Role Of The C-C Motif Chemokine Ligand 5 (CCL5) And Its Receptor, C-C Motif Chemokine Receptor 5 (CCR5) In The Genesis Of Aldosterone-induced Hypertension, Vascular Dysfunction, And End-organ Damage

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Abstract

Background: Aldosterone, a mineralocorticoid steroid hormone, has been described to initiate cardiovascular diseases by triggering exacerbated sterile vascular inflammation. The functions of C-C Motif Chemokine Ligand 5 (CCL5) and its receptor, C-C Motif Chemokine Receptor 5 (CCR5), are well known in infectious diseases, but their roles in the genesis of aldosterone-induced vascular injury and hypertension are unknown.

Methods: We analyzed the vascular profile, blood pressure, and renal damage in wild-type ($CCR5^{+/+}$) and CCR5 knockout ($CCR5^{-/-}$) mice treated with aldosterone (600 µg/kg/day for 14 days) while receiving 1% saline to drink.

Results: Here, we show that CCR5 plays a central role in aldosterone-induced vascular injury, hypertension, and renal damage. Long-term infusion of aldosterone in $CCR5^{+/+}$ mice resulted in exaggerated CCL5 circulating levels and vascular CCR5 expression. Aldosterone treatment also triggered vascular injury, characterized by endothelial dysfunction and inflammation, hypertension, and renal damage. Mice lacking CCR5 were protected from aldosterone-induced vascular damage, hypertension, and renal injury. Mechanistically, we demonstrated that CCL5 increased NADPH oxidase 1 (Nox1) expression, reactive oxygen species (ROS) formation, NFκB activation, and inflammation and reduced nitric oxide production in isolated endothelial cells. These effects were abolished by antagonizing CCR5 with Maraviroc. Finally, aortae incubated with CCL5 displayed severe endothelial dysfunction, which is prevented by blocking Nox1, NFκB, or with Maraviroc treatment.

Conclusions: Our data demonstrate that CCL5/CCR5, through activation of NFkB and Nox1, is critically involved in aldosterone-induced vascular and renal damage and hypertension. Our data place CCL5 and CCR5 as potential targets for therapeutic interventions in conditions with aldosterone excess.

Keywords: aldosterone; chemokines; chemokines receptors; NADPH oxidases; oxidative stress.

Nonstandard Abbreviations and Acronyms

4,5-Diaminofluorescein Diacetate (DAF-2 DA)

Acetylcholine (ACh)

Chemokine (C-C motif) Ligand 5 (CCL5)

Diastolic Blood Pressure (DBP)

Dulbecco's Modified Eagle's Medium (DMEM)

Enzyme-Linked Immunosorbent Assay (ELISA)

Lotus Tetragonolobus Lectin (LTA)

Mean arterial pressure (MAP)

Mineralocorticoid Receptors (MR)

Nuclear Factor Kappa B (NFκB)

PBS containing 0.1% Tween-20 (PBS-T)

Phenylephrine (PE)

Phosphate-Buffered Saline (PBS)

Reactive Oxygen Species (ROS)

Regulated on Activation Normal T Cell Expressed and Secreted (RANTES)

Sodium Nitroprusside (SNP)

Systolic Blood Pressure (SBP)

T-Regulatory Cells (T-Reg)

Vascular Smooth Muscle Cells (VSMC)

Introduction

Aldosterone is a mineralocorticoid steroid hormone, and it is principally generated by the adrenal glomerulosa[1], although other cell types, including adipocytes[2, 3], can be an alternative source of aldosterone. The primary function of aldosterone, via mineralocorticoid receptor (MR), is to regulate salt and water homeostasis in the body by acting on the distal tubule and collecting duct of nephrons in the kidney, which in turn leads to sodium and water reabsorption and potassium excretion[1, 4, 5]. However, MR are expressed in different cell types including vascular smooth muscle cells (VSMC)[6], endothelial cells[9], adipocytes that form perivascular adipose tissue[7], and immune cells[8, 9], where aldosterone can exert its deleterious effects including inflammation, oxidative stress, accelerated fibrosis, and proliferation[4, 5, 8, 10-13]. In this context, hyperaldosteronism and hyperactivation of MR signaling are key features in the pathogenesis of diabetes[2, 3, 10], obesity[2, 3, 10], atherosclerosis[14], stroke[13], and hypertension[15]. Although such evidence is well-established, the cellular and molecular mechanisms by which aldosterone induces cardiovascular injury are not fully elucidated.

Chemokines (or chemotactic cytokines) are a large family of small proteins that act through cell surface G protein-coupled chemokine receptors[16]. They are best known for their ability to stimulate the migration of cells, most notably immune cells into the injured area[17]. Conversely, chemokines can induce cellular changes independent of immune cell recruitment by signaling directly through their receptors in VSMC and endothelial cells[17-20].

Chemokine (C-C motif) ligand 5 (CCL5) (or regulated on activation normal T cell expressed and secreted - RANTES) is one of the most important chemokines secreted by T cells, macrophages, activated platelets, endothelial cells, and VSMC[17, 20, 21]. While CCL5 can bind to CCR1, CCR3, CCR4 and CCR5, it has the highest affinity to CCR5, which is expressed in endothelial cells as well[17, 18, 20, 21]. High levels of CCL5 have been identified in hyperlipidemia[22], atherosclerosis[23, 24], and hypertension[25]. *In vivo* and *in vitro* studies have revealed that lack or blockage of CCL5 or CCR5 significantly attenuates atherosclerosis[26-28], neointima formation[29], atherogenic phenotype switching in obesity[30], vascular inflammation in acquired lipodystrophy[31], and inflammation of perivascular adipose tissue in hypertension[25]. We previously reported that CCL5 via CCR5 induces VSMC proliferation and migration in a NADPH oxidase 1 (Nox1)-derived

reactive oxygen species (ROS) dependent-manner, whereas blockage of CCR5 *in vivo* attenuates vascular hypertrophy[19]. However, whether the effects of aldosterone-induced vascular injury and hypertension are mediated by exaggerated activity or increased levels of CCL5 and CCR5 is not known.

Herein, we addressed a new mechanism of aldosterone-induced endothelial dysfunction, hypertension, and renal damage. We found that CCL5 and CCR5 are connected to hyperaldosteronism- associated cardiovascular risk. Importantly, high levels of CCL5 in aldosterone-treated mice induces endothelial dysfunction and inflammation via nuclear factor kappa B (NFκB) and Nox1-derived ROS, whereas deficiency in CCR5 protects against aldosterone-induced vascular injury, hypertension, and end-organ damage. Therefore, we place CCL5 and CCR5 as a major trigger of cardiovascular disease in conditions associated with aldosterone excess such as primary aldosteronism, obesity, and hypertension.

Methods

Mice

Ten- to twelve-week-old male $CCR5^{+/+}$ (C57BL6/J) and $CCR5^{-/-}$ (Ccr5tm1Kuz/J Jax #005427) mice were used. All mice were fed with standard mouse chow and tap water was provided *ad libitum*. Mice were housed in an American Association of Laboratory Animal Care- approved animal care facility in Rangos Research Building at Children's Hospital of Pittsburgh (CHP) of University of Pittsburgh. The Institutional Animal Care and Use Committee approved all protocols (IACUC protocols #19065333 and #22061179). All experiments were performed in Rangos Research Building at CHP and were in accordance with the Guide Laboratory Animals for The Care and Use of Laboratory Animals.

Aldosterone treatment

$CCR5^{+/+}$ and $CCR5^{-/-}$ mice were infused with vehicle (saline) or aldosterone (600 μg/kg per day) for 14 days with ALZET osmotic minipumps (Durect, Cupertino, CA) while receiving 1% saline in the drinking water as described previously[8].

Circulating chemokine profile

Circulating chemokines levels were analyzed via the Proteome Profiler Mouse Cytokine Array from R&D System. Serum was pooled from six $CCR5^{+/+}$ mice treated with vehicle or aldosterone. Data were presented as fold changes by a Heat Map graph.

Circulating CCL5 levels

Circulating CCL5 levels were measured in serum from $CCR5^{+/+}$ mice treated with vehicle or aldosterone via Enzyme-Linked Immunosorbent Assay (ELISA) (R&D System).

Vascular function

As described previously[32 33], thoracic aortae from $CCR5^{+/+}$ and $CCR5^{-/-}$ mice treated with vehicle or aldosterone were dissected from connective tissues, separated into rings (2 mm), and mounted in a wire myograph (Danysh MyoTechnology) for isometric tension recordings with PowerLab software (AD Instruments). Rings were placed in tissue baths containing warmed (37 °C), aerated (95% O_2, 5% CO_2) Krebs Henseleit Solution (in mM: 130 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 0.03 EDTA, 1.6 $CaCl_2$, 14.9 $NaHCO_3$, 1.18 KH_2PO_4, and 5.5 glucose). After 30 min of stabilization, KCl (120 mM) was used to test arterial viability. Concentration-effect curves for phenylephrine (PE, α-1 adrenergic receptor-dependent vasoconstrictor), acetylcholine (ACh, endothelium-dependent vasodilator), and sodium nitroprusside (SNP, endothelium-independent vasodilator) were performed. Some experiments were performed in the presence of Nox1Ads (10 µM, specific Nox1 inhibitor) to analyze the involvement of Nox1 on aldosterone-induced vascular dysfunction.

Ex vivo protocol for isolated aortae incubation

Aortae were harvested from $CCR5^{+/+}$ mice, separated in 2 mm rings, and incubated with CCL5 (100 ng/mL) for 24 hours. Some aortic rings were treated with Maraviroc (40 µM, specific CCR5 antagonist), Nox1Ads (10 µM), or BMS-345541 (5 µM, NFκB signaling inhibitor). Then, experiments of endothelial function were performed.

Blood pressure analysis

$CCR5^{+/+}$ and $CCR5^{-/-}$ mice were instrumented with telemetry transmitters to record arterial pressure and heart rate (HD-X10, Data Sciences International). Transmitters were implanted as described previously[34-36]. After 7 days of recovery from surgery, necessary for the mice to gain their initial body weight, data were recorded for 4 days as baseline. Then, aldosterone was infused via osmotic mini-pump for 14 days as described[8], and blood pressure [systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP)] values were obtained for 2-3 hours per day between 12pm-5pm.

Immunohistochemical staining for renal injury

As described previously[37], kidneys were fixed in 4% paraformaldehyde overnight, embedded in paraffin and sectioned at 4 µm. After deparaffinization, rehydration, and permeabilization in PBS containing 0.1% Tween-20 (PBS-T), antigen retrieval was performed by boiling the slides for 30 min in either 10 mM sodium citrate pH 6.0 buffer or Trilogy (Cell Marque). Sections were then blocked in 3% BSA before and incubated overnight with the appropriate primary antibody at 4°C. Then, sections were washed with PBS-T, incubated with secondary antibody, washed again with PBS-T and mounted in Fluoro Gel with DABCO™ (Electron Microscopy Science) before visualizing with either a Leica DM2500 microscope equipped with a Leica DFC 7000T camera and a LAS X software or with a Zeiss 710 confocal microscope with Zen software (Zeiss). The antibodies used for immunostaining are shown in Supplementary Table 1. Primary antibodies were visualized by staining with fluorescence-conjugated antibodies. Nuclei were counterstained with DAPI. Proximal tubules were visualized with fluorescein-labeled Lotus tetragonolobus lectin (LTA).

Proteinuria

Urine was collected during the tissue harvesting, stored at -80°C, and proteinuria was analyzed as previously described[38]. 15 µL of urine samples were separated on 10% SDS-PAGE gels

followed by staining with Coomassie Brilliant Blue (Sigma-Aldrich). Albumin (15 µg) was used as a control.

Endothelial cell culture

Mouse Mesenteric Endothelial Cells (MEC) were purchased from Cell Biologics. Cells were maintained in Complete Mouse Endothelial Cell Medium (Cell Biologics) containing Endothelial Cell Medium Supplement Kit (Cell Biologics). Cells were used between passage 4-8.

Protocol of endothelial cells stimulation

MEC were treated with CCL5 (100 ng/mL) for 24 hours in the presence of vehicle or Maraviroc (40 µM), Nox1Ads (10 µM), or BMS-345541 (5 µM). MEC were also treated with aldosterone (0.1 µM) with or without eplerenone (1 µM). MEC were incubated for 30 min with Inhibitors and antagonists prior to CCL5 or aldosterone treatments.

Macrophage adhesion assay

Endothelial macrophage adhesion was determined according to our previously described methods[11 39]. Briefly, MEC were cultured to confluence in 6-well plates and treated with CCL5 (100 ng/mL) for 24 hours in the presence of vehicle or Maraviroc (40 µM). Non-stimulated MEC served as controls. Macrophage cells (RAW 264.7, ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) contained 10% of Fetal Bovine Serum (FBS). For cell fluorescent labeling, macrophages (10^5 cells/mL) were suspended in 1% bovine serum albumin (BSA)-supplemented phosphate buffered saline containing 1 µM calcein-AM (Invitrogen) and incubated for 20 minutes at 37 °C. Labeled macrophages were washed twice with phosphate-buffered saline and suspended in Hanks' buffered salt solution. Fluorescence labeled cells (10^5 cells/well) were then added to both non-stimulated and stimulated MEC layers and were allowed to adhere for 30 minutes at 37 °C in 5% CO_2. After incubation, non-adhered cells were removed by gently washing with pre-warmed Hanks' buffered salt solution. The number of adherent cells was determined by images or fluorescence intensity via fluorescence microscopy (Leica Microsystems) and fluorimeter (SpectraMax i3x Multi-Mode Microplate Reader), respectively.

For the fluorescence intensity, cells were lysed with 0.1 M NaOH and transferred to a 96 well plate, then fluorescence intensity was measured.

ROS measurement

Aortae and MEC were collected in lysis buffer (Hank's Balanced Salt Solution with Complete Mini protease inhibitor and PhosSTOP phosphatase inhibitor) and were lysed by five freeze/thaw cyclesand passed through a 30-gauge needle five times to disrupt cells to measured ROS as described before[40]. The cell lysates were centrifuged at 1000 g for 5 min at 4 °C to remove unbroken cells, nuclei, and debris. Throughout all procedures, extreme care was taken to maintain the lysate at a temperature close to 0 °C. Lysates of aortae and MEC were resuspended in Amplex Red assay mixture (25 mM HEPES, pH 7.4, containing 120 mM NaCl, 3 mM KCl, 1 mM MgCl2, 0.1 mM Amplex red (Invitrogen), and 0.35 U/ml horseradish peroxidase (HRP) in the presence and absence of catalase (300 U/ml). The reaction was initiated by the addition of 36 µmol/l NADPH (MP Biomedicals). Florescence measurements were made using a Biotek Synergy 4 hybrid multimode microplate reader with a 530/25-exitation and a 590/35-emission filter. The reaction was monitored at 25 ˚C for 1 hour.

Nitric oxide measurement

Nitric oxide production was measured by 4,5-Diaminofluorescein diacetate (DAF-2 DA) probe. Briefly, MEC were treated with CCL5 (100 ng/mL) for 24 hours in the presence of vehicle or Maraviroc (40 µM). Then, media were replaced, and bradykinin (1 µM)[41] was used to stimulate nitric oxide production for 30 minutes. After the treatments, cells were washed with PBS and stained with DAF-2 DA (5 µM) for 30 minutes before the analysis. Bradykinin (5 µM) was used as positive control. Fluorescence intensity was measured in a fluorimeter (SpectraMax i3x Multi-Mode Microplate Reader) (Emission. 538 nm/Excitation. 485 nm).

Western Blot

Aortic protein was extracted using radioimmunoprecipitation assay buffer (RIPA) buffer (30 mM HEPES, pH 7.4,150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium

dodecyl sulfate, 5 mM EDTA, 1 mM $NaV0_4$, 50 mM NaF, 1 mM PMSF, 10% pepstatin A, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). Total protein extracts were centrifuged at 15,000 rpm/10 min and the pellet was discarded. Proteins from homogenates of aortae (25 µg) were used. MEC samples were directly homogenized using 2x Laemmli Sample Buffer and supplemented with 2-mercaptoethanol (β-mercaptoethanol) (BioRad Hercules). Proteins were separated by electrophoresis on a polyacrylamide gradient gel (BioRad Hercules) and transferred to Immobilon-P poly (vinylidene fluoride) membranes. Non-specific binding sites were blocked with 5% skim milk or 1% bovine serum albumin (BSA) in tris-buffered saline solution with tween for 1h at 24 °C. Membranes were then incubated with specific antibodies overnight at 4 °C as described in Supplementary Table 2. After incubation with secondary antibodies, the enhanced chemiluminescence luminol reagent (SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Fisher) was used for antibody detection.

Real-Time Polymerase Chain Reaction (RT-PCR)

mRNA from aortae and MEC were extracted using RNeasy Mini Kit (Quiagen). Complementary DNA (cDNA) was generated by reverse transcription polymerase chain reaction (RT-PCR) with SuperScript III (Thermo Fisher). Reverse transcription was performed at 58 °C for 50 min; the enzyme was heat inactivated at 85 °C for 5 min, and real-time quantitative RT-PCR was performed with the PowerTrack™ SYBR Green Master Mix (Thermo Fisher). Sequences of genes as listed in Supplementary Table 3. Experiments were performed in a QuantStudio™ 5 Real-Time PCR System, 384-well (Thermo Fisher). Data were quantified by 2ΔΔ Ct and are presented by fold changes indicative of either upregulation or downregulation.

Statistical analysis

For comparisons of multiple groups, one-way or two-way analysis of variance (ANOVA), followed by the Tukey post-test was used. Differences between the two groups were determined using Student's t-test. The vascular relaxation response is expressed as a percentage of relaxation based on the phenylephrine maximal response (1uM), whereas contractile response is presented as millinewton (mN). Maximal response (Emax) and negative logarithm of EC50 (pD_2) were

determined. Analyses were performed using Prism 10.0 software (GraphPad). A difference was considered statistically significant when $p \leq 0.05$.

RESULTS

Circulating CCL5 levels and vascular CCR5 expression are increased in aldosterone-treated mice and may play important role on aldosterone-induced endothelial dysfunction

We firstly observed that $CCR5^{+/+}$ mice treated with aldosterone displayed changes in circulating cytokines and chemokines (Fig. 1A). However, CCL5, which is considered the main CCR5 ligand, was the most elevated in the proteome cytokine array. In addition, we also used ELISA to confirm the high CCL5 circulating levels post-aldosterone treatment (Fig. 1B).

In aortae from aldosterone-treated mice, we found elevated CCR1, CCR5, and CCL5 gene expression, with no difference for CCR3 (Fig. 1C). To understand whether aldosterone regulates chemokine receptors expression, we treated endothelial cells with aldosterone and observed a significant increase in CCR1, CCR5, and CCL5 levels, which was dependent on MR activation, since eplerenone blunted the aldosterone effects (Fig. 1D). These data indicate that aldosterone increases CCL5 production and CCR5 expression in endothelial cells in a MR dependent manner.

We recently demonstrated that CCL5 induces vascular inflammation and proliferation dependent on CCR5. Although CCL5 has a large affinity by CCR5, other chemokine receptors can recognize CCL5, such as CCR1 and CCR3. Therefore, we investigated whether CCL5 is leading to endothelial dysfunction via CCR5. By incubating aortae from $CCR5^{+/+}$ mice with CCL5, with or without a specific CCR5 antagonist (Maraviroc), and performing studies of endothelial function by myography, we observed that CCL5 triggered severe endothelial dysfunction in aortae, which was partially protected by antagonizing CCR5 (Fig. 1E). Furthermore, CCL5 incubation for 24h elevated CCR1 and CCL5 expressions in isolated aortae (Supplementary Fig. 1A). CCL5 triggered vascular hypercontractile to phenylephrine in aortae, which was protected by antagonizing CCR5 (Supplementary Fig. 1B). No difference was observed for SNP (endothelium-independent vasodilator) (Supplementary Fig. 1C).

CCR5 deficiency protects from aldosterone-induced endothelial dysfunction and vascular inflammation

Since we found that CCL5 and CCR5 are elevated in aldosterone-treated mice and CCL5 induces endothelial dysfunction in a CCR5-dependent manner, we treated $CCR5^{+/+}$ and $CCR5^{-/-}$ mice with aldosterone to analyze whether lack in CCR5 might confer protection against vascular injury. Interestingly, we found that aldosterone promoted severe vascular dysfunction characterized by an impaired endothelium-dependent relaxation to ACh (Fig. 2A) and hypercontractility to phenylephrine (Supplementary Fig. 2A); these changes were prevented by CCR5 deficiency. No difference was observed for SNP (Fig. 2B).

We found that aldosterone treatment augmented NFkB phosphorylation in aortae from $CCR5^{+/+}$ mice, but not in $CCR5^{-/-}$ mice (Fig. 2C). These effects were accompanied by increased expression of inflammatory genes (IL-1β, TNF-α, and VCAM expression) (Fig. 2D), which was not detected in $CCR5^{-/-}$ mice. *Ex vivo* experiments in aortae revealed that CCL5 incubation for 24h increased NFkB activation (Fig. 2E), whereas NFkB inhibition prevented CCL5-induced endothelial dysfunction (Fig. 2F). Furthermore, CCL5 incubation for 24h increased expression of inflammatory genes (IL-1β, TNF-α, ICAM and VCAM expression) in isolated aortae (Supplementary Fig. 2B).

To confirm that CCL5/CCR5 leads to endothelial inflammation, we interrogated whether CCL5 triggers endothelial inflammation by treating MEC with CCL5 in the presence or absence of Maraviroc and analyzing inflammatory markers and RAW264.7 adhesion. CCL5 augmented IL1β, TNF-α, ICAM and VCAM gene expression and increased RAW264.7 adhesion in MEC at 24 hr, whereas Maraviroc prevented these effects (Fig. 3A and B).

CCR5 deficiency prevents aldosterone-induced hypertension and kidney damage

Endothelial dysfunction is a key feature in the genesis and progression of hypertension and end-organ damage. Therefore, we investigated whether CCR5 deficiency could protect from aldosterone-induced hypertension and renal injury. Aldosterone induced hypertension is characterized by increased SBP, DBP, and MAP. Interestingly, $CCR5^{-/-}$ mice were protected against aldosterone-induced hypertension (Fig. 4A-C).

In kidneys, aldosterone augmented the expression of the renal injury markers, Kidney injury molecule-1 (KIM-1) and Neutrophil gelatinase-associated lipocalin (NGAL) by qRT-PCR (Fig. 4E). Furthermore, immunostaining analyses indicated elevated tubulointerstitial deposition of fibrotic markers, αSMA and collagen III (Fig. 4D). Increased collagen III levels were also confirmed by qRT-PCR (Fig. 4E). Interestingly, aldosterone-treated mice exhibited reduced expression of the podocyte-specific marker synaptopodin (measured by immunofluorescence and RT-PCR) (Fig. 4D and Fig. 4E), and this was accompanied by augmented proteinuria (Fig. 4F). No changes in the expression of the endothelial cell marker, endomucin, were detected (Fig. 4D and Fig. 4E). All these deleterious effects caused by aldosterone (renal damage and proteinuria) were abolished in $CCR5^{-/-}$ mice. Finally, no structural difference was observed in the afferent and efferent glomerular arterioles (Supplementary Fig. 3). These data suggest that CCL5/CCR5 play major role on aldosterone-associated hypertension and renal damage.

Aldosterone induces endothelial injury and Nox1-derived ROS and impairs nitric oxide formation via CCL5 and CCR5

Aldosterone has been associated with Nox activation in different cell types. Herein, we demonstrated that aldosterone treatment increases aortic Nox1 (but not Nox2 and Nox4) (Fig. 5A) and induces ROS production (Fig. 5B) in $CCR5^{+/+}$ mice. $CCR5^{-/-}$ mice were protected from these effects. To understand whether aldosterone is leading to endothelial dysfunction via Nox1 activation, we incubated arteries prior to ACh curves with a selective Nox1 inhibitor, NoxA1ds[40], and observed that Nox1 inhibition blunted the endothelial dysfunction in $CCR5^{+/+}$ mice (Fig.5C).

Next, we assessed whether CCL5 induces endothelial dysfunction via Nox1 activation by multiple approaches. Firstly, we treated isolated aortae from $CCR5^{+/+}$ mice with CCL5 for 24h, and we observed an increase in the Nox1 expression (Fig. 5D), but not Nox2 and Nox4 (Supplementary Fig. 4A-B), and in the ROS generation (Fig. 5D). This increase in ROS generation was abolished in the presence of a selective Nox1 inhibitor, NoxA1ds (Fig. 5D). In addition, in presence of Nox1Ads, we performed studies of endothelial function to examine whether CCL5 induces endothelial dysfunction via Nox1 and found Nox1 inhibition conferred protection against CCL5-induced endothelial dysfunction (Fig. 5E).

Secondly, we treated MEC with CCL5 in presence of Maraviroc to confirm that CCL5 increases endothelial Nox1 and ROS production. We observed that CCL5 treatment for 24h augmented Nox1 expression and induced ROS formation, which were blunted by blocking CCR5 (Maraviroc)or inhibiting NOX1 (NOXA1ds) (Fig. 5F).

Lastly, we treated MEC with CCL5 to analyze whether it can affect nitic oxide formation, as displayed in Supplementary Figures 4C and 4D. CCL5 (100ng/mL, 24h) decreased eNOS phosphorylation at Ser^{1177} and impaired bradykinin-induced nitric oxide formation at 24h, which were prevented by antagonizing CCR5 with Maraviroc.

Aldosterone induces Nox1 expression and endothelial dysfunction via CCL5/CCR5 and NFkB

The association between aldosterone, Nox1, and NFκB, as well as NFκB modulating Nox1 expression has been described before, but whether such communications are dependent on CCL5 and CCR5 are unknown. *In vitro*, we found that CCL5 induces an increase in Nox1 expression, which is prevented by the presence of the NFκB pathway inhibitor (Fig. 6A). Furthermore, the presence of the NFκB inhibitor blocks ROS generation (Fig. 6B). By a feedback mechanism, CCL5 increases NFκB phosphorylation, which was reverted in the presence of the Nox1 inhibitor (Fig. 6C), indicating that ROS formed by Nox1 increase NFκB activity, while NFκB activity can regulate NOX1 expression and ROS formation. Therefore, we can suggest that CCL5 induces endothelial injury via a continuous and positive feedback between Nox1 and NFκB.

Discussion

Cardiovascular diseases are the leading cause of death globally. Aldosterone contributes to the endocrine basis of the development and progression of multiple cardiovascular disease processes, including hypertension, chronic kidney disease, coronary artery disease, and congestive heart failure[4, 42]. The association between aldosterone and genesis of hypertension is particularly strong[3, 4, 15, 43]. Several studies place this mineralocorticoid hormone as a major trigger to the development and severity of hypertension, even in the absence of classically defined primary aldosteronism. For instance, blockage of the aldosterone receptor, MR, decreases blood pressure in rodent models of hypertension[44], improves cardiovascular and renal

outcomes associated with obesity and diabetes[12, 45-49], restores obesity-associated hypertension[46, 50]. Although the beneficial hemodynamic effects of aldosterone blockage have been described, the molecular and cellular mechanisms remain not fully elucidated.

Aldosterone is a potent inflammatory hormone, and several of its deleterious cardiovascular effects is mediated by its inflammatory capacity, which in turn can lead to dysfunction, fibrosis, and remodeling in the heart, vasculature, and kidney, as well as hypertension[51]. We previously demonstrated that aldosterone induces VSMC inflammation via oxidative stress[11] and triggers vascular injury and hypertension dependent on NLRP3 inflammasome and IL-1β formation in immune compartment[8]. Others have demonstrated that aldosterone treatment impairs T-regulatory cells (T-Reg) recruitment into injury sites, impairing a tuned innate and adaptive immune response and impacting vascular function and blood pressure control[52]. In atherosclerosis, lack of endothelial MR confers protection against leukocyte-endothelial interactions, plaque inflammation, and expression of adhesion proteins[14]. Therefore, elucidating how aldosterone initiates an inflammatory response is highly important to generate new therapeutic approaches for aldosterone excess-associated cardiovascular disease.

In this study, we are describing a novel mechanism by which aldosterone induces vascular injury, hypertension, and end-organ damage. We are demonstrating that aldosterone (1) increases vascular CCR5 via MR activation and circulating CCL5 levels in mice, (2) induces vascular dysfunction, inflammation, and remodeling in a CCR5-dependent manner, (3) promotes hypertension and renal damage via CCR5, and (4) activates a continuous and positive feedback between NFκB and Nox1-derived ROS in the vasculature via CCL5 and CCR5. These data reveal a remarkable participation of CCL5 and CCR5 in aldosterone-associated cardiovascular damage and hypertension.

Hypertension is associated with immune cell activation and their migration into the kidney, vasculature, heart, and brain; such mechanisms are central for blood pressure regulation and convincing targets for pharmacological intervention[53]. Because chemokines and their receptors (chemokine receptors) help recruit immune cells into the inflamed or injured areas, they are believed to be a leading pathway in the development and progression of hypertension,

but whether any interaction between chemokines and their receptors can directly propagate vascular injury, changes in blood pressure, and end-organ damage is unknown.

CCR5 and CCL5 are elevated in cardiovascular events[19, 27-29, 31] including hypertension[54-56]. Aligned with these previous reports, we observed that aldosterone-treated mice display increased circulating CCL5 and vascular CCR5, which seems to be mediated by MR activation, since aldosterone-induced CCR5 expression in endothelial cells is blunted by eplerenone. Although CCR5 and CCL5 are increased in different models of hypertension, whether they can modulate vascular function, blood pressure control, and renal injury is controversial[25, 55, 57] or not fully described. Furthermore, it is not clear whether CCR5 can participate in the development and progression of any model of hypertension, or if it is model dependent.

The role of CCR5 and CCL5 on hypertension has been explored before, but not in the aldosterone model. Krebs et al[55] demonstrated that CCR5 deficient mice are not protected from cardiac and renal injury and hypertension induced by DOCA salt (50mg) + angiotensin II infusion (1500ng/Kg/min), whereas Rudemiller et al[57] showed that CCL5 knockout mice demonstrate a worse renal outcome post angiotensin II treatment (1000ng/Kg/min) with no changes in blood pressure. Furthermore, Mikolajczyk et al[25] showed that CCL5 knockout mice or pharmacological intervention with CCL5 blockage (met-RANTES) does not affect blood pressure in angiotensin II-induced hypertension model (490ng/Kg/min), but improves vascular function and decreases vascular ROS, and T-cells infiltration into perivascular adipose tissue. In contrast, we found that CCR5 deficient mice are protected from aldosterone-induced vascular injury, hypertension, and renal damage. This discrepancy may be due to the highly potent model of hypertension, DOCA salt plus a very high dose of angiotensin II treatment used by Krebs et al[55]. On the other hand, Rudemiller et al[57] or Mikolajczyk et al[25] used CCL5 deficient mice, and not CCR5. Other ligands than CCL5 can activate CCR5 including CCL3 and CCL4[16], thus these intriguing studies do not rule out the effect of other chemokines activating CCR5. Finally, none of these studies used an aldosteronism model to induce hypertension and injury, so involvement of CCR5 may be specific to this model, and not for angiotensin II for example. Further studies are necessary to dissect any difference amongst the hypertension models including 2-kidneys 1-clip and genetic models.

Aldosterone increases cardiac and renal CCR5[51, 54, 56], but it is not clear if it does the same in arteries or endothelium and if CCR5 propagates any changes in the vasculature. Herein, we confirmed that aldosterone increases CCL5 and CCR5 and described CCL5 and CCR5 as novel regulators of endothelial homeostasis via adjusting Nox1 and NFκB in the vasculature. These findings corroborate our previous study[19], where we demonstrated that CCL5, via CCR5, induces VSMC proliferation and migration via Nox1-derived ROS likely dependent on NFκB activation. The involvement of NADPH oxidases and NFκB activation in the genesis of aldosterone-induced cardiovascular injury has been described before[8, 11, 58]. NADPH oxidases are the major source of ROS in the vasculature and have been implicated in vascular dysfunction, inflammation, and remodeling by regulating NFκB, an important redox sensitive transcript factor, which regulates multiple inflammatory genes[59]. Furthermore, others have reported that NFκB can also regulate NADPH oxidase expression[40, 60] indicating positive feedback between NADPH oxidases and NFκB.

While CCL5 can bind to CCR1, CCR3, CCR4 and CCR5, it has the highest affinity to CCR5[16]. By *in vitro* and *ex vivo* experiments, we examined whether CCL5 induces endothelial dysfunction and inflammation via CCR5. By treating arteries or endothelial cells with CCL5 with or without Maraviroc (CCR5 antagonist), we found that CCL5 impairs endothelial function and inflammation and increases NFkB activation and NOX1-derived ROS dependent on CCR5. Therefore, we can suggest that increases in circulating CCL5 might be activating CCR5 and triggering vascular injury and perhaps hypertension and renal damage in aldosterone treated mice.

It is important to acknowledge the limitations of the current study. First, we did not analyze immune cell infiltration into the arteries or kidney, which might be an additional mechanism whereby deficiency in CCR5 protects the vasculature and kidney. Instead, we mostly focused on a direct mechanism of CCL5 on endothelial cells. Second, we used aortae, and not resistance arteries. Blood pressure is normally regulated by small arteries, but multiple mouse models of hypertension are associated with vascular dysfunction in large[25, 61, 62] and small vessels[8, 63, 64], including aldosterone[8, 61, 62], therefore we can suggest that vascular dysfunction might be displayed in small arteries too. Finally, we did not evaluate the source of CCL5. CCL5 is produced by many cell types including immune and vascular cells, therefore we cannot confirm that increases in CCL5 produced by aldosterone treatment is generated by an immune

compartment or any other cell type. Future experiments including CCL5 deficiency in cell specific population or bone marrow transplant (CCL5 knockout mice into wild-type mice) could overcome this limitation.

Despite these limitations, our study provides the first evidence that aldosteronism impairs vascular function, induces vascular inflammation, and triggers hypertension and end-organ damage via regulating CCL5 and CCR5, which in turn activates NFkB and NOX1-derived ROS and diminishes nitric oxide formation. Taken together, these results indicate that blockage of CCR5 may confer protection against high levels of aldosterone-associated cardiovascular damage and hypertension.

Perspective

Overall, our findings provide novel mechanistic insights into the underlying causes of vascular damage, hypertension, and end-organ injury in the pathophysiology of aldosteronism. In addition, our data present CCR5 blockage, e.g., Maraviroc, a drug broadly used in human immunodeficiency virus $(HIV)^{+}$ patients[65], as a possible new avenue for the treatment of cardiovascular dysfunction and hypertension associated with high levels of aldosterone such as aldosteronism, obesity, and hypertension.

What Is New?

- Increase in aldosterone levels, a key characteristic of primary aldosteronism, obesity, and hypertension, augments circulating CCL5 and vascular CCR5, whereas CCR5 deficiency protects from aldosterone-induced vascular injury, hypertension, and renal damage.
- CCL5/CCR5 triggers endothelial dysfunction and vascular inflammation via NFκB and NOX1 activation.

What Is Relevant?

- Our study has clinical implications by suggesting that CCR5 blockage can prevent the vascular and renal injuries and hypertension in diseases associated with high levels of aldosterone.

Clinical/Pathophysiological Implications?

- Maraviroc, a selective CCR5 antagonist approved by FDA for the treatment of HIV infection, may demonstrate clinical implications also to treat cardiovascular diseases specially associated with high levels of aldosterone.

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Disclosures

None

Legends

Figure 1. Aldosterone-induced CCL5 high levels promotes endothelial dysfunction. Inflammatory profile in plasma, measured by proteome profiler mouse cytokine array and presented as heat map (A); CCL5 levels, measured by ELISA (B); and chemokine receptors expression, measured by RT-PCR (C), from $CCR5^{+/+}$ mice treated with vehicle or aldosterone (600 µg/kg/day for 14 days). Chemokine receptors expression, measured by RT-PCR, in endothelial cells (MEC) treated with vehicle or aldosterone (0.1µM) in the presence of Eplerenone (1µM) (D). Concentration-effect curves to acetylcholine in aortae from $CCR5^{+/+}$ mice incubated with vehicle

or CCL5 (100ng/ml, 24h) in the presence of Maraviroc (40µM) (E). Values represent means ± SEM (n= 3-7). Student t test or ANOVA test. *p<0.05 *vs.* Vehicle; #p<0.05 *vs.* CCL5.

Figure 2. CCR5 deficiency prevents endothelial dysfunction and vascular inflammation induced by aldosterone. Concentration-effect curves to acetylcholine (A) and sodium nitroprusside (B); phosphorylated (p65 subunit) and total NFκB expression, analyzed by western blot to (C); inflammatory markers, measured by RT-PCR (D), in aortae from $CCR5^{+/+}$ and $CCR5^{-/-}$ mice treated with vehicle or aldosterone (600 µg/kg/day + saline for 14 days). Phosphorylated (p65 subunit) and total NFκB expression analyzed by western blot in aortae from $CCR5^{+/+}$ mice incubated with vehicle or CCL5 (100ng/ml, 24h) (E) and concentration-effect curves to acetylcholine (F) in aortae from $CCR5^{+/+}$ mice incubated with vehicle or CCL5 (100ng/ml, 24h) in the presence of BMS-345541 (5µM). Values represent means ± SEM (n= 4-7). Student t test or ANOVA test. *p<0.05 *vs.* $CCR5^{+/+}$ or Vehicle; #p<0.05 *vs.* $CCR5^{+/+}$_Aldo or CCL5.

Figure 3. CCL5 via CCR5 induces endothelial cell activation and immune cell adhesion. Photomicrography and fluorescence intensity depicting labeled macrophages (calcein-AM probe, green) and endothelial cells (MEC, DAPI, blue). MEC were treated with vehicle, Maraviroc (40µM, 30 minutes prior CCL5 incubation), CCL5 (100ng/ml, 24h) or CCL5 plus Maraviroc-stimulated MEC. Scale bar = 100µm (A). Inflammatory markers, measured by RT-PCR, in MEC treated with vehicle or CCL5 (100ng/ml) in the presence of Maraviroc (40µM) (B). Values represent means ± SEM (n= 4). ANOVA test.

Figure 4. Aldosterone via CCR5 promotes increased blood pressure and kidney injury. Systolic blood pressure (A), diastolic blood pressure (B), and mean arterial pressure (C), measured via radiotelemetry, in $CCR5^{+/+}$ and $CCR5^{-/-}$ mice treated with vehicle or aldosterone (600 µg/kg/day and saline for 14 days). Immunofluorescence for podocyte marker synaptopodin (Synap; green), endothelial marker endomucin (Endom; red), and nuclei (DAPI, blue) represented by letters A to D, α-smooth muscle actin (α-SMA; green) and nuclei (DAPI, blue) represented by letters E to H, fibrosis marker and proximal tubules were visualized with fluorescein-labeled collagen III (red)

and Lotus tetragonolobus lectin (LTA; green), which are represented by letters I to L. Images were obtained from kidney sections of $CCR5^{+/+}$ and $CCR5^{-/-}$ mice treated with vehicle or aldosterone. The images shown are representative of three independent experiments. Scale bar = 20 or 50µm (D). Kidney injury markers, measured by RT-PCR in $CCR5^{+/+}$ and $CCR5^{-/-}$ mice treated with vehicle or aldosterone (E), and proteinuria levels in $CCR5^{+/+}$ and $CCR5^{-/-}$ mice treated with vehicle or aldosterone measured by 10% SDS-PAGE gels followed by staining with Coomassie Brilliant. Albumin was used as a control analyzed. Values represent means ± SEM (n= 3-7). Student t test or ANOVA test. *p<0.05 *vs.* $CCR5^{+/+}$.

Figure 5. Aldosterone, via CCR5, promotes endothelial dysfunction by NOX1-dependent mechanisms. Representative western blot (A) to NOX1 (i), NOX2 (ii) and NOX4 (iii) expression; ROS generation, measured Amplex red assay (B); and concentration-effect curves to acetylcholine, in the presence of NOXA1ds (10µM) (C), in aortae from CCR5+/+ and CCR5-/- mice treated with vehicle or aldosterone (600 µg/kg/day for 14 days). Representative western blot to NOX1 (i); ROS generation, measured by Amplex red assay (ii) (D); and concentration-effect curves to acetylcholine, in the presence of NOXA1ds (10µM) (E), in aortae from CCR5+/+ mice incubated with vehicle or CCL5 (100ng/ml). Representative western blot to NOX1 (i) and ROS generation, measured by Amplex red (ii), in endothelial cells (MEC) treated with vehicle or CCL5 (100ng/ml) in the presence of Maraviroc (40µM) and NOXA1ds (10µM) (F). Values represent means ± SEM (n= 4-7). ANOVA test. *p<0.05 *vs.* $CCR5^{+/+}$ or Vehicle; #p<0.05 *vs.* $CCR5^{+/+}$_Aldo or CCL5.

Figure 6. CCL5 induces an increase in NOX1 expression and NFKB activity by a positive feedback mechanism in endothelial cells. Representative western blot to NOX1 expression (A) and ROS generation, measured by Amplex red (B), in endothelial cells (MEC) treated with vehicle or CCL5 (100ng/ml), in the presence of BMS-345541 (5µM). Representative western blot to phosphorylated and total NFκB expression in MEC treated with vehicle or CCL5, in the presence of NOXA1ds (10µM) (C). Values represent means ± SEM (n= 4). ANOVA test.

References

1. Scott JH, Menouar MA, Dunn RJ. Physiology, aldosterone. *Statpearls*. Treasure Island (FL); 2023.
2. Briones AM, Nguyen Dinh Cat A, Callera GE, Yogi A, Burger D, He Y, Correa JW, Gagnon AM, Gomez-Sanchez CE, Gomez-Sanchez EP, Sorisky A, Ooi TC, Ruzicka M, Burns KD, Touyz RM. Adipocytes produce aldosterone through calcineurin-dependent signaling pathways: Implications in diabetes mellitus-associated obesity and vascular dysfunction. *Hypertension*. 2012;59:1069-1078
3. Dinh Cat AN, Friederich-Persson M, White A, Touyz RM. Adipocytes, aldosterone and obesity-related hypertension. *J Mol Endocrinol*. 2016;57:F7-F21
4. Calhoun DA. Aldosterone and cardiovascular disease: Smoke and fire. *Circulation*. 2006;114:2572-2574
5. He BJ, Anderson ME. Aldosterone and cardiovascular disease: The heart of the matter. *Trends Endocrinol Metab*. 2013;24:21-30
6. Biwer LA, Lu Q, Ibarrola J, Stepanian A, Man JJ, Carvajal BV, Camarda ND, Zsengeller Z, Skurnik G, Seely EW, Karumanchi SA, Jaffe IZ. Smooth muscle mineralocorticoid receptor promotes hypertension after preeclampsia. *Circ Res*. 2023;132:674-689
7. Costa RM, Neves KB, Tostes RC, Lobato NS. Perivascular adipose tissue as a relevant fat depot for cardiovascular risk in obesity. *Front Physiol*. 2018;9:253
8. Bruder-Nascimento T, Ferreira NS, Zanotto CZ, Ramalho F, Pequeno IO, Olivon VC, Neves KB, Alves-Lopes R, Campos E, Silva CA, Fazan R, Carlos D, Mestriner FL, Prado D, Pereira FV, Braga T, Luiz JP, Cau SB, Elias PC, Moreira AC, Camara NO, Zamboni DS, Alves-Filho JC, Tostes RC. Nlrp3 inflammasome mediates aldosterone-induced vascular damage. *Circulation*. 2016;134:1866-1880
9. Man JJ, Lu Q, Moss ME, Carvajal B, Baur W, Garza AE, Freeman R, Anastasiou M, Ngwenyama N, Adler GK, Alcaide P, Jaffe IZ. Myeloid mineralocorticoid receptor transcriptionally regulates p-selectin glycoprotein ligand-1 and promotes monocyte trafficking and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2021;41:2740-2755
10. Huby AC, Antonova G, Groenendyk J, Gomez-Sanchez CE, Bollag WB, Filosa JA, Belin de Chantemele EJ. Adipocyte-derived hormone leptin is a direct regulator of aldosterone secretion, which promotes endothelial dysfunction and cardiac fibrosis. *Circulation*. 2015;132:2134-2145
11. Bruder-Nascimento T, Callera GE, Montezano AC, Belin de Chantemele EJ, Tostes RC, Touyz RM. Atorvastatin inhibits pro-inflammatory actions of aldosterone in vascular smooth muscle cells by reducing oxidative stress. *Life Sci*. 2019;221:29-34
12. Silva MA, Cau SB, Lopes RA, Manzato CP, Neves KB, Bruder-Nascimento T, Mestriner FL, Montezano AC, Nguyen Dinh Cat A, Touyz RM, Tostes RC. Mineralocorticoid receptor blockade prevents vascular remodelling in a rodent model of type 2 diabetes mellitus. *Clin Sci (Lond)*. 2015;129:533-545
13. Harvey A, Montezano AC, Lopes RA, Rios F, Touyz RM. Vascular fibrosis in aging and hypertension: Molecular mechanisms and clinical implications. *Can J Cardiol*. 2016;32:659-668

14. Moss ME, Lu Q, Iyer SL, Engelbertsen D, Marzolla V, Caprio M, Lichtman AH, Jaffe IZ. Endothelial mineralocorticoid receptors contribute to vascular inflammation in atherosclerosis in a sex-specific manner. *Arterioscler Thromb Vasc Biol*. 2019;39:1588-1601
15. McCurley A, Pires PW, Bender SB, Aronovitz M, Zhao MJ, Metzger D, Chambon P, Hill MA, Dorrance AM, Mendelsohn ME, Jaffe IZ. Direct regulation of blood pressure by smooth muscle cell mineralocorticoid receptors. *Nat Med*. 2012;18:1429-1433
16. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. *FEBS J*. 2018;285:2944-2971
17. Zeng Z, Lan T, Wei Y, Wei X. Ccl5/ccr5 axis in human diseases and related treatments. *Genes Dis*. 2022;9:12-27
18. Li P, Wang L, Zhou Y, Gan Y, Zhu W, Xia Y, Jiang X, Watkins S, Vazquez A, Thomson AW, Chen J, Yu W, Hu X. C-c chemokine receptor type 5 (ccr5)-mediated docking of transferred tregs protects against early blood-brain barrier disruption after stroke. *J Am Heart Assoc*. 2017;6
19. Singh S, Bruder-Nascimento A, Belin de Chantemele EJ, Bruder-Nascimento T. Ccr5 antagonist treatment inhibits vascular injury by regulating nadph oxidase 1. *Biochem Pharmacol*. 2022;195:114859
20. Jones KL, Maguire JJ, Davenport AP. Chemokine receptor ccr5: From aids to atherosclerosis. *Br J Pharmacol*. 2011;162:1453-1469
21. Zhang Z, Wang Q, Yao J, Zhou X, Zhao J, Zhang X, Dong J, Liao L. Chemokine receptor 5, a double-edged sword in metabolic syndrome and cardiovascular disease. *Front Pharmacol*. 2020;11:146
22. Feng X, Gao X, Jia Y, Zhang H, Xu Y, Wang G. Ppar-alpha agonist fenofibrate decreased rantes levels in type 2 diabetes patients with hypertriglyceridemia. *Med Sci Monit*. 2016;22:743-751
23. Lv YB, Jing J, Li JM, Zhong JP, Fang L, Yang B. Assessment of rantes levels as the indicators of plaque vulnerability in rabbit models of atherosclerosis. *Pathol Res Pract*. 2014;210:1031-1037
24. Podolec J, Kopec G, Niewiara L, Komar M, Guzik B, Bartus K, Tomkiewicz-Pajak L, Guzik TJ, Plazak W, Zmudka K. Chemokine rantes is increased at early stages of coronary artery disease. *J Physiol Pharmacol*. 2016;67:321-328
25. Mikolajczyk TP, Nosalski R, Szczepaniak P, Budzyn K, Osmenda G, Skiba D, Sagan A, Wu J, Vinh A, Marvar PJ, Guzik B, Podolec J, Drummond G, Lob HE, Harrison DG, Guzik TJ. Role of chemokine rantes in the regulation of perivascular inflammation, t-cell accumulation, and vascular dysfunction in hypertension. *FASEB J*. 2016;30:1987-1999
26. Braunersreuther V, Zernecke A, Arnaud C, Liehn EA, Steffens S, Shagdarsuren E, Bidzhekov K, Burger F, Pelli G, Luckow B, Mach F, Weber C. Ccr5 but not ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*. 2007;27:373-379
27. Zernecke A, Liehn EA, Gao JL, Kuziel WA, Murphy PM, Weber C. Deficiency in ccr5 but not ccr1 protects against neointima formation in atherosclerosis-prone mice: Involvement of il-10. *Blood*. 2006;107:4240-4243

28. Cipriani S, Francisci D, Mencarelli A, Renga B, Schiaroli E, D'Amore C, Baldelli F, Fiorucci S. Efficacy of the ccr5 antagonist maraviroc in reducing early, ritonavir-induced atherogenesis and advanced plaque progression in mice. *Circulation*. 2013;127:2114-2124
29. Czepluch FS, Meier J, Binder C, Hasenfuss G, Schafer K. Ccl5 deficiency reduces neointima formation following arterial injury and thrombosis in apolipoprotein e-deficient mice. *Thromb Res*. 2016;144:136-143
30. Lin CS, Hsieh PS, Hwang LL, Lee YH, Tsai SH, Tu YC, Hung YW, Liu CC, Chuang YP, Liao MT, Chien S, Tsai MC. The ccl5/ccr5 axis promotes vascular smooth muscle cell proliferation and atherogenic phenotype switching. *Cell Physiol Biochem*. 2018;47:707-720
31. Bruder-Nascimento T, Kress TC, Kennard S, Belin de Chantemele EJ. Hiv protease inhibitor ritonavir impairs endothelial function via reduction in adipose mass and endothelial leptin receptor-dependent increases in nadph oxidase 1 (nox1), c-c chemokine receptor type 5 (ccr5), and inflammation. *J Am Heart Assoc*. 2020;9:e018074
32. Bruder-Nascimento T, Faulkner JL, Haigh S, Kennard S, Antonova G, Patel VS, Fulton DJR, Chen W, Belin de Chantemele EJ. Leptin restores endothelial function via endothelial ppargamma-nox1-mediated mechanisms in a mouse model of congenital generalized lipodystrophy. *Hypertension*. 2019;74:1399-1408
33. Costa RM, Alves-Lopes R, Alves JV, Servian CP, Mestriner FL, Carneiro FS, Lobato NS, Tostes RC. Testosterone contributes to vascular dysfunction in young mice fed a high fat diet by promoting nuclear factor e2-related factor 2 downregulation and oxidative stress. *Front Physiol*. 2022;13:837603
34. Bruder-Nascimento T, Butler BR, Herren DJ, Brands MW, Bence KK, Belin de Chantemele EJ. Deletion of protein tyrosine phosphatase 1b in proopiomelanocortin neurons reduces neurogenic control of blood pressure and protects mice from leptin- and sympatho-mediated hypertension. *Pharmacol Res*. 2015;102:235-244
35. Bruder-Nascimento T, Ekeledo OJ, Anderson R, Le HB, Belin de Chantemele EJ. Long term high fat diet treatment: An appropriate approach to study the sex-specificity of the autonomic and cardiovascular responses to obesity in mice. *Front Physiol*. 2017;8:32
36. DelVechio M, Alves JV, Saiyid AZ, Singh S, Galley J, Awata WMC, Costa RM, Bruder-Nascimento A, Bruder-Nascimento T. Progression of vascular function and blood pressure in a mouse model of kawasaki disease. *Shock*. 2023;59:74-81
37. Cerqueira DM, Hemker SL, Bodnar AJ, Ortiz DM, Oladipupo FO, Mukherjee E, Gong Z, Appolonia C, Muzumdar R, Sims-Lucas S, Ho J. In utero exposure to maternal diabetes impairs nephron progenitor differentiation. *Am J Physiol Renal Physiol*. 2019;317:F1318-F1330
38. Tsuji K, Paunescu TG, Suleiman H, Xie D, Mamuya FA, Miner JH, Lu HAJ. Re-characterization of the glomerulopathy in cd2ap deficient mice by high-resolution helium ion scanning microscopy. *Sci Rep*. 2017;7:8321
39. da Silva JF, Alves JV, Silva-Neto JA, Costa RM, Neves KB, Alves-Lopes R, Carmargo LL, Rios FJ, Montezano AC, Touyz RM, Tostes RC. Lysophosphatidylcholine induces oxidative stress in human endothelial cells via nox5 activation - implications in atherosclerosis. *Clin Sci (Lond)*. 2021;135:1845-1858

40. Li Y, Kracun D, Dustin CM, El Massry M, Yuan S, Goossen CJ, DeVallance ER, Sahoo S, St Hilaire C, Gurkar AU, Finkel T, Straub AC, Cifuentes-Pagano E, Pagano PJ. Forestalling age-impaired angiogenesis and blood flow by targeting nox: Interplay of nox1, il-6, and sasp in propagating cell senescence. *Proc Natl Acad Sci U S A*. 2021;118
41. Kichuk MR, Zhang X, Oz M, Michler R, Kaley G, Nasjletti A, Hintze TH. Angiotensin-converting enzyme inhibitors promote nitric oxide production in coronary microvessels from failing explanted human hearts. *Am J Cardiol*. 1997;80:137A-142A
42. Ferrario CM, Strawn WB. Role of the renin-angiotensin-aldosterone system and proinflammatory mediators in cardiovascular disease. *Am J Cardiol*. 2006;98:121-128
43. Hall JE, do Carmo JM, da Silva AA, Wang Z, Hall ME. Obesity-induced hypertension: Interaction of neurohumoral and renal mechanisms. *Circ Res*. 2015;116:991-1006
44. Virdis A, Neves MF, Amiri F, Viel E, Touyz RM, Schiffrin EL. Spironolactone improves angiotensin-induced vascular changes and oxidative stress. *Hypertension*. 2002;40:504-510
45. Palacios-Ramirez R, Lima-Posada I, Bonnard B, Genty M, Fernandez-Celis A, Hartleib-Geschwindner J, Foufelle F, Lopez-Andres N, Bamberg K, Jaisser F. Mineralocorticoid receptor antagonism prevents the synergistic effect of metabolic challenge and chronic kidney disease on renal fibrosis and inflammation in mice. *Front Physiol*. 2022;13:859812
46. Verdonschot JAJ, Ferreira JP, Pizard A, Pellicori P, Brunner La Rocca HP, Clark AL, Cosmi F, Cuthbert J, Girerd N, Waring OJ, Henkens M, Mariottoni B, Petutschnigg J, Rossignol P, Hazebroek MR, Cleland JGF, Zannad F, Heymans SRB, Consortium HHOiA. The effect of spironolactone in patients with obesity at risk for heart failure: Proteomic insights from the homage trial. *J Card Fail*. 2022;28:778-786
47. Schjoedt KJ, Rossing K, Juhl TR, Boomsma F, Tarnow L, Rossing P, Parving HH. Beneficial impact of spironolactone on nephrotic range albuminuria in diabetic nephropathy. *Kidney Int*. 2006;70:536-542
48. Ferreira NS, Bruder-Nascimento T, Pereira CA, Zanotto CZ, Prado DS, Silva JF, Rassi DM, Foss-Freitas MC, Alves-Filho JC, Carlos D, Tostes RC. Nlrp3 inflammasome and mineralocorticoid receptors are associated with vascular dysfunction in type 2 diabetes mellitus. *Cells*. 2019;8
49. Silva MA, Bruder-Nascimento T, Cau SB, Lopes RA, Mestriner FL, Fais RS, Touyz RM, Tostes RC. Spironolactone treatment attenuates vascular dysfunction in type 2 diabetic mice by decreasing oxidative stress and restoring no/gc signaling. *Front Physiol*. 2015;6:269
50. Huby AC, Otvos L, Jr., Belin de Chantemele EJ. Leptin induces hypertension and endothelial dysfunction via aldosterone-dependent mechanisms in obese female mice. *Hypertension*. 2016;67:1020-1028
51. Brown NJ. Aldosterone and vascular inflammation. *Hypertension*. 2008;51:161-167
52. Kasal DA, Barhoumi T, Li MW, Yamamoto N, Zdanovich E, Rehman A, Neves MF, Laurant P, Paradis P, Schiffrin EL. T regulatory lymphocytes prevent aldosterone-induced vascular injury. *Hypertension*. 2012;59:324-330
53. Mikolajczyk TP, Szczepaniak P, Vidler F, Maffia P, Graham GJ, Guzik TJ. Role of inflammatory chemokines in hypertension. *Pharmacol Ther*. 2021;223:107799
54. Rickard AJ, Morgan J, Bienvenu LA, Fletcher EK, Cranston GA, Shen JZ, Reichelt ME, Delbridge LM, Young MJ. Cardiomyocyte mineralocorticoid receptors are essential for

deoxycorticosterone/salt-mediated inflammation and cardiac fibrosis. *Hypertension*. 2012;60:1443-1450

55. Krebs C, Fraune C, Schmidt-Haupt R, Turner JE, Panzer U, Quang MN, Tannapfel A, Velden J, Stahl RA, Wenzel UO. Ccr5 deficiency does not reduce hypertensive end-organ damage in mice. *Am J Hypertens*. 2012;25:479-486
56. Brown NJ. Contribution of aldosterone to cardiovascular and renal inflammation and fibrosis. *Nat Rev Nephrol*. 2013;9:459-469
57. Rudemiller NP, Patel MB, Zhang JD, Jeffs AD, Karlovich NS, Griffiths R, Kan MJ, Buckley AF, Gunn MD, Crowley SD. C-c motif chemokine 5 attenuates angiotensin ii-dependent kidney injury by limiting renal macrophage infiltration. *Am J Pathol*. 2016;186:2846-2856
58. Callera GE, Touyz RM, Tostes RC, Yogi A, He Y, Malkinson S, Schiffrin EL. Aldosterone activates vascular p38map kinase and nadph oxidase via c-src. *Hypertension*. 2005;45:773-779
59. Queisser N, Schupp N. Aldosterone, oxidative stress, and nf-kappab activation in hypertension-related cardiovascular and renal diseases. *Free Radic Biol Med*. 2012;53:314-327
60. Wu W, Li L, Su X, Zhu Z, Lin X, Zhang J, Zhuang Z, Cai H, Huang W. Nuclear factor-kappab regulates the transcription of nadph oxidase 1 in human alveolar epithelial cells. *BMC Pulm Med*. 2021;21:98
61. Briet M, Barhoumi T, Mian MOR, Coelho SC, Ouerd S, Rautureau Y, Coffman TM, Paradis P, Schiffrin EL. Aldosterone-induced vascular remodeling and endothelial dysfunction require functional angiotensin type 1a receptors. *Hypertension*. 2016;67:897-905
62. Rodrigues D, Costa TJ, Silva JF, Neto JTO, Alves JV, Fedoce AG, Costa RM, Tostes RC. Aldosterone negatively regulates nrf2 activity: An additional mechanism contributing to oxidative stress and vascular dysfunction by aldosterone. *Int J Mol Sci*. 2021;22
63. Cau SB, Bruder-Nascimento A, Silva MB, Ramalho FNZ, Mestriner F, Alves-Lopes R, Ferreira N, Tostes RC, Bruder-Nascimento T. Angiotensin-ii activates vascular inflammasome and induces vascular damage. *Vascul Pharmacol*. 2021;139:106881
64. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C, Harrison DG. Role of the t cell in the genesis of angiotensin ii induced hypertension and vascular dysfunction. *J Exp Med*. 2007;204:2449-2460
65. Woollard SM, Kanmogne GD. Maraviroc: A review of its use in hiv infection and beyond. *Drug Des Devel Ther*. 2015;9:5447-5468