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Title

Aldosterone induces albuminuria via matrix metalloproteinase dependent glomerular endothelial glycocalyx damage

Running title

Aldosterone damages the glomerular endothelial glycocalyx

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Abstract

Aldosterone contributes to end organ damage in heart failure and renal disease. Mineralocorticoid

receptor (MR) inhibitors limit MR activation by aldosterone and slow disease progression but side

effects, including hyperkalemia, limit their use clinically. Damage to the endothelial glycocalyx (a

luminal biopolymer layer) has been implicated in the pathogenesis of endothelial dysfunction and

albuminuria, but to date no one has investigated if the glomerular endothelial glycocalyx is affected

by aldosterone. *In vitro* human glomerular endothelial cells exposed to 0.1nM aldosterone and

145mMol NaCl exhibited reduced cell surface glycocalyx components (heparan sulphate and

syndecan 4) and disrupted shear sensing consistent with glycocalyx damage. *In vivo* 0.6μg/g/day

of aldosterone (subcutaneous minipump) and 1% NaCl drinking water increased glomerular matrix

metalloproteinase 2 activity, reduced syndecan 4 expression, and caused albuminuria. Intravital multiphoton imaging confirmed that aldosterone caused glomerular endothelial glycocalyx damage and increased the glomerular sieving coefficient for albumin. Targeting matrix metalloproteinases 2/9 with a specific gelatinase inhibitor preserved the glycocalyx, blocked the rise in glomerular sieving coefficient and prevented albuminuria. Together these data suggest that preservation of the glomerular endothelial glycocalyx may represent a novel strategy for limiting the pathological effects of aldosterone.

Introduction

Chronic kidney disease (CKD) affects one in eight adults globally and its prevalence is increasing due to the rising incidence of diabetes, hypertension and obesity.[1] Blocking the renin-angiotensin-aldosterone (RAAS) pathway, blood pressure control and salt restriction delay the progression of CKD and are widely recommended in clinical guidelines.[2] High aldosterone levels are commonly seen in Conn's disease and syndrome,[3] but they are also seen in subset of patients taking angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blocker (ARB) medications,[4] and in patients with hypertension,[3] obesity,[3] CKD[5] and obstructive sleep apnoea.[3] In patients consuming a salt rich western diet, aldosterone levels above the population median value are associated with an increased risk of developing albuminuria, systemic endothelial dysfunction and CKD even when adjustments are made for other known risk factors.[6-8] Indigenous, seasonal diets with low sodium content result in very high serum aldosterone levels, however they do not result in demonstrable end-organ damage.[9, 10] Thus it appears that the balance between aldosterone and salt may alter the result of aldosterone exposure. Blockade of aldosterone systemically via mineralocorticoid receptor (MR) inhibition reduces albuminuria, prevents cardiac fibrosis and protects individuals with heart failure from progressive myocardial dammage.[11] However, due to the phenomenon of aldosterone escape blockade of the RAAS at levels 'upstream' of MR may not be effective at limiting MR stimulation.[12] The addition of MR blockade to ACEi or ARB in chronic kidney disease is currently under investigation,[13] however side effects, including potentially life-threatening hyperkalemia, limit the use of MR antagonists and have made clinicians reluctant to adopt this therapeutic strategy.[14-16] Thus novel tissue specific therapeutic targets 'down stream' of MR stimulation need to be sought.

Mineralocorticoid receptors are widely expressed outside of the renal tubules.[17-25] One site of action that has generated considerable research interest is the vascular endothelium.[18, 26-30] However, the actions of aldosterone on these specialized cells has yet to be fully investigated. The endothelial glycocalyx is a potential downstream target of aldosterone. This anionic biopolymer on

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the luminal surface of endothelial cells consists of anchored components including proteoglycans and sialoproteins, with adsorbed elements from the circulating plasma.[31] The anionic charge is largely due to the expression of the glycosaminoglycans: heparan sulphate (HS) and chondroitin sulphate (CS), which are covalently bound to core proteins including syndecans.[31] Many of the specialist functions performed by the endothelium are dependent on a 'healthy' glycocalyx.[31] To date the endothelial glycocalyx has been found to function as a permeability regulator, shear sensor, immune cell regulator and clotting modulator but further roles are under investigation.[31]

We hypothesized that glomerular endothelial cells (highly specialized fenestrated vascular endothelial cells) expressed MR, and that salt and aldosterone exposure would lead to pathological remodeling of the glomerular glycocalyx and contribute to albuminuria.

Results

Glomerular endothelial cells expressed MR and lost key glycocalyx components in response to salt and aldosterone

Mineralocorticoid receptors are expressed by freshly isolated human glomerular endothelial cells and conditionally immortalized human glomerular endothelial cells (CiGEnC) (figure 1A). Cultured monolayers of CiGEnC swapped from osmotically balanced media (containing mannitol and 125mM NaCl) to media containing 145mM NaCl and 0.1nM aldosterone for 5 days expressed significantly lower concentrations of syndecan 4. This effect was prevented by spironolactone, suggesting the pathway was dependent on MR activation (figure 1B). Glycocalyx components HS and the HS proteoglycan syndecan 4 can be detected on the surface of CiGEnC (supplementary figure 1). Under iso-osmotic conditions CiGEnC exposed to 145mM NaCl and 0.1nM aldosterone for 5 days lost significant quantities of syndecan 4 and HS from their cell surface (figure 1C and 1D respectively). In contrast the addition of aldosterone to osmotically balanced media containing 125mM NaCl had no measurable effect. Media containing 145mM NaCl had only a small effect in the absence of aldosterone (figures 1C and D).

Exposing CiGEnC to salt and aldosterone resulted in MMP up-regulation. MMP inhibition and MR inhibition were equally effective in preserving the glycocalyx

Matrix metalloproteinases (MMPs) 2 and 9 cleave syndecan-4 from the endothelial cell surface and have been implicated in glomerular disease.[32] We have shown that MMPs are important in glycocalyx shedding in response to TNFα.[33] We therefore conducted time course experiments to study changes at the mRNA level for these key enzymes. Under iso-osmotic conditions 145mM NaCl and 0.1nM aldosterone for 10 hours increased MMP2 and 9 mRNA expression significantly (figure 2A). 145mM NaCl and 0.1nM aldosterone exposure for 5 days also significantly increased in MMP2 activity in conditioned cell media. This effect was prevented by MR antagonism with spironolactone (figure 2B). HS and syndecan 4 loss from the CiGEnC glycocalyx was prevented by MR antagonism or MMP inhibition with Batimastat. The effects on glycocalyx preservation of these two remote drug classes were equivalent; suggesting the MMP inhibition may be effective in preserving the glycocalyx from MR mediated damage.

MMP inhibition maintained glycocalyx function

The endothelial glycocalyx contributes to the transduction of laminar shear stress (LSS) to induce intracellular signaling events and cellular responses.[31] Krupple like factor 2 (KLF2) is a key gene associated with cellular remodeling in response to LSS and here we used this as a functional measure of glycocalyx integrity.[34] Under standard conditions CiGEnC respond to 10dyn/cm^2 LSS with a dramatic increase in KLF2 mRNA expression at 24 hours.[35] Following 5 days 145mM NaCl and 0.1nM aldosterone KLF2 up-regulation in response to shear was significantly impaired relative to cells maintained in osmotically balanced control media, however, this effect was prevented in the presence of Batimastat (figure 3A). Enzymatic degradation of the glycocalyx, with a corresponding reduction in syndecan 4, had the same effect on CiGEnC, confirming that glycocalyx damage resulted in impairment of KLF2 up-regulation (supplemental figure 2). Syndecan 4 contributes to endothelial cell alignment in response to LSS.[36] We therefore investigated if the observed glycocalyx damage also affected CiGEnC alignment. Following 24 hours of 10dyn/cm^2 LSS CiGEnC maintained in osmotically balanced control media aligned their actin fibers parallel to the flowing media but cells maintained for 5 days in media containing

145mM NaCl and 0.1nM aldosterone failed to significantly align. Batimastat restored actin alignment (figures 3B and 3C). Together these results suggest that MMP inhibition preserved CiGEnC glycocalyx function.

Mice given salt and aldosterone developed significant albuminuria and glomerular inflammation in the absence of detectable hypertension or other detectable alterations to the glomerular filtration barrier

Mice receiving 1% NaCl drinking water and 0.6μg/g/day aldosterone delivered subcutaneously via minipump continued to gain weight at a normal rate (figure 4A). However, following 28 days of salt and aldosterone, adult male DBA2J mice developed significant albuminuria (figure 4B). The systolic blood pressure of awake, trained mice did not increase over this period (figure 4C). The glomerular expression of syndecan-4, TNFα, VCAM-1 and MMP2 mRNA all significantly increased relative to controls by day 28 suggesting glomerular inflammation (figure 4D). A syndecan 4 ectodomain ELISA confirmed that glomerular syndecan 4 expression was reduced despite the increased mRNA expression (figure 5A). These findings are consistent with an increased rate of shedding of syndecan 4 from the glomerular glycocalyx. Syndecan 4 levels did not measurably increase in the plasma, however, increased ectodomain concentrations in the urine were detected (figure 5B). Syndecan 4 ectodomains have been shown to act as signaling molecules, altering podocyte mobilization via TRPC6 channels,[37] whether this observation represents direct endothelial-podocyte communication warrants further investigation. Consistent with the glomerular mRNA results, a MMP2 activity assay confirmed an increased level of activated MMP2 within the glomeruli and urine of mice exposed to salt and aldosterone (figures 5C and 5D). We also found that glomerular and urine heparanase activity increased in response to salt and aldosterone exposure (supplemental figure 3A and 3B). However CiGEnC exposed salt and aldosterone *in vitro* did not increase heparanse activity, and Batimastat had no effect on heparanase activity (figure 3C). It seemed unlikely therefore that the effects on the glycocalyx seen in response to salt and aldosterone were the result of increased heparanase activity in this model. In addition, following blinded analysis of electron micrographs, we found no evidence of damage to the glomerular basement membrane, or podocyte foot process effacement. These findings suggest that damage

may be limited to the glycocalyx at this early (28 days) time point (supplemental figures 4A-E), however other undetectable damage to the GFB cannot be excluded. Images suggested that glycocalyx depth may have been reduced following salt and aldosterone exposure, but this was not quantified due to inconsistent staining in this group of animals.

Multiphoton microscopy can be used to directly measure changes in glomerular albumin leakage over time

To confirm that detected albuminuria was due to increased glomerular albumin leakage (and not alterations in tubular albumin uptake) multiphoton microscopy was utilized. Mice were lightly anaesthetized before Z-stack Images of the same glomeruli were acquired on days 0,5 +/-10. The glomerular sieving coefficient for albumin (GSCalb) was calculated as the ratio of Alexa 594 albumin signal intensity within the glomerular capillaries to that in Bowman's space (figures 6A and 6B). Young DBA2J mice developed significant albuminuria by day 10 of salt and aldosterone exposure (figure 6C). The GSCalb was significantly increased by day 5 (figure 6D). Glomerular depth is known to increase in mice with age and vary between strains.[38] We found DBA2J mice were not ideal for multiphoton glomerular imaging due to the scarcity of superficial glomeruli. A total of 20 male DBA2J mice were imaged but only five mice had sufficiently superficial glomeruli on days 0 and 5 (and only 1 mouse on day 10, data not shown) to be included in the study. For this reason we used C57 BL/6 mice for subsequent experiments.

Salt and aldosterone caused albuminuria in C57 BL/6 mice with an associated increase in glomerular albumin leakage and glomerular glycocalyx damage caused by MMPs

Following 10 days exposure to 1% NaCl (drinking water) and 0.6µg/g/day aldosterone (via subcutaneous minipump) the uACR had significantly increased (figure 7A). No detectable systolic blood pressure change had occurred by day 12 of salt and aldosterone exposure (figure 7B). Figure 7C illustrates the changes in recorded GSCalb for each individual mouse with time on a logarithmic scale. Figure 7D presents the same data with statistical comparisons highlighting the significantly increased GSCalb at day 5 and 10.

Wheat germ agglutinin (WGA) binds to the glomerular endothelial glycocalyx in rats.[39] WGA has also been shown to bind to the glycocalyx in systemic vessels in mice.[40] Consistent with these findings we found that intravenously administered FITC-WGA bound to the endothelial glycocalyx within mouse glomeruli (figure 8A). We developed two methods to quantify changes to the glycocalyx using multiphoton images. Firstly the fluorescence intensity of FITC-WGA bound to GEnC glycocalyx was taken to represent the number of available binding sites for WGA and therefore the amount of glycocalyx.[40, 41] Secondly measurement of the anatomical distance between peak FITC-WGA fluorescence and peak plasma albumin intensity (peak to peak measurement), representing the restriction of albumin's access to the endothelial cell membrane by the glycocalyx, provided an index of glycocalyx thickness (figure 8B). Significant reductions in the glomerular WGA signal intensity and peak-to-peak measurement were evident by day 10 of salt and aldosterone exposure (figure 8C and 8D).

Following on from earlier work highlighting increased glomerular MMP2 mRNA and activity after 28 days of salt and aldosterone exposure, we investigated if MMP 2 blockade could prevent glycocalyx damage *in vivo*. Again male C57 BL/6 mice were given salt and aldosterone but with either MMP 2/9 inhibitor or vehicle (DMSO saline) administered daily via intra-peritoneal (IP) injection. Mice were injected with FITC-WGA to image the glycocalyx on day 0 and day 5, allowing us to study changes in the glomerular endothelial glycocalyx from baseline (in the same glomeruli) as well as between groups. This shortened protocol ensured images were taken whilst glomeruli were very superficial to maximize the resolution of generated images. The MMP 2/9 inhibitor did not measurably alter the systolic blood pressure of mice by day 12 in mice receiving salt and aldosterone (figure 9A). However, MMP 2/9 inhibitor successfully prevented significant increases in the uACR and GSCalb from occurring (figure 9B and 9C). Significant glycocalyx damage was visible in vehicle-treated mice by day 5 (relative to baseline and relative to the MMP2/9-treated group) (figure 9D). MMP2/9 inhibitor prevented any detectable decrease in glomerular capillary WGA intensity and peak-to-peak measurement (figures 9E and 9F).

Discussion

We have demonstrated for the first time that up-regulation of MMPs in response to salt and aldosterone resulted in GEnC glycocalyx damage and a detectable impairment of glycocalyx function both *in vitro* and *in vivo*. To achieve this we used cutting edge techniques to simultaneously study the glomerular endothelial glycocalyx and glomerular albumin permeability in live perfused mice for the first time. In addition we tracked how the GSCalb in individual glomeruli changed with time and correlated these changes with albuminuria, giving us a unique insight into how glomerular and tubular albumin handling change within this model. Together these data suggest that direct glycocalyx protection could be a novel therapeutic strategy in patients unable to tolerate mineralocorticoid inhibition.

Glomerular endothelial cells expressed MR, and responded to aldosterone. *In vitro* the combination of sodium chloride (145mM), and aldosterone (0.1nM) resulted in the loss of key glycocalyx components. Atomic force microscopy has previously demonstrated that the glycocalyx on *ex vivo* human umbilical artery endothelial cells reduced in height by 50% following salt and aldosterone exposure closely matching the 53% reduction seen in glycocalyx depth on day 10 in the current study.[42] The combination of elevated salt intake and exogenous aldosterone has been noted previously to cause albuminuria before alterations in blood pressure occur.[43-45] We saw no detectable changes in systolic blood pressure in either mouse strain, or when the MMPi was used, however, tail cuff photoplethysmography cannot be used for 24h BP recording and so intermittent or nocturnal hypertension cannot be excluded. Our data are consistent with that generated by other groups using this model and with clinical studies suggesting that aldosterone can mediate damage via hypertension-independent mechanisms.[8, 45]

The glycocalyx limits albumin permeability and acts as a mechanosensor.[35, 39, 46, 47] Enzymatic degradation of the glycocalyx *in vitro* impaired cells ability to remodel in response to physiological shear stress suggesting that this function is glycocalyx dependent in CiGEnC. Consistent with our findings murine syndecan-4 knock down prevented endothelial cell alignment suggesting syndecan-4 may be a key glycocalyx mechanosensor.[36] In addition to MMPs, heparanase

exposure has been shown to result in syndecan-4 loss, this action is thought to be due to increased MMP access to the syndecan-4 cleavage site.[48] Interestingly glomerular heparanase activity did increase in this model, but we found no evidence that it was produced from glomerular endothelial cells. Podocytes have been shown to produce heparanase in response to aldosterone, so it is possible that other cell types within the glomerulus were responsible for this increase.[49] The possibility that remotely produced heparanase contributed to the loss of HS and syndecan 4 from the glycocalyx in this model warrants further investigation, but the effectiveness of MMP inhibition in this model suggests that heparanase had a limited direct effect. Syndecan 4 levels dramatically increased in the urine, whilst falling in the glomeruli. We could not detect a significant increase in plasma levels but this may have been confounded by the increased urinary protein leakage seen after 4 weeks of salt and aldosterone. With the increased glomerular syndecan 4 mRNA level seen in these mice, and based on our previous studies in vitro,[33] we feel it is likely that syndecan 4 is shed from the glycocalyx in this model and largely excreted in the urine, however rapid metabolism of the ectodomain in the plasma cannot be excluded.

The key mechanism of damage in this model appears to be MMP induction. Salt and aldosterone have been shown to up regulate MMP2 mRNA and protein expression in murine hearts and renin over-expressing rats been shown to have elevated glomerular levels of MMP2 and MMP9 mRNA suppression, consistent with our findings.[50, 51] We have shown that salt and aldosterone increased glomerular MMP2 activity in mice. MMP2 is a member of the gelatinase class of MMPs. Its substrates include syndecan-4 (a core protein for HS) and so the loss of HS seen *in vitro* could be explained by syndecan-4 loss.[33, 52] At the early time point studied we did not see evidence of damage to other components of the GFB, suggesting the glycocalyx damage may be an early initiating step in the development of albuminuria in this model. MMP inhibition prevented detectable damage to the glycocalyx, reduced glomerular albumin leakage and prevented significant increases in uACR. The use of FITC-WGA in mice with kidney imaging windows facilitated repeated measurements of glomerular glycocalyx depth and WGA binding site density to be made in the same glomeruli over time. When combined with serial GSCalb measurements in the same glomeruli these measurements provided a unique insight into how remodeling of the glomerular

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glycocalyx affected the glomerular filtration of albumin. Measuring GSCalb also allowed comparisons to be made with uACR results. Using these data it is possible to derive a measure of how tubular albumin uptake / degradation may have affected the uACR. Comparison of the changes in sieving coefficient between day 0 and 5 (5 fold) and 5 and 10 (a further 1.4 fold) with the corresponding changes in uACR highlighted a possible role for early tubular albumin re-uptake/degradation in this model, a finding that warrants further investigation and validation.

In summary we have demonstrated that MMP inhibition prevented damage to the glomerular endothelial glycocalyx in response to elevated salt and aldosterone. More broadly we have shown that preserving the glycocalyx appears to limit glomerular albumin leakage. In patients where MR blockade is deemed unsafe novel approaches to protect the glomerular endothelial glycocalyx may have exciting therapeutic potential.

Methods

The conditionally immortalised human glomerular endothelial cells (CiGEnC) used in this study have been used extensively to study the glomerular endothelial glycocalyx.[33, 35, 53] CiGEnC expresses fenestrations, which increase in response to VEGF, and endothelial surface proteins.[54] At the permissive temperature of 33°C cells divide to form 80% confluent monolayers prior terminally differentiating at 37°C. All cells (passages 20-26) were cultured in EGM2 media (Lonza, UK) with the EGM2-MV bullet kit in the absence of supplied VEGF and gentamicin. To avoid acute osmotic stress, all cells were swapped into media containing mannitol 5 days before experiments began. During the experimental phase mannitol (to maintain osmolarity) was continued or NaCl (to make a final concentration of 145mM) were added to cell media +/- 0.1nM aldosterone (Sigma UK A9477). When used, 0.1 µM spironolactone (Sigma UK S3378) or 5µM Batimastat (Sigma UK SML0041) were given 2 hours prior to, and during, salt and aldosterone exposure. Experiments typically lasted 5 days (with the exception of RNA harvest, which was conducted at 10 hours). When indicated 10 dyn/cm^2 shear stress was applied to cells cultured in 10cm round dishes using an orbital shaker (SSM1 Stuart UK) for 24h.[35] To image actin alignment following shear stress, cells were cultured on fibronectin coated coverslips at the periphery of 10cm tissue culture dishes.

Shear stress was applied for 24h before cells were stained using phaloidin conjugate (1:200 Invitogen USA 10135092) diluted in 1% BSA solution and imaged using a Leica DMI6000B microscope. All experiments were conducted using matched controls cultured simultaneously and maintained in the same incubators. A standard technique for western blotting was employed using mineralocorticoid receptor antibody (Abcam, UK, ab62532).[35] To quantify glycocalyx components using immunofluorescence cells were grown in 96 well plates or on fibronectin-coated coverslips (for representative images). Following 5 days experimental exposure, cells were incubated at room temperature with 4% PFA before blocking with 5% BSA. Primary antibodies – anti heparan sulphate (1:100 BIO-RAD UK 1698) and anti syndecan-4 antibody (1:100 Milipore ABT157), were incubated at 4°C for 12h. Secondary antibodies were applied for 1 hour at room temperature. Cells were imaged using an automated plate reader (96 well) (Dynex Opsys MR USA), or fixed on coverslips using Vectashield (Vectashield laboratories, UK, Cat no H-1000) and imaged manually (Leica DMI6000B with Leica CTR7000) to confirm cell surface staining. Comparison was made to cells incubated with heaparatinase III (1mU/ml) (Sigma UK H-8891) for 3h to confirm HS antibody specificity and IgG controls (supplementary figure 1).

RNA was extracted from lysed conditionally immortalized glomerular endothelial cells using the standard protocol supplied with the Qiagen RNeasy Kit (Cat No 74104). To collect RNA from mice, sections of renal cortex were passed through sequential sieves to extract glomeruli before they were lysed in the supplied buffer. To aid cellular lysis the glomeruli were drawn repeatedly into a 1ml syringe via sequentially smaller needles to break the glomeruli into single cell fragments. Following lysis RNA was extracted using the Qiagen RNeasy Kit (Cat No 74104). RNA concentrations were measured using nanophotometer™ (Pearl Implen) and normalized before cDNA conversion. To convert RNA to cDNA a standard protocol was employed using a high capacity mRNA to cDNA kit (Applied Biosystems, USA, ref 4387406) Once primer specificity and optimal concentrations had been established, a standard protocol was used for all qPCR using Fast Sybr™ Green master mix (Applied Biosystems, USA, ref 438612) (sequences listed in supplemental figure 6). All statistics and errors were calculated on delta CT values, all data are displayed as fold change + SEM. A human syndecan 4 ELISA (USCN Life Science Inc, USA

SEB939Hu) was used to quantify syndecan 4 loss in cell culture. Mouse syndecan 4 levels were assessed using mouse ELISA kit, (Cusabio, USA, CSB-EL020891MO). MMP2 activity was studied using the MMP-2 Biotrack Activity assay (GE healthcare, UK, RPN2631). Heparanase activity was studied using a heparanase degrading enzyme assay kit (Takara, Japan, MK412). For all commercially available assays the manufacturer's instructions were followed in full.

All animal protocols were approved by the Home Office (UK) or the University of Southern California animal care committee and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Time lines of the experimental protocols used are included (supplemental figure 7). All mice were male and purchased from Jackson laboratory (C57 BL/6 aged 2/52 were purchased with nursing mothers, remaining mice were purchased aged 3/52). All mice had free access to food and water/saline throughout the study. To administer aldosterone 0.6µg/g/day (Sigma A9477) or vehicle (ethanol saline) Alzet minipumps (models 2004 or 1002) were implanted subcutaneously under isoflurane anesthesia according to the manufacture's guidelines. The dose selected has previously been shown to increase blood aldosterone levels in mice from 4.42±0.83 nmol/l to 11.09±2.07 nmol/l.[45]1% NaCl solution was given as the only source of drinking water to all mice receiving aldosterone. To confirm that MMP induction mediated the glycocalyx damage seen *in vivo,* MMP 2/9 inhibitor (5mg/kg) (EMD Milipore 444241) was dissolved in DMSO saline and given via daily i.p. injection and comparisons made to vehicle (DMSO saline) treated mice. Blood pressure was recorded after 5 days training using Visitech systems, Apex, USA, BP2000 system and the protocols used are outlined in the data supplement (supplemental figure 7). Intravital multiphoton microscopy was performed using a Leica TCS SP5 multiphoton confocal fluorescence imaging system powered by a Chameleon Ultra-II MP laser (Coherent Inc., Santa Clara, CA). Standardised settings were used to gain12 bit 1.5µm Z-stack images of all superficial glomeruli. Imaging duration for glomerular permeability was limited to 30m. Animals were anaesthetised using isoflurane before 40µl of Alexa Flour 594 BSA (invitogen 1812249) was injected into the retro-orbital sinus (prepared using Nanosep 30K omega spin columns to remove free dye). 1.5 micrometre Z-stack images were then taken of all superficial glomeruli. Images were selected for blinded analysis provided the Bowman's space could be clearly defined and there was

sufficient area to analyse florescence at two sites with no capillary loops within 4.5 micrometres (3 frames in z-stack). Imaging the same glomeruli on day 0,5 and 10 allowed us to focus on glomerular changes resulting from salt and aldosterone exposure (fold change from baseline permeability for each glomerulus was calculated before averages were calculated for each mouse). FITC-WGA (2µg/g; Sigma L4895 in PBS) was given as a 20µl bolus via the retro-orbital sinus only after glomerular sieving coefficients had been calculated. Images were taken 10 minutes after the bolus up to 40 minutes post bolus. WGA staining intensity was quantified within the glomerular capillaries and normalised to adjacent proximal tubular auto-florescence to negate the effects of tissue depth/ overlying light absorption. In addition peak to peak analysis of the WGA labelled glycocalyx was use to derive a direct measure of glycocalyx depth. Having established that the glomerular sieving coefficient rapidly increased (within 5 days) in response to salt and aldosterone exposure the protocol was amended before testing the effectiveness of MMP 2/9 inhibitor. Amendments included baseline assessment of the glycocalyx using WGA and a shortened exposure to salt and aldosterone to optimize image resolution for glycocalyx depth calculations (data supplement figure 7A and B).

All image analysis was conducted using Leica Application Suite. All statistics were calculated using Prism 7. Normality was assessed visually and using the Shapiro-Wilk test. Normally distributed data were compared using T-test and ANOVA, where normality could not be assumed Mann-Whitney, Wilcoxon, Kruskall-Wallis and Friedman tests have been used. Significance was set at $p < 0.05$.

Figure 1. Glomerular endothelial cells express MR and loose key glycocalyx components in response to salt and aldosterone. A. Western blot confirming MR expression in renal cortex, FACS isolated human glomerular endothelial cells and CiGEnC . **B.** Syndecan 4 ELISA confirming that salt and aldosterone in combination result in syndecan 4 loss from CiGEnC lysates (ANOVA p=0.0011, Tukey's correction displayed, n=4). **C.** Syndecan-4 surface expression on CiGEnC declined following 5 days exposure to salt and aldosterone (ANOVA p=0.0021, Tukey's correction displayed, n=5). **D.** HS surface expression on CiGEnC declined following 5 days exposure to 145mM NaCl and combined 145mM NaCl and 0.1nM aldosterone exposure (n=5) (ANOVA p=0.012, Tukey's correction displayed, n=5). (salt indicates NaCl concentration increased to 145mMol   salt indicates mannitol added to balance osmolarity.   aldosterone indicates 0.1nM aldosterone added to media,   indicates vehicle alone added to media.   spiro

indicates 0.1µm spironolactone added to media, spiro = vehicle alone added to media)
Significance relative to control unless indicated. All error bars = SEM. *p<0.05; **p<0.01;
***p<0.005; ****p<0.001.

Figure 2. Exposing CiGEnC to salt and aldosterone results in MMP up-regulation whilst MMP inhibition and MR inhibition are equally effective at preserving the glycocalyx. A. mRNA response, assessed by qPCR, following 10h salt and aldosterone showing significant up regulation of MMP2 and 9 (ANOVA p<0.0001, Tukey's correction displayed, n=3). **B**. MMP2 activity assay confirmed increased MMP2 activity following salt and aldosterone. The increased activity was prevented by MR inhibition. (ANOVA p=0.02, Tukey's correction displayed, n=5). **C.** and **D**. Cells maintained in media containing Batimastat (MMP inhibitor) or Spironolactone did not loose significant HS (ANOVA p=0.0164, Tukey's correction displayed, n=3) or Syndecan 4 from their surface (ANOVA p=0.0015, Tukey's correction, n=3). (✔salt indicates NaCl concentration increased to 145mMol ✘salt indicates mannitol added to balance osmolarity. ✔aldosterone indicates 0.1nM aldosterone added to media, ✘indicates vehicle alone added to media. ✔spiro indicates 0.1µm spironolactone added to media, ✔batimastat = 5µm added to media, ✘ spiro or batimastat = vehicle alone added to media)

Figure 3. MMP inhibition using Batimastat preserved the CiGEnC glycocalyx-dependent response to shear. A. In cells exposed to salt and aldosterone for 5 days the inclusion of 5µM Batimastat in the cell media significantly increased cells ability to up-regulate KLF2 mRNA in response to 24 hours LSS. Batimastat did not affect KLF2 expression under static conditions (comparison to static controls (first column and dotted line) (n=3) (ANOVA with Tukey's correction). **B.** Measurement of actin alignment relative to the direction of applied laminar shear stress (LSS). Actin alignment did not alter after 24h LSS in CiGEnC exposed to salt and aldosterone for 5 days. Control cells, and cells exposed to salt and aldosterone in the presence of Batimastat, demonstrated significant actin alignment in response to shear with no detectable difference in the response (n=3) (ANOVA with Tukey's correction). **C.** CiGEnC with nuclear staining (DAPI), and actin staining (Alexa Fluor 568 phaloidin) highlighting actin cytoskeleton alignment. This process is visibly impaired in cells previously exposed to salt and aldosterone for 5 days, but restored in the presence of Batimastat (arrows indicate direction of flow and 100µm for scale). (shear = 10 dyn/cm^2 shear stress for 24h,   shear = static conditions in the same incubator,   salt + aldosterone indicates 0.1nM aldosterone added to media containing 145mMol NaCl,   salt + aldosterone indicates vehicle alone added to media with mannitol to balance osmolarity,   Batimastat = 5µM Batimastat added to media   Batimastat = vehicle only added to media)

Figure 4. Male DBA2J mice given salt and aldosterone developed significant albuminuria and glomerular inflammation in the absence of detectable systolic hypertension. A. The weight of mice receiving salt and aldosterone remained equivalent to control mice (ANOVA (time matched comparison) p=0.232) **B**. Mice given 28 days salt and aldosterone developed significant albuminuria (t-test p<0.0001). **C.** Mice were trained for 5 days before daily BP measurements were made from days 24-28, salt and aldosterone did not increase systolic blood pressures measured using the tail cuff method (t-test p=0.55 n=6). **D.** Key glomerular mRNA changes occur following 28 days exposure to salt and aldosterone with significant up regulation of MMP2, VCAM, TNFα and syndecan 4. Fold change relative to control mice, T-test displayed, n=8)   salt + aldosterone = 1% NaCl in drinking water (ad libitum) and 0.6µg/g/day aldosterone delivered s.c. via minipump   salt and aldosterone = standard drinking water (ad libitum) and vehicle (ethanol saline) filled minipump.

Figure 5. Salt and aldosterone result in loss of glomerular syndecan 4 and an increase in glomerular MMP 2 activity. A. Glomerular syndecan 4, measured by ELISA, decreased significantly following 28 days salt and aldosterone. (t-test, p=0.0016, n=6) **B.** Urine syndecan 4 creatinine ratios increased significantly. (t-test p=0.0339, n=8) **C.** Active glomerular MMP2, measured by activity assay, increased following 28 days salt and aldosterone (T-test, p=0.0061, N=6). **D** Urine active MMP2/creatinine ratios increased following salt and aldosterone (t-Test p=0.0012, n=6).   salt + aldosterone = 1% NaCl in drinking water (ad libitum) and 0.6µg/g/day aldosterone delivered s.c. via minipump   salt and aldosterone = standard drinking water (ad libitum) and vehicle (ethanol saline) filled minipump.

Figure 6. Multiphoton microscopy can be used to measure changes in glomerular albumin leakage. A. Simultaneous measurement of Alexa 594 albumin florescence intensity in the Bowman's space (BS) and within plasma filled glomerular capillary loops (CL) allowed calculation of the glomerular sieving coefficient for albumin (GSCalb). Multiphoton Images were selected for analysis provided the Bowman's space could be clearly defined and two sites with no capillary loops within 4.5µm (X Y and Z plane) could be sampled, minimising background florescence. The same glomeruli were imaged on day 0 and day 5 allowing calculation of the fold change in GSCalb in each glomerulus before calculating the mean for each mouse (Bar= 50µm). **B.** The formula used to calculate the GSCalb for each glomerulus. Fluorescence intensity (FI) was measured at two points within the BS and at 3 points within the capillary lumens on each image, 4 images were analysed for each glomerulus. The final GSCalb for each mouse was taken as the average result from all included glomeruli. **C.** Young DBA2J mice developed significant albuminuria following 10 days of salt and aldosterone exposure (ANOVA, p=0.176, Tukey's correction displayed, n=5). **D.** Fold change in the glomerular albumin sieving coefficients for young DBA2J mice following 5 days of salt and aldosterone (Mann-Whitney, p=0.0079, n=5). Day 10 measurements were attempted, but glomeruli were too deep for reliable assessment.
    salt + aldosterone = 1% NaCl in drinking water (ad libitum) and 0.6µg/g/day aldosterone delivered s.c. via minipump   salt and aldosterone = standard drinking water (ad libitum) and vehicle (ethanol saline) filled minipump.

Figure 7. Salt and aldosterone exposure resulted in albuminuria and increased glomerular albumin leakage with associated glycocalyx damage in young C57 BL/6 mice. A. Young C57 BL/6 mice developed significant albuminuria after 10 days exposure to salt and aldosterone compared to controls and baseline values (ANOVA p=0.0191, Sidak's comparison used for matched time points and displayed) **B.** Male C57 BL6 mice receiving salt and aldosterone do not develop significant systolic hypertension by day 12 (measured via tail cuff plethysmography in awake trained mice) T-test p=0.9694 (n=5). **C.** GSCalb values for control mice do not alter significantly (non parametric Friedman test of paired data with of repeated measures p=0.0864) although there was a trend for GSCalb to fall between day 0 and day 5. GSCalb values for mice receiving salt and aldosterone increased significantly (non parametric Friedman test of paired data with of repeated measures p=0.0085, n=5). **B.** Time match comparisons between control mice and those receiving salt and aldosterone confirmed significant increases in GSCalb (Mann-Whitney day 5 p=0.0079, day 10 p=0.0079, n=5).   salt + aldosterone = 1% NaCl in drinking water (ad libitum) and 0.6µg/g/day aldosterone delivered s.c. via minipump   salt and aldosterone = standard drinking water (ad libitum) and vehicle (ethanol saline) filled

minipump

Figure 8. Multiphoton microscopy confirmed that the glomerular endothelial glycocalyx is damaged following 10 days salt and aldosterone exposure. A. A multiphoton image of a FITC-WGA perfused control glomerulus on day 10 (after GSCalb images had been taken) (ROI = 11µm). Glycocalyx measurement relied on the selection of capillary segments where a clear plasma peak signal could be seen (free from circulating erythrocytes). Only cross sectioned (round) capillary profiles, and the level at 1⁄2 the capillary depth (deduced from the Z-stack) were analysed to ensure the glycocalyx was measured perpendicular to the endothelial membrane **B.** Plasma Alexa 594 albumin concentrations are maximal towards the periphery of glomerular capillaries (Fahraeus-Lindqvist effect). The plasma concentration of labeled albumin falls as it meets the glycocalyx (the first component of the glomerular filtration barrier). The distance between the maximal FITC-WGA glycocalyx signal and the adjacent peak Alexa 594 plasma albumin signal (indicated by the two vertical dotted lines) can therefore be used as an index of glycocalyx depth. **C.** The intensity of glomerular capillary FITC-WGA signal was assessed using standardised settings and normalised to adjacent tubular auto-florescence. Ten days exposure to salt and aldosterone reduced glomerular glycocalyx florescence intensity (Mann-Whitney p=0.0079, n=5) **D.** Peak to peak assessment of the glomerular endothelial glycocalyx on day 10 demonstrated a significant reduction in glycocalyx thickness in mice exposed to salt and aldosterone (t-test p=0.0013, n=5)

Figure 9. MMP 2/9 inhibitor prevented the effects of salt and aldosterone in vivo, without affecting animals blood pressure. A. MMP2/9 inhibitor did not alter the systolic blood pressure of male C57 BL6 mice receiving salt and aldosterone measured on day 12 using tail cuff plethysmography. (t-test p=0.435, n=5) **B.** uACR values increased significantly in mice receiving salt, aldosterone and vehicle by day 5. No significant change was detected in mice receiving salt, aldosterone and MMP2/9 inhibitor. Comparison between the groups on day 0 was not significant but comparison on day 5 confirmed that mice receiving salt, aldosterone and MMP2/9 inhibitor had significantly lower uACR levels (ANOVA p=0.0010, Tukey's analysis displayed, n=5). **C.** Mice receiving salt, aldosterone and MMP2/9 inhibitor had significantly lower GSCalb fold change values by day 5 compared to mice receiving salt, aldosterone and vehicle. (Mann-Whitney p=0.0079). **D.** Representative images of the same glomeruli day 0 and day 5 with FITC-WGA labeled glomerular glycocalyx (green) and circulating Alexa 594 albumin labeled plasma. **E.** Quantitative analysis of FITC-WGA florescence intensity confirmed MMP2/9 inhibition preserved the endothelial glycocalyx (Mann-Whitney p=0.0079, n=5). **F.** Peak to peak measurement of glomerular endothelial glycocalyx depth demonstrated a significant thinning of glycocalyx during the first 5 days exposure to salt and aldosterone. In the presence of MMP2/9 inhibitor no alteration was seen (ANOVA p<0.0001, Tukey's correction displayed, n=5). *salt + aldosterone = 1% NaCl in drinking water (ad libitum) and 0.6µg/g/ day aldosterone delivered S.C. via minipump   salt and aldosterone = standard drinking water (ad libitum) and vehicle (ethanol saline) filled minipump.*

MMP2/9i = 5mg/kg I.P. injection once daily,   MMP2/9i = daily vehicle (DMSO saline).

Disclosure statement

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