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Dapagliflozin protects the kidney in a non-diabetic model of cardiorenal syndrome

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ABSTRACT

Cardiorenal syndrome encompasses a spectrum of disorders involving heart and kidney dysfunction, and sharing common risk factors, such as hypertension and diabetes. Clinical studies have shown that patients with and without diabetes may benefit from using sodium-glucose cotransporter 2 inhibitors to reduce the risk of heart failure and ameliorate renal endpoints. Because the underlying mechanisms remain elusive, we investigated the effects of dapagliflozin on the progression of renal damage, using a model of non-diabetic cardiorenal disease. Dahl salt-sensitive rats were fed a high-salt diet for five weeks and then randomized to dapagliflozin or vehicle for the following six weeks. After treatment with dapagliflozin, renal function resulted ameliorated as shown by decrease of albuminuria and urine albumin-to-creatinine *ratio*. Functional benefit was accompanied by a decreased accumulation of extracellular matrix and a reduced number of sclerotic glomeruli. Dapagliflozin significantly reduced expression of inflammatory and endothelial activation markers such as NF-κB and e-selectin. Upregulation of pro-oxidant-releasing NADPH oxidases 2 and 4 as well as downregulation of antioxidant enzymes were also counteracted by drug treatment. Our findings also evidenced the modulation of both classic and non-classic renin-angiotensin-aldosterone system (RAAS), and effects of dapagliflozin on gene expression of ion channels/transporters involved in renal homeostasis. Thus, in a non-diabetic model of cardiorenal syndrome, dapagliflozin provides renal protection by modulating inflammatory response, endothelial activation, fibrosis, oxidative stress, local RAAS and ion channels.

1. Introduction

Heart failure often coexists with comorbidities in which declining renal function is of particular importance. The complex interaction between the heart and kidney in this setting involves interdependent mechanisms including hemodynamic alterations and activation of neurohormonal systems as well as pro-inflammatory signals [1–3]. Accumulation of immune cells in the renal vascular beds is associated with a release of cytokines, including transforming growth factor β (TGFβ), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), which

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cause renal damage and vascular dysfunction, resulting in avid sodium retention and elevated vascular resistance [4].

Long-term activation of the renin-angiotensin-aldosterone system (RAAS) contributes to renal injury by impairing mitochondrial function and increasing oxidative stress, which in turn can lead to sodium chloride and water retention [4,5]. The systemic and local RAAS are extremely sensitive to dietary salt intake, which is associated with high blood pressure and progression of kidney dysfunction. Experimental and clinical studies have demonstrated that salt intake paradoxically suppresses systemic renin release while activating the intrarenal RAAS, perhaps by enhancing tissue production of reactive oxygen species (ROS) [6–8], although the role of the relative contribution and balance between classic and non-classic RAAS in cardiorenal context is not completely understood. Little is known about the involvement of cytokines and RAAS in modulating the activity of specific renal ion channels, such as chloride (ClC-K) and potassium channels (Kir4.1/Kir5.1) that cooperate in salt reabsorption and blood pressure control [9]. Preclinical studies have demonstrated that chloride efflux mediated by Kir4.1-ClC-K channels is influenced by a direct interaction of T cells with renal cells, and is central for sodium retention and development of salt-sensitive hypertension [10]. Accordingly, ClC-K channel gain of function polymorphisms could predispose to human hypertension [11] while a ClC-K variant can increase heart failure risk [12]. In addition, a defective activity or expression of Cl^-/H^+ ClC-5, an electrogenic exchanger that contributes to the reabsorption and processing of albumin and low-molecular-weight proteins filtered from the glomerulus, is associated with proteinuria in various renal diseases [13,14].

Clinical studies have shown that sodium-glucose cotransporter 2 inhibitors (SGLT2i) reduce the risk of heart failure and ameliorate renal endpoints regardless of baseline renal function in patients with and without type 2 diabetes [15–19]. Specifically, CREDENCE and DAPA-CKD studies have evaluated the effects of canagliflozin and dapagliflozin, respectively, in diabetic and non-diabetic patients with renal failure, showing a slowing of renal functional decline. While the underlying mechanisms remain elusive, the hypothesis is that the renoprotective effects mediated by SGLT2i could be due to local actions at level of nephron tubule cells, as well as improvements in renal hemodynamics and glycaemic control. The need to advance a still incomplete understanding of the effects of these drugs on the kidney is increasingly important in view of the expanding use of SGLT2i in patients with HF, also in absence of diabetes. Therefore, we investigated the effects of the SGLT2i dapagliflozin on the development of renal dysfunction in a model of chronic non-diabetic cardiorenal disease.

2. Material and methods

2.1. Animal protocol

The *in vivo* experiments were conducted on six-week-old male Dahl salt-sensitive rats (Charles River Laboratories). The animals were fed laboratory chow containing 8% NaCl (high-salt diet) or 0.3% NaCl (low-salt diet). The first batch of samples was obtained after five weeks from animals on high-salt (HS 11 weeks; n = 5) and low-salt diet (LS 11 weeks; n = 5). Functional analysis and molecular biology assays were performed to characterize early-onset functional and molecular modifications prior to dapagliflozin treatment. Remaining animals on the high-salt diet were randomized into two groups and treated with dapagliflozin (0.1 mg/kg/day; HS+DAP; n = 10) or vehicle (HS 17 weeks; n = 10) for the following six weeks by oral gavage. Rats maintained on a low-salt diet served as age-matched control (LS 17 weeks; n = 5) (Suppl. Fig. 1). Analysis at this time-point served to assess the effects of dapagliflozin treatment on renal condition highlighting significant differences to untreated animals. Systolic and diastolic blood pressures were measured in conscious animals by the tail-cuff method (BP-2000; Visitech Systems). Urinary proteins, creatine and electrolyte values were determined by the Architect ci8200 Chemistry Analyzer (Abbott Diagnostics). Euthanasia was done by an overdose of anesthetic agents [intraperitoneal injection of ketamine (300 mg/kg) and medetomidine (0.75 mg/kg)]. *In vivo* procedures were carried out in accordance with the National ethical guidelines and the guidelines from Directive 2010/63/EU of the European Parliament. The present study complies with the ARRIVE guidelines and has been approved by the Ministry of Health (protocol n. 582/2015-PR) and by the local ethics committee.

2.2. Sample preparation

For histology, kidneys were embedded in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen and stored at − 80 °C; 10 μm thick tissue sections were cut with a CM3050 S cryostat (Leica Microsystems). Sample preparation of cryosections was carried out as previously described [20]. For molecular biology, samples were snap-frozen in liquid nitrogen and stored at − 80 °C.

2.3. Histology

Masson's trichrome staining was used to detect fibrosis. The extent of glomerulosclerosis was determined by counting sclerotic glomeruli and expressed as a percentage of sclerotic glomeruli over the total number of glomeruli. To evaluate oxidative stress, ROS levels were examined with dihydroethidium, damage at membrane level was determined with 4-hydroxynonenal antibody (Abcam), peroxynitrite formation was assessed by 3-nitrotyrosine immunostaining (Merck Millipore). NF-κB phosphorylation on Ser536 was analyzed (Sigma-Aldrich). Fluorescein isothiocyanate-conjugated secondary antibodies were used (Jackson ImmunoResearch). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Samples were analyzed with a LSM700 confocal microscope (Zeiss).

2.4. RNA isolation and real-time PCR

Total RNA from the kidney (separated into cortex and inner medulla) was isolated in TRIzol reagent according to the manufacturer's recommendations (Ambion, Life Technologies). Real-time PCR analysis was performed as previously described [21]. Samples were loaded in triplicate using the real-time PCR 7500 Fast system (Applied Biosystems). Normalization for RNA quantity was performed to the best housekeeping gene selected from 2-beta-microglobulin (2Bm) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh). TaqMan hydrolysis primer and probe gene expression assays were obtained from Life Technologies with the following assay IDs: BSND: Rn00564503_m1; Kcnj10: Rn04219568_m1; Kcnj16: Rn01465215_m1; Src Kinase: Rn00676848_m1; Clcnk2: Rn00687467_m1; ClcnK1: Rn00677135_m1; Clcn5: Rn00567357_m1; 2Bm: Rn00560865_m1; Gapdh: Rn01775763_g1. Absolute quantification was performed using the standard curve method. The real-time PCR protocols were performed in line with the guidelines for qPCR [22].

2.5. Western blotting

Tissue was lysed in ice-cold RIPA buffer (Santa Cruz Biotechnology), and protein concentration was determined with Bradford assay (Bio-Rad Laboratories). Protein extracts were separated by 6%−12% SDS-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene fluoride membrane [23]. Membranes were probed with primary antibodies against IL-1β, IL-6, total nuclear factor-κB (NF-κB), vascular cell adhesion molecule-1 (VCAM-1), e-selectin, in order to evaluate inflammatory process and endothelial activation. NADPH oxidases 2 and 4 (NOX2 and NOX4) and antioxidant enzymes [catalase and manganese superoxide dismutase (MnSOD)] were measured to establish the involvement of oxidative stress. Pro-fibrotic pathway was analyzed through TGFβ. Classical and non-classical renal RAAS was assessed by

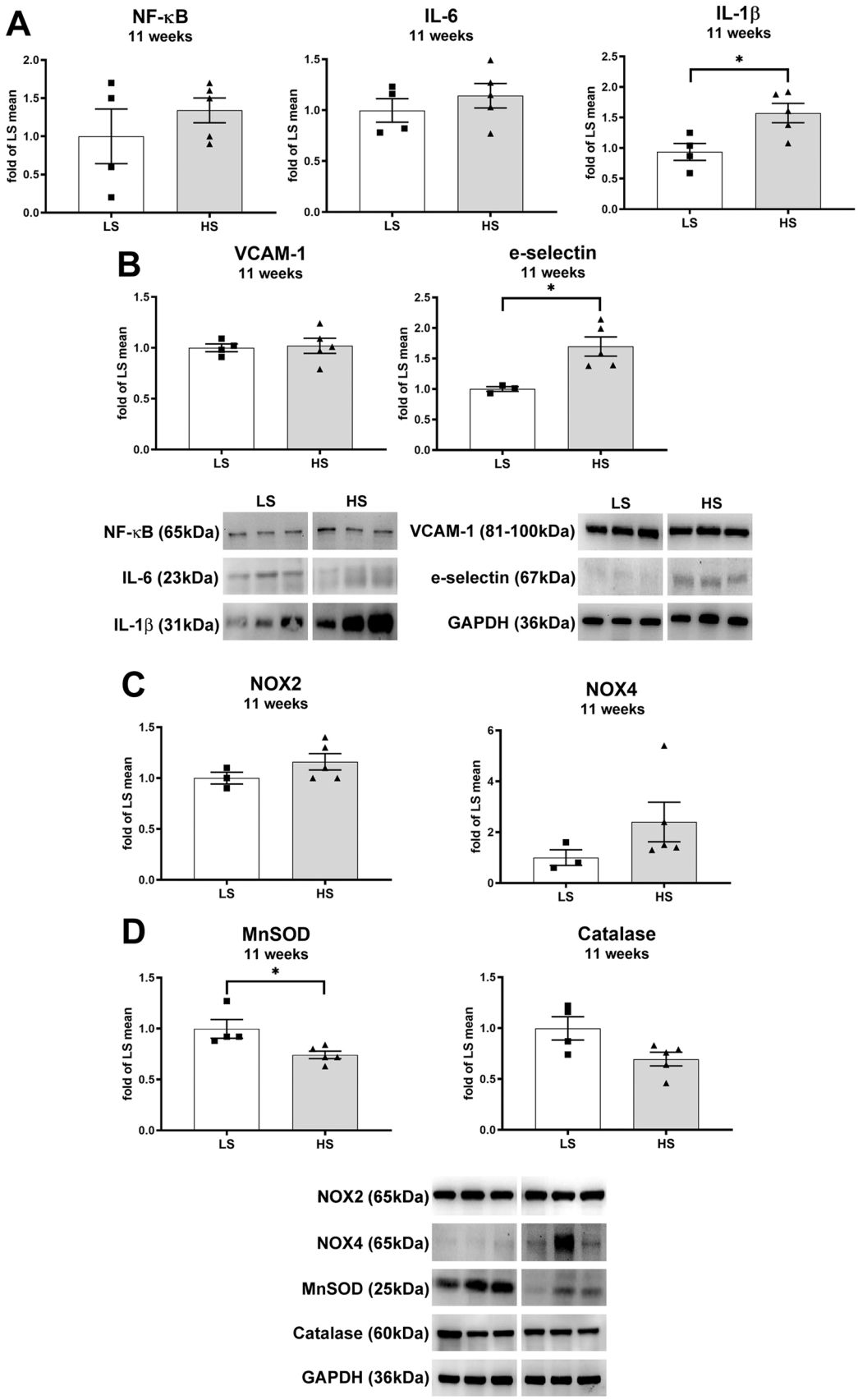

Fig. 1. Inflammation, endothelial function and oxidative stress at 11 weeks of age. Protein expression analysis by western blotting of (A) total nuclear factor-κB (NF-κB), interleukin-6 (IL-6), IL-1β, (B) vascular cell adhesion molecule-1 (VCAM-1), e-selectin, (C) NADPH oxidases 2 (NOX2), NOX4, (D) manganese superoxide dismutase (MnSOD), catalase. Western blotting data from 3 to 4 LS and 5 HS kidney samples. Western blotting gel bands represents 3 selected biological samples. * $P < 0.05$.

measuring ACE, angiotensin II type 1 and type 2 receptor (AT1R and AT2R), and MAS1 receptor. Loading conditions were determined with GAPDH. Antibody binding was visualized by chemiluminescence, and images were collected and analyzed using a Chemidoc Imaging System (Bio-Rad Laboratories).

2.6. Gel zymography

Zymography was performed according to the manufacturer's protocol (Bio-rad Laboratories). Frozen tissue was homogenized in lysis buffer including protease inhibitors. Equal amounts of non-denatured total cellular proteins were separated by electrophoresis. Gel was incubated in an appropriate buffer, stained with 0.5% Coomassie Blue R-250 in 30% methanol and 10% acetic acid and successively destained in the same solution without Coomassie Blue. Area of matrix metalloproteinase-2 (MMP2) gelatinolytic activity was shown as white bands against a blue background.

2.7. Statistical analysis

Results were presented as fold of control mean ± standard error of the mean unless stated otherwise. Each value has been normalized to the mean value of the control group, dividing the raw value (from each experimental group) by the value of the mean of the control values. This generates a Gaussian dataset that can be analysed by parametric statistics, allowing to show the standard error in the control group as well. Data were analyzed by using GraphPad Prism Software. Significance between two comparisons was determined by Student's t-test and, for multiple comparisons, by one-way ANOVA and Bonferroni's post-test. A P-value < 0.05 was considered statistically significant.

3. Results

As stated above, a subset of animals (indicated as "11 weeks") was analyzed at 11 weeks of age, that is, after five weeks of high-salt diet and before beginning treatment with dapagliflozin.

3.1. Inflammation, endothelial function and oxidative stress after five weeks of high-salt diet

After five weeks of high-salt diet (at 11 weeks of age) a mild inflammatory process and endothelial dysfunction were revealed in kidney of HS rats by a slight increase of inflammatory markers NF-κBand IL-6 of, with a significant overexpression of IL-1β (Fig. 1A) together with a significant increase of e-selectin (Fig. 1B). These findings areconsistent with the inflammation and endothelial activation as early-activated aspects of renal injury [24]. Although not statistically significant, a slight increase of NOX2 and a 2-fold increase of NOX4 (two principal enzymes producing ROS) were observed in kidney of HS rats (Fig. 1C). Of note, the capacity of ROS scavenging was already compromised as shown by the decrease of MnSOD expression (Fig. 1D). At this time point, additional relevant pathophysiological changes were detectedd. Analysis addressed local RAAS, involved in target-organ damage [25,26], renal gene expression of ion channels/transporters, and the effects of cytokines on HEK293 cells expressin renal ClC-Ka/barttin channels [27] (See Suppl. Results). Given the progressive organ damage in cardiorenal disease, noxious stimulus of high-salt intake was continued for the next six weeks with or without treatment with dapagliflozin.

3.2. Effect of dapagliflozin on animal well-being, blood pressure and renal function

In untreated HS animals at 17 weeks of age (that is after 11 weeks of high-salt diet), body weight was significantly lower than that of LS rats. Notably, the treatment with dapagliflozin positively affected general health of animals as shown by greater body weight compared to untreated HS rats. Kidney weight and kidney weight over tibia length ratio that were higher in HS than in LS rats, showed a restoration trend following dapagliflozin although not reaching statistical significance (Table 1). Compared to the previous time-point, blood pressure continued to rise in HS rats. However, after six weeks of treatment with dapagliflozin, a significant reduction of systolic and diastolic pressures was observed, although the rats remained severely hypertensive (Fig. 2A). Urine analysis revealed elevated proteinuria, albuminuria and UAC *ratio* in HS rats compared to LS rats. Treatment with dapagliflozin partially protected renal integrity as shown by efficacious decreasing albuminuria and UAC *ratio*. (Fig. 2B). Urinary concentrations of sodium, chloride and calcium were elevated in HS group and were not modulated by dapagliflozin. Potassium and magnesium remained unchanged (Fig. 2C).

3.3. Effect of dapagliflozin on fibrosis

Masson's trichrome staining evidenced massive fibrotic remodeling in HS animals, showing tubulointerstitial and glomerular fibrosis. Accumulation of extracellular matrix was remarkably suppressed upon dapagliflozin treatment as revealed by the reduced extension of glomerulosclerosis (Fig. 3A, B). Consistently, WB blotting showed the upregulation of TGFβ induced by high-salt diet, which was significantly reduced by dapagliflozin (Fig. 3C). Finally, MMP2 gelatinolytic activity in renal tissue was increased in HS rats and significantly reduced in dapagliflozin-treated animals (Fig. 3D). These observations evidenced a progressive tissue remodeling (not detectable at the previous time-point) that was positively regulated by dapagliflozin.

3.4. Effect of dapagliflozin on inflammation and endothelial activation

At this second time-point, kidney inflammation was evident. Pro-inflammatory cytokines, such as IL-6 and IL-1β, were overexpressed in HS rats. Also, the protein level of NF-κB, master regulator of inflammatory response, was elevated in the kidneys of HS rats. Treatment with dapagliflozin exerted an anti-inflammatory effect. Although the levels of IL-6 and IL-1β were only partly reduced, the expression of NF-κB was significantly diminished in HS+DAPA group (Fig. 4A; Suppl. Fig. 7). Moreover, activation of NF-κB evidenced by phosphorylation in p65 subunit was reduced by dapagliflozin with respect to untreated rats (Suppl. Fig. 8).

The evaluation of vascular endothelium showed a marked degree of activation/dysfunction in HS animals that was reverted following administration of dapagliflozin. The treatment restored the basal levels of e-selectin, important adhesion molecule for recruiting circulating immune cells, which was significantly overexpressed in untreated HS rats. Expression of VCAM-1 did not change (Fig. 4B).

3.5. Effect of dapagliflozin on oxidative stress

Evidence of ROS production documented the contribution of oxidative stress to the progression of renal damage. Immunofluorescence documented increased protein nitration and membrane peroxidation by 3-nitrotyrosine and 4-hydroxynonenal formation, respectively. Free

Table 1
Effects of dapagliflozin on body and kidney weight. Results are expressed as mean ± standard deviation. ** $p < 0.0005$ *vs* LS (17 weeks); ° $p < 0.05$ *vs* HS (17 weeks).

Groups	LS 17 weeks	HS 17 weeks	HS+DAP 17 weeks
Body weight (g)	396 ± 12	342 ± 18 **	365 ± 14°
Kidney weight (g)	1.46 ± 0.21	1.86 ± 0.15 **	1.70 ± 0.14
Kidney weight/tibia lenght (g/cm)	0.348 ± 0.050	0.448 ± 0.048 **	0.412 ± 0.025

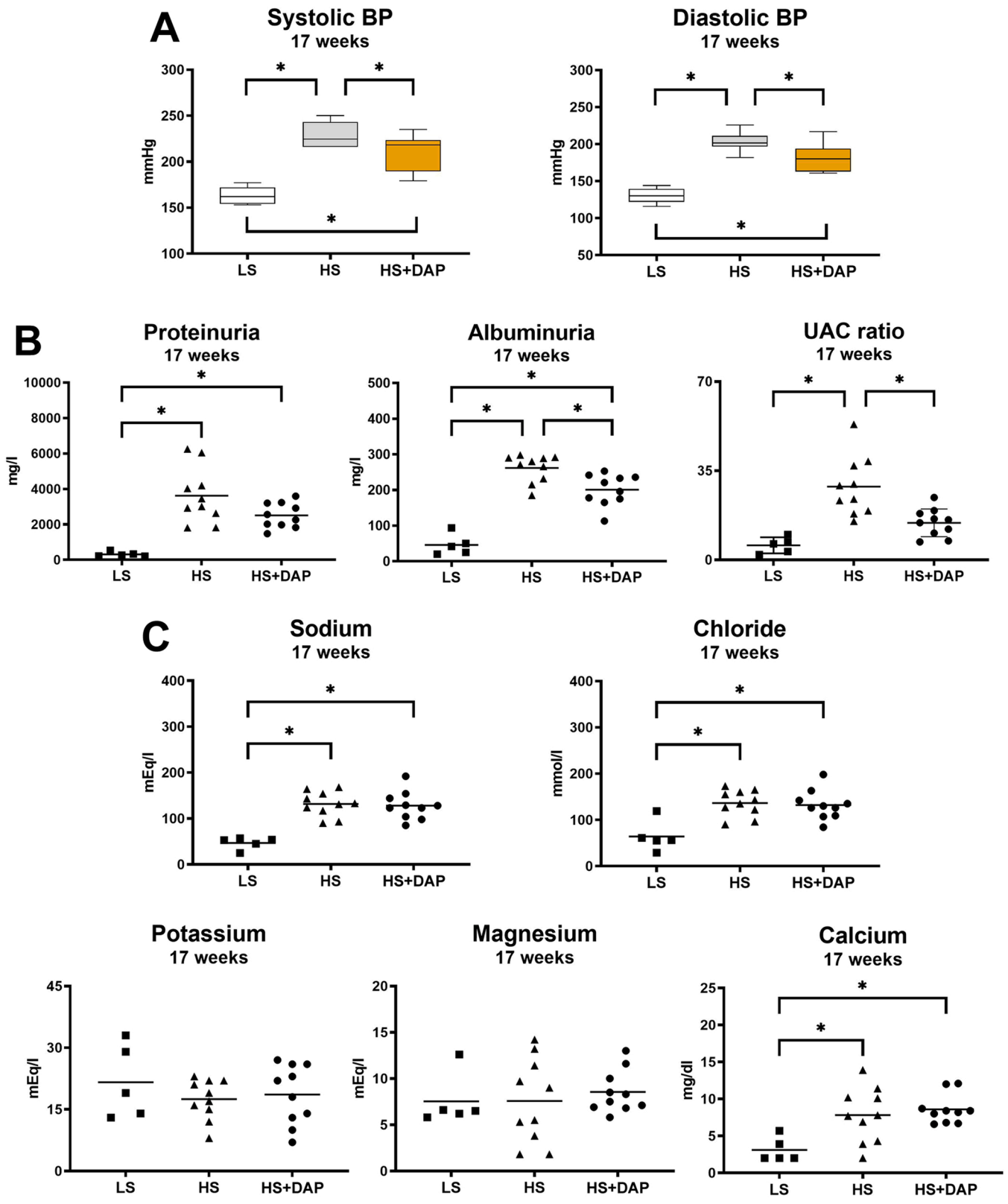

Fig. 2. Effect of dapagliflozin on blood pressure and renal function. (A) Systolic and diastolic blood pressure. Analysis of (B) proteinuria, albuminuria, urine albumin-to-creatinine (UAC) *ratio,* (C) sodium, chloride, potassium, magnesium and calcium concentration. Data from 5 LS, 10 HS and 10 HS+DAP rats. * $P < 0.05$.

radical generation was also confirmed by increased nuclear dihydroethidium. The oxidative damage was weakened following the drug treatment (Fig. 5A). The expression of NOX2 and NOX4 that progressively increased in HS animals, resulted decreased in dapagliflozin-treated rats (Fig. 5B). Interestingly, the ability to scavenge reactive species was completely restored by dapagliflozin administration as documented by increased expression level of MnSOD and catalase (Fig. 5C).

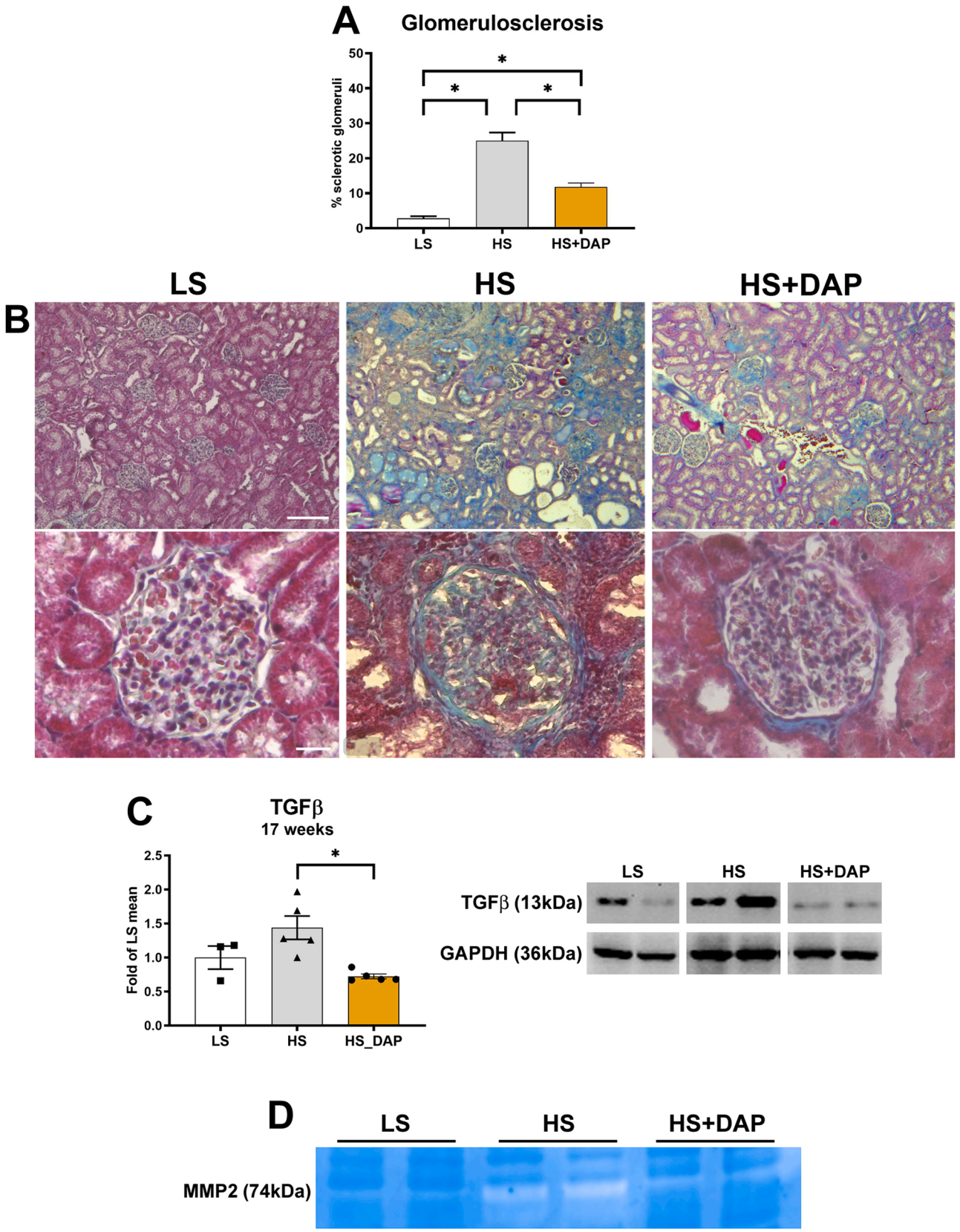

Fig. 3. Effect of dapagliflozin on fibrosis. (A) Percentage of sclerotic glomeruli, determined by counting sclerotic glomeruli over the total number of glomeruli. (B) Masson's trichrome staining showing collagen deposition (blue) and sclerotic glomeruli. Scale bars: upper panels 200 μm, lower panels 25 μm. (C) Protein expression analysis by western blotting of transforming growth factor β (TGFβ). Western blotting data from 3 LS, 5 HS and 5 HS+DAP kidney samples. Western blotting gel bands represents 2 selected biological samples. (D) Matrix metalloproteinase-2 (MMP2) gelatinase activity shown by gel zymography assay. * $P < 0.05$.

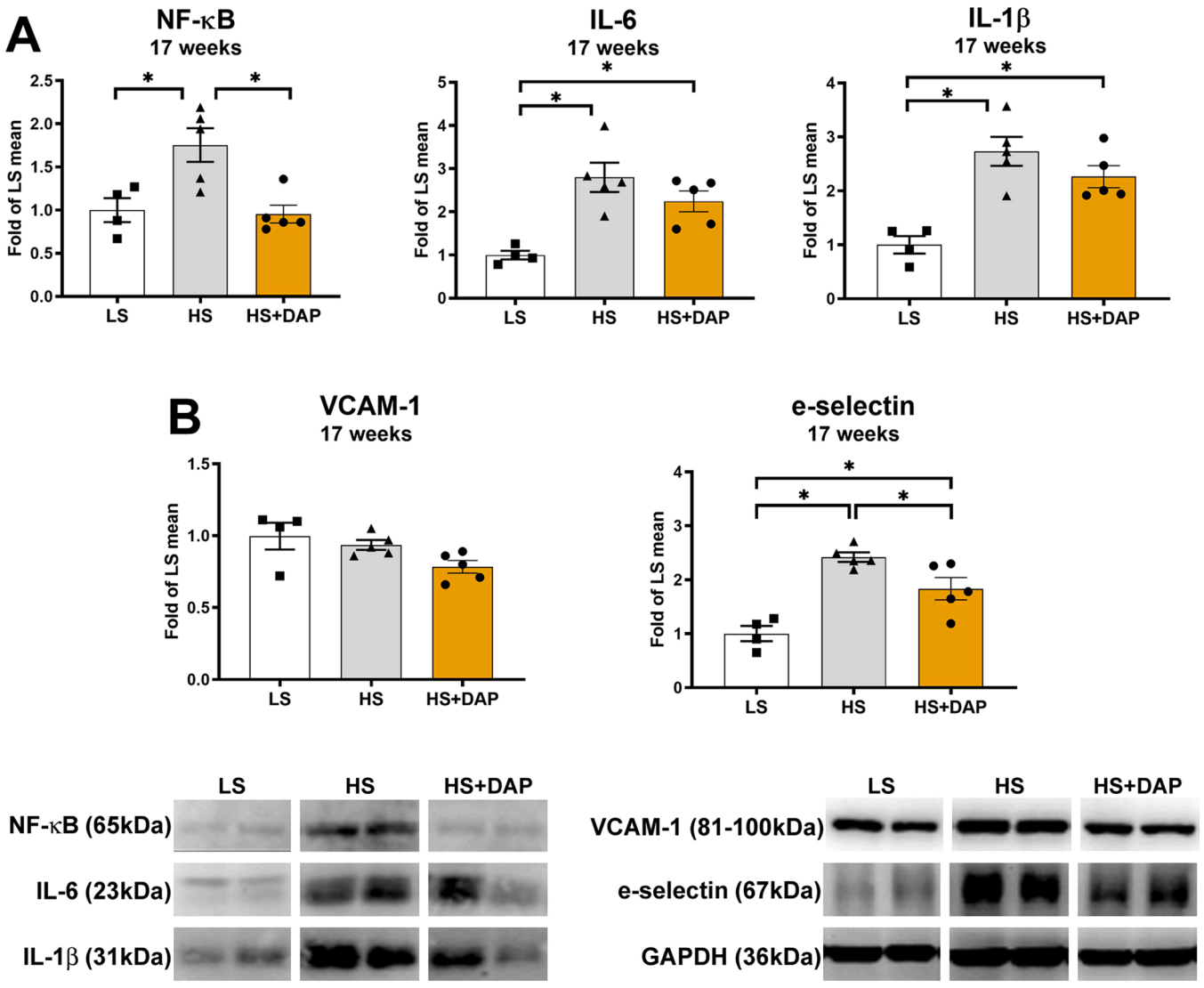

Fig. 4. Effect of dapagliflozin on inflammation and endothelial activation. Protein expression analysis by western blotting of (A) total nuclear factor-κB (NF-κB), interleukin-6 (IL-6), IL-1β, (B) vascular cell adhesion molecule-1 (VCAM-1), e-selectin. Western blotting data from 4 LS, 5 HS and 5 HS+DAP kidney samples. Western blotting gel bands represents 2 selected biological samples. *P < 0.05.

3.6. Effect of dapagliflozin on renal RAAS signaling

Persistent alterations of both classic and non-classic RAAS pathways were present in a diseased kidney. In HS group, increased protein levels of ACE and AT1R, and lower expression of AT2R and MAS1 were documented. Dapagliflozin counteracted these changes, as shown by a decrease in ACE and AT1R together with the increase in AT2R and MAS1 (Fig. 6A, B). The potentiated alternative RAAS pathway could actively promote anti-inflammatory and anti-fibrotic mechanisms.

3.7. Effects of dapagliflozin on gene expression profile of ion channels/transporters involved in salt and low-molecular weight proteins reabsorption

At 17 weeks of age, HS rats showed a significant downregulation of *CLCNK1 and Kcnj10* mRNA levels in kidney inner medulla. Furthermore, a significant reduction of *Kcnj16* mRNA levels in both kidney cortex and inner medulla of HS rats was observed. No significant alteration was detected for *CLCNK2*, *BSND* and *Src*. Dapagliflozin was able to fully or partially restore gene expression of *Kcnj16* in cortex, and *CLCNK1* and *Kcnj10* in inner medulla, respectively. In contrast, *CLCN5* mRNA levels resulted still downregulated in the kidney cortex of HS rats after dapagliflozin treatment (Fig. 7 A, B).

4. Discussion

The incidence and prevalence of chronic kidney disease (CKD) is steadily increasing with recent estimates of nearly 700 million persons concerned [28,29]. Renal and cardiovascular diseases often share risk factors, such as hypertension and type 2 diabetes, and a strict pathophysiological and clinical relationship between kidneys and heart constitutes the cardiorenal axis, whereby a dysfunction of one organ may induce a dysfunction in the other [30]. Accordingly, trials on diabetic and non-diabetic patients at risk of cardiovascular events have tested the efficacy of SGLT2i not only on cardiovascular outcomes but also on renal function, demonstrating that the beneficial effects were largely independent of the blood glucose-lowering action, and prompting dedicated studies to renal outcomes [17–19, 31]. While experimental and clinical data on diabetic nephropathy has pointed out that dapagliflozin (and other SGLT2i) exerts protective effects on renal physiology by improving endothelial dysfunction and inflammation [32–35], research on renal effects of SGLT2i in non-diabetic conditions are still limited and

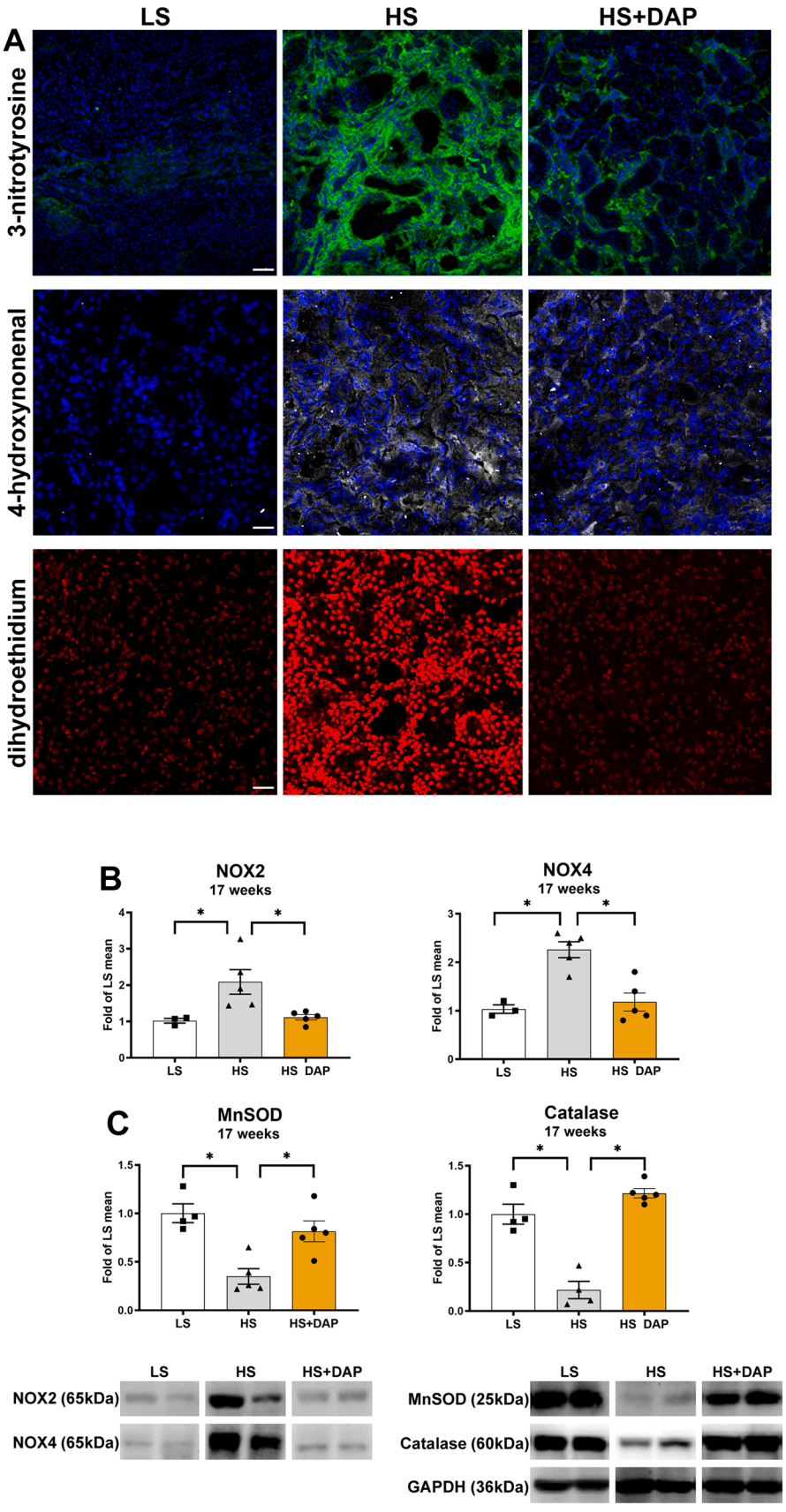

Fig. 5. Effect of dapagliflozin on oxidative stress. (A) Immunofluorescence showing 3-nitrotyrosine (upper panels, green), 4-hydroxynonenal (central panels, white) and dihydroethidium (red). Nuclei were counterstained with DAPI (blue). Scale bars: upper panels 50 μm, central panels 25 μm, lower panels 25 μm. Protein expression analysis by western blotting of (B) NADPH oxidases 2 (NOX2) and 4 (NOX4), (C) manganese superoxide dismutase (MnSOD), catalase. Western blotting data from 3 to 4 LS, 5 HS and 5 HS+DAP kidney samples. Western blotting gel bands represents 2 selected biological samples. * $P < 0.05$.

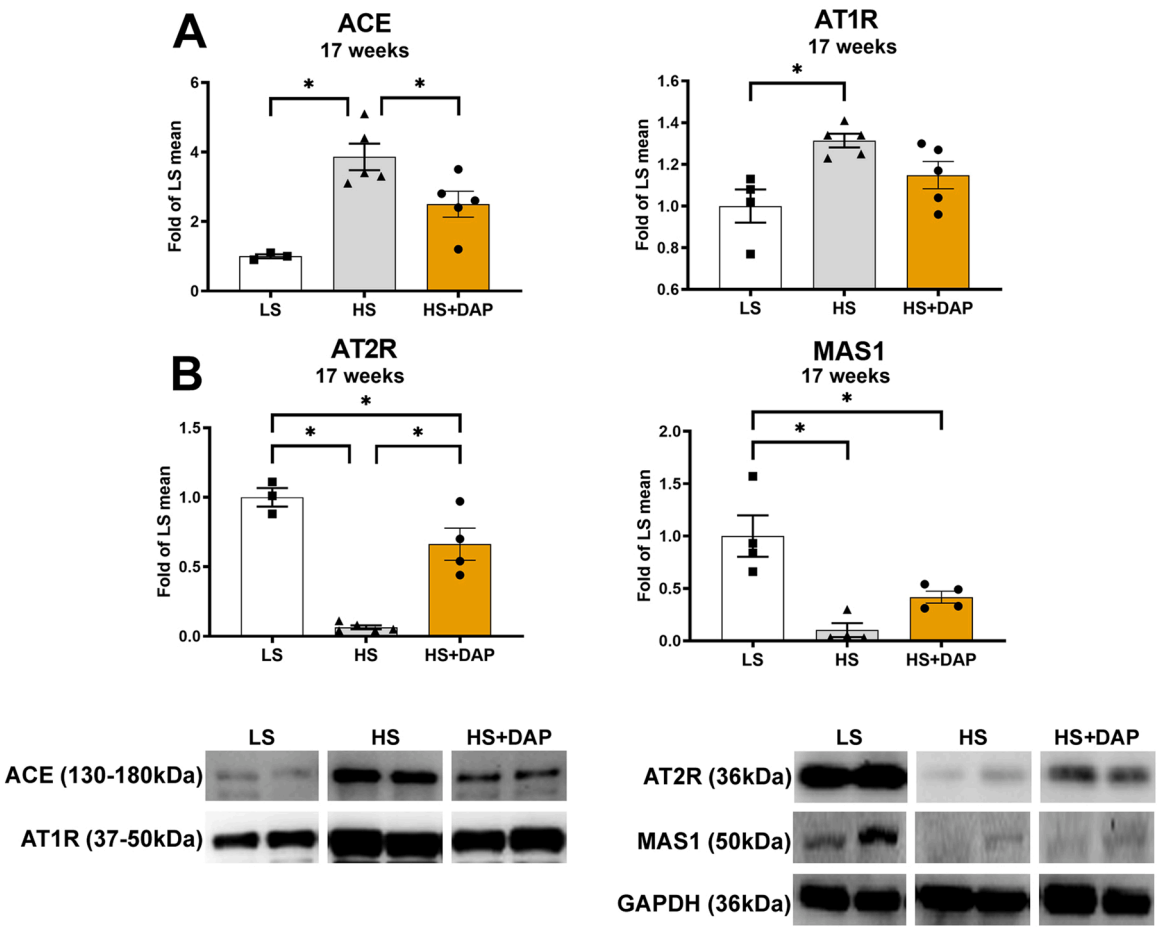

Fig. 6. Effect of dapagliflozin on kidney renin-angiotensin-aldosterone system signaling. Protein expression analysis by western blotting of (A) angiotensin-converting-enzyme (ACE), angiotensin II type 1 receptor (AT1R), (B) angiotensin II type 1 receptor (AT2R), MAS1. Western blotting data from 3 to 4 LS, 5 HS and 5 HS+DAP kidney samples. Western blotting gel bands represents 2 selected biological samples. * $P < 0.05$.

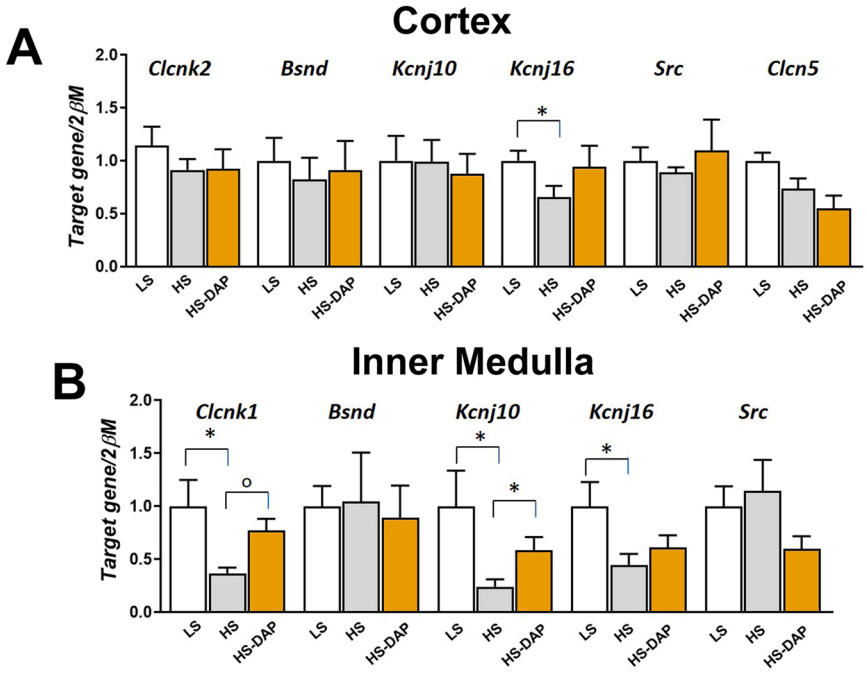

Fig. 7. Effect of dapagliflozin on the expression of selected genes encoding for ion channels/transporters. The relative content of transcript levels in kidney cortex (A) and inner medulla (B) for *Clcnk1* (encoding for ClC-K1), *Clcnk2* (encoding for ClC-K2), *Bsnd* (encoding for barttin), *Kcnj10* (encoding for Kir4.1), *Kcnj16* (encoding for Kir5.1) and *Src* (encoding for Src kinase) and Clcn5 (encoding for Clc5). Real-time PCR, normalized to β-2-microglobulin, data from 5 LS, 5 HS and 5 HS+DAP kidney samples. * $P < 0.05$.

inconclusive [36–39].

In the present study, we reported the effects of SGLT2i dapagliflozin on the progression of renal damage in a non-diabetic model of salt-induced hypertension and cardiorenal syndrome, in which renal disease is associated with the development of heart failure with preserved ejection fraction [3, 40–42]. The major and novel findings are that dapagliflozin attenuated renal inflammation and endothelial activation, partially regulated ion channel expression, suppressed ROS formation, promoted antioxidant defence, and modulated RAAS by decreasing classic and enhancing non-classic pathway. These events translated into reduction of renal injury, and evidenced a renoprotective role of this anti-diabetic in the absence of diabetes.

Chronic low-grade inflammation and endothelial dysfunction are two recognized determinants of renal dysfunction, characterized by a continuous production of pro-inflammatory cytokines that correlates with a worsening of clinical status of CKD patients [43–46]. Accordingly, a progressive involvement of endothelial dysfunction and pro-inflammatory markers, limited at 11 weeks and significant at 17 weeks of age, was evident in our model. Of note, dapagliflozin significantly weakened the expression of adhesion molecule e-selectin, and attenuated pro-inflammatory components.

Although the undoubted role played by inflammatory mediators in causing avid sodium chloride retention and water imbalance in heart failure, very little is known about the involvement of these effectors in modulating the activity of specific renal ion channels, the actual actors of the tubular salt and water reabsorption. Our study provides novel evidence that altered expression of renal ion channels may constitute a mechanism for kidney dysfunction. Particularly, our findings showed changes of gene expression encoding for Kir4.1/Kir5.1 potassium channels and ClC-K chloride channels, all components of a pathway that is triggered by infiltrate inflammatory immune cells and involved in the development of salt-sensitive hypertension [10]. The cytokine-induced increase of ClC-K activity observed in *in vitro* experiments on heterologously expressed chloride channels, could lead, in the animal model resembling a pathological chronic state, a compensatory change in gene expression. Importantly, the gene expression reduction of ClC-K1, Kir4.1 and Kir5.1 paralleled with the urine electrolyte profiles detected in HS rats. Dapagliflozin conferred benefits in diseased rats by partially preventing gene expression alteration. Nevertheless, the partial restoration of gene expression is not sufficient to reestablish an electrolyte balance, remaining the sodium and chloride amounts elevated in urine of HS animals. This could be because dapagliflozin increases glucose and sodium excretion without secondary effects on the expression and function of Na^+ channels and transporters along the nephron [47]. The gene expression analysis also revealed previously unknown involvement, in cardiorenal context, of the Cl^-/H^+ ClC-5 electrogenic exchanger, a protein predominantly located in the proximal tubule, that contributes together with cubulin and megalin to the reabsorption of albumin and low-molecular-weight proteins filtered from the glomerulus [13]. In fact, the decreased expression of ClC-5 observed in kidney cortex of HS rats correlates with proteinuria and albuminuria in this model. The lack of effect of dapagliflozin in ameliorating proteinuria could reflect, among the other mechanisms, the lack of effect in restoring ClC-5 gene expression.

RAAS signaling, as fundamental component of renal and cardiovascular pathophysiology, is essential in maintaining arterial blood pressure, and sodium and water balance [24,25]. Furthermore, RAAS is strongly influenced by diet [6,7]. In Dahl salt-sensitive rats or in hypertensive patients, the elevated sodium intake, leading to increased glomerular ACE/ACE2 *ratio* or excessive AT1R activation promote nitro-oxidative stress and proteinuria, and mediate deleterious effects by inducing vasoconstriction, vascular hypertrophy and fibrosis [48,49]. In the same scenario, the protective route, which *via* MAS1 activation leads to natriuresis and diuresis, seems to be suppressed. Indeed, non-classic RAAS is viewed as an essential element in counterbalancing the deleterious effects of angiotensin II, when angiotensin II-derived peptides [*i.e.* angiotensin[1–7]], by activating Mas receptor, induce vasodilation and anti-proliferative effects, and attenuate albuminuria and proteinuria [50]. Our model showed a markedly decreased expression of renoprotective AT2R and MAS1 receptor along with an increased level of deleterious ACE and AT1R in the kidney of high-salt fed rats. Notably, dapagliflozin had a significant impact on the modulation of the local RAAS, as shown by the upregulation of AT2R and MAS1 and the concomitant decrease of ACE and AT1R expression. There is a close parallelism between SGLT2 and RAAS inhibitors displaying similar patterns of effects on renal function in diabetic patients, when the treatment with either one drug class or the other leads to a decrease in microalbuminuria and similarly affects glomerular filtration rate [51]. However, the potential mechanism of interaction between SGLT2 and RAAS is matter of debate for the paucity of clinical and experimental studies, and it has not yet been clarified whether the treatment with dapagliflozin or other SGLT2i can affect kidney RAAS.

It should be underlined that activation of AT1 receptor by angiotensin II increases ClC-K2 activity in the intercalated cells by stimulating NOX and following ROS production. Based on the remarkably steep pH dependence of ClC-K activity, it has been proposed that angiotensin II-AT1R-NOX pathway promotes generation of superoxide to increase cytosolic pH to activate ClC-K2 [52]. An increase of ClC-K activity could, in a chronic state such as HF, lead to a compensatory reduction of gene expression. Thus, the alteration of activity and gene expression of renal ion channels characterizing our model could be also related to the observed RAAS components dysfunction. Accordingly, dapagliflozin effects on gene expression could reflect the drug capability of modulating RAAS components.

Another key player in the progression of kidney damage is a low-grade systemic inflammation and production of ROS that, in turn, aggravate endothelial activation and inflammation [53]. Activation of ROS-generating enzymes in endothelial and vascular smooth muscle cells results in redox signaling that activates pro-inflammatory transcription factors [25]. High dietary salt intake may impair kidney disease boosting oxidative stress *via* NAPDH oxidases, main sources of superoxide anion and hydrogen peroxide, and blunting anti-oxidant defenses [8].

Indeed, our study showed augmentation of ROS and nitrosative species with an alteration of NAPDH oxidases 2 and 4 along with a significant downregulation of MnSOD and catalase in HS rats. Dapagliflozin treatment markedly decreased the expression of pro-oxidant enzymes and restored anti-oxidant scavengers. These findings represent a novel and largely unexplored mechanism, since a role of SGLT2 inhibitors as modulators of oxidative damage in the kidney has mainly been studied in a diabetic setting by using diabetic murine models and *in vitro* hyperglycemic conditions [54–57].

The possibility that renoprotection was driven by the reduction in blood pressure needs consideration. Clinical evidence has confirmed the effects of SGLT2 inhibitors in reducing blood pressure in patients with CKD, dissociating anti-hypertensive from anti-hyperglycemic effects. The anti-hypertensive action might be attributed to weight loss, by means of glycosuria and increased fat utilization, and to diuresis and natriuresis [58–60]. Despite these aspects, the fall in blood pressure is insufficient to account for all the benefits [61]. In fact, in our model, at 17 weeks of age, both dapagliflozin-treated and untreated rats on high-salt diet were hypertensive although treatment with dapagliflozin mildly but significantly reduced systolic and diastolic blood pressure. While non-specific pressure-mediated structural effects cannot be excluded, molecular responses to treatment were evident during continuous hypertensive insult, strongly suggesting hypertension-independent mechanisms.

5. Conclusions

In summary, this study provides evidence that dapagliflozin has renoprotective effects in a non-diabetic model of cardiorenal syndrome.

In addition to inflammation, endothelial activation and oxidative damage, dapagliflozin modulated tubular ion channels expression and non-classic RAAS. These data may be viewed as a step in comprehending the full potential of this class of drugs in the cardiorenal continuum.

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CRediT authorship contribution statement

Konrad Urbanek: Conceptualization, Formal analysis, Writing – review & editing, Writing. **Donato Cappetta**: Conceptualization, Formal analysis, Writing – review & editing, Writing. **Gabriella Bellocchio**: Investigation, Formal analysis. **Maria Antonietta Coppola**: Investigation. **Paola Imbrici:** Investigation, Formal analysis. **Marialucia Telesca:** Investigation. **Maria Donniacuo**: Investigation. **Maria Antonietta Riemma:** Investigation. **Eleonora Cianflone**: Investigation, Formal analysis. **Silvio Naviglio**: Formal analysis. **Elena Conte**: Investigation. **Giulia Maria Camerino:** Formal analysis. **Marco Mele:** Formal analysis. **Mariarosaria Bucci**: Supervision. : Supervision. **Giuseppe Castaldo**: Supervision. **Annamaria De Luca** Supervision. **Francesco Rossi:Liberato Berrino:** Funding acquisition, Supervision. **Antonella Liantonio**: Conceptualization, Funding acquisition, Writing – review & editing, Writing. **Antonella De Angelis:** Conceptualization, Funding acquisition, Writing – review & editing, Writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106659.

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