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Empagliflozin prevents angiotensin II-induced hypertension related micro and macrovascular endothelial cell activation and diastolic dysfunction in rats despite persistent hypertension: Role of endothelial SGLT1 and 2

Christophe Bruckert [a,b,1], Kensuke Matsushita [a,b,c,1], Ali Mroueh [a], Said Amissi [a,b], Cyril Auger [a,b], Ursula Houngue [a,b], Lamia Remila [a,b], Ahmed Bey Chaker [a,b], Sin-Hee Park [a,b], Paola Algara-Suarez [a,b], Eugenia Belcastro [a,b], Laurence Jesel [a,c], Patrick Ohlmann [c], Olivier Morel [a,c], Valérie B. Schini-Kerth [a,b,*]

[a] INSERM (French National Institute of Health and Medical Research), UMR 1260, Regenerative Nanomedicine, FMTS, Strasbourg, France
[b] Université de Strasbourg, Faculté de Pharmacie, Strasbourg, France
[c] Hôpitaux Universitaires de Strasbourg (HUS), Service de Cardiologie, Strasbourg, France

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ABSTRACT

SGLT2 inhibitors (SGLT2i) showed pronounced beneficial effects in patients with heart failure but the underlying mechanisms remain unclear. We evaluated the effect of empagliflozin, selective SGLT2i, on hypertension-induced cardiac and vascular dysfunction.

Male Wistar rats received diet with or without empagliflozin (30 mg/kg/day). After 1 week, a hypertensive dose of Ang II (0.4 mg/kg/day) was administered using osmotic mini-pumps for 4 weeks. Systolic blood pressure was determined by sphygmomanometry, the cardiac function by echocardiography and *ex vivo* (coronary microvascular endothelial cell activation, LV remodeling and fibrosis responses), and the systemic micro and macrovascular endothelial cell activation *ex vivo*. Empagliflozin treatment did not affect the Ang II-induced hypertensive response. Ang II treatment increased LV mass and induced LV diastolic dysfunction, fibrosis, collagen I and ANP expression, and infiltration of macrophages. In the vasculature, it caused eNOS upregulation in the aorta and down-regulation in mesenteric microvessels associated with increased oxidative stress, ACE, AT1R, VCAM-1, MCP-1, MMP-2, and MMP-9 and collagen I expression, increased endothelial SGLT1 staining in the aorta, mesenteric and coronary microvessels, increased SGLT1 and 2 protein levels in the aorta. All Ang II-induced cardiac and vascular responses were reduced by the empagliflozin treatment.

Thus, the SGLT2i effectively attenuated the deleterious impact of Ang II-induced hypertension on target organs including cardiac diastolic dysfunction and remodeling, and endothelial cell activation and pro-atherosclerotic, pro-fibrotic and pro-remodeling responses in macro and microvessels despite persistent hypertension.

1. Introduction

The gliflozin family was originally developed as antidiabetic drugs for treatment of patients with type 2 diabetes by selectively inhibiting the sodium-glucose cotransporter 2 (SGLT2) in the kidney. [1] The meta-analysis of large cardiovascular outcome trials with SGLT2 inhibitors (SGLT2i) in type 2 diabetes indicated consistent benefits with a ~ 30% reduction in the risk of heart failure (HF) hospitalization and with a ~ 40–50% reduction in the risk of serious adverse kidney events, and a moderate reduction on cardiovascular death by ~15%. [2] Recent meta-analysis of two large-scale trials with SGLT2i that enrolled patients with HF with reduced ejection fraction (HFrEF) showed a ~ 25% reduction of the combined risk of cardiovascular death or hospitalization for HF, and improved renal outcomes. [3] These benefits are observed regardless of the diabetic status and across all ages. [3]

SGLT2i showed also cardiovascular protective effects in several diabetic and non-diabetic experimental models of cardiovascular diseases. SGLT2i attenuated doxorubicin-induced HF and reduced fibrosis

* Corresponding author at: UMR1260 INSERM, University of Strasbourg, CRBS, 1 rue Eugène Boeckel, 67084 Strasbourg, France.
E-mail address: valerie.schini-kerth@unistra.fr (V.B. Schini-Kerth).
[1] Co-first authors

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in mice, [4] improved cardiac function and remodeling in rats after myocardial infarction in both diabetic and nondiabetic rats, [5,6] and ameliorated systolic and diastolic functions, left ventricle (LV) fibrosis and stiffness in a nondiabetic porcine model of HFrEF after myocardial infarction. [7,8] In addition, in a rat model of metabolic syndrome with HF with preserved ejection fraction (ZSF1 rat, HFpEF), empagliflozin (a selective SGLT2i) prevented hypertrophy and remodeling of the heart as well as endothelial dysfunction. [9] Despite the remarkable cardiovascular protective effect of SGLT2i, the underlying mechanisms at the heart and vasculature still remain to be clarified.

Both clinical and experimental studies have highlighted that angiotensin II (Ang II) contributes to the development of major types of cardiovascular diseases including hypertension and HF. Many of the pathophysiological actions of Ang II are mediated *via* activation of the angiotensin type 1 receptor (AT1R) to stimulate reactive oxygen species (ROS) generation through activation of NADPH oxidases, and, as a consequence, to induce endothelial dysfunction, vascular fibrosis, inflammation and calcification, cardiac dysfunction and remodeling, and also the progression of atherosclerotic lesions. [10] Recently, this group has shown that Ang II is also a strong inducer of SGLT1 and SGLT2 expression in endothelial cells through a redox-sensitive mechanism to promote premature endothelial senescence and dysfunction. [11] The fact that empagliflozin and sotagliflozin, a dual SGLT1 and 2i, prevented the Ang II-induced pro-oxidant response, up-regulation of SGLT1 and 2 expression and induction of endothelial dysfunction implies a determinant role of SGLT1 and 2. [11] Moreover, experiments performed using rat arteries showed that SGLT1 and SGLT2 are overexpressed at arterial sites of risk (aortic arch versus thoracic aorta) where the local angiotensin system is particularly activated and characterized by high levels of oxidative stress and endothelial dysfunction, and also in aorta segments following their *ex vivo* exposure to Ang II. [11] Therefore, the aim of the present study is to evaluate the potential effect of empagliflozin on Ang II-induced hypertension and its deleterious impact on both the vasculature and the heart in normoglycemic rats, and to characterize the underlying mechanisms.

2. Methods

An expanded Material and Methods section is available in online supplementary Appendix S1. All animal care and experimental procedures complied with the rules of the European Union Directive of September 2010 (2010/63/EU) and the French Legislation. The study was approved by the local animal Ethic Committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg) and authorized by the French Ministry of Higher Education, Research and Innovation (authorization #15155–201805181704700). Rats were divided into four groups: control, empagliflozin (Empa, 30 mg/kg/day), Ang II (0.4 mg/kg/day) and Ang II (0.4 mg/kg/day) + empagliflozin (30 mg/kg/day). Empagliflozin treatment was provided in the diet for five weeks, and Ang II was infused using osmotic mini pumps during the last four weeks. Urine samples as well as echocardiography were performed the day before euthanasia, and blood samples were collected by terminal cardiac puncture. Non-invasive blood pressure was determined during the five-week period. After 5 weeks organ morphometry, the generation of ROS, mRNA and protein expression levels, fibrosis, hypertrophy, and macrophages infiltration were quantified in the aorta, mesenteric and coronary microcirculation and in the LV. To characterize the vascular formation of ROS, cryosections were exposed to N^G-Nitro-L-arginine (300 μM, NO synthase inhibitor (NOS)) to determine the role of uncoupled eNOS, to losartan (1 μM, AT1R antagonist) and VAS-2870 (1 μM, NADPH oxidase inhibitor) for the local AT1R/NADPH oxidases pathway, and to sotagliflozin (100 nM) and empagliflozin (100 nM) for SGLT1 and SGLT2 for 30 min before the determination of the level of ROS. Data are expressed as mean ± SEM. Analysis was done using analysis of variance (ANOVA) followed by Bonferroni's multiple comparison.

3. Results

3.1. Empagliflozin does not affect the hypertensive response to Ang II in rats

Ang II treatment increased the level of systolic blood pressure by about 40 mmHg compared to the control group (Fig. 1). The empagliflozin treatment affected minimally the systolic blood pressure level under control conditions and the hypertensive response to Ang II (Fig. 1).

3.2. Effect of empagliflozin on body weight and the weight of different organs

The morphometric evaluation of rats has indicated that the control group, the empagliflozin group and the Ang II group had similar body weights whereas a significantly reduced body weight was observed in the Ang II + empagliflozin group (Table 1). In the empagliflozin group, the weight of both the left and right kidneys was significantly increased compared to the control group whereas no such effect was observed in the Ang II group and the Ang II + empagliflozin group (Table 1). The weight of the liver and the LV + septum was similar in all 4 groups (Table 1). The Ang II treatment increased the whole heart weight and the right ventricle weight which were not observed in the Ang II + empagliflozin group (Table 1).

3.3. Empagliflozin attenuates the Ang II-induced diastolic dysfunction

Assessment by echocardiography of the LV ejection fraction (LVEF), the fractional shortening (FS), the cardiac output (CO) and the stroke volume (SV) has indicated that neither the Ang II treatment nor the empagliflozin treatment affected the heart systolic function (Table 1). In contrast, the diastolic function determined by e' velocity, E/e' ratio and isovolumic relaxation time (IVRT) were significantly affected by the Ang II treatment compared to the control group and the empagliflozin group (Table 1). The empagliflozin treatment significantly prevented the Ang II-induced increased E/e' ratio and normalized the e' velocity and the IVRT (Table 1). As a consequence, the IVSTd and the LV mass were significantly increased in the Ang II group but not in the Ang II + empagliflozin group compared to the control group (Table 1). The

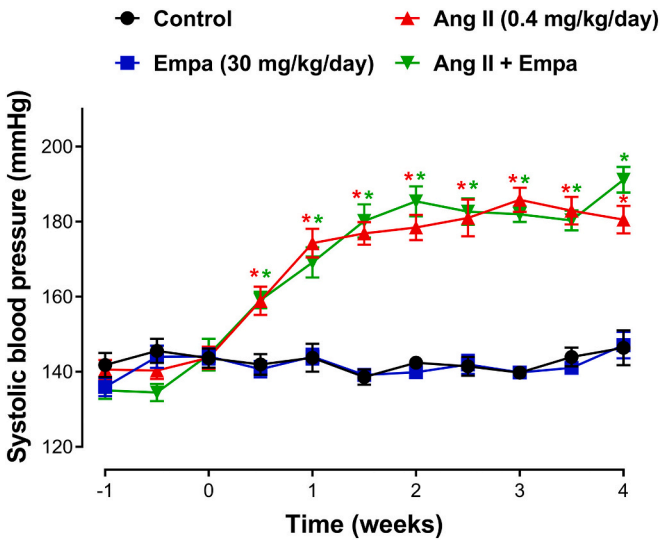

Fig. 1. Effect of a 4-week infusion of Ang II and/or oral intake of empagliflozin (Empa) on systolic blood pressure in rats. Systolic blood pressure is assessed by the tail cuff method. Values are expressed as mean ± SEM of $n = 12$ per group. * $P < 0.05$ vs control group. Statistical analysis is performed by 2-way ANOVA (Bonferroni's post hoc test).

Table 1
Effect of empagliflozin treatment on body weight and relative weight of different organs in control and Ang II-treated rats, and on Ang II-induced hypertension and related cardiac diastolic dysfunction.

	Control	Empa	Ang II	Ang II + Empa
Morphometric parameters				
Body weight (g)	367.00 ± 12.00	360.00 ± 12.00	379.00 ± 12.00	340.00 ± 13.00$^{\#}$
Tibia length (mm)	46.61 ± 0.57	45.60 ± 1.19	47.36 ± 1.07	48.27 ± 0.75
Heart/tibia (mg/mm)	25.92 ± 0.88	24.69 ± 0.17	29.66 ± 1.26*	26.66 ± 0.30
LV + septum/tibia (mg/mm)	12.56 ± 1.01	11.88 ± 0.76	14.05 ± 0.90	12.82 ± 0.60
Right ventricle/tibia (mg/mm)	7.30 ± 0.41	6.09 ± 0.60	9.26 ± 0.60*	7.98 ± 0.53
Liver/tibia (mg/mm)	208.90 ± 5.28	242.31 ± 17.48	209.81 ± 6.84	199.00 ± 7.12
Left kidney/tibia (mg/mm)	27.82 ± 0.86	31.29 ± 1.30*	26.53 ± 1.10	28.46 ± 1.46
Right kidney/tibia (mg/mm)	28.88 ± 0.05	32.66 ± 1.32*	26.62 ± 1.11	29.19 ± 0.92
LV diastolic function				
e' velocity (cm/s)	8.49 ± 0.37	7.79 ± 0.40	6.48 ± 0.41*	7.73 ± 0.44
E/e' (ratio)	11.98 ± 0.44	12.93 ± 0.59	17.54 ± 0.99*	14.11 ± 0.94$^{\#}$
IVRT (s)	0.019 ± 0.001	0.019 ± 0.001	0.015 ± 0.001*	0.020 ± 0.001$^{\#}$
LV systolic function				
LVEF (%)	80.04 ± 1.91	80.20 ± 1.96	80.58 ± 2.04	78.45 ± 2.14
FS (%)	50.94 ± 2.02	50.66 ± 2.19	51.18 ± 2.23	49.07 ± 2.25
CO (mL/min)	178.50 ± 22.70	170.30 ± 17.66	192.90 ± 13.72	194.70 ± 33.83
SV (mL)	0.49 ± 0.06	0.47 ± 0.04	0.54 ± 0.04	0.53 ± 0.09
Cardiac structure				
IVSTd (mm)	1.55 ± 0.07	1.63 ± 0.06	2.10 ± 0.11*	1.82 ± 0.07
LV mass (mg)	624.50 ± 32.42	602.30 ± 32.76	824.00 ± 48.85*	767.40 ± 39.35

Organs are weighted and indexed to the respective tibial length. All the cardiac parameters are measured by echocardiography. Values are expressed as mean ± SEM of $n = 9$–12 per group. *$P < 0.05$ vs control group and $^{\#}P < 0.05$ vs Ang II group. Statistical analysis is performed by one-way ANOVA (Bonferroni's post hoc test). LV, left ventricle; IVRT, isovolumic relaxation time; LVEF, left ventricular ejection fraction; FS, fractional shortening; CO, cardiac output; SV, stroke volume; IVSTd, interventricular septum thickness in diastole.

empagliflozin treatment alone had minimal effects (Table 1).

3.4. Empagliflozin improves the eNOS/ROS balance in both macro and microvessels of Ang II-treated rats

The formation of ROS as assessed using dihydroethidium was markedly increased throughout the arterial wall in both the aorta and mesenteric microvessels of the Ang II group compared to the control group whereas only a small but significant increase was observed in the Ang II + empagliflozin group (Fig. 2A). As expected, the antioxidant *N*-acetylcysteine reduced the ROS level in all groups (Fig. 2A). The characterization of the pro-oxidant response in the Ang II group indicated a significant inhibitory effect of the NOS inhibitor N^{G}-nitro L-arginine, the AT1R antagonist losartan and the NADPH oxidase inhibitor VAS-2870 indicating the involvement of uncoupled eNOS and the AT1R/NADPH oxidase pathway (Fig. 2A). In addition, the vascular pro-oxidant response was also significantly reduced by sotagliflozin and abolished by empagliflozin suggesting that SGLT2, and, possibly also SGLT1, have a pivotal role (Fig. 2A).

To evaluate the consequence of the pro-oxidant level in the arterial wall, the expression level of eNOS and nitrotyrosine was assessed by immunofluorescence, Western blot and/or RT-qPCR analysis. The Ang II treatment was associated with an increased eNOS signal and levels of eNOS mRNA and protein in the aorta compared to those of the control group possibly as part of a compensatory mechanism subsequent to the degradation of NO by superoxide anions (Fig. 2B). In contrast, in mesenteric microvessels the eNOS signal was decreased by about 2.5-fold suggesting that microvessels are impacted to a greater extent (Fig. 2C). These effects of Ang II on eNOS expression were not observed in the Ang II + empagliflozin group indicating that the endothelium has been preserved (Figs. 2 B, C). An unbalanced eNOS/ROS is also indicated by the enhanced nitrotyrosine protein level and signal observed throughout the aorta wall in the Ang II group compared to the control group whereas no such effects were observed in the Ang II + empagliflozin group (Fig. 2C). The empagliflozin treatment alone affected minimally all these signals (Figs. 2 B, C). Thus, the empagliflozin treatment effectively attenuated the Ang II-induced vascular oxidative stress and the impact on the endothelium.

3.5. Empagliflozin prevents the activation of the ACE/AT1R/NADPH oxidases pathway in both macro and microvessels of Ang II-treated rats

Consistent with previous observations [12,13], the Ang II-induced hypertension is associated with an increased ACE immunofluorescence signal predominantly in the endothelium in both the aorta (by about 3-fold) and mesenteric microvessels (by about 2-fold) associated with increased ACE mRNA and protein levels in the aorta compared to the control group (Fig. 3A). It was also associated with an increased AT1R mRNA level in the aorta and enhanced immunofluorescence signals throughout the arterial wall in both the aorta and mesenteric microvessels compared to the control group (Fig. 3B). The empagliflozin treatment markedly prevented the Ang II-induced increased ACE and AT1R expression in the aorta and normalized those in mesenteric microvessels (Figs. 3 A, B). The empagliflozin treatment alone had little effect (Figs. 3 A, B). Since the NAPDH oxidase inhibitor VAS-2780 markedly reduced the level of oxidative stress in the Ang II group, the expression level of NADPH oxidase subunits was evaluated by RT-qPCR in the aorta. Increased mRNA levels of p47phox, p22phox, NOX1, and NOX2 associated with a reduced NOX4 level were observed in the Ang II group compared to the control group (Fig. 3C). The empagliflozin treatment significantly prevented the Ang II-induced changes of the mRNA level of p47phox, p22phox, NOX1 and NOX4 but not of NOX2 (Fig. 3C). The empagliflozin treatment alone had only minor effects (Fig. 3C).

3.6. Empagliflozin prevents the expression of pro-arteriosclerotic, pro-fibrotic and pro-remodeling markers in both macro and microvessels of Ang II-treated rats

In the Ang II group, increased VCAM-1 and MCP-1 immunofluorescence signals are observed predominantly in the endothelium of mesenteric microvessels compared to those of the control group (Figs. 4 A, C). Increased ICAM-1 and MCP-1 mRNA levels and an increased VCAM-1 protein level were also observed in the aorta of the Ang II group (Figs. 4A-C). In addition, the Ang II treatment significantly increased the collagen I signal by about 4-fold in the microvessel wall, and also those of the matrix metalloproteinases MMP-2 and MMP-9 by about 5- and 4-fold, respectively, predominantly in the endothelium (Figs. 4D-F). Increased MMP-2 mRNA level and MMP-9 protein level were also observed in the aorta of the Ang II group (Figs. 4E,F). The empagliflozin treatment prevented the stimulatory effect of Ang II on all these markers (Fig. 3). Thus, these findings indicate that the empagliflozin treatment attenuated pro-arteriosclerotic, pro-fibrotic and pro-remodeling responses in both the macro and microvessls of the Ang II group.

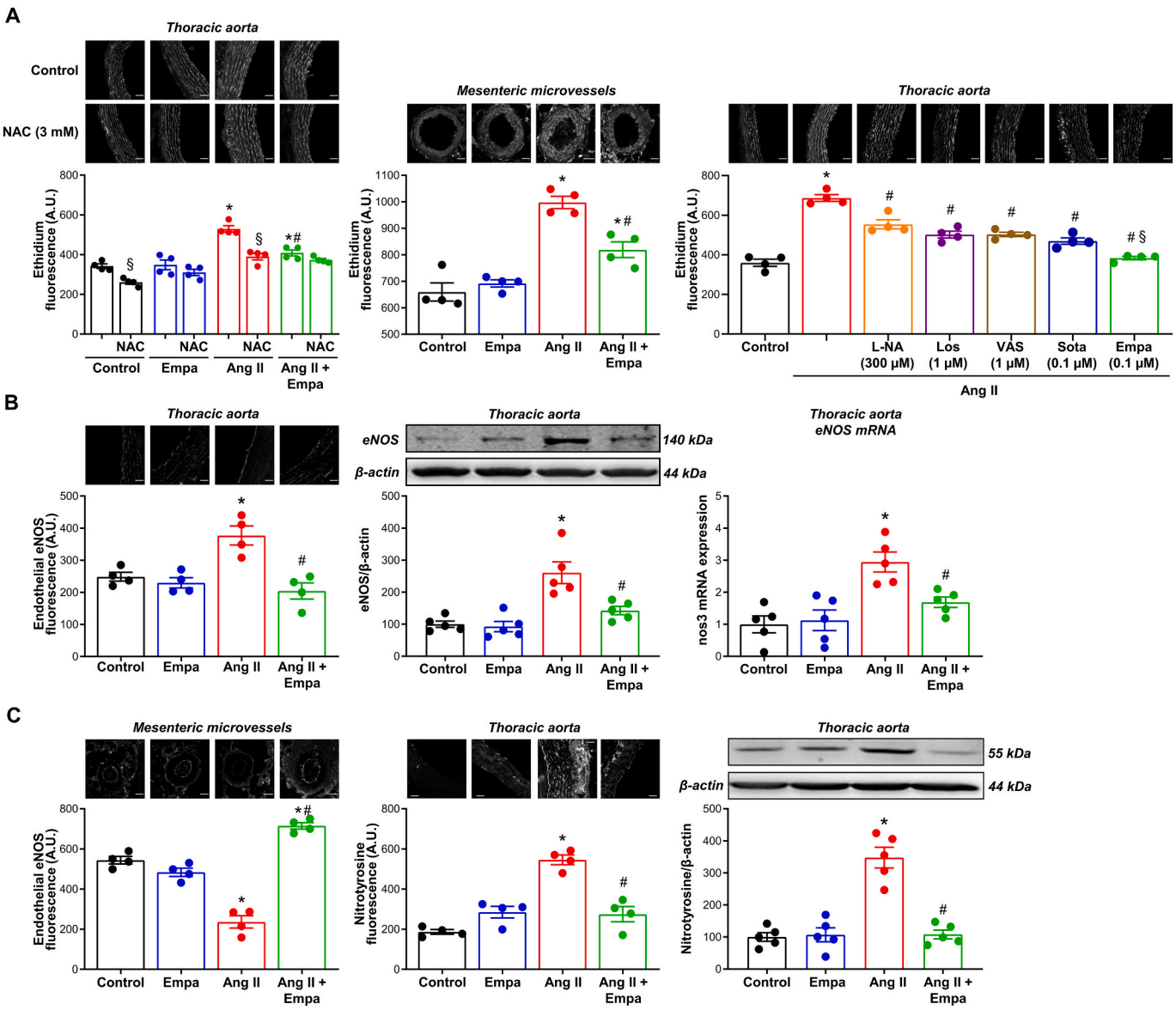

Fig. 2. The empagliflozin treatment improves the eNOS/ROS balance in both macro and microvessels of Ang II-treated rats. (A) The level of oxidative stress is assessed in aorta and secondary branch of mesenteric artery cryosections from the control, empagliflozin (Empa), Ang II and Ang II + Empa group. In some experiments, sections are incubated with *N*-acetyl cysteine (NAC, an antioxidant) at 3 mM for 2 h before exposure to dihydroethidium. Aorta cryosections from Ang II rats are treated with either N^{ω}-nitro-L-arginine (L-NA, an inhibitor of NO synthase, 300 µM), Losartan (Los, an AT1R antagonist, 1 µM), VAS-2870 (VAS, a NADPH oxidase inhibitor, 1 µM), sotagliflozin (Sota, a dual SGLT1 and SGLT2 inhibitor, 100 nM) or Empa (a selective SGLT2 inhibitor, 100 nM) for 30 min before dihydroethidium staining. (B and C) Protein immunofluorescence signals are determined in fixed cryosections of the aorta and mesenteric microvessels using confocal microscopy, and mRNA and protein levels in the aorta by RT-qPCR and Western blot analysis, respectively. Results are shown as representative signals (upper panels) and corresponding cumulative data (lower panels). Values are expressed as mean ± SEM of $n = 4$ per group. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs Ang II group, and $^{\S}P < 0.05$ vs respective control. Statistical analysis is performed by 1-way ANOVA (Bonferroni's post hoc test).

3.7. Empagliflozin prevents the expression of SGLT1 and SGLT2 in the endothelium of Ang II-treated rats

Ang II has been shown to be a strong inducer of SGLT1 and 2 expression in coronary endothelial cells and isolated rat aorta. [11] Therefore the expression levels of SGLT1 and SGLT2 were assessed by immunofluorescence staining and Western blot analysis. An increased SGLT1 immunofluorescence signal was observed predominantly in the endothelium of the aorta, and also of both mesenteric and coronary microvessels in the Ang II group compared to the control group (Fig. 5A). The empagliflozin treatment markedly prevented the Ang II-induced increased SGLT1 signals (Fig. 5A). The empagliflozin treatment alone significantly increased the SGLT1 signal in the aorta but neither in the mesenteric nor in the coronary microvessels (Fig. 5A). In contrast to SGLT1, no SGLT2 signal was detected possibly due to an expression level below the detection limit (data not shown). Nevertheless, increased SGLT1 and SGLT2 protein levels were observed in the aorta of the Ang II group but not in the empagliflozin + Ang II group (Fig. 5B). The empagliflozin treatment alone did affect neither the basal protein expression level of SGLT1 nor that of SGLT2 (Fig. 5B).

3.8. Empagliflozin prevents pro-fibrotic and pro-remodeling responses, cardiomyocyte hypertrophy and macrophages infiltration in the left ventricle of Ang II-treated rats

Since the empagliflozin treatment prevented the increased heart

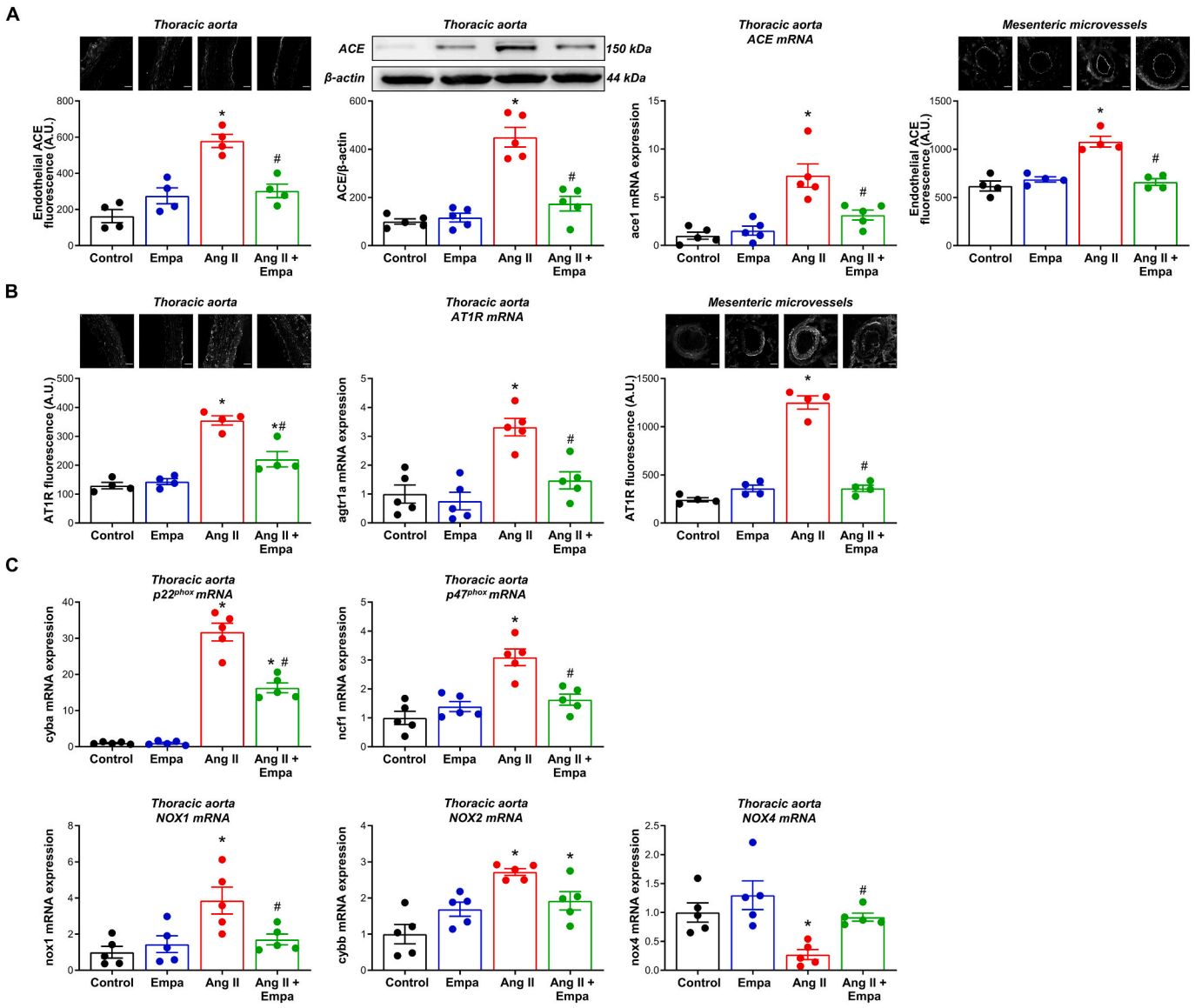

Fig. 3. The empagliflozin treatment prevents up-regulation of ACE, AT1R and NADPH oxidase subunits in both macro and microvessels of Ang II-treated rats. (A to C) Protein immunofluorescence signals are determined in fixed cryosections of the aorta and mesenteric microvessels using confocal microscopy, and mRNA and protein levels in the aorta by RT-qPCR and Western blot analysis, respectively. Results are shown as representative signals (upper panels) and corresponding cumulative data (lower panels). Values are expressed as mean ± SEM of n = 4 per group. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs Ang II group. Statistical analysis is performed by 1-way ANOVA (Bonferroni's post hoc test).

weight and LV mass in the Ang II group (Table 1), the effect of the SGLT2i on LV fibrosis and remodeling was evaluated. In the control group and the empagliflozin group no significant fibrosis was observed whereas in the Ang II group the fibrotic area amounted to about 28% (Fig. 6A). The stimulatory effect of Ang II was markedly reduced by the empagliflozin treatment (Fig. 6A). Increased signals of collagen I and atrial natriuretic peptide (ANP, a marker of cardiac stress) were observed in the LV of the Ang II group, which were not observed in the Ang II + empagliflozin treatment (Figs. 6B-C). The cardiomyocyte area was increased in the Ang II group compared to the control group, and this effect was prevented by the empagliflozin treatment indicating effective prevention of cardiomyocyte hypertrophy (Fig. 6D). In addition, the empagliflozin treatment prevented the increased LV infiltration of macrophages observed in the Ang II group but not in the control group (Fig. 6E).

4. Discussion

The major findings of the present study indicate that the empagliflozin treatment effectively prevented the deleterious impact of Ang II-induced hypertension on both the macro and microvascular endothelial cell activation and the cardiac function despite persistent hypertension in rats. The beneficial effects involve at the level of blood vessels an improved balance between the eNOS pathway and the AT1R/SGLT1/2 pro-oxidant pathway, a blunted local angiotensin system and an attenuation of pro-atherosclerotic and pro-remodeling responses, and at the level of the LV improved diastolic function, reduced pro-fibrotic and pro-remodeling responses, and also reduced macrophages infiltration. Recently, Ang II has been shown to be a strong inducer of SGLT1 and 2 expression in endothelial cells to perpetuate the formation of ROS, which in turn leads to endothelial dysfunction and the subsequent development of the pro-atherosclerotic responses. [11] Thus, the ability of the empagliflozin treatment to blunt the stimulatory effect of Ang II-induced hypertension on SGLT1 and 2 pathways in the endothelium of

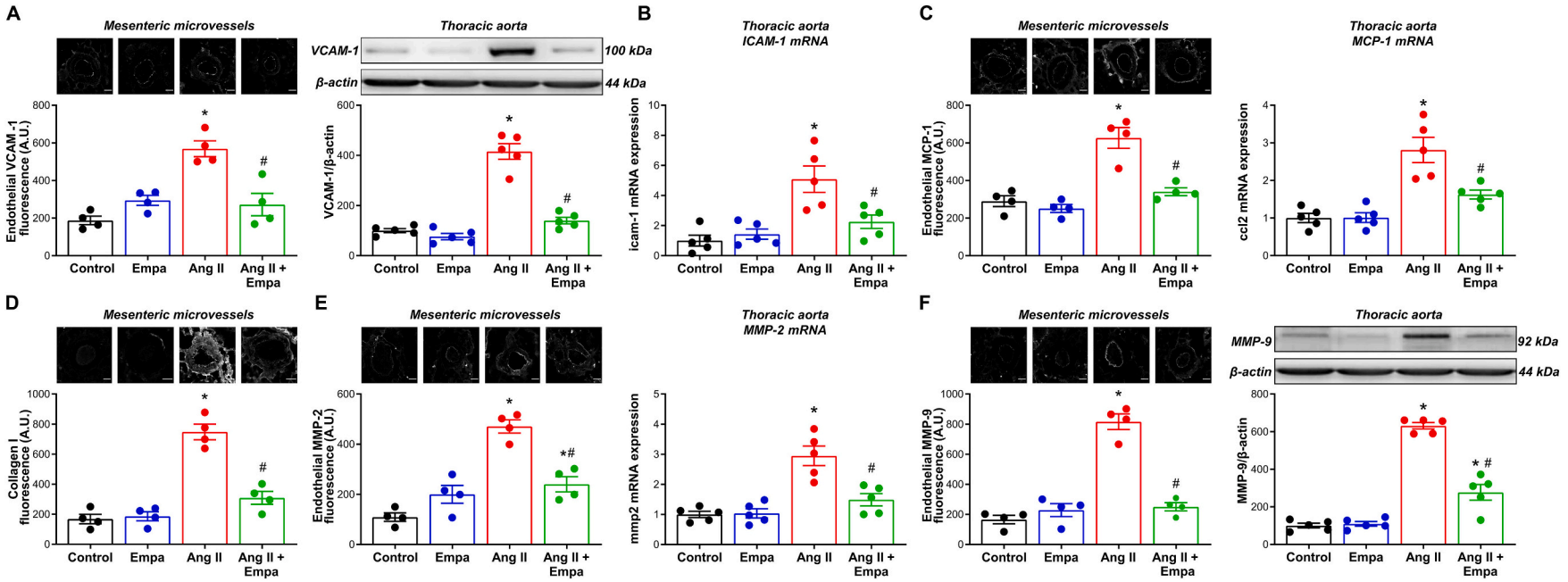

Fig. 4. The empagliflozin treatment prevents pro-atherosclerotic and pro-remodeling responses in both macro and microvessels of Ang II-treated rats. (A to F) Protein immunofluorescence signals are determined in fixed cryosections of the mesenteric microvessels using confocal microscopy, and mRNA and protein levels in the aorta by RT-qPCR and Western blot analysis, respectively. Results are shown as representative signals (upper panels) and corresponding cumulative data (lower panels). Values are expressed as mean ± SEM of n = 4 per group. $^{*}P < 0.05$ vs control group and $^{\#}P < 0.05$ vs Ang II group. Statistical analysis is performed by 1-way ANOVA (Bonferroni's post hoc test).

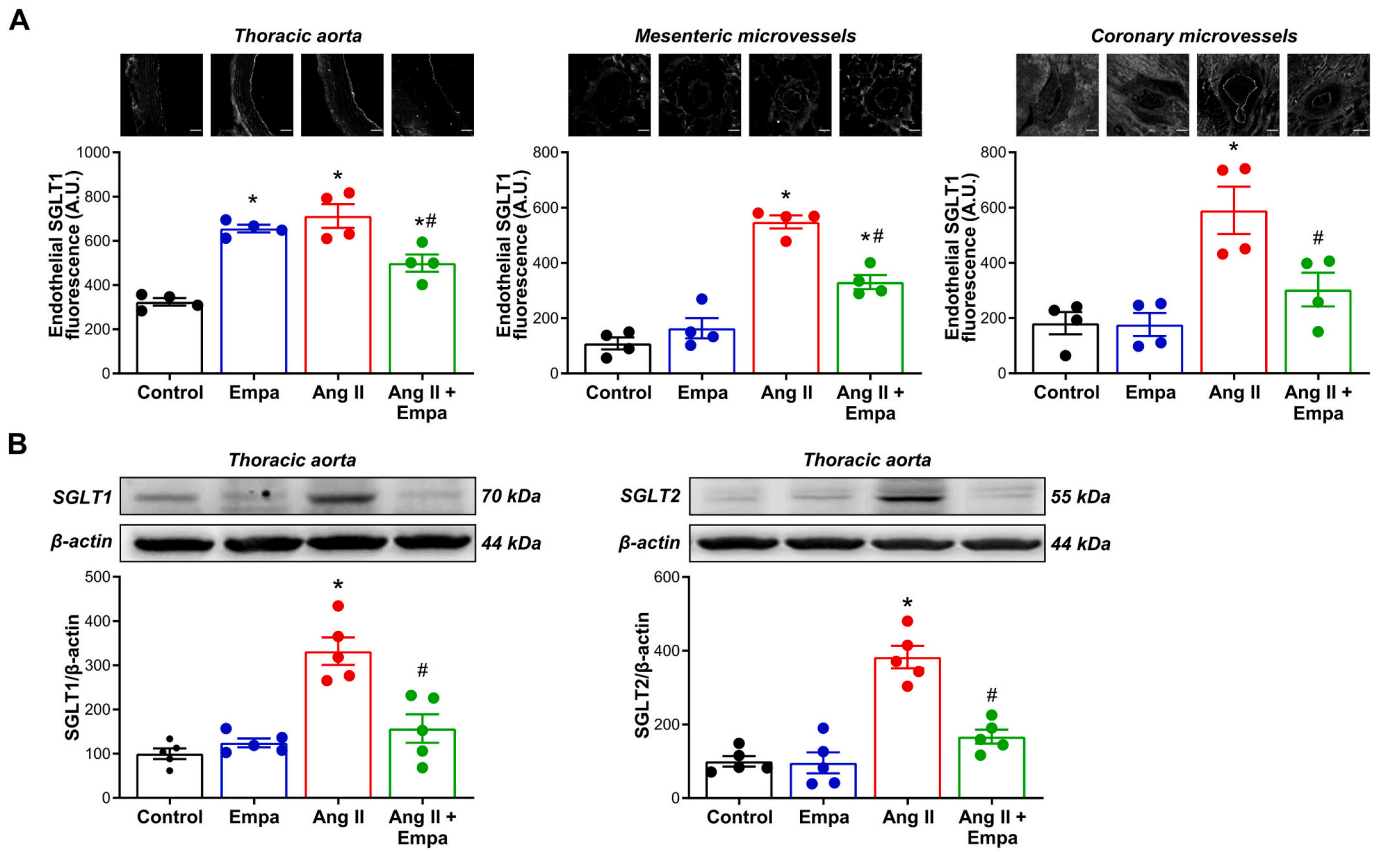

Fig. 5. The empagliflozin treatment prevents the expression of SGLT1 and SGLT2 in both macro and microvessels of Ang II-treated rats. (A) Protein immunofluorescence signals are determined in fixed cryosections of the aorta, mesenteric microvessels, and coronary microvessels of the LV. (B) Expression levels of SGLT1 and SGLT2 proteins in the aorta as assessed by Western blot analysis. Values are expressed as mean ± SEM of n = 4 per group. $^{*}P < 0.05$ vs respective control, $^{\#}P < 0.05$ vs Ang II group. Statistical analysis is performed by 1-way ANOVA (Bonferroni's post hoc test).

both large arteries and microvessels including the coronary microcirculation contributes to explain the beneficial effects on the cardiovascular system.

Several large-scale trials have in a consistent manner shown that SGLT2i exert a favorable effect on the heart and the kidney that cannot be explained by their moderate glucose-lowering effect. [2,3] These benefits include a lower risk of cardiovascular death or hospitalization for HF, and a reduced progression of chronic kidney disease in patients with type 2 diabetes, and in patients with HF and a reduced ejection fraction regardless of the presence or absence of diabetes. [2,3] Besides improved heart and kidney function, and of importance, SGLT2i resulted also in an improvement in quality-of-life and showed excellent tolerability in patients with HFrEF. [14,15] However, the mechanisms of the cardiac benefits remain unclear.

Recent experimental studies have indicated that empagliflozin improves LV systolic function, ameliorates adverse cardiac remodeling, and enhances myocardial energetics in a nondiabetic porcine model of HF. [7,8] Besides the systolic function, SGLT2i also improved the LV diastolic function in genetic models of type 2 diabetes including the ob/ob mice and the db/db mice, and this effect is associated with reduced LV hypertrophy and interstitial fibrosis. [16,17] An improved LV diastolic function is also observed in a nondiabetic HFrEF porcine model after myocardial infarction and is associated with reduced LV and cardiomyocyte stiffness, [8] and in a nondiabetic deoxycorticosterone acetate hypertensive salt model of HFpEF in rats associated with reduced LV mass, improved wall stress and hemodynamics. [18] Moreover, a study with myocardial fibers from patients and rats with diastolic HF indicated that empagliflozin causes direct beneficial effects on the myocardium by improving LV diastolic stiffness and hence diastolic function. [19]

Left ventricle diastolic dysfunction is highly prevalent in T2D patients, and is a major causative factor that is associated with adverse outcomes in HFpEF, and also predicts adverse prognosis in HFrEF. [20,21] Thus, an improvement of diastolic function with SGLT2i is of major clinical importance. The development of diastolic dysfunction involves several pathophysiological mechanisms including an increased myocardial collagen deposition that promotes interstitial myocardial fibrosis and increased LV stiffness, inflammation and a pro-oxidant state that causes coronary microvascular endothelial dysfunction leading to a reduced NO and cyclic GMP-protein kinase G signaling in adjacent cardiomyocytes. As a consequence, the phosphorylation level of titin, the major molecular spring within the cardiomyocyte, is decreased thereby promoting stiffening of cardiomyocytes. [20,22]

Both clinical and experimental studies have evidenced that the angiotensin system has a central role in the development of HF and LV diastolic dysfunction, endothelial dysfunction in response to cardiovascular risk factors such as hypertension, inflammation and also in cardiovascular remodeling, fibrosis and stiffness. [10,23] The role of the Ang II system in HF and LV diastolic dysfunction is underlined by the clinical benefit provided by therapeutic classes targeting the angiotensin system such as ACE inhibitors, AT1R blockers, as well as mineralocorticoid receptor antagonists. [24] The deleterious impact of Ang II on the CV system is mediated by the activation of AT1R and the subsequent stimulation of NADPH oxidases to promote an increased formation of ROS, which, in turn, induces pro-atherosclerotic, pro-inflammatory and pro-remodeling responses. [10] The pro-oxidant response will also induce endothelial dysfunction by reducing the bioavailability of NO and by promoting uncoupling of eNOS, and as a consequence eNOS will no longer generate NO but instead superoxide anions thereby further exaggerating oxidative stress. [25]

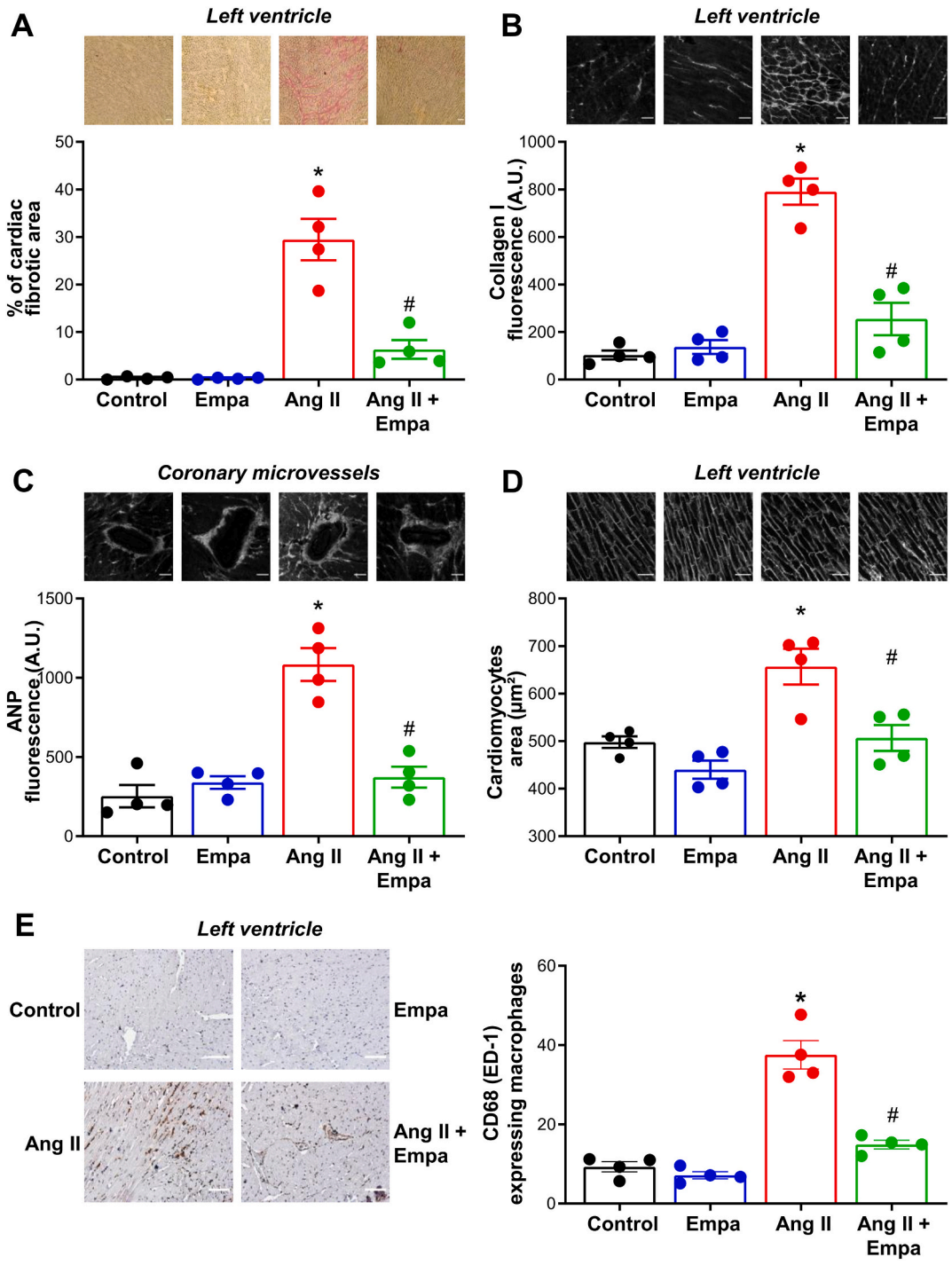

Fig. 6. The empagliflozin treatment reduces pro-fibrotic and pro-remodeling responses, and cardiac stress in the LV of Ang II-treated rats. (A) Extent of fibrosis determined using Sirius red; (B, C) Protein immunofluorescence signals; (D) Cardiomyocytes cell area determined using wheat germ agglutinin, and (E) infiltration of macrophages as assessed by CD68. All samples are observed by confocal microscopy. Results are shown as representative staining (upper panels) and corresponding cumulative data (lower panels). Values are expressed as mean ± SEM of $n = 3$–4 per group. $^{*}P < 0.05$ vs control group and $^{\#}P < 0.05$ vs Ang II group. Statistical analysis is performed by 1-way ANOVA (Bonferroni's post hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This group has recently highlighted that SGLT1 and 2 are part of a feed-forward loop enhancing the deleterious impact of Ang II of the endothelial function. [11,26] Indeed, inhibition of the local Ang II system prevented the high glucose-induced redox-sensitive expression of SGLT1 and 2 expression in coronary endothelial cells that leads to the subsequent induction of endothelial senescence and dysfunction. [26] Moreover, exposure of coronary endothelial cells and isolated aorta of rats to Ang II caused an up-regulation of SGLT1 and 2 expression that is mediated by the AT1R/NADPH oxidases/ROS pathway. [11] Furthermore, since inhibition of SGLT2 effectively prevented the Ang II-induced sustained (24 h) but not short-term (30 min) pro-oxidant response, the up-regulation of the local angiotensin system and the induction of endothelial dysfunction, the AT1R/NADPH oxidases/SGLT1 and 2 pathway perpetuates the deleterious activator signal. [11,26,27] It is

also consistent with the fact that SGLT2i prevented the Ang II-induced upregulation of SGLT1 and 2 expression in endothelial cells demonstrating a positive feed-forward loop. [11]

The present findings indicate that the Ang II-mediated hypertension in the rat has a deleterious impact on the vasculature as indicated by several markers of endothelial cell activation including an excessive activation of the local Ang II system, a pro-oxidant response, and expression of pro-atherosclerotic, pro-remodeling and pro-fibrotic markers, and also on the heart with diastolic dysfunction and pro-remodeling and pro-fibrotic responses as well as macrophages infiltration. All these effects were effectively prevented by the empagliflozin treatment despite persistent hypertension. The characterization of the underlying mechanism has indicated that the empagliflozin treatment markedly reduced the Ang II-induced generation of ROS in both the macro and microvessels, and normalized the eNOS mRNA and protein expression levels and activity in the aorta as indicated by nitrotyrosine levels similar to those in the control group. The observed increased eNOS level in the aorta of the Ang II group is most likely part of a compensatory mechanism to preserve the protective endothelial function. In contrast, a decreased eNOS level is observed in mesenteric microvessels suggesting that the microcirculation might be more sensitive to the deleterious impact of Ang II-induced hypertension. The empagliflozin treatment also resulted in the normalization of levels of Ang II-stimulated vascular targets that are controlled by oxidative stress including markers of the angiotensin system, pro-atherosclerotic, pro-fibrotic and pro-remodeling responses, and LV targets including markers involved in pro-fibrotic and pro-remodeling responses and cardiomyocytes hypertrophy. Of interest, the empagliflozin treatment prevented the stimulatory effect of Ang II on the up-regulation of the mRNA level of several NADPH oxidase subunits including p47phox, p22phox and NOX1 mainly considered as deleterious and the down-regulation of NOX4, a subunit increasingly recognized as vaso-protective [28,29], in the aorta. The beneficial effect of the empagliflozin treatment resulted also in the normalization of the SGLT1 and SGLT2 expression levels in the macrocirculation, and of SGLT1 in the microcirculation that were upregulated in the Ang II-induced hypertensive group. Since increased SGLT1 and 2 levels mediate the sustained Ang II-induced formation of ROS in endothelial cells that is dependent on extracellular glucose and Na^+, [11] it implies that the Ang II/NADPH oxidases/SGLT1 and SGLT2 pro-oxidant pathways contribute to perpetuate the pathological activation of the cardiovascular system.

In good agreement with the present findings, the empagliflozin treatment normalized the endothelial function and reduced oxidative stress in aortic vessels of a streptozotocin rat model of type 1 diabetes, [30] and improved the NO-mediated endothelium-dependent relaxations and prevented endothelium-dependent contractile responses to acetylcholine in a rat model of metabolic syndrome, the ZSF1 rat. [9] In addition, SGLT2i improved markers of oxidative stress and fibrosis in the heart of a genetic model of type 2 diabetes, the KK-Ay mice, through inhibition of the transforming growth factor β/Smad pathway and activation of the Nrf2/ARE signaling. [31]

The ability of SGLT2i to maintain a protective endothelial eNOS/ROS balance might also involve blockade of inflammatory macrophages infiltration as observed in the renal tissue associated with reduced renal fibrosis, [32] and, as shown in the present study, in the LV of Ang II-treated hypertensive rats. Indeed, pro-inflammatory factors such as TNFα caused oxidative stress in cardiac microvascular endothelial cells leading to reduced bioavailability of NO and, as a consequence, resulted in impaired contractility and relaxation of cardiomyocytes. [33]

5. Conclusions

The present findings indicate that the empagliflozin treatment prevented diastolic dysfunction, cardiac remodeling, fibrosis, infiltration of macrophages, and endothelial cell activation in response to Ang II-induced hypertension. They further suggest that the cardiovascular benefit results from the preservation of the eNOS/ROS balance by preventing the activation of the deleterious Ang II/NADPH oxidases/SGLT1- and 2 pro-oxidant pathways in endothelial cells, thereby allowing endothelial cells to perpetuate their pivotal role in the control of vascular and cardiac homeostasis. Altogether, the findings support the concept that SGLT2 inhibition appears as a promising approach to preserve the diastolic function by preventing endothelial cell activation in both the macro and coronary microcirculation despite persistent hypertension and regardless of the presence or absence of diabetes.

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Author contributions

Conceptualization, V.B. S.-K. and O.M.; Formal Analysis, C.B., K.M. and V.B. S.-K.; Funding Acquisition, V.B.S.-K.; Investigation, C.B., K.M., A.M., S.A., C.A., U.H., L.R., A.B.C., S.-H. P., P.A.-S., E.B.; Methodology, V.B. S.-K., O.M. and C.A.; Project Administration, V.B.S.-K., C.A. and O. M.; Supervision, V.B.S.-K., C.A. and O.M.; Validation, V.B. S.-K., C.A. and O.M.; Visualization, C.B., K.M., A.M., C.A. and V.B. S.-K.; Writing – Original Draft Preparation, C.B., K.M. and V.B. S.-K.; Writing – Review & Editing, C.B., K.M., C.A., L.J., P.O., O.M. and V.B.S.-K.

Declaration of Competing Interest

V.S.-K. has received research grants from Boehringer Ingelheim Pharma GmbH & Co. KG, and O.M. from AstraZeneca. All other authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2022.107095.

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