylserine equivalent

(nM PhtdSer eq). The phosphatidylserine content of MPs captured onto annexin-V were detected at 405 nm using a chromogenic substrate for thrombin.

Cell culture

Porcine hearts were obtained from the local slaughterhouse (SOCOPA, Holtzheim, France) and ECs were isolated from porcine left circumflex coronary arteries as described previously.[30] Briefly, porcine left circumflex coronary arteries were dissected and cleaned of connective tissues. After washing with phosphate-buffered saline solution (PBS) without calcium to remove remaining blood, ECs were isolated by type I collagenase (Invitrogen) treatment at 1 mg/ml for 15 min at 37 °C and cultured in a T25 flask containing MCDB 131 medium (Invitrogen) supplemented with 15 % fetal calf serum, fungizone (2.5 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM, all from Lonza, Levallois-Perret, France) and grown to 80-90 % confluence for 48-72 h (passage 0). All experiments were performed with cultured ECs at passage 1, which were treated 15 h after passaging. ECs were exposed to serum-free culture medium for 2 h before the addition of Ang II or CAD-MPs. In some experiments, ECs were pretreated with a pharmacological modulator for 30 min before the addition of Ang II or CAD-MPs. In experiments CAD-MPs, ECs were incubated with CAD-MPs (10 nM PhtdSer eq) for 48 h.

Western blot analysis

After treatment, ECs were washed with cold PBS and lysed in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5), NaCl 150, Na_3VO_4 1, $Na_4P_2O_7$ 10, NaF 20, okadaic acid 0.01, 1 % Triton X-100 and protease inhibitor cocktail (Complete Mini, Roche)). Total proteins (15 µg) were separated on 8 or 12 % SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membrane (GE Healthcare Life Sciences). After

blocking with 5 % bovine serum albumin in Tris-buffered saline (TBS) containing 0.1 % Tween 20 for 1 h at room temperature, membranes were incubated with a primary antibody against either rabbit polyclonal anti-SGLT1 (1:1,000, Abcam, ab14685), rabbit polyclonal anti-SGLT2 (1:1,000, Abcam, ab37296), rabbit polyclonal anti-angiotensin-converting enzyme (ACE, 1:1,000, Abbiotec, 250450), rabbit polyclonal anti-angiotensin type 1 receptor (AT1R, 1:1,000, Abcam, ab124505), mouse monoclonal anti-eNOS (1:5,000, BD Transduction Laboratories, 610297), rabbit monoclonal anti-VCAM-1 (1:10,000, Abcam, ab134047), rabbit polyclonal anti-MCP-1 (1:1,000, Abcam, ab25124), rabbit polyclonal anti-tissue factor (TF, 1:1,000, Sekisui Diagnostics, 4509) or mouse monoclonal anti-β-tubulin (1:20,000, Sigma-Aldrich, T7816) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin G, 1:10,000, Cell Signaling Technology, #7074, #7076, respectively) for 1 h at room temperature. The immunoreactive bands were developed by enhanced chemiluminescence (ECL, Amersham) using ImageQuant LAS 4000 (GE Healthcare).

Immunofluorescence staining

ECs were cultured on 8 well Lab-Tek® chambers and exposed to either H_2O_2 (100 μM) or Ang II (100 nM) for 24 h. Cells were fixed during 30 min with 4 % (w/v) paraformaldehyde and then incubated with blocking/permeabilizing buffer (PBS containing 1 % BSA (w/v) and 0.5 % Triton X-100 (w/v)) for 30 min at room temperature. After buffer removal, cells were incubated with 1:100 dilution of either rabbit anti-SGLT1 or SGLT2 for 1 h at 4 °C. After washing 3 times with PBS, they were further incubated with a 1:250 dilution of a polyclonal goat anti rabbit immunoglobulin G coupled to CF 633 (Alexa Fluor 633 conjugate, Invitrogen) for 1 h at room temperature in the dark. After washing 3 times

with PBS, cells were incubated with 1 mg/ml 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, Thermo Fisher) during 3 min at room temperature, in order to counterstain nuclei. After disassembling, slides mounted with fluorescent mounting medium. Images were acquired using a Leica TCS SPE confocal microscope.

Cellular level of oxidative stress

ECs were cultured on 8 well Lab-Tek® chambers and pretreated with either an antioxidant (N-acetyl cysteine, 1 mM) for 2 h, a NADPH oxidase inhibitor (VAS-2870, 1 μM), a cyclooxygenase inhibitor (indomethacin, 30 μM), a mixture of the mitochondrial respiratory chain inhibitors (myxothiazol, KCN and rotenone, 0.5, 1, 1 μM, respectively), an AT1R antagonist (losartan, 1 μM), a dual SGLT1 and SGLT2 inhibitor (sotagliflozin, 100 nM) or a selective SGLT2 inhibitor (empagliflozin, 100 nM) for 30 min before the addition of Ang II for 30 min or 24 h. To determine the contribution of glucose and sodium, ECs after 24-h treatment period with Ang II were exposed to different concentrations of glucose (0, 0.344, 1.375, 5.5, 10, 15, 20 and 25 mM) for 1 h in either sodium-containing buffer (mM: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, KH_2PO_4 1, and HEPES 10, pH 7.4) or sodium-free buffer with NaCl replaced by N-methyl-D-glucamine (NMDG)-Cl. To evaluate the role of glucose metabolism, a non-metabolizable glucose analogue, methyl α-D-glucopyranoside (AMG, 25 mM) was tested and mannitol (25 mM) was used to rule out an osmotic effect. In some experiments, ECs were also incubated with a Na^+/H^+ exchanger (NHE)-1 inhibitor (cariporide, 10 μM), a Na^+/Ca^{2+} exchanger (NCX) inhibitor (KB-R7943, 10 μM) or a Na^+/K^+-ATPase (NKA) inhibitor (ouabain, 10 nM). Thereafter, cells were exposed to dihyroethidium (5 μM), a redox-sensitive fluorescent dye for 30 min at 37 °C in the dark. After washing 3 times with PBS, cells were mounted with fluorescent mounting medium. Images were acquired using a Leica TCS SPE confocal microscope.

Determination of senescence-associated β-galactosidase (SA-β-gal) activity

Fluorescence-based SA-β-gal activity was determined in ECs using 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a membrane-permeable fluorogenic substrate of β-galactosidase, by flow cytometry as described previously.[31] ECs were pretreated with either VAS-2870 (1 μM), losartan (1 μM), sotagliflozin (100 nM), empagliflozin (100 nM), cariporide (10 μM) or KB-R7943 (10 μM) for 30 min before the addition of Ang II (100 nM) for 24 h. ECs were exposed to chloroquine (300 μM), a lysosomal inhibitory drug, for 1 h before the addition of C_{12}FDG (33 μM) for 1 h. After washing twice with PBS, ECs were harvested by trypsinization and centrifuged at 10,000 rpm for 10 min at 4 °C followed by resuspension in ice-cold PBS. The relative SA-β-gal activity was estimated using the MFI of the population determined by BD FACSCelesta flow cytometer.

Cellular level of nitric oxide (NO)

ECs were cultured on 8 well Lab-Tek® chambers and pretreated with either sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min before the addition of Ang II for 24 h. ECs were exposed to DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluororescein diacetate, 1 μM), a NO-sensitive fluorescent dye for 20 min at 37 °C in the dark. NO synthesis was induced by stimulation with bradykinin (100 nM) for 15 min. After washing 3 times with PBS, cells were mounted with fluorescent mounting medium. Images were acquired using a Leica TCS SPE confocal microscope.

Statistical analysis

Values are expressed as means ± SEM. Statistical analysis was assessed by one-way analysis of variance followed by Tukey's multiple comparison *post hoc* test using GraphPad Prism (Version 7). The differences between groups were considered statistically significant at $P < 0.05$.

Results

Ang II induces expression of SGLT1 and 2 in ECs

Control ECs expressed low levels of SGLT1 and SGLT2 proteins of about a 65 kDa as assessed by Western blot analysis (Figure 1). Exposure of ECs to Ang II caused a time-dependent increase in the protein expression level of SGLT1 and 2 reaching both about a 1.8-fold increase after a 24-h stimulatory period (Figure 1A). The stimulatory effect of Ang II at 24 h was concentration-dependent with a significant increase observed at concentrations of or greater than 10 nM, and reaching about 3.7- and 4.7-fold at 100 nM, respectively (Figure 1B). In addition, increased SGLT1 and 2 fluorescence signals by about 2.8- and 2.0-fold, respectively, were observed in ECs in response to Ang II (100 nM), and by about 2.3- and 1.6-fold, respectively to H_2O_2 (100 μM, Figure 1C).

Role of SGLT1 and 2 in the Ang II-induced pro-oxidant response in ECs

Since Ang II is a potent inducer of the generation of reactive oxygen species (ROS) in vascular cells including ECs,[32] the possibility that SGLT1 and 2 contribute to the pro-oxidant response was evaluated. For this purpose, a first series of experiments has characterized the Ang II-induced generation of ROS using the redox-sensitive fluorescent probe dihydroethidium (DHE) by confocal microscope. Ang II increased the level of ethidium fluorescence after 30 min and the stimulatory effect persisted up to 24 h (Figure 2A and B). Both the short and sustained pro-oxidant responses to Ang II were abolished by the antioxidant N-acetyl cysteine, and the AT1R antagonist losartan (Figures 2A and B). The characterization of the Ang II-triggered sources of ROS has indicated that the NADPH oxidase inhibitor, VAS-2870, the cyclooxygenase inhibitor, indomethacin and inhibitors of the mitochondrial respiratory chain (combination of myxothiazol, KCN and rotenone) all markedly inhibited both the short-term and long-term pro-oxidant response, indicating the

involvement of several sources including NADPH oxidase, cyclooxygenases and the mitochondrial respiratory chain (Figure 2A and B).

Next the possibly that SGLT1 and 2 contribute to the pro-oxidant response to Ang II in ECs was determined using a dual SGLT1 and 2 inhibitor, sotagliflozin and a selective SGLT2 inhibitor, empagliflozin. As shown in Figure 2C and D, the pro-oxidant response to Ang II was abolished by sotagliflozin and empagliflozin after a 24-h incubation period and not affected after 30 min (Figure 2C and D), indicating that although SGLT1 and 2 do not contribute to the early pro-oxidant response, they have a crucial role in perpetuating oxidative stress. In addition, sotagliflozin and empagliflozin alone affected the low basal formation of ROS in ECs neither after 30 min nor 24 h (Figure 2C and D).

To further determine the role of SGLT1 and 2 in the pro-oxidant response to Ang II, the contribution of glucose and sodium was assessed by reducing the level of glucose, that of Na^+ being replaced by NMDG, or both. The pro-oxidant response of Ang II was significantly reduced by decreasing the concentration of glucose and by replacing extracellular Na^+ by NMDG, and abolished in the absence of extracellular glucose and Na^+ (Figure 3A), indicating a key role of both extracellular glucose and Na^+ possibly subsequent to their entry via SGLT1 and 2.

In addition, exposure of ECs to increasing concentrations of glucose from 5.5 to 25 mM for 24 h resulted in an enhanced level of oxidative stress with a significant effect observed at concentrations of or greater than 20 mM (Figure 3B). Moreover, the combined treatment of ECs with Ang II and high glucose resulted in an additive effect of the pro-oxidant responses (Figure 3B). Besides glucose, a similar potentiating effect of the pro-oxidant response to Ang II was also observed in response to a non-metabolizable glucose analogue, AMG, which has been shown to enter cells via SGLTs.[33, 34] In contrast, no such effect was observed with mannitol ruling out an osmotic effect (Figure 3B). Thus, these

findings indicate that Ang II and high glucose act together to promote an excessive level of oxidative stress that is independent of glucose metabolism. Moreover, the sustained pro-oxidant response to Ang II was markedly inhibited by cariporide, an inhibitor of NHE-1 and by KB-R7943, an inhibitor of NCX, and not affected by ouabain, an inhibitor of NKA, suggesting the involvement, besides SGLT1 and 2, also of NHE-1 and NCX (Figure 3C).

SGLT1 and 2 are part of a vicious cycle to exacerbate the stimulatory effect of the Ang II/AT1R/NADPH oxidase/ROS pathway on SGLT1 and 2 expression in ECs

Since H_2O_2 has been shown to induce the expression of SGLT1 and 2 in ECs,[27] the possibility that oxidative stress mediates Ang II-induced SGLT1 and 2 protein expression was evaluated in ECs. N-acetyl cysteine, VAS-2870, indomethacin and the mitochondrial respiratory chain inhibitors all significantly blunted the stimulatory effect of Ang II on SGLT1 and 2 protein expression levels (Figure 4A and B) demonstrating the involvement of a crucial redox-sensitive mechanism. Moreover, the fact that sotagliflozin and empagliflozin abolished the Ang II-induced up-regulation of both SGLT1 and 2 (Figure 4C and D) suggests that SGLT1 and 2 act in a feed forward manner to promote the redox-sensitive expression of SGLT1 and 2.

Role of SGLT1 and 2 in Ang II-induced endothelial senescence and dysfunction

Since Ang II is potent redox-sensitive inducer of endothelial senescence in ECs,[35] the role of SGLT1 and 2 was evaluated using SA-β-gal activity and C_{12}FDG. Ang II increased SA-β-gal activity, which was significantly inhibited by losartan and VAS-2870, and also by sotagliflozin and empagliflozin, but not by cariporide and KB-R7943 (Figure 5A), indicating the involvement of the AT1R/NADPH oxidase/SGLT1 and 2 pathway in the pro-senescence response. Consistent with an increased SA-β-gal activity, Ang II upregulated the expression

level of senescence makers including p53, p21 and p16, and this effect was abolished by both sotagliflozin and empagliflozin (Figure 5B-D).

Since endothelial senescence is an upstream signaling event promoting endothelial dysfunction[36] in ECs, the role of SGLT1 and 2 in Ang II-induced endothelial dysfunction was determined. Ang II caused a down-regulation of the protein expression level of eNOS and an up-regulation of that of VCAM-1, MCP-1 and tissue factor (Figure 6). The Ang II-induced down-regulation of eNOS was prevented by N-acetyl cysteine and the mitochondrial respiratory chain inhibitors but not by VAS-2870 and indomethacin, and the upregulation of VCAM-1 by N-acetyl cysteine, VAS-2870, indomethacin and the mitochondrial respiratory chain inhibitors (Figure 6A and B). In addition, the fact that both sotagliflozin and empagliflozin prevented the Ang II-induced down-regulation of eNOS and up-regulation of VCAM-1, MCP-1 and tissue factor indicates a determinant role of SGLT1 and 2 in the induction of endothelial dysfunction (Figure 6C-F). Moreover, the Ang II-induced up-regulation of ACE and AT1R were abolished by both sotagliflozin and empagliflozin (Figure 6G and H), suggesting that the SGLT1 and 2 are part of an amplifying loop to exacerbate the Ang II/AT1R/NADPH oxidase pro-oxidant stimulatory signal.

To further confirm whether the fact that both sotagliflozin and empagliflozin prevented the Ang II-induced down-regulation of eNOS is associated with the increase in NO production in ECs stimulated by Ang II, intracellular NO levels were measured using the NO-sensitive fluorescent probe DAF-FM diacetate by confocal microscope. NO synthesis was stimulated by treating ECs with bradykinin (100 nM) for 15 min. Ang II decreased bradykinin-induced NO production and this effect was abolished by sotagliflozin and empagliflozin (Figure 6I), demonstrating the involvement of a crucial NO-sensitive mechanism.

Circulating MPs from CAD patients induce expression of SGLT1 and 2 in ECs to promote endothelial dysfunction

Since previous observations have indicated that circulating MPs from patients with acute coronary syndrome induce endothelial senescence and thrombogenicity involving the local pro-oxidant angiotensin system,[21] experiments were performed to determine whether circulating MPs from CAD patients induce SGLT1 and 2 expression in ECs and, if so, to clarify their role in the induction of endothelial dysfunction. Clinical characteristics of the CAD patients are provided in Table 1. The CAD-MPs from 7 out of 10 patients increased the protein expression level of both SGLT1 and 2, and from 10 out of 10 patients VCAM-1, and from 7 out of 10 patients down-regulated that of eNOS (Figure 7A-D). All the CAD-MPs-induced effects were prevented by VAS-2870, losartan, sotagliflozin and empagliflozin (Figure 7E-H), suggesting that the AT1R/NADPH oxidase pathway promotes the expression of SGLT1 and 2, which, in turn, act in a feed forward manner to induce endothelial dysfunction.

Discussion

The major findings of the present study indicate that Ang II and MPs derived from CAD patients up-regulates SGLT1 and 2 expression to promote endothelial senescence and dysfunction. The stimulatory effect of Ang II leads to an early oxidative stress involving NADPH oxidase, cyclooxygenases and mitochondrial respiratory chain, which, in turn, triggers the increase in SGLT1 and 2 expression associated with both glucose- and sodium-mediated sustained oxidative stress to ultimately induce endothelial senescence. Ang II-induced premature senescence results in endothelial dysfunction characterized by eNOS down-regulation, reduced NO production, enhanced oxidative stress and up-regulation of pro-atherosclerotic factors including VCAM-1, MCP-1 and TF. Furthermore, inhibition of

SGLT1 and/or SGLT2 prevented CAD-MPs-induced NADPH oxidase- and local angiotensin system-mediated expression of SGLT1 and 2, VCAM-1 and down-regulation of eNOS. Altogether, these findings suggest that SGLT1 and 2 contribute to the Ang II-induced endothelial senescence and dysfunction, possibly by acting as a crucial amplifying system, to promote the further deterioration of the vascular system and, hence, SGLT1 and 2 appear as novel targets for vascular protection.

The EMPA-REG OUTCOME trial (2010–2015) has shown remarkable cardioprotective effects of the selective SGLT2 inhibitor empagliflozin by significantly lowering the mortality from cardiovascular causes (38% relative risk reduction), all-cause death (32% relative risk reduction) and hospitalization for heart failure (35% relative risk reduction) in T2DM patients with established cardiovascular diseases.[24] In addition, several experimental studies have demonstrated that ipragliflozin improved endothelial dysfunction, and restored phosphorylation of Akt and eNOS, decreased ROS production, and expression of MCP-1, VCAM-1 and ICAM-1 in the abdominal aorta from STZ-induced diabetic mice,[37] empagliflozin improved endothelium-dependent relaxations in STZ-induced diabetic rats[38] and reduced atherosclerotic plaque formation in $ApoE^{-/-}$ mice by ameliorating inflammation and insulin resistance.[39] However, the underlying mechanism of the protective effects on the endothelial function remains to be clarified. Recent studies have indicated that the improvement of cardiovascular outcomes by SGLT2 inhibitors is independent of glycemic control.[25] Moreover, high glucose induced premature endothelial senescence via Ang II-mediated redox-sensitive SGLT1 and 2 expression, suggesting a possible protective effect on the endothelial function.[27] Indeed, senescent ECs have been observed in human aortic arch[40] and coronary arteries[41] at sites overlapping atherosclerotic plaques. Moreover, since targeted expression of the senescent marker p53 in ECs promoted endothelial dysfunction and reduced formation of NO,[42] cellular senescence pathway appears to act as a key upstream

signaling event promoting endothelial dysfunction. Moreover, endothelial dysfunction associated with aging and major cardiovascular diseases such as CAD is related to a rise in shedding of MPs considered as a potential diagnostic biomarker and bioeffector of vascular dysfunction.[16, 17, 43] Indeed, circulating MPs from patients with ACS abolished *ex vivo* endothelium-dependent relaxations in rat aortic rings[44] and caused the induction of premature senescence in ECs via the Ang II-dependent NADPH oxidase-mediated oxidative stress, resulting in endothelial dysfunction.[21] In light with these previous observations and the fact that Ang II is a stimulator of premature senescence through increased oxidative stress in cultured ECs,[35] the role of SGLT1 and 2 in Ang II- and CAD-MPs-induced endothelial senescence and dysfunction was investigated.

The present findings indicate that the stimulatory effect of Ang II caused a time- and concentration-dependent increase in SGLT1 and 2 protein level, associated with an increased SGLT1 and 2 immunofluorescence staining in ECs. Ang II induced sustained oxidative stress involving several sources including NADPH oxidase, cyclooxygenases and the mitochondrial respiratory chain. Using a dual SGLT1 and 2 inhibitor sotagliflozin and a selective SGLT2 inhibitor empagliflozin, it was observed that SGLT1 and 2 contribute to the Ang II-induced sustained oxidative stress in senescent ECs but not to the early response. Since SGLTs transport glucose and sodium into the cell driven by the sodium gradient, the role of glucose and sodium in the Ang II-induced pro-oxidant response was determined. For this purpose, ECs were incubated with different glucose concentrations in the absence and presence of sodium, before the Ang II-induced oxidative stress was evaluated. The pro-oxidant response of Ang II was reduced by decreasing the glucose concentration and also in the absence of sodium. Moreover, the Ang II-induced oxidative stress was further increased by higher concentrations of glucose. In addition, incubation of ECs with the non-metabolizable glucose analogue, AMG, which enters the cells primarily via SGLTs,[33, 34] mimicked the detrimental

effect of high glucose. Altogether, these findings suggest that both glucose and sodium are involved in the Ang II-induced oxidative stress possibly through SGLT1 and 2. They further suggest that SGLT1 and 2 act as a glucose sensor as has been already demonstrated in cardiomyocytes,[45] hypothalamic neurons[46] and rat mesangial cells.[47] SGLT1 and 2 might have a pivotal role in hyperglycemia-associated Ang II-mediated vascular complications. The characterization of the sodium pathway in Ang II-induced oxidative stress in ECs has indicated the involvement of NHE and NCX. In mammalian cells, a low level of intracellular Na^+ ($[Na^+]_i$) is maintained by actively expelling Na^+ through the NKA, and alterations in $[Na^+]_i$ critically influence the function of several transporters including NHE and NCX. An increase in $[Na^+]_i$ is coupled to the subsequent secondary rise of $[Ca^{2+}]_i$ and diminished mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) involving NCX in the plasma and mitochondrial membrane, respectively, in cardiac myocytes, which leads to disturbance of mitochondrial homeostasis, including deteriorated energetics and enhanced ROS generation.[48, 49] Elevated $[Ca^{2+}]_i$ has been identified in LPS- and hyperglycemia-induced endothelial dysfunction,[50, 51] in cardiomyocytes during IR injury[52] and artrial fibrillation.[53]

In line with these observations, the Ang II-induced SGLT1 and 2 protein expression was abolished by inhibition of oxidative stress sources including NADPH oxidase, cyclooxygenases and the mitochondrial respiratory chain, and also by a dual SGLT1 and 2 inhibitor and a selective SGLT2 inhibitor. In addition, Ang II-induced upregulation of both ACE and AT1R was prevented by sotagliflozin and empagliflozin. These observations imply that the SGLT1 and 2 promote the local angiotensin system, which acts in a feed forward manner to sustain the Ang II-induced oxidative stress. Several studies have supported that Ang II-mediated oxidative stress is a key inducer of premature endothelial senescence.[11, 35] The present findings indicate that Ang II-induced endothelial senescence via the activation of NADPH oxidase and AT1R was prevented by inhibition of SGLT1 and/or SGLT2, but not by

inhibition of NHE and NCX, as evaluated by SA-β-gal activity and expression of senescence makers including p53, p21 and p16. Consistent with previous findings, Ang II-induced oxidative stress-mediated endothelial senescence promoted endothelial dysfunction as indicated by the down-regulation of eNOS, the reduced NO production and the up-regulation of pro-atherothrombotic makers including VCAM-1, MCP-1 and TF. Since the dual SGLT1 and 2 inhibitor and the selective SGLT2 inhibitor abolished the Ang II-induced senescence and endothelial dysfunction, SGLT2 and possibly also SGLT1 appear as novel targets to protect the endothelial function, and, hence, the cardiovascular system. The potential clinical implication of these findings is supported by the fact that CAD-MPs up-regulated the protein levels of SGLT1 and 2 and that this effect involves the activation of NADPH oxidase and the local angiotensin system to promote endothelial dysfunction as indicated by the down-regulation of eNOS and up-regulation of VCAM-1, and also by the fact that those effects were prevented by inhibition of SGLT1 and/or 2.

In conclusion, the present findings indicate that Ang II and circulating MPs from CAD patients via the activation of the local angiotensin system are potent inducers of SGLT1 and 2 expression to promote endothelial senescence and dysfunction, subsequent to the induction of glucose- and sodium-dependent oxidative stress leading to pro-atherosclerotic responses. They further suggest that inhibition of SGLT1 and/or SGLT2 might be an attractive therapeutic strategy to protect endothelial function, and the subsequent development of cardiovascular diseases.

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Figure legends

Figure 1. Ang II induces a time- and concentration-dependent expression of SGLT1 and SGLT2 protein levels and an increased SGLT1 and 2 immunofluorescence staining in ECs. (A) ECs at P1 are exposed to 100 nM Ang II for the times indicated. (B) ECs at P1 are exposed to increasing concentrations of Ang II for 24 h. Thereafter, the expression level of SGLT1 and SGLT2 protein is determined by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels). (C) ECs at P1 are exposed to either H_2O_2 (100 μM) or Ang II (100 nM) for 24 h before immunofluorescence staining for SGLT1 and SGLT2 using confocal microscope. SGLT1 and SGLT2 protein staining appears in red and nuclei are stained with DAPI (blue). Results are shown as representative immunofluorescence staining (left panels) and corresponding cumulative data (right panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control.

Figure 2. Ang II-induced oxidative stress in ECs involves NADPH oxidase, cyclooxygenases and the mitochondrial respiratory chain, and is sensitive to a dual SGLT1 and SGLT2 inhibitor, sotagliflozin, and a selective SGLT2 inhibitor, empagliflozin. ECs are incubated with either N-acetyl cysteine (NAC, an antioxidant, 1 mM) for 2 h, VAS-2870 (VAS, a NADPH oxidase inhibitor, 1 μM), indomethacin (INDO, a cyclooxygenase inhibitor, 30 μM), myxothiazol + KCN + rotenone (MKR, mitochondrial respiratory chain inhibitors, 0.5, 1, 1 μM, respectively) or losartan (LOS, an AT1R antagonist, 1 μM) for 30 min before the addition of Ang II (A) for 30 min (short-term) or (B) for 24 h (long-term). ECs are incubated with either sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min before the addition of Ang II (C) for 30 min (short-term) or (D) for 24 h (long-term), and the subsequent determination of dihydroethidium staining by confocal microscope. Results are shown as

representative micrography of dihydroethidium staining (left or upper panels) and corresponding cumulative data (right or lower panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control and $^{\#}P < 0.05$ vs Ang II.

Figure 3. Role of glucose and sodium in the Ang II-induced oxidative stress in ECs. ECs are incubated with Ang II for 24 h before being exposed to (A) the indicated glucose concentrations for 1 h in the presence or absence of sodium, (B) the indicated glucose concentrations, methyl α-D-glucopyranoside (AMG, a non-metabolizable glucose analogue, 25 mM), or mannitol (25 mM), and (C) cariporide (a NHE-1 inhibitor, 10 μM), KB-R7943 (a NCX inhibitor, 1 μM), or ouabain (a NKA inhibitor, 10 nM) for 1 h, and the subsequent dihydroethidium staining using confocal microscope. Results are shown as representative micrography of dihydroethidium staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control and $^{\#}P < 0.05$ vs Ang II.

Figure 4. SGLT1 and SGLT2 are part of a feed forward mechanism exacerbating the Ang II-induced redox-sensitive up-regulation of SGLT1 and 2 in ECs. ECs are incubated with either (A, B) NAC (1 mM) for 2 h, VAS (1 μM), INDO (30 μM) or MKR (0.5, 1, 1 μM, respectively) for 30 min, and (C, D) sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min before the addition of Ang II for 24 h. Thereafter, the expression level of SGLT1 and SGLT2 is assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control and $^{\#}P < 0.05$ vs Ang II.

Figure 5. Ang II-induced endothelial senescence involves the AT_1R/NADPH oxidase/SGLT1 and 2 pathway. ECs are incubated with either (A) VAS (1 μM), LOS (1 μM), sotagliflozin (100 nM), empagliflozin (100 nM), cariporide (10 μM) or KB-R7943 (10 μM) for 30 min before the addition of Ang II for 24 h, and the subsequent determination of SA-β-gal activity, and (B-D) sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min before the addition of Ang II for 24 h, and the subsequent determination of the expression level of p53, p21 and p16 as assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control and $^{\#}P < 0.05$ vs Ang II.

Figure 6. Ang II-induced expression of pro-atherothrombotic markers in ECs is dependent on oxidative stress and SGLT1 and 2. ECs are incubated with either (A, B) NAC (1 mM) for 2 h, VAS (1 μM), INDO (30 μM) or MKR (0.5, 1, 1 μM, respectively) for 30 min, and (C-I) sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min before the addition of Ang II for 24 h. Thereafter, (C-H) the expression level of eNOS, VCAM-1, MCP-1, Tissue factor (TF), ACE and AT1R is assessed by Western blot analysis or (I) the subsequent DAF-FM staining by confocal microscope. NO synthesis was stimulated by addition of bradykinin (100 nM) for 15 min. Results are shown as representative immunoblots or micrography of DAF-FM staining (upper or left panels, respectively) and corresponding cumulative data (lower or right panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control and $^{\#}P < 0.05$ vs (A-H) Ang II or (I) control + bradykinin.

Figure 7. Circulating MPs from patients with coronary artery diseases (CAD) up-regulate SGLT1, SGLT2 and VCAM-1, and down-regulate eNOS protein expression involving the AT1R/NADPH oxidase/SGLT1 and SGLT2 pathway in ECs. (A-D) ECs at P1 are exposed to

CAD-MPs (10 nM PhtdSer eq) from 10 different CAD patients for 48 h. (E-H) ECs at P1 are incubated with either VAS (1 μM), LOS (1 μM), sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min before the addition of CAD-MPs (10 nM PhtdSer eq) pooled from 6 patients with CAD for 48 h. Thereafter, the expression level of SGLT1, SGLT2, eNOS and VCAM-1 is assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean ± SEM of n=3. $^{*}P < 0.05$ vs control and $^{\#}P < 0.05$ vs CAD-MPs.

Table 1. Clinical characteristics of CAD patients.

Patient	Age	Sex	BMI	Hypertension	Dyslipidemia	Diabetes	Smoking	Family history
CAD1	61	F	25.4	-	+	-	+	+
CAD2	67	M	29.6	+	+	-	+	+
CAD3	79	M	26.2	+	-	-	-	+
CAD4	66	M	24.5	+	+	-	+	+
CAD5	71	M	28.1	+	+	+	+	+
CAD6	59	M	28.7	+	+	-	-	-
CAD7	75	M	30.9	+	-	-	+	-
CAD8	88	M	18.8	+	+	-	-	-
CAD9	70	M		-	-	-	+	+
CAD10	67	M	29.1	+	-	+	-	-
CAD11	50	M	30.1	-	+	+	+	-
CAD12	81	F	31.3	+	-	-	+	+
CAD13	61	M	25.5	-	-	+	+	+
CAD14	83	M	38.0	+	-	+	+	-
CAD15	59	F	26.0	+	+	-	+	+
CAD16	64	M	30.9	+	+	-	+	-

CAD indicates coronary artery disease; F, female; M, male; BMI, Body mass index: body mass (kg)/ [height (m)]^2.

Figure 1

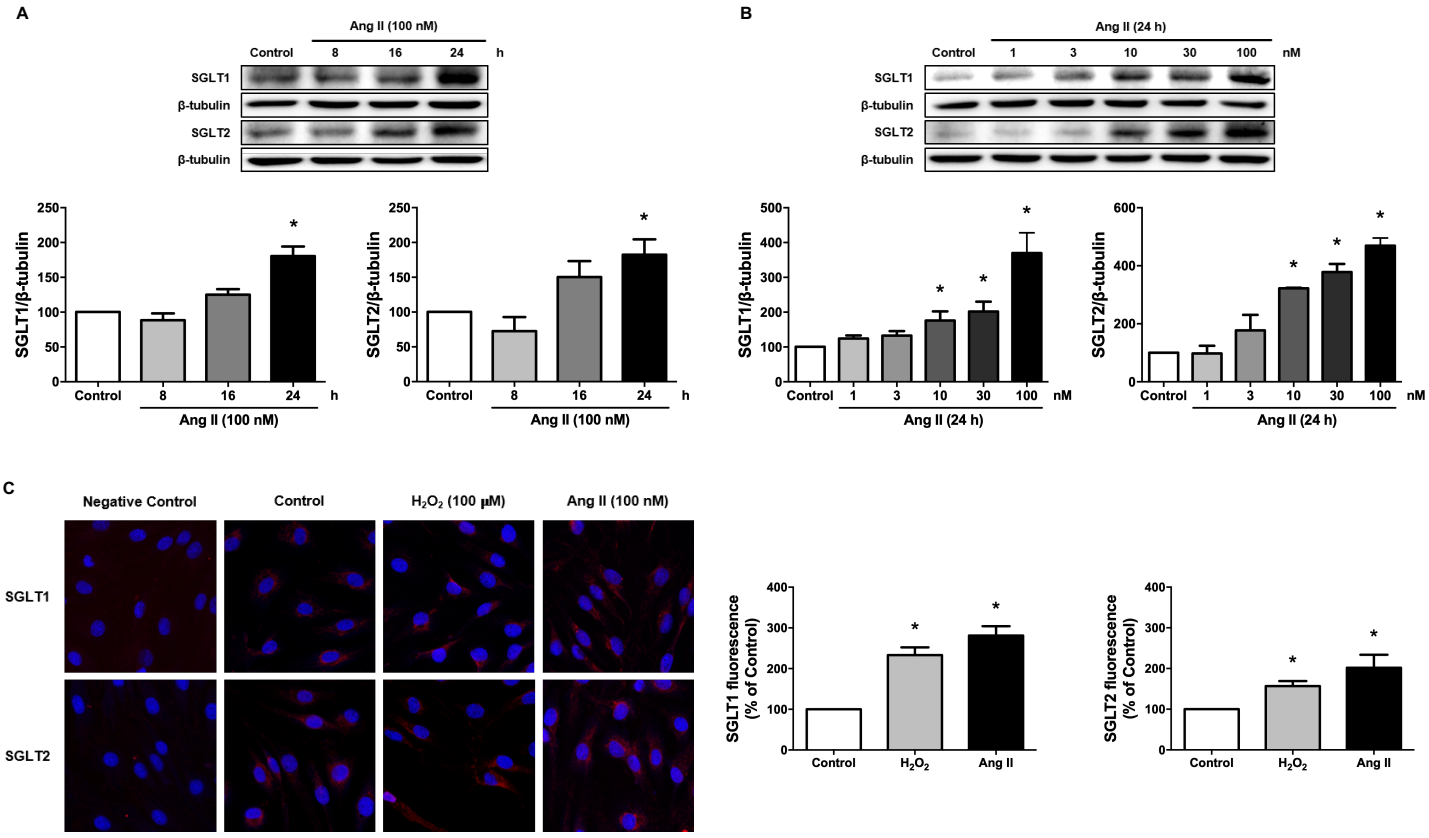

A
Ang II (100 nM)
Control
8
16
24
h
SGLT1
β-tubulin
SGLT2
β-tubulin
SGLT1/β-tubulin
SGLT2/β-tubulin
Ang II (100 nM)
B
Ang II (24 h)
Control
1
3
10
30
100
nM
SGLT1
β-tubulin
SGLT2
β-tubulin
SGLT1/β-tubulin
SGLT2/β-tubulin
Ang II (24 h)
C
Negative Control
Control
H2O2 (100 μM)
Ang II (100 nM)
SGLT1
SGLT2
SGLT1 fluorescence (% of Control)
SGLT2 fluorescence (% of Control)
Control
H2O2
Ang II

Figure 2

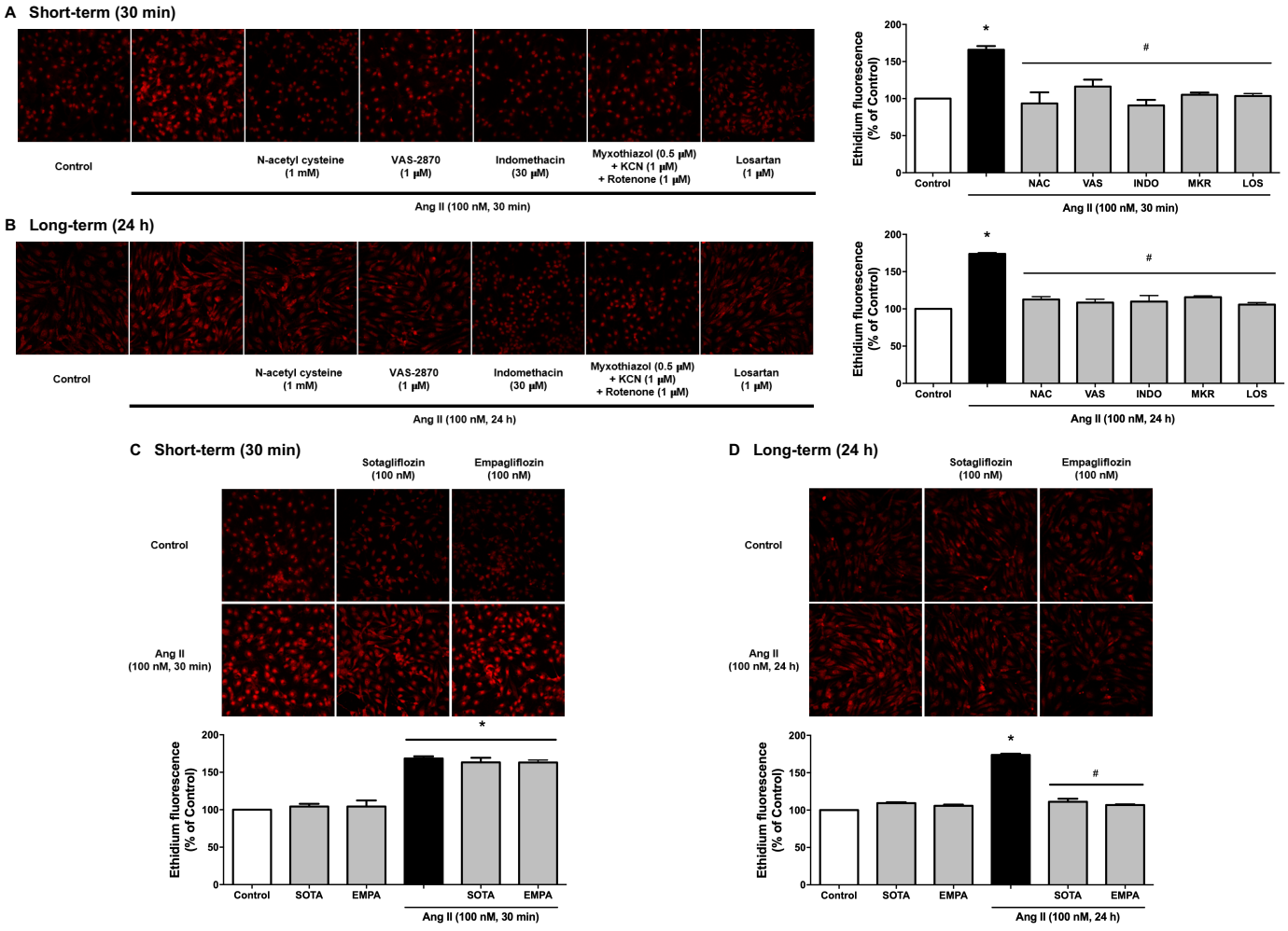

A Short-term (30 min)
Control
N-acetyl cysteine (1 mM)
VAS-2870 (1 μM)
Indomethacin (30 μM)
Myxothiazol (0.5 μM) + KCN (1 μM) + Rotenone (1 μM)
Losartan (1 μM)
Ang II (100 nM, 30 min)
Ethidium fluorescence (% of Control)
NAC
VAS
INDO
MKR
LOS
B Long-term (24 h)
Ang II (100 nM, 24 h)
C Short-term (30 min)
Sotagliflozin (100 nM)
Empagliflozin (100 nM)
Ang II (100 nM, 30 min)
SOTA
EMPA
D Long-term (24 h)
Ang II (100 nM, 24 h)

Figure 3

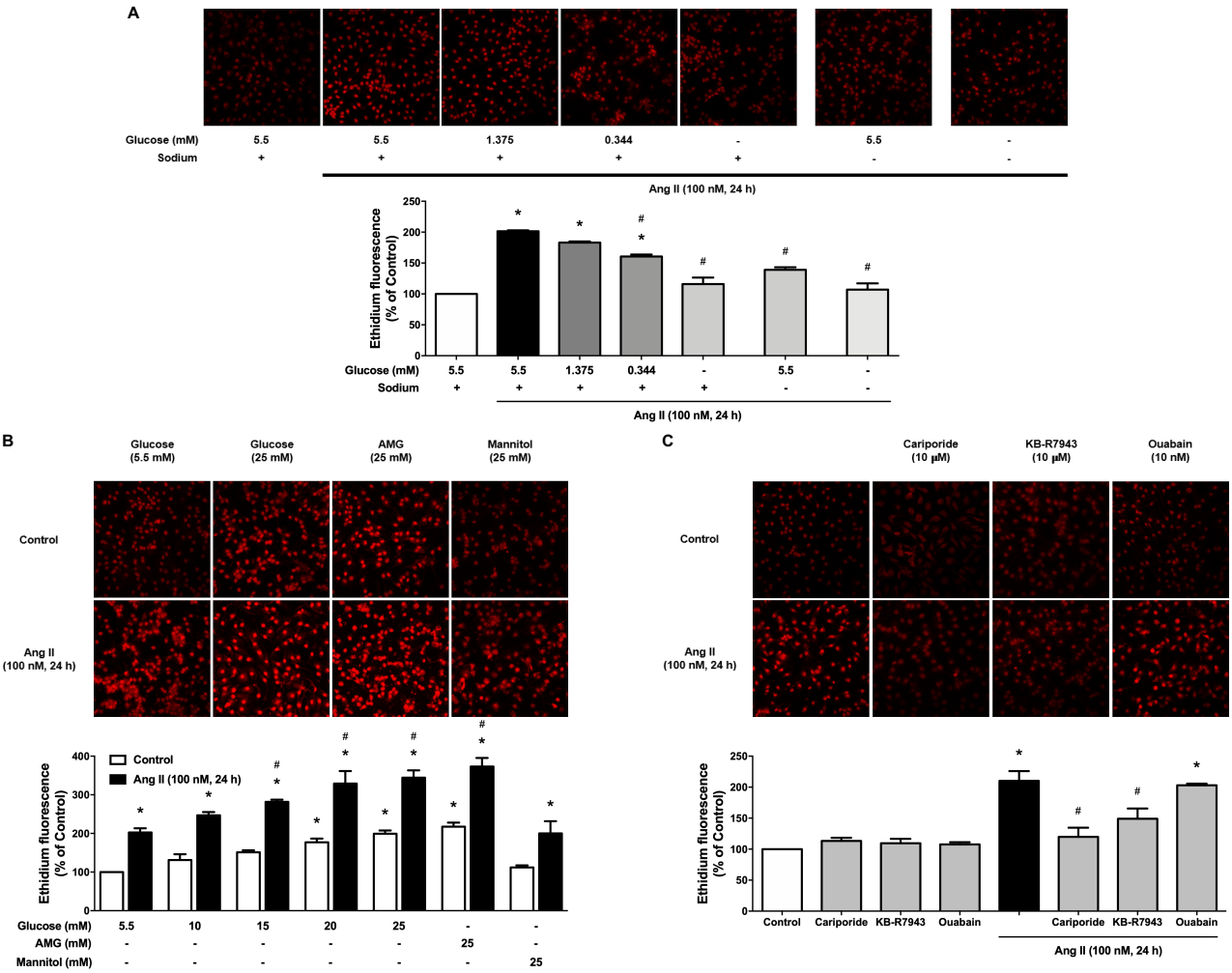

A
Glucose (mM) 5.5 5.5 1.375 0.344 - 5.5 -
Sodium + + + + + - -
Ang II (100 nM, 24 h)
Ethidium fluorescence (% of Control)
Glucose (mM) 5.5 5.5 1.375 0.344 - 5.5 -
Sodium + + + + + - -
Ang II (100 nM, 24 h)
B
Glucose (5.5 mM)
Glucose (25 mM)
AMG (25 mM)
Mannitol (25 mM)
Control
Ang II (100 nM, 24 h)
Control
Ang II (100 nM, 24 h)
Ethidium fluorescence (% of Control)
Glucose (mM) 5.5 10 15 20 25 - -
AMG (mM) - - - - - 25 -
Mannitol (mM) - - - - - - 25
C
Cariporide (10 μM)
KB-R7943 (10 μM)
Ouabain (10 nM)
Control
Ang II (100 nM, 24 h)
Ethidium fluorescence (% of Control)
Control Cariporide KB-R7943 Ouabain Cariporide KB-R7943 Ouabain
Ang II (100 nM, 24 h)

Figure 4

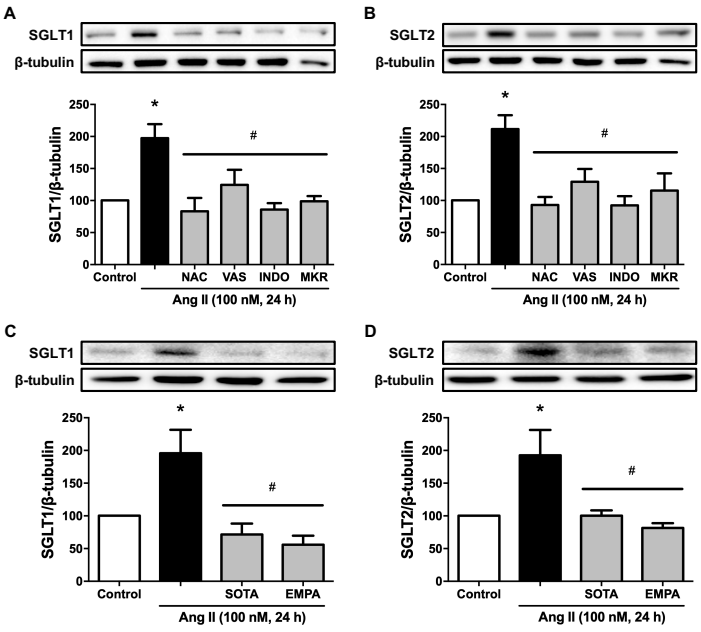

A
SGLT1
β-tubulin
SGLT1/β-tubulin
250
200
150
100
50
0
*
#
Control
NAC
VAS
INDO
MKR
Ang II (100 nM, 24 h)
B
SGLT2
β-tubulin
SGLT2/β-tubulin
250
200
150
100
50
0
*
#
Control
NAC
VAS
INDO
MKR
Ang II (100 nM, 24 h)
C
SGLT1
β-tubulin
SGLT1/β-tubulin
250
200
150
100
50
0
*
#
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
D
SGLT2
β-tubulin
SGLT2/β-tubulin
250
200
150
100
50
0
*
#
Control
SOTA
EMPA
Ang II (100 nM, 24 h)

Figure 5

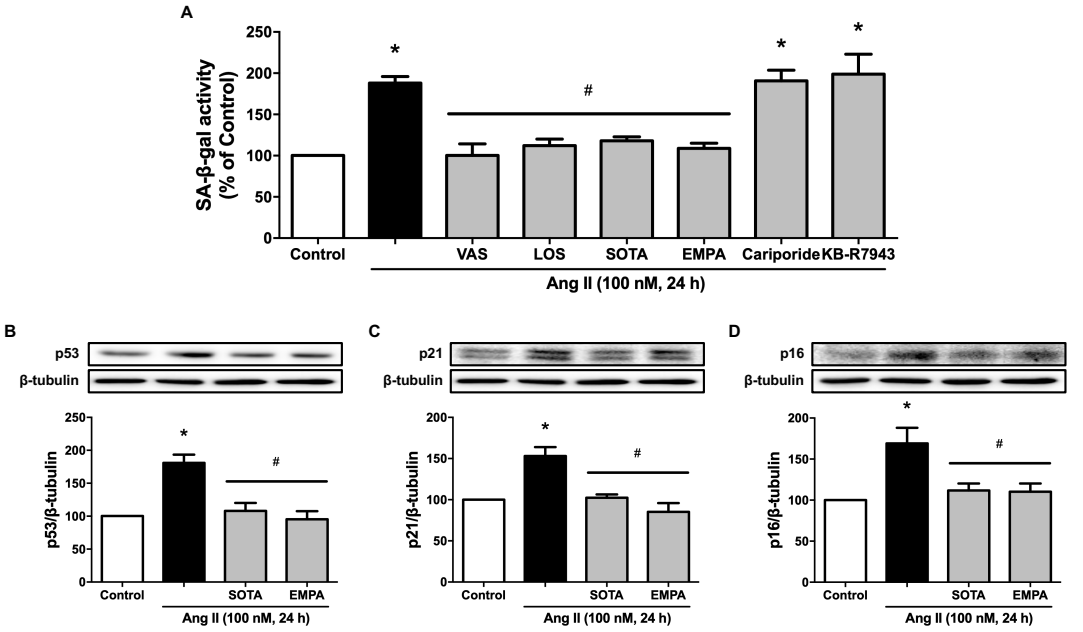

A
SA-β-gal activity (% of Control)
250
200
150
100
50
0
*
*
*
#
Control
VAS
LOS
SOTA
EMPA
Cariporide
KB-R7943
Ang II (100 nM, 24 h)
B
p53
β-tubulin
p53/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
C
p21
β-tubulin
p21/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
D
p16
β-tubulin
p16/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)

Figure 6

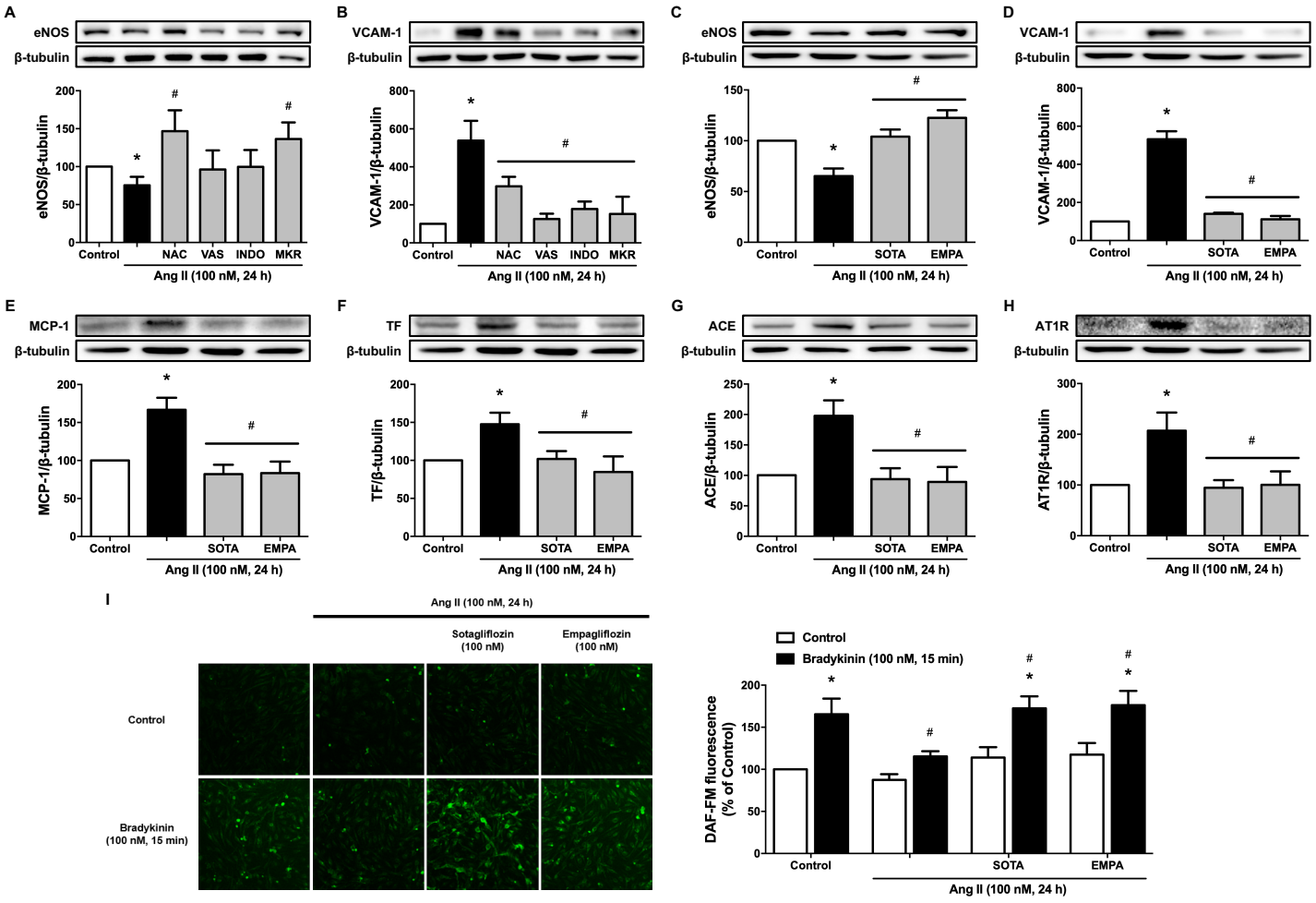
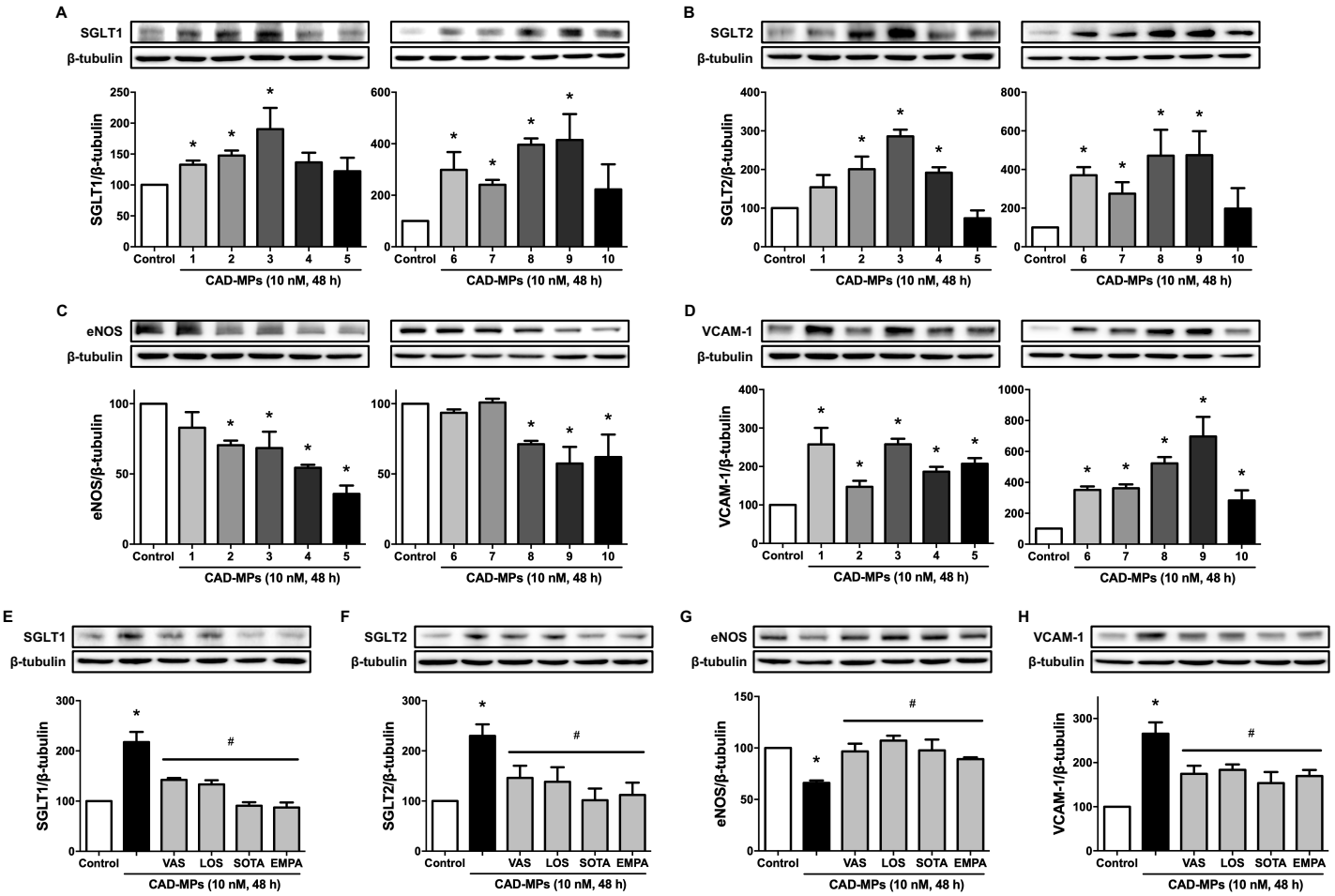

A
eNOS
β-tubulin
eNOS/β-tubulin
Control
NAC
VAS
INDO
MKR
Ang II (100 nM, 24 h)
B
VCAM-1
β-tubulin
VCAM-1/β-tubulin
Control
NAC
VAS
INDO
MKR
Ang II (100 nM, 24 h)
C
eNOS
β-tubulin
eNOS/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
D
VCAM-1
β-tubulin
VCAM-1/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
E
MCP-1
β-tubulin
MCP-1/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
F
TF
β-tubulin
TF/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
G
ACE
β-tubulin
ACE/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
H
AT1R
β-tubulin
AT1R/β-tubulin
Control
SOTA
EMPA
Ang II (100 nM, 24 h)
I
Ang II (100 nM, 24 h)
Sotagliflozin (100 nM)
Empagliflozin (100 nM)
Control
Bradykinin (100 nM, 15 min)
Control
Bradykinin (100 nM, 15 min)
DAF-FM fluorescence (% of Control)
Control
SOTA
EMPA
Ang II (100 nM, 24 h)

Figure 7


A
SGLT1
β-tubulin
SGLT1/β-tubulin
Control
CAD-MPs (10 nM, 48 h)
B
SGLT2
β-tubulin
SGLT2/β-tubulin
Control
CAD-MPs (10 nM, 48 h)
C
eNOS
β-tubulin
eNOS/β-tubulin
Control
CAD-MPs (10 nM, 48 h)
D
VCAM-1
β-tubulin
VCAM-1/β-tubulin
Control
CAD-MPs (10 nM, 48 h)
E
SGLT1
β-tubulin
SGLT1/β-tubulin
Control
VAS
LOS
SOTA
EMPA
CAD-MPs (10 nM, 48 h)
F
SGLT2
β-tubulin
SGLT2/β-tubulin
Control
VAS
LOS
SOTA
EMPA
CAD-MPs (10 nM, 48 h)
G
eNOS
β-tubulin
eNOS/β-tubulin
Control
VAS
LOS
SOTA
EMPA
CAD-MPs (10 nM, 48 h)
H
VCAM-1
β-tubulin
VCAM-1/β-tubulin
Control
VAS
LOS
SOTA
EMPA
CAD-MPs (10 nM, 48 h)

Article II

Article II

Empagliflozin, a sodium-glucose cotransporter 2 inhibitor, improved heart remodeling, endothelial and vascular dysfunction in the metabolic syndrome ZSF1 rats

The EMPA-REG OUTCOME trial has shown remarkable cardioprotective effects of the selective SGLT2 inhibitor empagliflozin by significantly lowering the mortality from cardiovascular causes, all-cause death and hospitalization for heart failure in T2DM patients with established cardiovascular diseases. The protective effect on the cardiovascular system appears to be independent of glycemic control. However, the underlying mechanism of the protective effects on the endothelial and vascular function remains to be clarified. The study aims to determine the effect of empagliflozin on both the endothelial and vascular function, and the heart structure and function in an experimental model of metabolic syndrome with heart failure with preserved ejection fraction (HFpEF), the obese ZSF1 rat.

Empagliflozin treatment resulted in improved hyperglycemia, total cholesterol and triglycerides levels and body weight in the ZSF1 group. The weight of the heart and all the four cardiac cavities together with the left ventricle area were increased in the ZSF1 group, an effect that was significantly blunted by the empagliflozin treatment, except for left auricle and septum. LV ejection fraction and cardiac output were similar in all groups. An up-regulation of senescence markers (p53, p21, p16), tissue factor, VCAM-1, SGLT1 and SGLT2 and a down-regulation of eNOS were observed in the inner curvature of aortic arch compared to the outer one in the control group, which were prevented by the empagliflozin treatment. In the mesenteric artery of ZSF1 group, acetylcholine-induced endothelium-dependent relaxations were slightly but significantly blunted and EDCFs were increased compared to control group. The cyclooxygenase inhibitor indomethacin improved endothelium-dependent relaxations to acetylcholine, and abolished EDCFs in the ZSF1 rats.

In conclusion, the major findings of the study indicate that empagliflozin protects the heart and the vascular system, and that the treatment is particularly effective to delay premature vascular senescence known to promote the development of cardiovascular diseases at arterial sites at risk of atherogenesis.

Article II

L'empagliflozine, un inhibiteur du cotransporteur sodium-glucose 2, améliore le remodelage cardiaque, la dysfonction endothéliale et vasculaire dans le syndrome métabolique chez les rats ZSF1

L'essai EMPA-REG OUTCOME a montré des effets cardioprotecteurs remarquables de l'empagliflozine, inhibiteur sélectif du SGLT2, en réduisant de manière significative la mortalité par cause cardiovasculaire, le décès toutes causes confondues et l'hospitalisation pour insuffisance cardiaque chez des patients atteints de DT2 avec une maladie cardiovasculaire établie. L'effet protecteur cardiovasculaire semble être indépendant du contrôle glycémique. Cependant, le mécanisme sous-jacent des effets protecteurs sur la fonction endothéliale et vasculaire reste à clarifier. L'étude vise à déterminer l'effet de l'empagliflozine sur les fonctions endothéliale et vasculaire, ainsi que sur la structure et la fonction du cœur dans un modèle expérimental de syndrome métabolique avec insuffisance cardiaque avec fraction d'éjection préservée (HFpEF), le rat obèse ZSF1.

Le traitement à l'empagliflozine a entraîné une réduction de la glycémie, des taux de cholestérol total et de triglycérides et du poids corporel dans le groupe ZSF1. Le poids du cœur et des quatre cavités cardiaques ainsi que la zone du ventricule gauche étaient augmentés dans le groupe ZSF1 en comparaison au rat contrôles, un effet significativement atténué par le traitement à l'empagliflozine, à l'exception de l'auricule et du septum gauches. La fraction d'éjection du VG et le débit cardiaque étaient similaires dans tous les groupes. Une régulation à la hausse des marqueurs de sénescence (p53, p21, p16), du facteur tissulaire, de VCAM-1, de SGLT1 et SGLT2 et une régulation à la baisse de eNOS ont été observées dans la courbure interne de la crosse aortique par rapport à celle externe du groupe témoin. Ces effets ont été prévenus par le traitement à l'empagliflozine. Dans l'artère mésentérique du groupe ZSF1, les relaxations dépendantes de l'endothélium induites par l'acétylcholine

étaient légèrement altérées alors que les facteurs constricteurs dépendants de l'endothélium augmentaient par rapport au groupe témoin. L'indométhacine, un inhibiteur de la cyclooxygénase, améliorait les relaxations induites par l'acétylcholine et éliminaient les facteurs constricteurs dépendants de l'endothélium chez les rats ZSF1.

En conclusion, l'étude indiquent que l'empagliflozine protège le cœur et le système vasculaire et retarde la sénescence vasculaire prématurée connue pour favoriser le développement de maladies cardiovasculaires au niveau des sites artériels présentant un risque d'athérogenèse.

Empagliflozin, a sodium-glucose cotransporter2 inhibitor, improved systolic blood pressure, endothelial dysfunction and heart remodeling in the metabolic syndrome ZSF1 rat

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Abstract

Background: Empagliflozin (empa), a selective sodium-glucose cotransporter (SGLT)2 inhibitor, reduced cardiovascular mortality and hospitalization for heart failure in patients with type 2 diabetes at high cardiovascular risk independent of glycemic control. The possibility that empa protects the cardiovascular system was evaluated in an experimental model of metabolic syndrome with heart failure with preserved ejection fraction (HFpEF), the obese ZSF1 rat, and its' lean control.

Methods: Rats received either control diet or diet containing empa (30 mg/kg/day) for 6 weeks. Vascular reactivity was assessed using mesenteric artery rings, systolic blood pressure by tail-cuff sphygmomanometry, heart function and structural changes by echocardiography, and protein expression levels by Western blot analysis.

Results: Obese ZSF1 rats were characterized by increased systolic blood pressure, and blunted acetylcholine-induced endothelium-dependent relaxations associated with the appearance of endothelium-dependent contractile responses (EDCFs) compared to control lean rats. These effects were prevented by the empa treatment, and an improvement of the endothelial function was also observed with the cyclooxygenase inhibitor indomethacin. Obese ZSF1 rats showed increased weight of the heart and of the left ventricle volume with a preserved left ventricle ejection fraction and cardiac output, which were improved by the empa treatment. An increased expression level of senescence markers (p53, p21, p16), tissue factor, VCAM-1, SGLT1 and SGLT2 and a down-regulation of eNOS were observed in the aortic inner curvature compared to the outer one in the control lean rats, which were prevented by the empa treatment. In the obese ZSF1 rats, no such effects were observed. The empa treatment reduced the increased body weight and weight of lungs, spleen, liver and perirenal fat, hyperglycemia and the increased levels of total cholesterol and triglycerides in

obese ZSF1 rats, and increased blood ketone levels and urinary glucose excretion in control lean and obese ZSF1 rats.

Conclusion: The empa treatment improved both endothelial function and cardiac remodeling in the obese ZSF1 rat, and the increased expression level of pro-senescence and atherothrombotic markers at arterial sites at risk in the control lean rat. These protective cardiovascular effects were associated with improved hyperglycemia, lipid profile and body weight.

Keywords: Empagliflozin – SGLT2 – endothelial function – heart function – heart structure – senescence – ZSF1 – metabolic syndrome

Background

Metabolic syndrome is a critical health state comprising central obesity, dyslipidemia, hypertension, and glucose intolerance that carries increased risk for both type 2 diabetes (T2D) and cardiovascular disease (CVD) [1-4]. Over the past three decades, the prevalence rate of metabolic syndrome has increased rapidly with age worldwide and its prevalence ranges from <10 % up to 84 % [5, 6]. The direct and indirect effects of hyperglycemia in T2D on the vascular tree promote both macrovascular complications including stroke, coronary artery diseases, and peripheral vascular diseases, and microvascular complications including diabetic retinopathy, nephropathy, and neuropathy [7]. One of the earliest impact of hyperglycemia on the cardiovascular system leads to the induction of an endothelial dysfunction characterized by a reduced nitric oxide (NO) component and often also endothelium-dependent hyperpolarization (EDH) component, and the induction of endothelium-dependent contractile responses (EDCFs) [8-14]. Even though the entire vascular tree is subjected to cardiovascular risk factors, phenotypic alterations leading to a dysfunctional endothelium are revealed initially at specific regions of arteries such as curvatures and bifurcations, where disturbed flow and low shear stress take place and which are prone to early atherogenesis. Senescent endothelial cells (ECs), which are characterized by endothelial nitric oxide synthase (eNOS) down-regulation and a reduced formation of NO, are observed in the human aortic arch [15] and coronary artery at sites overlying atherosclerotic plaques [16]. The fact that the selective expression of the senescence marker p53 in the endothelium leads to diminished endothelium-dependent relaxations and NO formation in aortic rings of rats [17] suggests that the induction of cellular senescence acts as a critical upstream signaling pathway to promote endothelial dysfunction.

Gliflozins including empagliflozin, dapagliflozin, and canagliflozin are a novel class of antidiabetic agents used for the treatment of T2D that selectively inhibit the

sodium-glucose cotransporter (SGLT)2 to prevent glucose reabsorption in the renal proximal tubule. In the cardiovascular EMPA-REG OUTCOME trial including 7,020 T2D patients with established CVD, empagliflozin reduced cardiovascular death by 38 % and heart failure (HF) hospitalization by 35 % [18]. In T2D patients with established CVD, canagliflozin reduced the composite of cardiovascular cause of death including nonfatal myocardial infarction or nonfatal stroke by 14 % and HF hospitalization by 33 % [19], and dapagliflozin lowered the rate of cardiovascular death or HF hospitalization, with a 27 % lower rate of HF hospitalization [20]. Subsequent analysis of the EMPA-REG OUTCOME data indicated that the cardioprotective effect of empagliflozin appears to be independent of glycemic control [21], suggesting that mechanisms, in addition to glycemic control, are involved. Experimental studies have shown that empagliflozin improved endothelium-dependent relaxations in streptozotocin-induced diabetic rats [22], and decreased left ventricular weight, cardiomyocytes size, and cardiac interstitial fibrosis and macrophage infiltration in genetic prediabetic rats with metabolic syndrome [23]. In addition, a redox-sensitive up-regulation of SGLT1 and 2 has been observed in coronary artery ECs in response to high glucose and H_2O_2 leading to enhanced glucose uptake and induction of atherothrombotic responses [24]. Altogether, these findings support the concept that gliflozins protect the cardiovascular system, besides the improvement of the glycemic control, possibly also by targeting the pivotal protective endothelial function.

Therefore, the aim of the present study was to investigate the effect of a 6-week oral intake of empagliflozin on the cardiovascular system in the obese ZSF1 rat, an established experimental model of metabolic syndrome with preserved ejection fraction (HFpEF) and systolic hypertension, and its' lean control. In particular, the effect of the empagliflozin treatment was evaluated on: 1) the metabolic status, 2) the level of systolic blood pressure, 3)

the endothelial function in the isolated mesenteric artery, and 4) the structure and function of the heart.

Materials and Methods

Materials

Empagliflozin was provided by Boehringer Ingelheim Pharma GmbH & Co KG (Biberach an der Riss, Germany). All other chemicals were from Sigma-Aldrich (Sigma-Aldrich Chemie SARL, St Quentin Fallavier, France) unless otherwise specified.

Animals and *in vivo* treatment

The experimental model of metabolic syndrome studied was the obese Zucker diabetic fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1)-HFpEF rats and its' lean control. A total number of 20 obese ZSF1 rats and respective 20 lean control rats were obtained from Charles River Laboratories. At an age of 12 weeks, rats were divided into four groups of n = 10 per group: lean control rats, lean control rats + empagliflozin (30 mg/kg/day), obese ZSF1 rats and obese ZSF1 rats + empagliflozin (30 mg/kg/day) provided in the feed. After a 6-week treatment period, rats were euthanized by IP injection of an overdose of ketamine and xylazine (120 and 20 mg/kg, i.p.).

Biochemical analysis

Urine samples were collected from rats for 24 h in metabolic cages on the day before sacrifice and blood glucose and ketone levels were assessed in tail bleed from overnight fasted rats using the blood glucose and β-ketone meter (GLUCOFIX® Premium, A. Menarini diagnostics). Blood samples were collected by terminal cardiac puncture in heparin containing tubes and plasma was prepared after centrifugation at 7,000 rpm for 10 min at room temperature. Thereafter aliquots were stored at -80 °C until use. Plasma parameters were determined using an Advia 20400 automatic analyzer (Siemens Healthineers) and plasma lipid levels using an AU480 chemistry analyzer (Beckman Coulter). Total cholesterol

was assessed using OSR6116 (Enzymatic Color Test CHOD-PAP Method), HDL using cholesterol OSR6187 (Immunoinhibition / enzymatic color test), LDL cholesterol using OSR6183 (enzymatic color test CHOD-PAP method) and triglycerides using OSR61118 (enzymatic color test GPO-PAP method).

Blood pressure measurements

Systolic blood pressure was determined by tail-cuff sphygmomanometry twice weekly during 5 weeks using the blood pressure analysis system (BP-2000 Serie II, Visitech Systems). Prior to start of blood pressure monitoring, rats were trained daily for one week to get used to the system.

Echocardiography

After 5 weeks of treatment, rats were anaesthetized by inhalation of isoflurane (5 % induction and 2 % for maintenance, 5 L/min of air plus 2 L/min of O_2). Cardiac structure and function were determined by transthoracic echocardiography using the Phillips Sonos 5500 machine equipped with a probe 12 MHz transducer. Two-dimensional short axis views of the left ventricle and M-mode tracings were recorded through anterior and posterior left ventricular walls at the papillary muscle level.

Morphological characterization of the cardiac left ventricle was performed following the determination of the parameters: left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and posterior diastolic wall thickness (PWT). Left ventricular volume, E/E' ratio, cardiac output and left ventricular ejection fraction were subsequently obtained from these parameters.

Western blot analysis

Inner and outer segments of the aortic arch were homogenized in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5), NaCl 150, Na_3VO_4 1, $Na_4P_2O_7$ 10, NaF 20, okadaic acid 0.01, 1 % Triton X-100 and protease inhibitor cocktail (Complete Mini, Roche)). Total proteins (15 μg) were separated on 8 or 12 % SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membrane (GE Healthcare Life Sciences). After blocking with 5 % bovine serum albumin in Tris-buffered saline (TBS) containing 0.1 % Tween 20 for 1 h at room temperature, membranes were incubated with primary antibodies against rabbit polyclonal anti-p53 (1:1,000; Santa Cruz Biotechnology; sc-6243), mouse monoclonal anti-p21 (1:1,000; Santa Cruz Biotechnology; sc-817), rabbit polyclonal anti-p16-INK4 (1:500; Abbiotec; 250804), murine monoclonal anti-tissue factor (TF; 1:1,000; Sekisui Diagnostics; 4509), mouse monoclonal anti-eNOS/NOS (1:5,000; BD Transduction Laboratories; 610297), rabbit monoclonal anti-VCAM-1 (1:10,000; Abcam; ab134047), rabbit polyclonal anti-SGLT1 (1:1,000; Santa Cruz Biotechnology; sc-98974), rabbit polyclonal anti-SGLT2 (1:1,000; Santa Cruz Biotechnology; sc-98975) or mouse monoclonal anti-β-tubulin (1:20,000; Sigma-Aldrich; T7816) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin G; 1:10,000; Cell Signaling Technology; #7074, #7076, respectively) for 1 h at room temperature. The immunoreactive bands were developed by enhanced chemiluminescence (ECL, Amersham) using ImageQuant LAS 4000 (GE Healthcare).

Vascular reactivity study

Vascular reactivity study was performed using the main mesenteric artery as described previously [25]. Briefly, the main superior mesenteric arteries were cleaned of

connective tissue, and cut into rings (2-3 mm in length). Then, rings were suspended in organ baths containing oxygenated (95 % O_2, 5 % CO_2) Krebs bicarbonate solution (mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and D-glucose 11, pH 7.4) at 37 °C for the assessment of changes in isometric tension. Following equilibration for at least 60 min at a stable resting tension of 1 g, rings were exposed to 80 mM of KCl-containing Krebs solution. Thereafter, rings were contracted with phenylephrine (1 μM) before the induction of a relaxation to acetylcholine (1 μM), to clarify the presence of a functional endothelium. After washout and a 30-min resting time, rings were contracted with phenylephrine (1 μM) prior to the construction of a concentration-relaxation curve in response to acetylcholine (1 nM-10 μM) or sodium nitroprusside (0.1 nM-10 μM). In some experiments, rings were exposed to an inhibitor for 20 min before the addition of phenylephrine. To evaluate NO-mediated relaxation, rings were incubated in the presence of indomethacin (10 μM), and TRAM-34 (1 μM) plus UCL-1648 (1 μM) to exclude the formation of vasoactive prostanoids and EDH-mediated relaxation, respectively. The EDH-mediated relaxation was evaluated in rings incubated with indomethacin and N^{ω}-nitro-L-arginine (L-NA; 300 μM) to exclude the formation of vasoactive prostanoids and NO, respectively. To evaluate EDCFs, rings were exposed to L-NA and TRAM-34 plus UCL-1648 for 20 min to prevent the formation of NO and EDH, respectively before the induction of about a 20-30 % pre-contraction with phenylephrine followed by the construction of a concentration-contraction curve to acetylcholine.

Statistical analysis

Values are expressed as means ± SEM of different rats. Statistical analysis was assessed by one-way analysis of variance followed by Tukey's multiple comparison *post hoc*

test using GraphPad Prism (Version 7). The differences between groups were considered statistically significant at $P < 0.05$.

Results

Effect of empagliflozin treatment on metabolic parameters in ZSF1 rats

Plasma parameters have indicated an increased level of glycemia, plasma proteins and albumin, AST, ALT and ALP whereas plasma creatinine and bilirubin were decreased in the obese ZSF1 group compared to the lean control group (Table 1). The empagliflozin treatment significantly decreased glycemia and ALP in the obese ZSF1 group but not in the lean control group except creatinine, which was slightly but significantly decreased (Table 1). In addition, the empagliflozin treatment increased urea in both the lean control and obese ZSF1 groups (Table 1). The obese ZSF1 group had an altered plasma lipid profile with increased levels of total cholesterol, HDL, LDL and triglycerides compared to the lean control group (Table 1). Among them, the level of total cholesterol and triglycerides was significantly decreased by the empagliflozin treatment (Table 1). Blood ketone levels were similar in the lean control and obese ZSF1 groups, and both were significantly increased by the empagliflozin treatment (Table 1). Both glycosuria and proteinuria were significantly increased in the obese ZSF1 group compared to the lean control group (Table 1). The empagliflozin treatment increased glycosuria in both the lean control and obese ZSF1 groups, and markedly reduced proteinuria in the obese ZSF1 group without affecting that in the lean control group (Table 1).

Effect of empagliflozin treatment on morphometric parameters in ZSF1 rats

The morphometric evaluation of rats and organs has indicated that the weight of several organs including spleen, liver, kidney, perirenal fat, lung, heart and body weight were higher in the obese ZSF1 group than the lean control group (Table 2). The empagliflozin treatment significantly decreased the weight of the spleen, liver, perirenal fat, lung, heart and body weight in the obese ZSF1 group (Table 2). In the control group, empagliflozin significantly decreased the weight of the heart and body (Table 2).

Empagliflozin treatment reduces systolic blood pressure in obese ZSF1 rats

Systolic blood pressure was higher by about 14.5 mmHg in the obese ZSF1 group compared to that in the lean control group (Figure 1). The empagliflozin treatment significantly reduced systolic blood pressure by about 3.6 mmHg (reduction from 173.9±0.7 to 170.3±0.9 mmHg) in the obese ZSF1 group after 5 weeks without affecting that in the lean control group (Figure 1).

Empagliflozin prevents alteration of heart structure and function in obese ZSF1 rats

Evaluation of the structure of the different parts of the heart by weight and echocardiography has indicated that the left ventricle plus septum, right ventricle, left auricle plus septum and right auricle weights, the left ventricular and auricular volume and the left ventricular posterior diastolic wall thickness (PWT) were increased in the obese ZSF1 group compared to those of the lean control group (Figure 2). The empagliflozin treatment significantly reduced the left ventricle plus septum, right ventricle and right auricle weights, and also the left ventricular and auricular volumes in the obese ZSF1 group (Figure 2). The empagliflozin treatment also reduced the left ventricle plus septum weight in the control lean group, whereas the other parameters were not affected (Figure 2).

The cardiac output and left ventricular ejection fraction were similar in the lean control and the obese ZSF1 groups, and not affected by the empagliflozin treatment (Figure 2). The E/E' ratio, an indicator of the function of left ventricle filling, was slightly increased in the obese ZSF1 group compared to that of the lean control group, however, this effect did not reach statistical significance (Figure 2).

Empagliflozin treatment improves endothelium-dependent relaxations and reduces endothelium-dependent contractile responses in ZSF1 rats: role of cyclooxygenases

The endothelial and vascular function were assessed by vascular reactivity studies of the main mesenteric artery. The concentration-dependent relaxation curve to acetylcholine was slightly but significantly shifted to the right in mesenteric artery rings with endothelium of the obese ZSF1 compared to that of the lean control group (Figure 3A). The blunted endothelium-dependent relaxation in the ZSF1 group was associated with increased endothelium-dependent contractile responses to acetylcholine (Figure 3B). The empagliflozin treatment restored normal endothelium-dependent relaxations and blunted endothelium-dependent contractile responses to acetylcholine (Figures 3A, B). The empagliflozin treatment did affect neither relaxations nor the small endothelium-dependent contractile responses to acetylcholine in the control lean group (Figures 3A, B). In addition, relaxations to the NO donor sodium nitroprusside were similar in all groups (Figure 3C).

The characterization of the blunted endothelium-dependent relaxation to acetylcholine in mesenteric artery rings of the ZSF1 group has indicated a slight but significant increased relaxation in the presence of indomethacin, a non-selective inhibitor of cyclooxygenases indicating the involvement, to some extent, of vasoconstrictor prostanoids (Figure 4A). The role of endothelial NO in the relaxation to acetylcholine was assessed using the NO synthase inhibitor N^{ω}-nitro-L-arginine, and that of EDH by using TRAM-34 and UCL-1684, inhibitors of intermediate and small calcium-dependent K^+ channels, respectively, involved in EDH. Although the addition of TRAM-34 and UCL-1684 to indomethacin affected only slightly the relaxation to acetylcholine, the response was abolished in the presence of N^{ω}-nitro-L-arginine demonstrating the exclusive involvement of NO (Figure 4A). In addition, the endothelium-dependent contractile response to acetylcholine in mesenteric artery rings of the obese ZSF1 group was abolished in the presence of indomethacin (Figure 4B).

Empagliflozin prevents the expression of senescence and pro-atherothrombotic markers, and also SGLT1 and 2 at arterial sites at risk

Since senescence has been identified as an early event promoting endothelial dysfunction [17], the expression level of the senescence markers p53, p21 and p16 was evaluated by Western blot analysis in the outer aortic arch curvature, an arterial site exposed to a high level of shear stress and at low risk, and the inner aortic arch curvature, an arterial site exposed to a low level of shear stress and at high risk [26]. The expression level of p53, p21 and p16 were significantly higher in the inner than outer aortic arch curvatures in the lean control group (Figure 5). The empagliflozin treatment reduced the expression level of p53, p21 and p16 in the inner curvature to a similar level as those observed in the outer curvature of the aortic arch in the lean control group (Figure 5). The empagliflozin treatment also reduced significantly the expression level of p16 in the outer aortic arch curvature whereas those of p53 and p21 were not affected in the lean control group (Figure 5).

In contrast to the lean control group, the obese ZSF1 group showed low levels of p53 and p21 which were similar in the inner and outer aortic arch curvatures and not affected by the empagliflozin treatment (Figures 5A, B). Regarding p16 levels, an increased level was observed in the inner compared to the outer curvatures, however, this difference did not reach statistical significance and was not affected by the empagliflozin treatment (Figure 5C).

To evaluate the endothelial dysfunction at arterial sites at risk, the expression level of pro-atherothrombotic markers including eNOS, VCAM-1 and tissue factor, the initiator of the coagulation cascade, was assessed in the inner and outer aortic arch curvatures by Western blot analysis. These investigations have indicated that eNOS is down-regulated and VCAM-1 and tissue factor are up-regulated in the inner versus outer aortic arch curvatures in the lean control group, and that these effects are normalized by the empagliflozin treatment (Figures 6A-C). In the ZSF1 group, eNOS levels were decreased in the inner versus outer aortic arch

curvatures, but this effect did not reach statistical significance (Figure 6A). The levels of VCAM-1 and tissue factor were similar in the inner and outer aortic arch curvatures of the ZSF1 group, and not affected by the empagliflozin treatment (Figures 6B, C)

Of interest, an increased expression level of SGLT1 and SGLT2 (immunoreactive bands of about 70 kDa) were observed in the inner compared to the outer aortic arch curvatures in the lean control group (Figures 6D, E). The empagliflozin treatment normalized the expression level of SGLT1 and SGLT2 in the inner aortic arch curvature to a level similar as that observed in the outer aortic arch curvature (Figures 6D, E). Similar expression levels of SGLT1 and 2 were observed in the inner and outer aortic arch curvatures in the ZSF1 group and the empagliflozin-treated ZSF1 group (Figures 6D, E).

Discussion

The major findings of the present study indicate that the selective SGLT2 inhibitor empagliflozin improves systolic blood pressure, heart remodeling and endothelial dysfunction in an experimental model of metabolic syndrome with HFpEF, the ZSF1 rat. The protective effect of empagliflozin on the heart of the ZSF1 rat with HFpEF involves normalization of the heart weight with an improvement of the left ventricle weight and volume, and also of the posterior wall thickness. The protective effect of empagliflozin on the endothelial function in the mesenteric artery involves normalization of NO-mediated endothelium-dependent relaxations and prevention of endothelium-dependent contractile responses to acetylcholine most likely by targeting the cyclooxygenase pathway. A protective effect of empagliflozin is also observed at arterial sites at risk (inner versus outer curvatures of the aortic arch) in the lean control group as indicated by the normalization of the expression level of senescence markers (p53, p21, p16), atherothrombotic markers, and SGLT1 and 2. Moreover, the empagliflozin treatment resulted in improved glycemic level, lipid profile and body weight. Altogether, the present findings indicate that the highly selective SGLT2 inhibitor is able to target several major components of the metabolic syndrome in the ZSF1 rat to improve the function of the cardiovascular system.

The EMPA-REG OUTCOME, the CANVAS and the DECLARE-TIMI58 trials showed that SGLT2 inhibitors reduced the risk of cardiovascular death or hospitalization for heart failure in T2D individuals with established CVD [18-20]. The subsequent analysis of the EMPA-REG OUTCOME data indicated that the cardiovascular benefits of empagliflozin is independent of glycemic control [21]. More recently, the DAPA-Heart Failure trial indicated that among patients with heart failure and a reduced ejection fraction, dapagliflozin reduced the risk of worsening heart failure or death from cardiovascular causes regardless of the presence or absence of diabetes [27]. Altogether, these findings suggest that SGLT2

inhibitors act on the cardiovascular system possibly through glucose-independent mechanisms, which however remain to be elucidated. Several potential mechanism, besides glycemic control, have been suggested such as the involvement of visceral adiposity, body weight, hyperinsulinemia, blood pressure, arterial stiffness, lipid profile, and albuminuria [28]. In addition, SGLT1 and 2 expression has been observed in cultured and native ECs under pathological conditions such as hyperglycemic state and oxidative stress, promoting endothelial senescence and dysfunction subsequent to excessive glucose entry [24]. Since all of these effects were inhibited by empagliflozin, SGLT2 inhibitors may possibly also contribute to protect the cardiovascular system by targeting the pivotal endothelial function [24]. Therefore, the present study has evaluated the impact of empagliflozin on the cardiovascular system in an experimental model of metabolic syndrome with HFpEF, the ZSF1 rat, which combines several major cardiovascular risk factors including obesity, hypertension, diabetes and dyslipidemia [29-31].

After 18 weeks, the obese ZSF1 rat showed many characteristics of metabolic risk such as visceral obesity as indicated by increased perirenal fat, elevated body weight, hyperglycemia, and dyslipidemia characterized by high levels of total cholesterol, LDL and triglycerides. In addition, an increased level of systolic blood pressure was observed in the obese ZSF1 rat compared to the lean rat. The present findings indicate a beneficial effect of the empagliflozin treatment promoting a reduction of the body weight and the weight of perirenal fat, and hyperglycemia, and an improvement of the lipid profile as well as of systolic blood pressure in the obese ZSF1 rat. Previous studies have also shown that empagliflozin reduced body weight and fat associated with an increased urinary glucose excretion in an animal model of diet-induced obesity [32], lowered plasma cholesterol and liver triglyceride levels in pre-diabetic ob/ob$^{-/-}$ mice [33], and decreased blood pressure by about 15 mmHg in the Cohen-Rosenthal diabetic hypertensive rat [34].

Consistent with previous studies, the obese ZSF1 rat, an experimental model of HFpEF, is characterized by pronounced heart remodeling affecting the left and right heart with a preserved heart function as indicated by normal cardiac output and ejection fraction. The present findings indicate that the empagliflozin treatment was able to prevent the hypertrophy of both the left and right heart as indicated by the normalization of the weight of the left and right ventricles and the right auricle, and also of the heart remodeling as indicated by the normalization of the volume of the left ventricle and auricle, and the posterior wall thickness of the left ventricle. A beneficial effect of empagliflozin on the heart has also been observed previously since empagliflozin reduced left ventricle weight, cardiomyocyte size, cardiac interstitial fibrosis and macrophage infiltration in genetic pre-diabetic rats with metabolic syndrome [23], improved cardiac function and ATP production in db/db mice associated with preserved cardiac glucose and lipid metabolism [35] and improved diastolic function, mitochondrial expansion, and myocardial fibrosis in female db/db mice [36]. In addition, Connelly et al. observed that empagliflozin preserved lung weight, ameliorated diastolic dysfunction, and attenuated cardiac hypertrophy using a rat model of uninephrectomy treated with deoxycorticosterone acetate and 1 % NaCl water to induce HFpEF [37].

Chronic inhibition of SGLT2 decreases extracellular fluid and plasma volumes as was obvious in the EMPA-REG OUTCOME trial with a 5.2 % rise in hematocrit level at the end of the study [18]. The diuretic effect subsequent to inhibition of the SGLT2-mediated glucose uptake in the kidney proximal tube will further reduce central aortic pressure and afterload, resulting in improved left ventricular function, and decreased cardiac workload and myocardial oxygen demand [38, 39]. Preload reduction by lower plasma volume most likely acts synergistically with afterload reduction to promote a reduction of cardiac events, especially in individuals with diabetes and impaired left ventricular function, ischemic heart disease, or congestive HF [38, 39].

Endothelial dysfunction caused by hyperglycemia is a critical initiator of the development of macro- and micro-vascular complications in T2D with high metabolic risk [7, 40]. Previous studies have indicated that hyperglycemia-related endothelial dysfunction is characterized often by blunted NO and EDH components of the endothelium-dependent relaxation as observed in the rat arterioles [14], rat aorta [13], and rabbit aorta [12]. In the obese ZSF1 rat, the endothelial dysfunction of the main mesenteric artery is characterized by blunted endothelium-dependent relaxations and the appearance of endothelium-dependent contractile responses to acetylcholine. Moreover, the characterization of the endothelial dysfunction to acetylcholine indicated that the NO component is significantly reduced in the mesenteric artery of the obese ZSF1 rat. Interestingly, empagliflozin treatment restored the protective endothelial function in the mesenteric artery as indicated by normal endothelium-dependent relaxations and blunted endothelium-dependent contractile responses to acetylcholine most likely by preventing the formation of cyclooxygenase-derived contractile prostanoids. Experimental investigations have also shown that empagliflozin improved endothelium-dependent relaxations in streptozotocin-induced diabetic rats and Zucker diabetic fatty rats [22, 41]. Canagliflozin has also been shown to increase SNP-dependent relaxation in coronary arteries from diabetic mice subsequent to vascular smooth muscle hyperpolarization involving potassium channels [42] and Ipragliflozin improved endothelium-dependent vasodilation in streptozocin-induced diabetic mice, in part, by an improvement of eNOS function as suggested by an increased phosphorylation level of eNOS at the activator site Ser1177 and Akt [43].

Premature endothelial senescence as indicated by senescence-associated β-galactosidase (SA-β-gal), p53 and p16 staining was observed in the aortas obtained from Zucker diabetic rats [44]. SA-β-gal-positive staining was also observed in atherosclerotic lesions of the human thoracic aorta at the intimal side as well as at sites of disturbed blood flow including

branches and bifurcations [45, 46]. Since the selective p53 overexpression in ECs resulted in impaired endothelium-dependent relaxation and NO bioavailability [17], premature endothelial senescence most likely acts as an upstream signaling event to promote endothelial dysfunction. Although the lean control ZSF1 rat is characterized by normal heart structure and function, and endothelial function in the mesenteric artery, a more precise analysis of the vascular tree has provided evidence of premature senescence at arterial sites at risk. Indeed, an increased level of senescence markers p53, p21 and p16 was observed in the inner compared to the outer aortic arch curvature, a potential site of early atherogenesis characterized by disturbed flow and low shear stress [26]. Moreover, the premature senescence was associated with the appearance of pro-atherothrombotic signals as indicated by a decreased expression level of eNOS and increased levels of tissue factor, the main activator of the coagulation cascade, and VCAM-1 involved in the recruitment of monocytes into the arterial wall. Furthermore, an increased expression level of SGLT1 and SGLT2 was observed at the arterial site at risk supporting the concept that possibly an excessive entry of glucose via SGLTs might contribute to promote structural and functional changes favoring the pathologic development of the arterial site at risk. The present findings indicate that the empagliflozin treatment was able to normalize the expression level of senescence markers, eNOS, tissue factor, VCAM-1 and SGLT1 and 2 in the inner aortic arch curvature to levels similar to those in the outer aortic arch curvature, which is protected by the exposure to high levels of shear stress. Thus, these observations suggest that empagliflozin might possibly contribute to protect arterial sites of atherogenesis by blunting the SGLT2-mediated pro-senescent and pro-atherothrombotic responses [24]. The fact that no such differences in the expression level of pro-atherothrombotic markers are observed in the inner and outer curvature of the obese ZSF1 rat suggests that the responsiveness of the arterial wall to local

blood flow behavior appears to be altered possibly due to the chronic exposure of metabolic stress.

Conclusions

The study indicates that empagliflozin prevents hypertrophy and remodeling of the heart, and also endothelial dysfunction as indicated by an improved NO-mediated relaxation and the prevention of cyclooxygenases-mediated endothelium-dependent contractile responses in an experimental model of metabolic syndrome with HFpEF. The beneficial effect involves an improvement of systolic blood pressure, glycemia, the lipid profile and body weight, and also of premature vascular senescence at arterial sites at risk.

Abbreviations

AIC: inner curvature of the aortic arch

ALP: alkaline phosphatase

ALT: alanine aminotransferase

AOC: outer curvature of the aortic arch

AST: aspartate aminotransferase

CVD: cardiovascular disease

ECs: endothelial cells

EDCFs: endothelium-dependent contractile responses

EDH: endothelium-dependent hyperpolarization

empa: empagliflozin

eNOS: endothelial nitric oxide synthase

HDL: high density lipoprotein

HF: heart failure

HFpEF: heart failure with preserved ejection fraction

L-NA: N^{ω}-nitro-L-arginine

LDL: low density lipoprotein

NO: nitric oxide

PWT: posterior diastolic wall thickness

SA-β-gal: senescence-associated β-galactosidase

SGLT: selective sodium-glucose cotransporter

T2D: type 2 diabetes

TBS: Tris-buffered saline

TF: tissue factor

ZSF1: obese Zucker diabetic fatty/Spontaneously hypertensive heart failure F1 hybrid

Declarations

Ethics approval and consent to participate

All animal procedures complied with the Guide of Care and the Use of Laboratory Animals published by the U.S. National Institutes of Health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996) and authorization was given by the French Ministry of Research (#10073-2017053013335510) after approval by the local Ethics Committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg, CE35).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

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Authors' contributions

S-HP, MAF, SG, CB, AWQ, H-HL, DB, and BP performed the experiments and analyzed the data. S-HP, DS, PO, J-ML, EM, CA, OM, and VS-K designed the study and wrote the paper.

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SCAVENGER EBSELEN? *Drug discovery today Therapeutic strategies* 2007, **4**(1):93-99.

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Figure legends

Figure 1. Effect of empagliflozin treatment on systolic blood pressure in the lean control and the ZSF1 groups. Values are shown as mean ± SEM of n = 9-10 per group. $^{*}P < 0.05$ vs control group and $^{\#}P < 0.05$ vs ZSF1 group.

Figure 2. Oral intake of empagliflozin prevents heart remodeling in ZSF1 rats. (A-D) All the four cavities of heart (left ventricle and septum, right ventricle, left auricle and septum and right auricle) were weighted and indexed to the respective tibial length. (E-J) The different cardiac markers related to cardiac function and morphology were assessed by echocardiography. Values are shown as mean ± SEM of n = 6-10 per group. $^{*}P < 0.05$ vs control group and $^{\#}P < 0.05$ vs ZSF1 group.

Figure 3. Effect of empagliflozin treatment on the endothelium-dependent relaxation and endothelium-dependent contractile response to acetylcholine in the lean control and ZSF1 groups. Arterial rings from the main mesenteric artery with endothelium were suspended in organ baths containing oxygenated Krebs buffer. For concentration-relaxation curves, rings were precontracted with phenylephrine (1 μM) before the addition of increasing concentrations of either acetylcholine (A) or the NO donor sodium nitroprusside (C). (B) Endothelium-dependent contractile responses (EDCF) were studied in the presence of N^{ω}-nitro-L-arginine (300 μM) and UCL-1684 plus TRAM-34 (1 μM each) to prevent the formation of NO- and EDH-mediated relaxations, respectively. Rings were precontracted to about 20-30 % of the maximal contraction with phenylephrine before the addition of increasing concentrations of acetylcholine. Values are shown as means ± SEM of 8-10 rats per group. $^{*}P < 0.05$ vs control group and $^{\#}P < 0.05$ vs ZSF1 group.

Figure 4. Characterization of endothelium-dependent relaxation and contractile responses to acetylcholine in mesenteric artery rings with endothelium of the ZSF1 group. Arterial rings from the main mesenteric artery with endothelium were suspended in organ baths containing oxygenated Krebs buffer. Pharmacological inhibitors were added 20 min before the contraction to phenylephrine to assess the role of the NO-mediated component (N^{ω}-nitro-L-arginine, L-NA, 300 μM), the EDH-mediated component (UCL-1684 plus TRAM-34, UCL+Tram, 1 μM each), and the formation of vasoactive prostanoids (indomethacin, Indo, 10 μM). Rings were contracted with phenylephrine (1 μM) (A) or to about 20-30 % of the maximal contraction (B) before the addition of increasing concentrations of acetylcholine. Values are shown as mean ± SEM of n = 8-10. $^{*}P < 0.05$ vs control group.

Figure 5. Effect of empagliflozin treatment on the expression level of senescence markers (p53, p21 and p16) in segments of the outer curvature (AOC), an arterial site at low risk, and in those of the inner curvature (AIC) of the aortic arch, an arterial site at high risk, in the lean control and ZSF1 groups as assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels). Values are shown as mean ± SEM of n = 3-4 per group. $^{*}P < 0.05$ vs AOC of control group and $^{\#}P < 0.05$ vs AIC of control group and $^{+}P < 0.05$ vs AOC of Empa group.

Figure 6. Effect of empagliflozin treatment on the expression level of eNOS, VCAM-1, TF, SGLT1 and SGLT2 in segments of the AOC and in those of the AIC of the lean control and ZSF1 groups as assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels). Values are

shown as mean ± SEM of n = 3-4 per group. $^{*}P < 0.05$ vs AOC of control group and $^{\#}P < 0.05$ vs AIC of control group.

Table 1. Effect of a 6-week oral intake of empagliflozin on plasma, blood and urine parameters in the lean control and ZSF1 groups.

	Control	**Empa**	**ZSF1**	**ZSF1+Empa**
Plasma				
Glycemia (mmol/l)	11.33±1.11	9.94±1.30	28.07±2.13*	20.81±0.78$^{*\#}$
Urea (mmol/l)	5.84±0.26	8.71±0.31*	6.86±0.30	7.56±0.28*
Plasma creatinine (µmol/l)	33.18±0.46	31.23±0.56*	24.21±0.80*	25.37±0.22
Plasma proteins (g/l)	58.89±0.99	58.88±0.83	64.88±0.90*	64.00±0.69*
Albumin (g/l)	36.49±0.49	37.16±0.49	39.46±0.48*	39.49±0.43*
Bilirubin (µmol/l)	0.57±0.02	0.61±0.06	0.25±0.16*	0.01±0.01*
AST (U/l)	130.90±12.34	171.00±7.80	220.60±35.64*	152.20±8.94
ALT (U/l)	63.78±2.74	108.80±7.56	161.20±19.02*	131.20±8.05
ALP (U/l)	62.78±4.06	72.13±8.60	136.60±13.24*	97.11±10.87$^{\#}$
Total cholesterol (mmol/l)	2.33±0.06	2.38±0.11	6.31±0.34*	4.90±0.30$^{*\#}$
HDL cholesterol (mmol/l)	1.37±0.02	1.43±0.05	2.51±0.09*	2.45±0.08*
LDL cholesterol (mmol/l)	0.63±0.03	0.69±0.02	0.75±0.04*	0.66±0.06
Triglycerides (mmol/l)	0.48±0.05	0.49±0.03	6.23±1.06*	2.92±0.28$^{*\#}$
Blood				
Ketone (mmol/L)	0.33±0.06	0.71±0.10*	0.32±0.06	0.56±0.10$^{\#}$
Glucose (mg/dL)	134.60±9.07	134.10±8.69	274.50±29.41*	196.70±22.16$^{\#}$
Urine				
Glycosuria (mmol)	0.89±0.40	12.03±1.55*	6.09±0.85*	17.47±0.98$^{*\#}$
Proteinuria (g/l)	1.59±0.17	0.74±0.06	11.35±0.84*	4.62±0.44$^{*\#}$
Volume (mL)	9.33±0.94	26.22±1.62*	22.11±2.31*	39.40±2.22$^{*\#}$

Metabolic parameters were measured in plasma except for ketone and glucose which were determined in whole blood, and glycosuria and proteinuria which were determined in urine

collected over a 24-h period. Values are shown as mean ± SEM of n = 5-10 per group. $^{*}P <$ 0.05 vs control group and $^{\#}P < 0.05$ vs ZSF1 group. Empa, empagliflozin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 2. Effect of empagliflozin on morphometric parameters of different organs and body weight in the lean control and ZSF1 groups.

	Control	Empa	ZSF1	ZSF1+Empa
Spleen ($mg \cdot mm^{-1}$)	17.45±0.38	16.55±0.62	20.90±0.32*	18.53±0.81$^{\#}$
Liver ($mg \cdot mm^{-1}$)	286.40±12.58	264.80±7.80	771.40±34.64*	547.90±21.37$^{*\#}$
Kidney ($mg \cdot mm^{-1}$)	40.52±1.33	41.58±1.34	55.22±1.46*	54.92±1.60*
Perirenal fat ($mg \cdot mm^{-1}$)	11.32±1.85	8.47±1.63	183.70±36.89*	100.60±27.25$^{*\#}$
Left lung ($mg \cdot mm^{-1}$)	11.06±0.20	10.65±0.65	12.92±0.69*	11.02±0.25$^{\#}$
Dry weight of left lung ($mg \cdot mm^{-1}$)	2.30±0.03	2.16±0.06	2.70±0.08*	2.42±0.06$^{\#}$
3 lobes of lung ($mg \cdot mm^{-1}$)	17.94±0.41	16.40±0.41	21.48±1.64*	17.23±0.37$^{\#}$
Heart ($mg \cdot mm^{-1}$)	31.93±0.66	28.74±0.75*	38.98±0.95*	35.40±0.63$^{*\#}$
Tibial length (mm)	41.76±0.35	42.54±0.53	40.22±0.42	39.89±0.43
Weight (g)	407.70±9.07	366.00±7.67*	523.80±19.93*	478.90±8.73$^{*\#}$

Organs were weighted and indexed to the respective tibial length. Values are shown as mean ± SEM of n = 7-10 per group. $^{*}P < 0.05$ vs control group and $^{\#}P < 0.05$ vs ZSF1 group. Empa, empagliflozin.

Figure 1

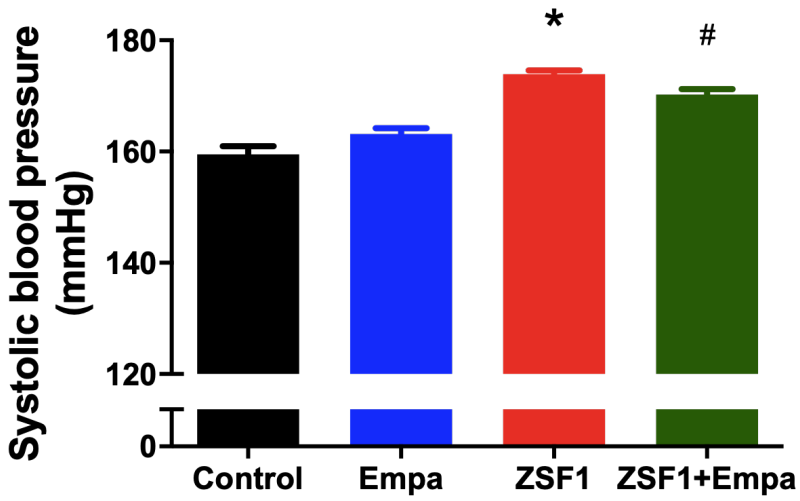

Systolic blood pressure (mmHg)
180
160
140
120
0
*
#
Control
Empa
ZSF1
ZSF1+Empa

Figure 2

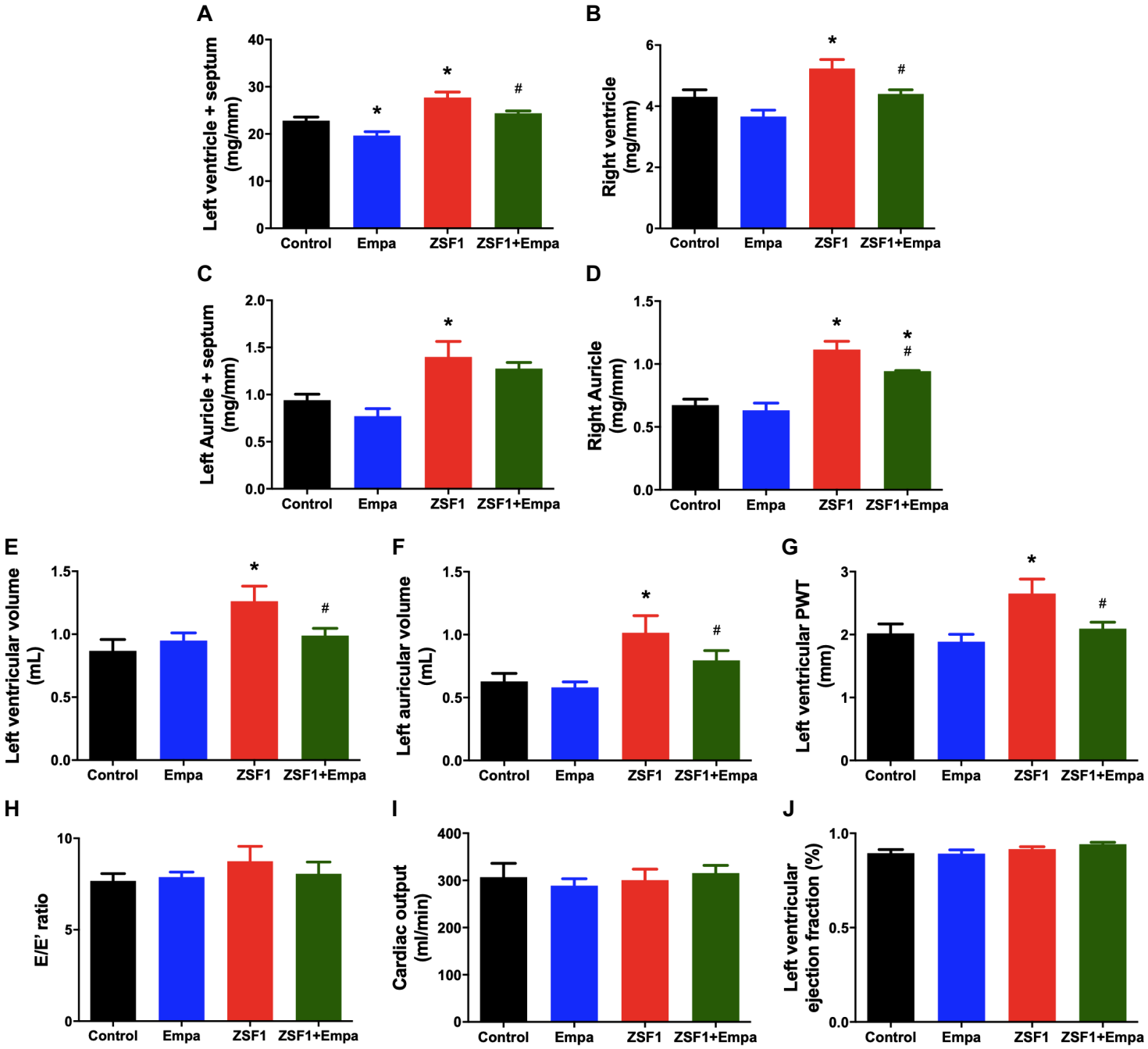

A
Left ventricle + septum (mg/mm)
40
30
20
10
0
*
*
#
Control
Empa
ZSF1
ZSF1+Empa
B
Right ventricle (mg/mm)
6
4
2
0
*
#
Control
Empa
ZSF1
ZSF1+Empa
C
Left Auricle + septum (mg/mm)
2.0
1.5
1.0
0.5
0.0
*
Control
Empa
ZSF1
ZSF1+Empa
D
Right Auricle (mg/mm)
1.5
1.0
0.5
0.0
*
*
#
Control
Empa
ZSF1
ZSF1+Empa
E
Left ventricular volume (mL)
1.5
1.0
0.5
0.0
*
#
Control
Empa
ZSF1
ZSF1+Empa
F
Left auricular volume (mL)
1.5
1.0
0.5
0.0
*
#
Control
Empa
ZSF1
ZSF1+Empa
G
Left ventricular PWT (mm)
3
2
1
0
*
#
Control
Empa
ZSF1
ZSF1+Empa
H
E/E' ratio
10
5
0
Control
Empa
ZSF1
ZSF1+Empa
I
Cardiac output (ml/min)
400
300
200
100
0
Control
Empa
ZSF1
ZSF1+Empa
J
Left ventricular ejection fraction (%)
1.0
0.5
0.0
Control
Empa
ZSF1
ZSF1+Empa

Figure 3

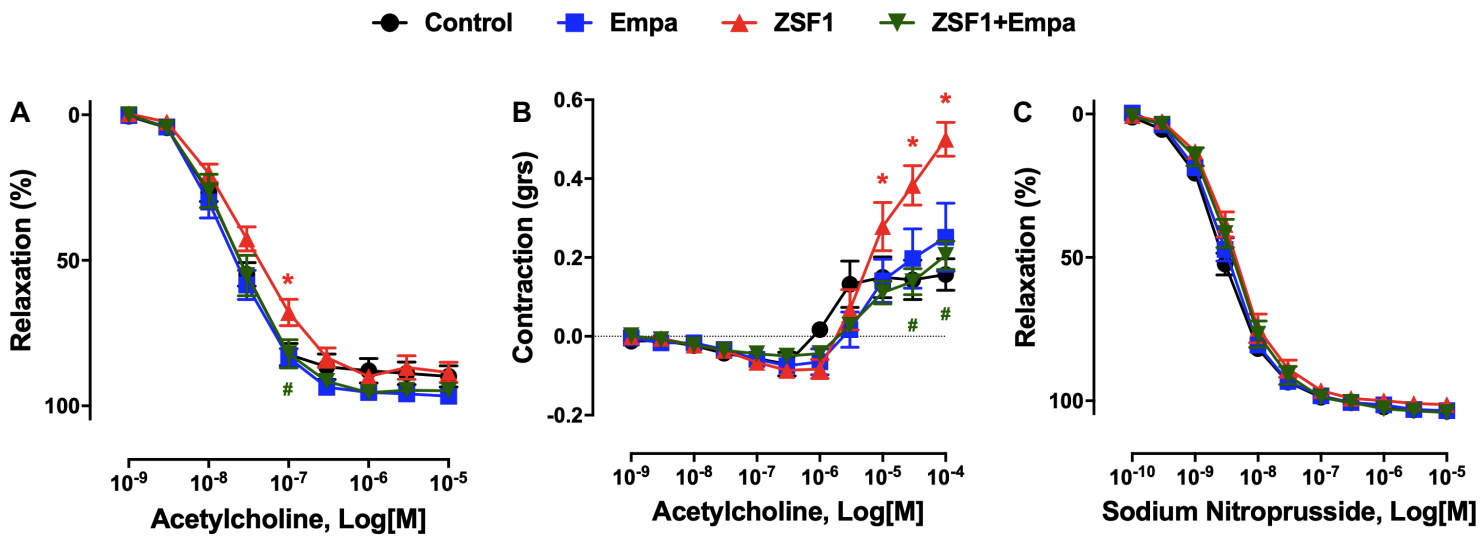

Control
Empa
ZSF1
ZSF1+Empa
A
Relaxation (%)
0
50
100
10-9
10-8
10-7
10-6
10-5
Acetylcholine, Log[M]
B
Contraction (grs)
0.6
0.4
0.2
0.0
-0.2
10-9
10-8
10-7
10-6
10-5
10-4
Acetylcholine, Log[M]
C
Relaxation (%)
0
50
100
10-10
10-9
10-8
10-7
10-6
10-5
Sodium Nitroprusside, Log[M]

Figure 4

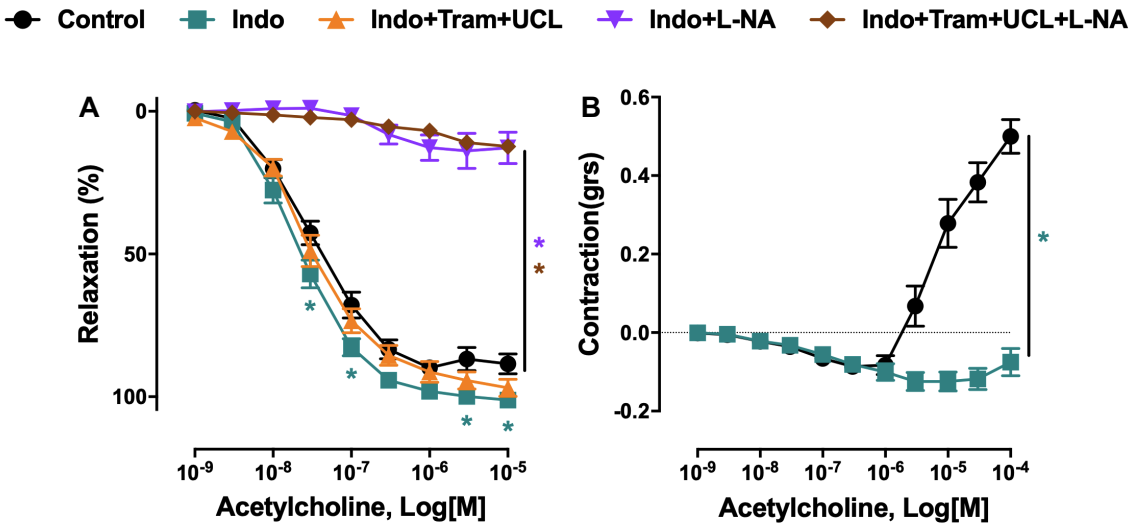

Control
Indo
Indo+Tram+UCL
Indo+L-NA
Indo+Tram+UCL+L-NA
A
Relaxation (%)
0
50
100
10-9
10-8
10-7
10-6
10-5
Acetylcholine, Log[M]
B
Contraction(grs)
0.6
0.4
0.2
0.0
-0.2
10-9
10-8
10-7
10-6
10-5
10-4
Acetylcholine, Log[M]

Figure 5

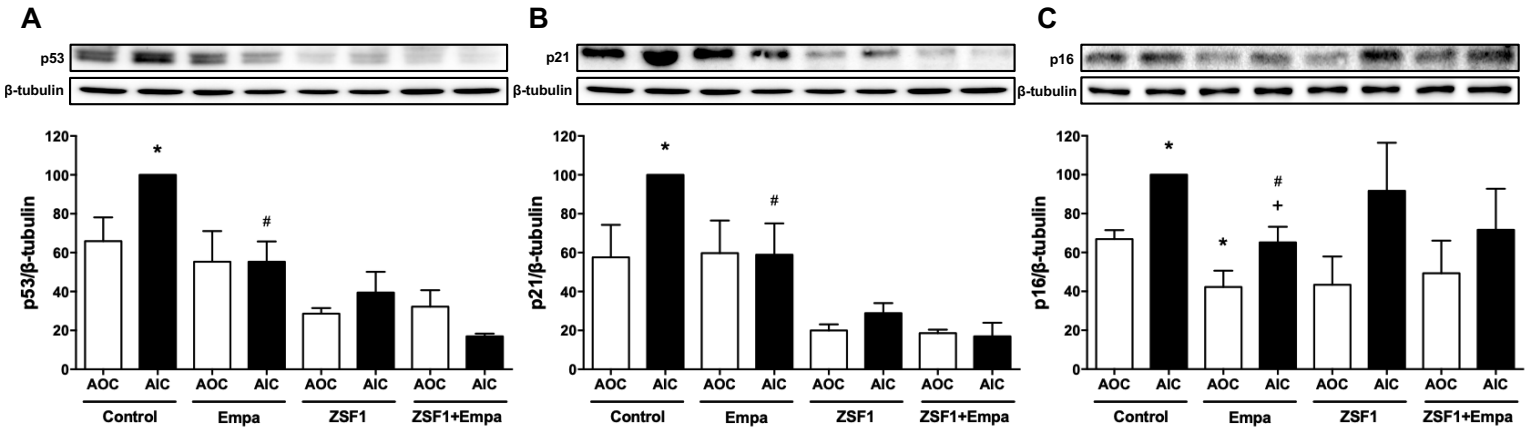
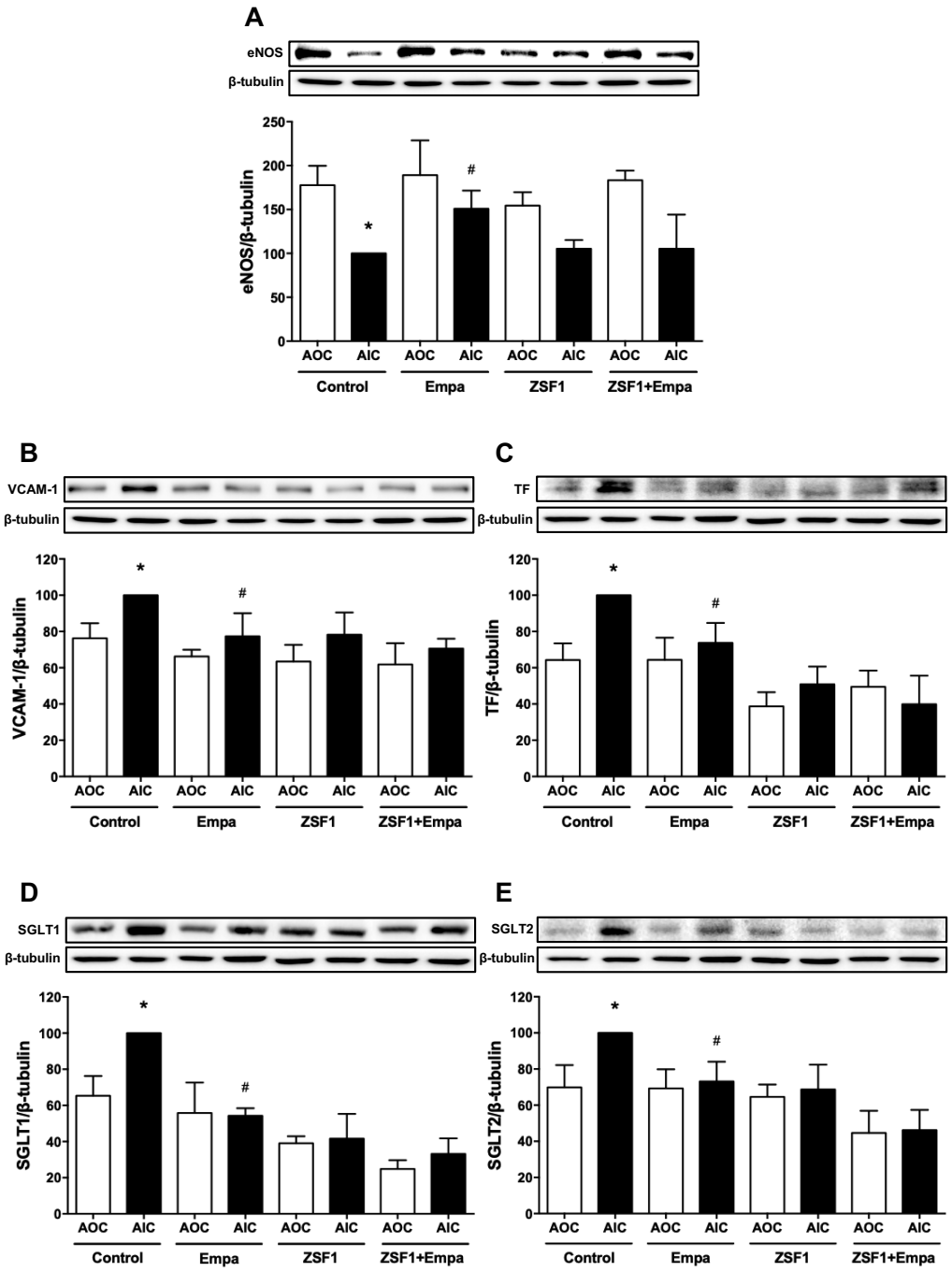

A
p53
β-tubulin
p53/β-tubulin
B
p21
β-tubulin
p21/β-tubulin
C
p16
β-tubulin
p16/β-tubulin
AOC
AIC
Control
Empa
ZSF1
ZSF1+Empa

Figure 6


A
eNOS
β-tubulin
eNOS/β-tubulin
250
200
150
100
50
0
*
#
AOC AIC AOC AIC AOC AIC AOC AIC
Control Empa ZSF1 ZSF1+Empa
B
VCAM-1
β-tubulin
VCAM-1/β-tubulin
120
100
80
60
40
20
0
*
#
AOC AIC AOC AIC AOC AIC AOC AIC
Control Empa ZSF1 ZSF1+Empa
C
TF
β-tubulin
TF/β-tubulin
120
100
80
60
40
20
0
*
#
AOC AIC AOC AIC AOC AIC AOC AIC
Control Empa ZSF1 ZSF1+Empa
D
SGLT1
β-tubulin
SGLT1/β-tubulin
120
100
80
60
40
20
0
*
#
AOC AIC AOC AIC AOC AIC AOC AIC
Control Empa ZSF1 ZSF1+Empa
E
SGLT2
β-tubulin
SGLT2/β-tubulin
120
100
80
60
40
20
0
*
#
AOC AIC AOC AIC AOC AIC AOC AIC
Control Empa ZSF1 ZSF1+Empa

GENERAL DISCUSSION

General discussion

1. Cardiovascular outcome trials of SGLT2 inhibitors

The EMPA-REG OUTCOME trial (2010–2015) involving 7,020 T2DM individuals with established CVD showed the cardioprotective effect of empagliflozin by significantly lowering the mortality from cardiovascular causes (38% relative risk reduction), all-cause death (32% relative risk reduction) and hospitalization for HF (35% relative risk reduction) (Zinman et al. 2015). The CANVAS trial (2009–2017) involving 10,142 patients with T2DM and a high risk of CVD reported canagliflozin significantly decreased the composite of cardiovascular-cause death, nonfatal myocardial infarction or nonfatal stroke (14% relative risk reduction) and hospitalization for HF (33% relative risk reduction) (Neal et al. 2017). In the DECLARE-TIMI 58 trial (2013–2019) including 17,160 patients with T2DM who had or were at risk for atherosclerotic CVD, treatment with dapagliflozin resulted in a lower rate of cardiovascular death or hospitalization for HF (27% relative risk reduction) (Wiviott et al. 2019). Altogether, the beneficial effects on cardiovascular outcomes appear to be a class effect of SGLT2 inhibitors. They further have been shown to be independent of glycemic control, suggesting that other effects are involved (Inzucchi et al. 2018). However, they still remain to be clarified.

2. Potential mechanisms of SGLT2 inhibitors on cardiovascular protective effects

1) Reduced blood pressure

The EMPA-REG OUTCOME trial has indicated that the empagliflozin reduced by about ~5/2 mmHg systolic and diastolic blood pressure, which could contribute to explain, to some extent, the beneficial cardiovascular outcome (Zinman et al. 2015). Experimental studies showed that canagliflozin significantly decreased mean blood pressure in STZ-induced

diabetic rats (Abu Seif et al. 2017). Canagliflozin also increased SNP-dependent relaxation in coronary arteries from diabetic mice via induction of hyperpolarization in the vascular smooth muscle, mediated by stimulation of potassium channels (Han et al. 2015)

2) Reduced plasma volume

SGLT2 inhibitors chronically decrease extracellular fluid and plasma volumes as was obvious in the EMPA-REG OUTCOME trial with a 5.2 % rise in hematocrit level at the end of the study (Zinman et al. 2015). The diuretic effect by SGLT2 inhibition would be expected to further reduce central aortic pressure and afterload, resulting in improved LV function, and decreased cardiac workload and myocardial oxygen demand. Preload reduction by lower plasma volume would be supposed to act synergistically with afterload reduction to decrease cardiac events, especially in individuals with diabetes and impaired LV function, ischaemic heart disease, or congestive HF (McMurray 2016; Kimura 2016). However, a reduction in intravascular volume can activate the renin–angiotensin–aldosterone system and worsen CVD by stimulating AT_1R. Nevertheless, more than 80 % of patients in the EMPA-REG OUTCOME trial were treated with an angiotensin-receptor blocker or an ACE inhibitor, which would be expected to favor the activation of the angiotensin 1-7 pathway via AT_2R, leading to vasodilation, anti-inflammatory, anti-proliferation of vascular SMCs, and positive ionotropic effects (Jiang et al. 2014).

3) Weight loss

Obesity and insulin resistance are independent risk factors for atherosclerotic CVD (DeFronzo 2010). As mentioned previously, SGLT2 inhibitors induced weight loss. However, the extent of the weight loss and rapid reduction in cardiac mortality and HF in the EMPA-REG OUTCOME trial suggest that weight reduction was not responsible for the cardiovascular benefit, which was observed early after initiation of empagliflozin therapy. It

is possible however, that weight loss could contribute to the progressive reduction in cardiovascular mortality and HF over 1-3 years.

4) Other mechanisms

A number of other mechanisms such as decreased plasma uric acid level, improved lipid profile, reduced inflammation and oxidative stress, improved insulin sensitivity, and diminished albuminuria have been suggested to explain the cardioprotective effect of SGLT2 inhibitors (DeFronzo et al. 2017). However, the possibly the SGLT2 contributes to the alleviation of the endothelial dysfunction, an early event in the development of cardiovascular disease has been poorly investigated.

3. Role of SGLT1 and 2 in endothelial senescence and dysfunction

Several experimental studies have shown the effect of SGLT2 inhibitors on endothelial and vascular function. In STZ-induced diabetic mice, the *in vivo* treatment with ipragliflozin improved the reduced phosphorylation of Akt and eNOS, ROS generation, and expression of MCP-1, VCAM-1 and ICAM-1 in the abdominal aorta and improved endothelium-dependent relaxations of aortic rings (Salim et al. 2016). Empagliflozin also improved endothelium-dependent relaxations and decreased vascular oxidative stress mediated by glucotoxicity in STZ-treated mice (Oelze et al. 2014). In $ApoE^{-/-}$ mice, empagliflozin decreased atherosclerotic plaque formation in the aortic arch/valve most likely by improving insulin resistance and inflammation (Han et al. 2017). However, it is unclear whether the vasoprotective effect of SGLT2 inhibitors is related to a direct effect at the arterial wall and/or other effects. Recent studies have indicated the expression of SGLT1 and SGLT2 in the vascular endothelium. In HUVECs, both SGLT1 and SGLT2 expressions were revealed at the protein and mRNA level, and the expression levels were upregulated by palmitic acid

(Li et al. 2018). The redox-sensitive upregulation of SGLT1 and 2 was observed in cultured and native coronary artery ECs under pathological conditions such as hyperglycemic state and oxidative stress, and involved in endothelial senescence and dysfunction by promoting excessive glucose entry (Khemais-Benkhiat et al. 2019). Indeed, premature endothelial senescence as indicated by SA-β-gal, p53 and p16 staining is observed in the aortas from Zucker diabetic rats, and also at the intimal site of atherosclerotic lesions of the human thoracic aorta as well as at sites of disturbed blood flow including branches and bifurcations (Chen et al. 2007; Hayashi et al. 2006). Moreover, the p53 overexpression selectively at ECs induced impaired endothelium-dependent vasorelaxation and NO bioavailability of rat aortic rings (Kumar et al. 2011), indicating premature cellular senescence as a key upstream signaling event to promote endothelial dysfunction.

Therefore, the present study investigated whether SGLT1 and 2 may contribute to endothelial senescence and dysfunction, and the subsequent development of cardiovascular diseases and, if so, their role in the cardiovascular system was determined.

4. Role of Ang II in endothelial senescence and dysfunction

The RAS is a vital component in regulating physiological and pathological processes of the cardiovascular system (Mehta & Griendling 2007). Previous findings have indicated a key role of both the circulating and the local angiotensin system in the induction of ED in experimental models of hypertension, diabetes and atherosclerosis, and in aging-related ED, and also in patients with cardiovascular risk factors (Dal-Ros et al. 2009; Dal-Ros et al. 2012; Idris-Khodja et al. 2012; Kane et al. 2010; Lee et al. 2011; Lee et al. 2013). ECs express high levels of ACE promoting Ang II formation (Wu et al. 2015). Ang II is a potent inducer of endothelial senescence contributing to ED by inducing vascular oxidative stress subsequent to the upregulation of the expression of NADPH oxidase (Harrison et al. 2003; Sunggip et al.

2013). The rise in oxidative stress stimulated by Ang II leads to diminished endothelium-dependent relaxation and ED in an experimental hypertensive rat model (Rajagopalan et al. 1996). In humans with elevated RAS activity, ROS-mediated ED associated with vascular growth and inflammation has been involved in the formation of atheroma (Kolakovic et al. 2017). Ang II also acts as a pro-atherosclerotic factor causing vasoconstriction and stimulates the expression not only of cell adhesion molecules (CD31/PECAM-1, VCAM-1, ICAM-1) but also of growth factors, cytokines, and chemokines within the vascular wall (Touyz & Schiffrin 1999; Touyz & Schiffrin 2000; Ushio-Fukai et al. 2002).

5. Circulating MPs act as bioeffectors in the development of CVD

The vascular endothelium plays a critical role in the pathogenesis of CAD which is the major clinical manifestation of atherosclerosis. During the past decades, although medical interventional procedures and secondary prevention medications, such as ACE inhibitors or statins, have substantially advanced and improved cardiovascular outcome, atherosclerosis still remains a principal contributor to cardiovascular mortality worldwide. Experimental and clinical studies indicate that ED is considered as an independent predictor of detrimental events in CAD patients (Schächinger et al. 2000; Heitzer et al. 2001), and that the plasma membrane-derived circulating MPs have emerged as a surrogate biomarker of ED and cardiovascular risk (Bulut et al. 2008; Werner et al. 2006). Moreover, circulating MPs in response to cellular injury or apoptosis act as a biological transcellular signal delivery system (Mause & Weber 2010). They consequently contribute to endothelial senescence and dysfunction especially by modulating the NO/ROS balance in favor of oxidative stress, which promotes procoagulant and proinflammatory responses (Brodsky et al. 2004; Mostefai et al. 2008). Indeed, previous studies have shown that circulating MPs from patients with acute

coronary syndrome abolished endothelium-dependent relaxation in rat aortic rings (Boulanger et al. 2001), and induced premature endothelial senescence and thrombogenicity through activation of the Ang II/AT_1R/NADPH oxidase pathway (Abbas et al. 2017).

The major findings of the present study indicate that MPs derived from CAD patients upregulate SGLT1 and 2 expression to facilitate the induction of endothelial senescence and dysfunction via the local Ang II/AT_1R/NADPH oxidase pathway. Ang II promoted an early oxidative stress involving NADPH oxidase, COXs and the mitochondrial respiratory chain, which, in turn, triggered the increase in SGLT1 and 2 expression associated with both glucose- and sodium-dependent sustained oxidative stress to ultimately induce endothelial senescence. Ang II-induced premature senescence resulted in ED characterized by eNOS down-regulation, enhanced oxidative stress and up-regulation of pro-atherosclerotic factors including VCAM-1, MCP-1 and TF. Furthermore, inhibition of SGLT1 and SGLT2, or SGLT2 prevented CAD MPs- and Ang II-induced local angiotensin system and NADPH oxidase-mediated expression of SGLT1 and 2, VCAM-1 and down-regulation of eNOS.

Altogether, the findings indicate that SGLT2 and most likely also SGLT1, by acting as a crucial amplifying pathway to promote the induction of endothelial senescence and dysfunction, contribute to further impair the function of the vascular system. They further suggest that inhibition of SGLT2 and/or SGLT1 might be an attractive strategy to protect the arterial wall and, hence, the development of cardiovascular diseases.

Several experimental studies have shown that the SGLT2 inhibitor empagliflozin improved endothelium-dependent relaxations in both streptozotocin-induced diabetic rats and Zucker diabetic fatty rats (Oelze et al. 2014; Steven et al. 2017), and significantly decreased LV weight, size of cardiomyocyte, cardiac interstitial fibrosis and macrophage infiltration in

genetic pre-diabetic rats with metabolic syndrome (Kusaka et al. 2016). Moreover, empagliflozin reduced atherosclerotic plaque formation by ameliorating inflammation and insulin resistance in $ApoE^{-/-}$ mice (Han et al. 2017), and improved HF morphology and attenuated HF markers expression including atrial natriuretic peptide and brain natriuretic peptide in aristolochic acid-induced HF in zebrafish embryos (Shi et al. 2017). However, the underlying mechanisms leading to the protective effect of empagliflozin on the endothelial and vascular function remain to be investigated.

In *ex vivo* blood vessels and *in vivo* an experimental model of metabolic syndrome with HFpEF, the treatment of ZSF1 rats with the SGLT2 inhibitor empagliflozin improved heart remodeling, endothelial and vascular dysfunction. The protective effects resulted in improved hyperglycemia, total cholesterol and triglycerides levels and body weight. In the ZSF1 group, the weight of the heart and all the four cavities of cardiac tissue, and the left ventricle area were increased, and significantly decreased by the empagliflozin treatment, except for left auricle plus septum weight. LV ejection fraction and cardiac output were similar in all groups. An up-regulation of senescence markers (p53, p21, p16), tissue factor, VCAM-1, SGLT1 and SGLT2 and a down-regulation of eNOS were observed in the inner curvature of aortic arch compared to the outer one in the control group, which were prevented by the empagliflozin treatment. In the mesenteric artery of ZSF1 group, acetylcholine-induced endothelium-dependent relaxations were slightly but significantly blunted and EDCFs were increased compared to control group. The cyclooxygenase inhibitor indomethacin improved endothelium-dependent relaxations to acetylcholine, and abolished EDCFs in the ZSF1 rats.

Thus, the major findings of the study indicate that empagliflozin protects the heart and the vascular system, and that the treatment is particularly effective to delay premature vascular senescence known to promote the development of cardiovascular diseases.

Discussion générale

1. Effets cardiovasculaires des inhibiteurs de SGLT2

L'essai clinique EMPA-REG OUTCOME (2010-2015) portant sur 7,020 sujets atteints de diabète de type 2 présentant une maladie cardio-vasculaire établie a montré l'effet cardioprotecteur de l'empagliflozine en réduisant de manière significative la mortalité par causes cardiovasculaires (réduction du risque relatif de 38%), les décès toutes causes confondues (réduction du risque relatif de 32%), et les hospitalisations pour insuffisance cardiaque (réduction du risque relatif de 35%) (Zinman et al. 2015). Dans l'essai clinique CANVAS (2009-2017) portant sur 10,142 patients atteints de DT2 présentant un risque élevé de maladies cardiovasculaires, la canagliflozine a significativement diminué les décès cardiovasculaires composites, infarctus du myocarde non mortel, accident vasculaire cérébral non mortel (réduction du risque relatif de 14%) et les hospitalisations pour insuffisance cardiaque (33% de réduction du risque relatif) (Neal et al. 2017). Dans l'essai DECLARE-TIMI 58 (2013-2019) incluant 17,160 patients atteints de DT2 à risque ou qui présentaient des maladies cardiovasculaires athérosclérotique, le traitement par la dapagliflozine a entraîné un taux de mortalité cardiovasculaire inférieur ou une hospitalisation pour insuffisance cardiaque (réduction de 27% du risque relatif) (Wiviott et al 2019). En résumé, les effets cardiovasculaires bénéfiques semblent être dus à un effet de classe des inhibiteurs de SGLT2. De plus, ils se sont révélés indépendants du contrôle glycémique, suggérant que d'autres mécanismes sont impliqués (Inzucchi et al. 2018). Cependant, ils doivent encore être clarifiés.

2. Mécanismes potentiels des inhibiteurs de SGLT2 sur les effets protecteurs cardiovasculaires

1) Pression artérielle réduite

L’essai EMPA-REG OUTCOME a montré que l’empagliflozine entrainait une réduction de la pression artérielle systolique et diastolique d’environ 5,2 mmHg, ce qui pourrait contribuer à expliquer, dans une certaine mesure, l’évolution bénéfique du système cardiovasculaire (Zinman et al. 2015). Des études expérimentales ont montré que la canagliflozine diminuait significativement la pression artérielle moyenne chez des rats diabétiques induits par la STZ (Abu Seif et al. 2017). La canagliflozine a également augmenté la vasorelaxation induite par le SNP dans les artères coronaires de souris diabétiques via l'induction d'une hyperpolarisation du muscle lisse vasculaire dépendante des canaux potassiques (Han et al. 2015).

2) Volume plasmatique réduit

Les inhibiteurs de SGLT2 diminuent de manière chronique les volumes de liquide extracellulaire et de plasma, comme cela a été mis en évidence dans l'essai EMPA-REG OUTCOME avec une augmentation de 5,2% du taux d'hématocrite (Zinman et al. 2015). L'effet diurétique de l'inhibition du SGLT2 devrait permettre de réduire davantage la pression aortique centrale et la postcharge, entraînant une amélioration de la fonction du VG et une diminution du travail cardiaque et de la demande en oxygène du myocarde. Une réduction de la précharge due à un volume plasmatique réduit serait censée agir en synergie avec une réduction après une charge pour réduire les événements cardiaques, en particulier chez les personnes atteintes de diabète et d'une altération de la fonction VG, de cardiopathie ischémique ou d'insuffisance cardiaque congestive (McMurray 2016; Kimura 2016). Cependant, une réduction de la volémie peut activer le système rénine-angiotensine-aldostérone et aggraver la condition cardiovasculaire en stimulant le récepteur AT1R. Néanmoins, plus de 80% des patients de l’étude EMPA-REG OUTCOME ont été traités avec un antagoniste des récepteurs de l’angiotensine ou un inhibiteur de l'ECA,

qui devrait favoriser l'activation de la voie de l'angiotensine 1-7 via AT2R, entraînant une vasodilatation, un effet anti-inflammatoire, une anti-prolifération des SMC vasculaires et des effets ionotropes positifs (Jiang et al. 2014).

3) Perte de poids

L'obésité et la résistance à l'insuline sont des facteurs de risque indépendants pour les maladies cardiovasculaires athéromateuses (DeFronzo 2010). Comme mentionné précédemment, les inhibiteurs de SGLT2 ont induit une perte de poids. Cependant, l'ampleur de la perte de poids et de la réduction rapide de la mortalité cardiaque et de l'insuffisance cardiaque dans l'essai EMPA-REG OUTCOME suggère que la réduction de poids n'était pas responsable du bénéfice cardiovasculaire rapidement observé après le début du traitement par empagliflozine. Il est possible cependant qu'une perte de poids contribue à la réduction progressive de la mortalité cardiovasculaire et de l'insuffisance cardiaque sur une période de 1 à 3 ans.

4) Autres mécanismes

D'autres mécanismes tels qu'une diminution du taux plasmatique d'acide urique, une amélioration du profil lipidique, une réduction de l'inflammation et du stress oxydatif, une amélioration de la sensibilité à l'insuline et une diminution de l'albuminurie ont été suggérés pour expliquer l'effet cardioprotecteur des inhibiteurs de SGLT2 (DeFronzo et al. 2017). Cependant, l'hypothèse selon laquelle le SGLT2 contribue à limiter la dysfonction endothéliale, un événement précoce dans le développement de la maladie cardiovasculaire, a été peu étudié.

3. Rôle des SGLT1 et 2 dans la sénescence et la dysfonction endothéliale

Plusieurs études expérimentales ont montré l'effet des inhibiteurs du SGLT2 sur la fonction endothéliale et vasculaire. Chez des souris diabétiques induites par la STZ, le traitement in vivo par l'ipragliflozine a permis de contrecarrer la phosphorylation réduite d'Akt, la diminution d'expression de la eNOS ainsi que celle de MCP-1, VCAM-1 et ICAM-1 et de réduire la génération de ROS au niveau de l'aorte abdominale. Ces changements étaient associés à une amélioration de la relaxation dépendante de l'endothélium (Salim et al. 2016). Un traitement par l'empagliflozine a également permis d'améliorer les relaxations dépendant de l'endothélium et de diminuer le stress oxydatif induit par la glucotoxicité chez les souris traitées à la STZ (Oelze et al. 2014). Chez les souris *ApoE*$^{-/-}$, un traitement par l'empagliflozine a permis de diminuer la formation de la plaque d'athérosclérose au niveau de la crosse aortique, probablement en améliorant la résistance à l'insuline et l'inflammation (Han et al. 2017). Cependant, il n'est pas clair si l'effet vasoprotecteur des inhibiteurs de SGLT2 est lié à un effet direct au niveau de la paroi artérielle et / ou à d'autres effets. Des études récentes ont permis de mettre en évidence une expression de SGLT1 et SGLT2 dans l'endothélium vasculaire. Dans les cellules HUVEC, les expressions SGLT1 et SGLT2 ont été détectées au niveau des protéines et de l'ARNm. Leurs niveaux d'expression sont augmentés par l'acide palmitique (Li et al. 2018). Une régulation des SGLT1 et 2 sensible au rédox a été mise en évidence dans des CE primaires d'artères coronaire dans des conditions pathologiques telles qu'un état d'hyperglycémie et un stress oxydant. Cette expression accrue est impliquée dans la sénescence et DE en favorisant une entrée excessive de glucose (Khemais-Benkhiat et al. 2019). En effet, une sénescence endothéliale prématurée, attestée par les marqueurs SA-β-gal, p53 et p16, peut être observée dans les aortes de rats diabétiques Zucker, au niveau de l'intima des lésions athérosclérotiques de l'aorte thoracique humaine ainsi que qu'au niveau des sites arteriels exposé à des régimes de flux turbulents comme les branches et des bifurcations (Chen et al. 2007; Hayashi et al.

2006). De plus, la surexpression de p53 sélectivement au niveau des CE induisait une réduction de la vasorelaxation dépendante de l'endothélium associée à une biodisponibilité réduite du NO dans les des anneaux aortiques de rat (Kumar et al. 2011), indiquant une sénescence cellulaire prématurée en tant qu'événement de signalisation clé précédent la DE.

Par conséquent, la présente étude a examiné si les SGLT1 et 2 pouvaient contribuer à la sénescence et au dysfonctionnement endothéliaux et au développement ultérieur de maladies cardiovasculaires et, dans l'affirmative, à leur rôle dans le système cardiovasculaire.

4. Rôle de l'Ang II dans la sénescence et le dysfonctionnement endothéliaux

Le système rénine angiotensine (SRA) est un élément essentiel de la régulation des processus physiologiques et pathologiques du système cardiovasculaire (Mehta & Griendling 2007). Des résultats antérieurs ont montré que le systèmes angiotensine circulant et locaux jouent un rôle clé dans l'induction de la DE dans des modèles expérimentaux d'hypertension, de diabète et d'athérosclérose et dans la DE liée au vieillissement, ainsi que chez les patients présentant des facteurs de risque cardiovasculaires (Dal-Ros et al., 2009; Dal-Ros et al., 2012; Idris-Khodja et al., 2012; Kane et al., 2010; Lee et al, 2011; Lee et al, 2013). Les CE expriment des niveaux élevés d'ECA favorisant la formation d'Ang II (Wu et al. 2015). L'Ang II est un puissant inducteur de la sénescence endothéliale, contribuant à la DE en induisant un stress oxydatif consécutif à la régulation positive de l'expression de la NADPH oxydase (Harrison et al. 2003; Sunggip et al. 2013). L'augmentation du stress oxydatif stimulée par l'Ang II entraîne une diminution de la relaxation dépendante de l'endothélium et la DE dans un modèle expérimental de rats hypertendus (Rajagopalan et al., 1996). Chez les humains présentant une activité SRA élevée, la DE médiée par les ROS, associée à la croissance et à l'inflammation vasculaires, est impliquée dans la formation d'athérome (Kolakovic et al. 2017). L'Ang II agit également en tant que facteur pro-athérosclérotique provoquant une

vasoconstriction et stimule l'expression non seulement des molécules d'adhésion cellulaire (CD31 / PECAM-1, VCAM-1, ICAM-1), mais également des facteurs de croissance, des cytokines et des chimiokines dans la paroi vasculaire (Touyz et Schiffrin 1999; Touyz et Schiffrin 2000; Ushio-Fukai et al. 2002).

5. Les MPs en circulation agissent comme des bio-facteurs dans le développement des MCV

L'endothélium vasculaire joue un rôle essentiel dans la pathogenèse de la coronaropathie, principale manifestation clinique de l'athérosclérose. Au cours des dernières décennies, bien que les interventions médicales et les médicaments de prévention secondaire, tels que les inhibiteurs de l'ECA ou les statines, aient considérablement progressé et amélioré le traitement des patients, l'athérosclérose reste le principal facteur de mortalité dans le monde. Des études expérimentales et cliniques indiquent que la DE est considérée comme un facteur prédictif indépendant des événements cliniques chez les patients atteints de coronaropathie (Schächinger et al. 2000; Heitzer et al. 2001). Les MPs circulantes, dérivées de la membrane plasmique, émergent comme biomarqueur de substitution du risque cardiovasculaire (Bulut et al. 2008; Werner et al. 2006). De plus, les MPs circulantes, émise en réponse à une lésion cellulaire ou lors de l'apoptose, agissent comme un système de transmission de signal biologique transcellulaire (Mause & Weber 2010). Les MPs contribuent par conséquent à la sénescence et DE, en particulier en modulant la balance NO/ROS en faveur du stress oxydatif, ce qui favorise les réponses procoagulantes et proinflammatoires (Brodsky et al. 2004; Mostefai et al. 2008). Des études antérieures ont montré que des MPs circulantes provenant de patients atteints d'un syndrome coronarien aigu abolissaient la relaxation dépendante de l'endothélium dans les anneaux aortiques de rat (Boulanger et al. 2001), et induisaient une

sénescence endothéliale prématurée et une thrombogénicité par l'activation de l'Ang II/AT1R /Voie de la NADPH oxydase (Abbas et al. 2017).

Les principales conclusions de la présente étude indiquent que les MPs dérivées de patients atteints de coronaropathie régulent positivement l'expression de SGLT1 et 2 afin de faciliter l'induction de la sénescence et la DE via la voie locale Ang II/AT1R/NADPH oxydase. L'Ang II favorise un stress oxydatif précoce dépendant de la NADPH oxydase, des COX et de la chaîne respiratoire mitochondriale, ce qui déclenche l'augmentation de l'expression des SGLT1 et 2 associée à un stress oxydatif prolongé dépendant du glucose et du sodium, pour finalement induire la sénescence endothéliale. La sénescence prématurée induite par l'Ang II a entraîné une DE caractérisée par une régulation à la baisse de la eNOS, un stress oxydatif accru et une induction endothéliale des facteurs pro-athérosclérotiques VCAM-1, MCP-1 et le facteur tissulaire. En outre, suite à une stimulation par les MPs de patients coronariens soit par l'Ang II , l'inhibition de SGLT1 et SGLT2 ou SGLT2 seul, a permis de prévenir l'induction du système angiotensine locale ainsi que l'expression dépendante de la NADPH oxydase des SGLT1 et 2, de VCAM-1 et la régulation à la baisse de la eNOS.

Globalement, ces résultats indiquent que SGLT2 et très probablement aussi SGLT1, agissent comme une voie d'amplification cruciale qui favorise l'induction de la sénescence et la DE et contribuent à majorer la condition cardiovasculaire. Ils suggèrent en outre que l'inhibition du SGLT2 et / ou du SGLT1 pourrait constituer une stratégie intéressante pour protéger la paroi artérielle dans le contexte des pathologies cardiovasculaires.

Plusieurs études expérimentales ont montré que l'empagliflozine, inhibiteur du SGLT2, améliorait la fonction endothéliale chez les rats diabétiques de type 1 (STZ) et de type 2 (Zucker) (Oelze et al. 2014; Steven et al. 2017). L'empagliflozine a permis d'entrainer

également diminution significative du poids du VG et de la taille des cardiomyocytes, de la fibrose interstitielle cardiaque et de l'infiltration de macrophages chez des rats pré-diabétiques présentant un syndrome métabolique (Kusaka et al. 2016). En outre, l'empagliflozine a permis de réduire la formation de plaques d'athérosclérose en diminuant l'inflammation et la résistance à l'insuline chez les souris $ApoE^{-/-}$ (Han et al. 2017). Elle a permis également d'améliorer la morphologie cardiaque ainsi que les marqueurs associés à l'insuffisance cardiaque, notamment le peptide natriurétique auriculaire et le peptide natriurétique cérébral dans un modele d'insuffisance cardiaque induite par l'acide Aristolochic dans les embryons de poisson-zèbre (Shi et al. 2017). Cependant, les mécanismes sous-jacents à l'effet protecteur de l'empagliflozine sur les fonctions endothéliale et vasculaire restent à élucider.

A la fois dans des vaisseaux sanguins ex vivo et dans un modèle expérimental de syndrome métabolique avec HFpEF in vivo, le traitement de rats ZSF1 par l'empagliflozine a permis d'améliorer le remodelage cardiaque, la DE et vasculaire. Les effets protecteurs ont entraîné une amélioration de l'hyperglycémie, des taux de cholestérol total et de triglycérides et du poids corporel. Dans le groupe ZSF1, le poids du cœur et des quatre cavités du tissu cardiaque ainsi que la région du ventricule gauche ont été augmentés et diminués de manière significative par le traitement à l'empagliflozine, à l'exception du poids de l'auricule gauche et du septum. La fraction d'éjection du VG et le débit cardiaque étaient similaires dans tous les groupes. Une régulation à la hausse des marqueurs de sénescence (p53, p21, p16), du facteur tissulaire, de VCAM-1, SGLT1 et SGLT2 et une régulation à la baisse de eNOS ont été observées dans la courbure interne de la crosse aortique par rapport à celle externe du groupe témoin, qui ont été prévenus par le traitement à l'empagliflozine. Dans l'artère mésentérique du groupe ZSF1, les relaxations dépendantes de l'endothélium induites par l'acétylcholine étaient légèrement émoussées et les facteurs constricteurs dérivés de

l’endothélium augmentaient par rapport au groupe témoin. L'indométhacine, un inhibiteur de la cyclooxygénase, améliore les relaxations dépendantes de l'endothélium induite par l’acétylcholine et élimine les facteurs constricteurs dérivés de l’endothélium chez les rats ZSF1.

Ainsi, les principaux résultats de l’étude indiquent que l’empagliflozine protège le cœur et le système vasculaire et que le traitement est particulièrement efficace pour retarder la sénescence vasculaire prématurée connue pour favoriser le développement de maladies cardiovasculaires.

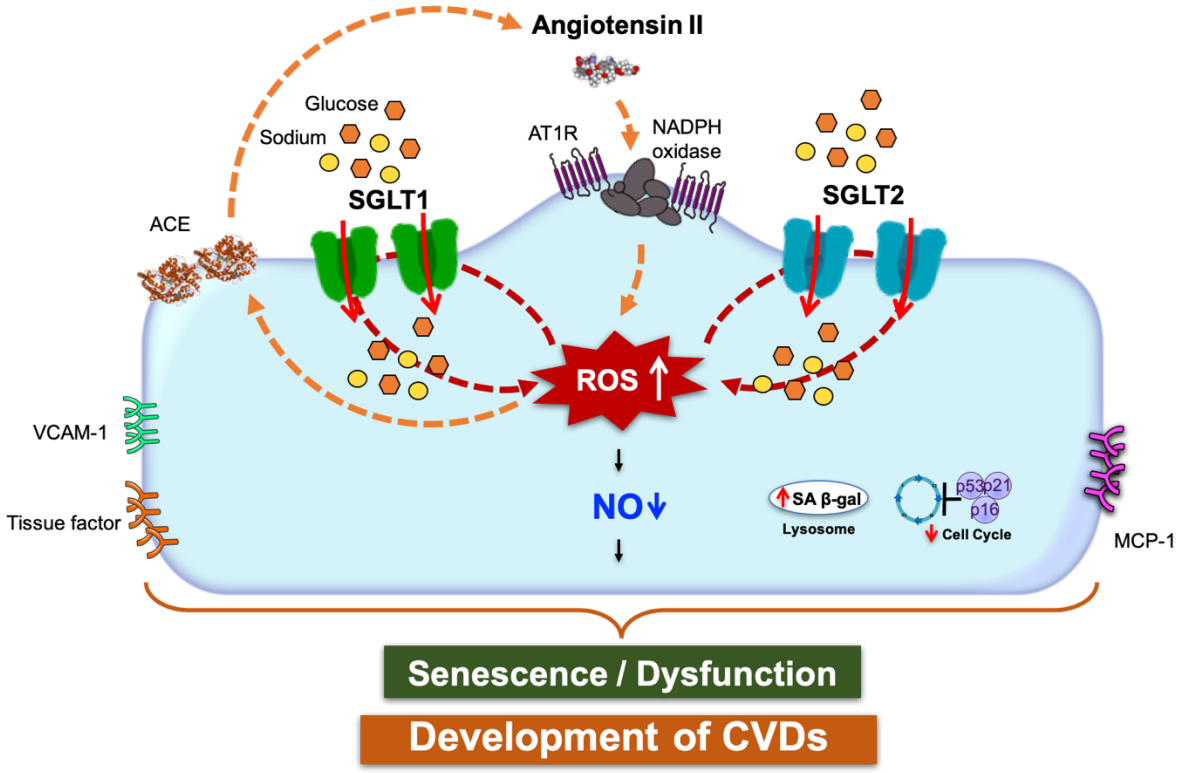


Figure 19. Schematic indicating the role of SGLT1 and 2 in the Ang II-induced endothelial senescence and dysfunction.

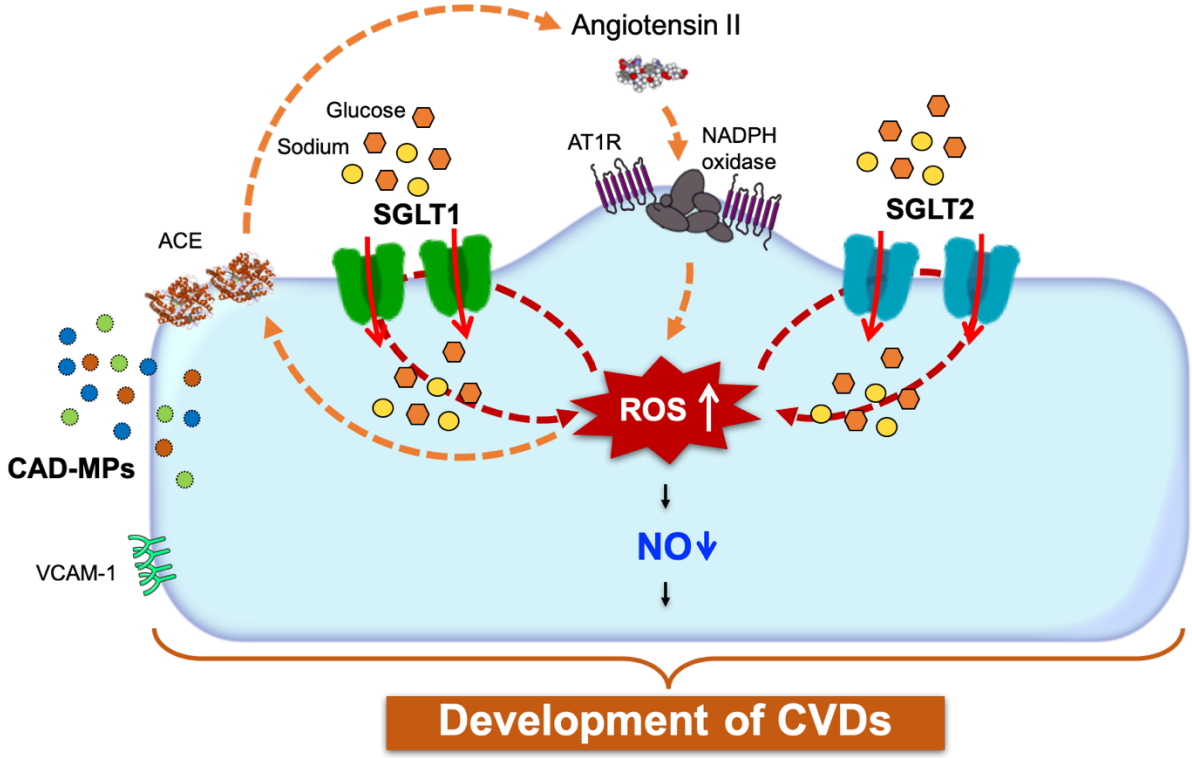


Figure 20. Schematic indicating the role of SGLT1 and 2 in the CAD MPs-induced pro-atherosclerotic responses via the local Ang II/AT_1R/NADPH oxidase pathway.

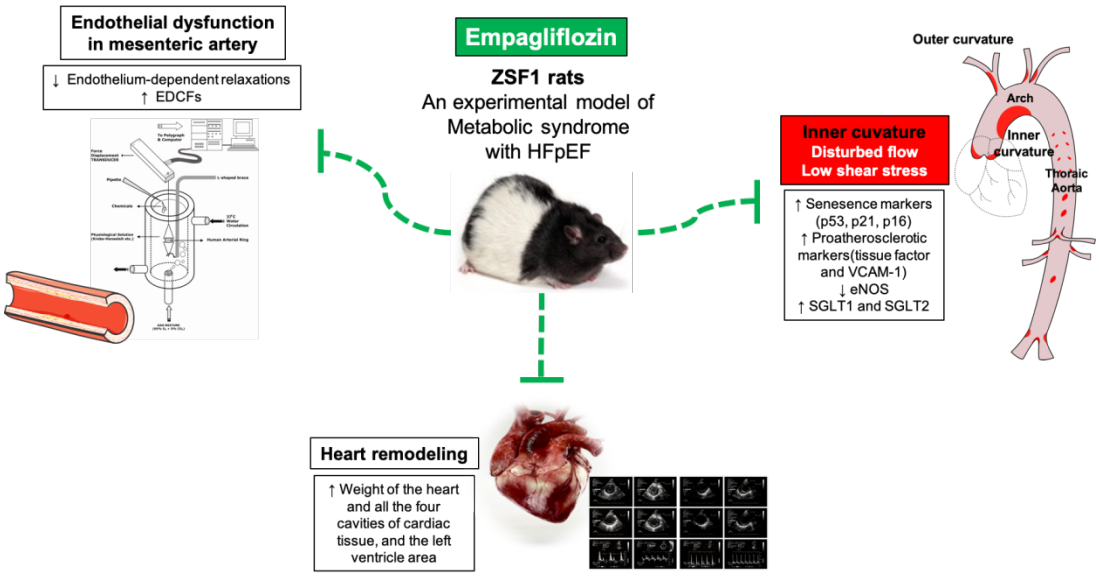


Figure 21. Schematic indicating the effect of a selective SGLT2 inhibitor empagliflozin on heart remodeling, endothelial and vascular dysfunction in a well characterized animal model of metabolic syndrome with HFpEF, the ZSF1.

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Evaluation du rôle des co-transporteurs sodium-glucose SGLT1 et 2 dans l'induction de la sénescence et de la dysfonction des cellules endothéliales à l'aide d'une approche *in vitro* et *in vivo*

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Résumé

Les inhibiteurs du cotransporteur sodium-glucose SGLT2 ont montré des effets protecteurs cardiovasculaires remarquables avec une réduction du risque de mortalité cardiovasculaire et d'hospitalisation pour insuffisance cardiaque chez des patients atteints de DT2 avec un risque cardiovasculaire élevé, un effet indépendant du contrôle de la glycémie. La possibilité que les inhibiteurs de SGLT1 et 2 protègent le système cardiovasculaire en ciblant la fonction endothéliale protectrice reste incertaine. La première étude *in vitro* indique que l'angiotensine II et les microparticules circulantes provenant de patients atteints de coronaropathie sont de puissants inducteurs de l'expression de SGLT1 et 2 via l'activation du système angiotensine local afin de promouvoir la sénescence et la dysfonction endothéliale. La deuxième étude *in vivo* indique que l'empagliflozine, un inhibiteur sélectif de SGLT2, protège le cœur et le système vasculaire, et que le traitement est particulièrement efficace pour retarder la sénescence vasculaire prématurée connue pour favoriser le développement de maladies cardiovasculaires au niveau des sites artériels présentant un risque athérogène. Dans l'ensemble, ces études suggèrent que l'inhibition de SGLT2 et/ou de SGLT1 pourrait constituer une stratégie thérapeutique attrayante pour la protection de la fonction endothéliale et le développement ultérieur de maladies cardiovasculaires.

Mots-clés: Cotransporteur sodium-glucose 2, Angiotensine II, Cellules endothéliales, Sénescence

Résumé en anglais

Sodium-glucose cotransporter (SGLT)2 inhibitors have shown remarkable cardiovascular protective effects with reduced risk of cardiovascular mortality and hospitalization for heart failure in T2DM patients with a high cardiovascular risk and that this effect is independent of glucose control. The possibility that SGLT1 and 2 inhibitors protect the cardiovascular system by targeting the pivotal protective endothelial function remains unclear. The first *in vitro* study indicates that angiotensin II and circulating microparticles from patients with coronary artery disease via the activation of the local angiotensin system are potent inducers of SGLT1 and 2 expression to promote endothelial senescence and dysfunction. The second *in vivo* study indicates that the selective SGLT2 inhibitor empagliflozin protects the heart and the vascular system, and that the treatment is particularly effective to delay premature vascular senescence known to promote the development of cardiovascular diseases at arterial sites at risk of atherogenesis. Altogether, these studies suggest that inhibition of SGLT2 and/or SGLT1 might be an attractive therapeutic strategy to protect the endothelial function, and the subsequent development of cardiovascular diseases.

Keywords: Sodium-glucose cotransporter 2, Angiotensin II, Endothelial cells, Senescence