Determinants of renal oxygen metabolism during low Na$^+$ diet: effect of angiotensin II AT$_1$ and aldosterone receptor blockade

Running title: Low Na$^+$ diet and renal oxygen metabolism: effect of RAAS blockade

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Key points:

- Reducing Na^+ intake reduces the partial pressure of oxygen in the renal cortex and activates the renin-angiotensin-aldosterone system.
- In the absence of high blood pressure, these consequences of dietary Na^+ reduction may be detrimental for the kidney.
- In a normotensive animal experimental model, reducing Na^+ intake for two weeks increased renal oxygen consumption that was normalized by mineralocorticoid receptor blockade. Furthermore, blockade of angiotensin II AT_1 receptor restored cortical partial pressure of oxygen by improving oxygen delivery.
- This shows that increased renin-angiotensin-aldosterone system contributes to increased oxygen metabolism in the kidney after two weeks of low Na^+ diet.
- The results provide insights to dietary Na^+ restriction in the absence of high blood pressure, and its consequences for the kidney.

Abstract

Reduced Na^+ intake reduces the partial pressure of oxygen (PO_2) in the renal cortex. Upon reduced Na^+ intake, reabsorption along the nephron is adjusted with activation of the renin-angiotensin-aldosterone system (RAAS). Thus, we studied the effect of reduced Na^+ intake on renal oxygen homeostasis and function in rats, and the impact of intrarenal angiotensin II AT_1 receptor blockade using candesartan and mineralocorticoid receptor blockade using canrenoic acid potassium salt (CAP).

Male Sprague-Dawley rats were fed standard rat chow containing normal (0.25%) and low (0.025%) Na^+ for two weeks. Animals were anesthetized (thiobutabarbital 120 mg/kg) and surgically prepared for kidney oxygen metabolism and function studies before and after acute intrarenal arterial infusion of candesartan (4.2μg/kg) or the intravenous infusion of CAP (20mg/kg).

Baseline mean arterial pressure and renal blood flow were similar in both dietary groups. Fractional Na^+ excretion and cortical oxygen tension were lower, and renal oxygen consumption was higher in low Na^+ groups. Neither candesartan nor CAP impacted upon arterial pressure. Renal blood flow increased in both groups after candesartan as well as

cortical oxygen tension, on low Na^+ group. Fractional Na^+ excretion was increased and oxygen consumption reduced on low Na^+ group after CAP.

These results suggest that blockade of angiotensin II AT_1 receptors have a major impact upon oxygen delivery during normal and low Na^+, while aldosterone receptors mainly affect oxygen metabolism following two weeks of low Na^+ diet.

Keywords: Low Na^+, kidney, oxygen consumption, hypoxia, angiotensin II, aldosterone.

Joint first author's photo and profile

Daniela Patinha is a Postdoctoral Research Fellow at the University of Exeter. Her work has focused on identifying new therapeutic targets for kidney disease. More specifically, she is interested in oxygen metabolism by the kidney and its impact for the onset and progression of acute and chronic kidney disease as well as its influence on blood pressure control.

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Introduction

Like in other organs, renal oxygen content is determined by delivery and demand. Renal autoregulation maintains renal blood flow (RBF) within a high amplitude of perfusion pressure to maintain a stable glomerular filtration rate (GFR). However, since RBF is not under metabolic control, increasing renal metabolism can have a detrimental impact in tissue integrity if not matched by oxygen delivery. The partial pressure of oxygen (PO_2) is heterogeneous in the renal parenchyma, it is relatively high in the highly perfused renal cortex and decreases towards the scarcely perfused inner renal medulla (Brezis *et al.*, 1984; Brezis *et al.*, 1994b). Low PO_2 is a common observation in end stage renal disease, regardless of its etiology (Hirakawa *et al.*, 2017). We and others have shown that prolonged increased oxygen consumption (QO_2), low PO_2 along the renal parenchyma and consequent renal hypoxia are associated with long-term renal impairment (Welch *et al.*, 2001; Manotham *et al.*, 2004; Matsumoto *et al.*, 2004; Palm *et al.*, 2004; Friederich-Persson *et al.*, 2013; Ow *et al.*, 2014; Franzen *et al.*, 2016; Emans *et al.*, 2018). In physiological conditions, the major determinant of renal oxygen metabolism is Na^+ transport (Brezis *et al.*, 1994a). Increased kidney QO_2 can result from increased tubular Na^+ reabsorption to either compensate for glomerular hyperfiltration (Körner *et al.*, 1994) or oxidative stress-induced reduction of electrolyte transport efficiency (Palm *et al.*, 2003; Welch *et al.*, 2003; Welch *et al.*, 2005). The majority of Na^+ reabsorption occurs in the proximal tubule. Its permeable epithelium allows for paracellular ion flux driven by active Na^+ transport, resulting in a highly energy efficient process (Cohen, 1986; Pei *et al.*, 2016). The downstream segments of the nephron have a tighter epithelium and account for refinement of Na^+ reabsorption with higher energy expenditure to conserve it (Cohen, 1986; O'Neill *et al.*, 2015; Palmer & Schnermann, 2015; Layton *et al.*, 2016; Pei *et al.*, 2016). Consequently, increasing Na^+ reabsorption in downstream segments of the nephron may lead increased total kidney QO_2.

While high Na^+ intake is associated with increased blood pressure and higher cardiovascular risk, there is lack of evidence for the beneficial effects of Na^+ reduction in diet in normotensive conditions (Committee on the Consequences of Sodium Reduction in *et al.*, 2013; Graudal *et al.*, 2014; Kong *et al.*, 2016; Lelli *et al.*, 2018). Reducing Na^+ intake can reduce high blood pressure, thereby decreasing the risk for cardiovascular events and mortality. However, an association of low Na^+ diet with overall increased cardiovascular disease and mortality has been reported (Midgley *et al.*, 1996; Stolarz-Skrzypek *et al.*, 2011; Graudal *et al.*, 2014; O'Donnell *et al.*, 2014; Lelli *et al.*, 2018). Low Na^+ intake results in

compensatory activation of the renin-angiotensin aldosterone system (RAAS) (Graudal *et al.*, 1998; Shao *et al.*, 2013) to the same proportion of Na^+ reduction (Graudal *et al.*, 1998). This can be detrimental for the kidney and would explain the relatively modest reductions in blood pressure observed after Na^+ restriction (Graudal *et al.*, 1998). Accordingly, following two weeks of Na^+ restriction, renal angiotensin II is increased resulting in both glomerular and tubulointerstial fibrosis in rats (Shao *et al.*, 2013). Activation of the RAAS affects both oxygen delivery and consumption in the kidney. Angiotensin II AT_1 receptor activation increases renal vascular tone, aldosterone production, Na^+ reabsorption mainly in the proximal tubule and reactive oxygen species (ROS) production (Onozato *et al.*, 2002; Kobori *et al.*, 2007; Banday & Lokhandwala, 2011; Patinha *et al.*, 2013). In addition, aldosterone signaling through mineralocorticoid receptors increases Na^+ reabsorption in the distal tubule and collecting duct and contributes to ROS formation (Garty, 2000; Miyata *et al.*, 2005; Udwan *et al.*, 2017; Frindt *et al.*, 2018). It would therefore be expected that reduced dietary Na^+ intake would increase kidney QO_2 due to elevated tubular sodium transport (T_{Na+}) to maintain electrolyte balance, which potentially could result in intrarenal tissue hypoxia.

The pioneering work of Stillman and coworkers demonstrated that the tissue PO_2 gradient in the kidney was inverted (Stillman *et al.*, 1994). Following chronic administration of a Na^+ depleted diet, the PO_2 was higher in the renal medulla and lower in the cortical region (Stillman *et al.*, 1994). This reversal of oxygen gradients may compromise kidney function and its long-term consequences are unknown. The mechanistic explanations of these alterations are presently undefined, but it may be speculated that the pronounced cortical hypoxia during Na^+ restriction may be initiated by augmented RAAS signaling in the kidney. Prolonged exposure to increased RAAS could potentially explain the reported detrimental effects of reduced dietary Na^+ intake (Stillman *et al.*, 1994; Midgley *et al.*, 1996; Graudal *et al.*, 2014; O'Donnell *et al.*, 2014). In the present study, we tested the hypothesis that low Na^+ intake results in RAAS activation and a compensatory increase in tubular Na^+ reabsorption. Furthermore, we hypothesized that low Na^+ diet affects renal QO_2 as a consequence of increased RAAS signaling, resulting in altered oxygen availability.

Materials and Methods

Ethical Approval

All experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and

approved by the local Animal Care and Use Committee (approval reference 5.8.18-13971/2018). Animals were housed in temperature, humidity and light controlled environment (12h light-dark cycle), had free access to water and were fed rat chow ad libitum. Investigators understand the ethical principles under which The Journal of Physiology operates and the present studies comply with its animal ethics checklist.

Animal model

Age matched (8-9 weeks-old) male Sprague-Dawley rats (Charles River, Sulzfeldt, Germany) were fed with either normal (0.25%) or low (0.025%) Na^+ diet (R36, Labfor, Stockholm, Sweden) during two weeks. Subsequently, animals in each group were subjected to functional renal assessments under anesthesia (see below) and further subdivided to yield four groups: normal Na^+ diet with intrarenal angiotensin II AT_1 receptor blockade using candesartan (n=13) or mineralocorticoid receptor blockade using canrenoic acid potassium salt (CAP, n=11) or low Na^+ diet with candesartan (n=12) or CAP (n=9).

Acute experimental protocol

The experimental protocol is depicted in Fig 1. Rats were terminally anesthetized with thiobutabarbital (Inactin, 120 mg/kg, *ip*), placed on a heating pad set at $37^{\circ}C$ and tracheostomized. Catheters were placed into the left carotid artery and the left femoral artery and vein for blood pressure measurement, blood collection and saline infusion (Ringer solution, 5 mL/kg/h), respectively. The bladder was catheterized for urinary drainage. The left kidney was exposed by a subcostal flank incision and immobilized in a plastic cup, as previously described (Patinha *et al.*, 2013) and the left ureter was catheterized for urine collection. A catheter was advanced ~1–2 mm into the left renal artery, through a lumbar artery, for precise intrarenal infusion (Lissamine green 10% solution was infused to confirm that catheter placement allowed precise intra-renal infusion with uniform distribution). After 45min of stabilization, measurements were performed before and after drug infusion (Fig 1). Candesartan (AstraZeneca, Mölndal, Sweden; 4.2 μg/kg in 200μL), the angiotensin II AT_1 receptor blocker, was slowly infused during 10 min into the kidney via renal artery, 10 min after the experimental period started, as previously detailed (Patinha *et al.*, 2013). In another set of experiments, the aldosterone antagonist CAP (bolus 20 mg/kg in 200 μL), was infused

intravenously. At the end of the experiments animals were terminated under anesthesia by infusion of saturated potassium chloride or perfusion solution.

Measurements of kidney hemodynamics and oxygen metabolism

Blood pressure was measured using a transducer (model P23dB; Statham Laboratories, Los Angeles, CA, USA) connected to the left carotid artery catheter. Total RBF was measured using an ultrasound flow probe (Transonic Systems Inc., Ithaca, NY, USA) placed around the left renal artery. These parameters were continuously recorded with a Power Lab instrument (AD Instruments, Hastings, UK) connected to a Personal Computer. Glomerular filtration rate was estimated by inulin clearance ([^{3}H]-Inulin, American Radiolabeled Chemicals, St. Louis, MO, USA, 185 kBq/kg/h). Blood gas parameters were measured from samples drawn from the left femoral artery and left renal vein using the iSTAT System (Abbott Laboratories, Abbott Park, IL, USA). The left renal vein blood sample was collected using a heparinized syringe, in a slow manner to avoid sampling from the vena cava.

Urine volume was measured gravimetrically, and urinary Na^+ concentration was quantified by flame spectrophotometry using a multianalyzer (model IL543; Instrumentation Lab, Milan, Italy). Protein concentrations were determined using DC Protein Assay according to manufacturer instructions (BioRad Laboratories, Hercules, CA, USA). Thiobarbituric acid reactive substances (TBARS) were measured in urine and plasma samples. Brifely, 50 μL of standards (malondialdehyde-bis-(diethylacetate; Merck-Schuchardt, Schuchardt, Germany) and samples were heated with 62.5 μL of thiobarbituric acid (0.67%), 97°C, 60 min. Samples were immediately cooled down on ice, precipitated with methanol/NaOH mmol/L (91:9) and centrifuged 3000 rpm, 5min, RT. Fluorescence was determined on the supernatant (excitation 532 nm; emission 553 nm; Tecan Safire^2, Männedorf, Switzerland).

Kidney PO_2 was determined during the clearance period under anesthesia using modified Clark-type oxygen microelectrodes with an outer diameter of the tip of less than 10 μm (Unisense, Aarhus, Denmark). Electrodes were two point calibrated in water saturated with either $Na_2S_2O_5$ ($PO_2 = 0$ mmHg) or air ($PO_2 = 147$ mmHg). The oxygen microelectrodes were positioned using a micromanipulator in order to measure the cortical and medullary PO_2 at 1 and 4 mm depth from kidney surface, respectively.

Tissue collection.

Animals (additional normal n=10 and low n=10 Na$^+$ diet) were anesthetized as previously described and the kidneys extracted after ice-cold saline perfusion. The left kidney was collected for angiotensin II extraction and quantification and the right kidney was dissected and placed in RNA preserving solution (Ambion RNAlater, ThermoFisher Scientific, Waltham, MA, USA) or snap frozen with liquid nitrogen.

Angiotensin II extraction and quantification.

Angiotensin II was extracted from the renal tissue as previously described (Fox *et al.*, 1992). Briefly, the left kidney was removed, weighed, rapidly homogenized in cold methanol (10%, wt/vol) and stored at -80°C. Samples were thawed, centrifuged (10 min, 4°C) and dried overnight in a vacuum centrifuge before extraction. The dried residue was reconstituted in sodium phosphate buffer (50 mmol/L, pH 7.4) and applied to phenyl-bounded solid phase extraction (SPE) column (Discovery® DSC-Ph SPE Tube, Sigma Aldrich) previously conditioned with methanol and equilibrated with water. After washing, angiotensin II was eluted from SPE column with 90% methanol in water and dried under vacuum. Angiotensin II was measured using Angiotensin II EIA kit (Peninsula Laboratories Inc., San Carlos, CA, USA) according to the manufacturer's instructions.

RNA extraction, cDNA Synthesis and quantification.

The kidney cortex sections preserved in RNAlater® were used for total RNA extraction using RNAqueous®-4PCR (Ambion, Waltham, MA, USA). iScript cDNA Synthesis Kit (BioRad Laboratories) was used to synthesize cDNA from total RNA and levels were assessed by real time quantitative PCR using SYBR green PCR reagent (LightCycler® FastStart DNA Master SYBR Green I, Roche, Mannheim, Germany) and the iCycler PCR system (BioRad Laboratories) according to the manufacturer's instructions. Briefly, amplification reactions consisted of 2 μL cDNA, 2 μL Mix (LightCycler® FastStart DNA Master SYBR Green I; Roche), 2.5 μL primers mix and 3.5 μL of H$_2$O. Relative gene expressions were calculated from $2^{-\Delta Ct}$ in relation to β-actin, which was used as housekeeping gene. Primer sequences are detailed in Table 1.

Calculations

Renal vascular resistance (RVR) was calculated as MAP/RBF. The filtration fraction (FF) was calculated using the formula FF=GFR/[RBF*(1-Hematocrite)]. *In vivo* kidney QO_2 was determined from the product of the arterio-venous difference in oxygen content (O_2ct) and RBF. Transported Na^+ (TNa) was calculated as TNa=[PNa]*GFR-[UNa]*Urine flow, where [PNa] and [UNa] are plasma and urine Na^+ concentration, respectively. Fractional Na^+ excretion (FE_{Na}) was estimated from [UNa] * [Pi]/[PNa] * [Ui], where [PNa], [UNa], [Pi] and [Ui] are plasma and urine Na^+ and inulin concentration, respectively.

All chemicals were from Sigma Aldrich (St. Louis, MO, USA) and of highest grade available if not otherwise stated.

Statistical analysis

All statistical analysis were performed using Graph Pad Prism (GraphPad Software, San Diego, CA, USA). Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test. Differences in angiotensin II concentration and mRNA expression of angiotensin II AT1a and AT1b receptors were assessed using unpaired *t* test. For all comparisons, $p < 0.05$ was considered statistically significant. Descriptive statistics are presented as mean±SD.

Results

Effect of Na$^+$ diet

Increased RAAS activity in low Na$^+$ group was confirmed by increased intrarenal tissue concentration of angiotensin II (Table 2). In the kidney cortex, mRNA expression of angiotensin II receptor AT$_{1a}$ was decreased while no alteration was observed in AT$_{1b}$ (Table 2). Body weight (396±8 vs 396±9 g; NS) and kidney weight (1.28±0.04 vs 1.27±0.03 g; NS) were similar between normal and low Na$^+$ groups. Likewise, no difference was observed in MAP and RBF (Figs. 2 and 6), GFR, FF, FE$_K$ and arterial gas status (Tables 3 and 4) between normal and low Na$^+$ groups at baseline. Arterial blood electrolytes, hemoglobin, hematocrit and pH, TBARS and protein excretion as well as plasma TBARS were also similar at baseline between normal and low Na$^+$ diet groups (Table 3 and 4). Oxygen delivery (Figs. 3A and 7A) was similar between normal and low Na$^+$ groups at baseline; however QO$_2$ (Figs. 3B and 7B) and fractional O$_2$ extraction (Figs. 3C and 7C) were more than 50% higher in low Na$^+$ group when compared with normal Na$^+$ at baseline. T$_{Na}$ (Fig 4B and 8B) was unaltered while FE$_{Na}$ (Figs. 4A and 8A) and T$_{Na}$/QO$_2$ (Figs. 4C and 8C) were more than 30% lower in low Na$^+$ groups. This coincided with more than 14% lower cortical (Figs. 5 A and 9A) and more than 25% higher medullary PO$_2$ (Figs. 5B and 9B) in the low Na$^+$ groups at baseline when compared with normal Na$^+$ at baseline.

Contribution of RAAS - Effect of angiotensin II AT$_1$ receptor blockade

Candesartan was used to block angiotensin II AT$_1$ receptors; Infusion of candesartan directly into the left kidney via renal artery did not alter MAP (Fig. 2A), GFR, arterial blood gas status, electrolytes, hemoglobin, hematocrit or pH (Table 3) in either dietary group. Intrarenal infusion of candesartan decreased FF in the normal Na$^+$ group but was without significant effect on this variable in the low Na$^+$ group (Table 3). Following candesartan infusion FE$_K$ and TBARS excretion were increased in the low Na$^+$ group but was without significant effect on these variables in the normal Na$^+$ group (Table 3). After candesartan infusion proteinuria was increased in both groups (Table 3). RBF (Fig. 2B) and renal oxygen delivery (Fig. 3A) had more than 35% increase in both normal and low Na$^+$ groups following intrarenal candesartan infusion. QO$_2$ (Fig. 3B) and fractional oxygen extraction (Fig. 3C) were more than doubled following candesartan infusion in normal Na$^+$ with no significant alteration in

low Na$^+$ group. Intrarenal candesartan infusion more than doubled FE$_{Na}$ in both normal and low Na$^+$ groups (Fig 4A). T$_{Na}$ (Fig. 4B) was not significantly altered following candesartan infusion in both groups and T$_{Na}$/QO$_2$ (Fig. 4C) was more than 40% decreased following candesartan infusion in the normal Na$^+$ group without significant effect in the low Na$^+$ group. Following candesartan infusion, PO$_2$ in the renal cortex of low Na$^+$ group significantly increased to levels comparable with the normal Na$^+$ group at baseline, without altering renal medulla PO$_2$ (Figs. 5A and B).

Contribution of RAAS - Effect of aldosterone mineralocorticoid receptor blockade

CAP was used to block aldosterone receptors; Following CAP infusion no effect was observed on MAP, RBF (Fig. 6), GFR or FF (Table 4) in either dietary group when compared with baseline levels. Similarly, arterial blood gas status, electrolytes, hemoglobin, hematocrit or pH (Table 4) remained unaltered following CAP infusion in normal and low Na$^+$ groups. Interestingly, FE$_K$ was increased by approximately 7-fold after CAP in the low Na$^+$ group with no significant alteration observed in the normal Na$^+$ group (Table 4). Urinary TBARS and protein excretion as well as plasma TBARS were not altered after CAP infusion in both groups. After CAP infusion no significant alteration was observed on oxygen delivery (Fig 7A) in either group; However, QO$_2$ (Fig. 7B) was reduced by 37% in low Na$^+$ group to levels that were comparable with the normal Na$^+$ group at baseline. Fractional oxygen extraction (Fig. 7C) was significantly reduced by 42% only on low Na$^+$ group. Following CAP infusion, no alteration was observed on FE$_{Na}$ (Fig. 8A) in the normal Na$^+$ group but it increased FE$_{Na}$ to levels that were comparable with the normal Na$^+$ group at baseline. No effect was observed on transported Na$^+$ (Fig. 8B) or T$_{Na}$/QO$_2$ on both groups after CAP. Finally, no significant effect was observed on tissue PO$_2$ in the cortex and medulla following CAP infusion in both normal and low Na$^+$ groups (Fig. 8).

Discussion

The main novel finding of the present study was that reducing Na^+ intake for two weeks to the extent that intrarenal angiotensin II is increased, in normotensive conditions, increased renal QO_2 that was completely normalized by mineralocorticoid receptor blockade. Furthermore, acute blockade of angiotensin II AT_1 receptor restored cortical PO_2 by improving oxygen delivery. Overall, these data suggest that heightened aldosterone signaling promotes an increase in renal QO_2 in the low Na^+ group. Interestingly, the normalization of cortical PO_2 in the low Na^+ group was only achieved during intrarenal angiotensin II AT_1 receptor blockade. This was mediated through an increase in oxygen delivery as opposed to alteration of renal QO_2, indicating that increased oxygen delivery regulation of renal PO_2 supersedes metabolic demand in this context.

Urinary Na^+ excretion is continuously adjusted to match intake, thus preserving osmotic balance. Accordingly, in the present study, normal and low Na^+ groups had similar plasma electrolyte concentration, MAP, RBF and GFR, but the low Na^+ group had lower FE_{Na} indicating that adjustment to low Na^+ intake occurred mainly within the tubular segments. The otherwise healthy kidneys of animals in the low Na^+ group exhibited altered oxygen homeostasis with increased QO_2 and decreased Na^+ transport efficiency. This is possibly associated with a redistribution of Na^+ reabsorption along the tubule (Udwan *et al.*, 2017; Frindt *et al.*, 2018), and consequent increase workload in less efficient segments of the nephron where the epithelia is tighter and the energy demand is higher when compared with the proximal tubule (Cohen, 1986; Palmer & Schnermann, 2015; Layton *et al.*, 2016). Indeed, during low Na^+ diet, Na^+ reabsorption is increased not only in the proximal tubule but also in the distal tubule/connecting tubule and collecting duct (Udwan *et al.*, 2017; Frindt *et al.*, 2018).

The observed increase in renal QO_2 may have long-term detrimental effects. Friederich-Persson showed that increasing kidney metabolism by increasing mitochondrial QO_2 leads to tubulointerstitial damage, proteinuria and infiltration of inflammatory cells (Friederich-Persson *et al.*, 2013). Also, low renal PO_2 precedes oxidative stress and proteinuria in diabetic mice (Franzen *et al.*, 2016). Likewise, reduced cortical PO_2 precedes renal injury in angiotensin II-dependent hypertension (Emans *et al.*, 2016). Indicating that the presently observed increased QO_2 and reduced cortical PO_2 induced by low Na^+ diet may lead to renal injury. Accordingly, tubulointerstitial fibrosis (Shao *et al.*, 2013) and proteinuria have been

documented in healthy animals fed a low Na^+ diet for two weeks. Furthermore, cortical hypoxia measured using BOLD-MRI was reported to be a prognostic marker in the progression of chronic kidney disease (Zhou *et al.*, 2018). In agreement with the previous report by Stillman *et al* (Stillman *et al.*, 1994), we also observed increased medullary PO_2 in the Na^+ restricted animals. It may only be speculated that increased proximal Na^+ reabsorption reduced medullary tubular Na^+ load resulting in reduced amount of active reabsorption by the medullary nephron segments. Indeed, reduced Na^+ intake for four weeks significantly reduces medullary thick ascending limb size (Stillman *et al.*, 1994), possibly indicating reduced workload in this part of the segment. This would imply that reduced Na^+ intake reduces the metabolic activity in the renal medulla and therefore also reduces QO_2 and increases PO_2 in this region of the kidney. Contrary, the increased reabsorption in cortical nephron segments would increase QO_2 and cause hypoxia. It has also been shown in normotensive humans with reduced Na^+ intake that the increased medullary oxygenation is due to enhanced proximal tubular Na^+ reabsorption resulting in reduced Na^+ delivery and workload to the distal, less efficient, tubular segments (Pruijm *et al.*, 2010).These alterations could mechanistically explain the abolished cortico-medullary PO_2 gradient during reduced Na^+ intake.

Low Na^+ diet increases intrarenal activity of the RAAS (Schmid *et al.*, 1997; Graudal *et al.*, 1998; Shao *et al.*, 2013), confirmed in the present study by increased tissue angiotensin II concentration in low Na^+ group. Low Na^+ diet decreased AT_{1a} receptor expression but did not affect AT_{1b} receptor expression, this differential regulation is in good agreement with previously reported data (Schmid *et al.*, 1997). The AT_{1a} receptor is critical for Na^+ handling and blood pressure control (Chen *et al.*, 1997; Oliverio *et al.*, 2000), its downregulation may be important to prevent blood pressure increase in the context of high angiotensin II, while the AT_{1b} receptors may be more relevant for Na^+ conservation in low Na^+ conditions. Indeed, during low Na^+, AT_{1b} receptors mediate RAAS-dependent stimulation of aldosterone production in the adrenal gland (Kitami *et al.*, 1992; Schmid *et al.*, 1997). The Angiotensin (1-7)/Mas receptor axis may also be an important contributor for Na^+ balance in low Na^+ conditions. Indeed, the diuretic and natriuretic actions of angiotensin 1-7 are enhanced following low Na^+ diet, although the blockade of the Mas receptor had no effect on basal Na^+ excretion (O'Neill *et al.*, 2013; O'Neill *et al.*, 2017). Rearrangements of Na^+ reabsorption along the tubule during low Na^+ diet are mainly attributed to proximal tubule, late distal tubule/connecting tubule and collecting duct adaptations (Udwan *et al.*, 2017; Frindt *et al.*,

2018). In these tubular segments, NHE_3 and epithelial Na^+ channel (ENaC) are major transporters (Palmer & Schnermann, 2015) and under strict regulation by the RAAS (Geibel *et al.*, 1990; Garty, 2000; Peti-Peterdi *et al.*, 2002; Banday & Lokhandwala, 2011). We therefore tested the effect of angiotensin II AT_1 and aldosterone mineralocorticoid receptor blockade on oxygen metabolism after two weeks of low Na^+ diet.

Mean arterial pressure remained stable in both groups after angiotensin II AT_1 receptor blockade, confirming that candesartan infusion into the kidney was accurate and that the observed effects were blood pressure independent. RBF and oxygen delivery were increased to a similar extent and GFR unaltered, after angiotensin II AT_1 receptor blockade in both groups. Intriguingly, in the normal Na^+ group this was accompanied by an increase in QO_2 while no alteration was observed in the low Na^+ group. Also, FE_{Na} was increased in both groups. Angiotensin II AT_1 receptor activation directly influences Na^+ reabsorption namely by 1) stimulation Na^+/K^+ ATPase pump in the basolateral membrane (Yingst *et al.*, 2004); 2) stimulation Na^+/H^+ exchange in the luminal membrane, mainly by activating NHE_3 in the proximal tubule (Geibel *et al.*, 1990); and 3) Na^+/HCO_3^- co-transport in the basolateral membrane (Geibel *et al.*, 1990). Additionally, the vasoconstrictor effect on efferent arteriole reduces the blood flow to peritubular capillaries thus increasing Na^+ net reabsorption, especially in the proximal tubule. Hence, we propose that angiotensin II AT_1 receptor blockade mainly affected Na^+ reabsorption in the proximal region of the tubule of normal Na^+ group, promoting higher downstream delivery where Na^+ reabsorption is less effective. This premise is in line with the effect of inhibiting angiotensin converting enzyme that rapidly reduced Na^+ reabsorption in the proximal tubule mainly by reducing NHE_3, without altering blood pressure or GFR (Leong *et al.*, 2006). Our findings also in part corroborate the mathematical modeling predictions of NHE_3 inhibition resulting in lower Na^+ transport efficiency in the otherwise healthy kidney of rats (Layton *et al.*, 2016). The effect of Angiotensin II AT_1 receptor activation in the stimulation Na^+ reabsorption in the cortical collecting duct via ENaC (Peti-Peterdi *et al.*, 2002) may also contribute, to a smaller extent, to the observed effects. The present results refer to the whole kidney, one cannot exclude that there might be heterogeneity in these parameters at the nephron level (Källskog *et al.*, 1976; Inscho *et al.*, 1997)].

The lack of effect of angiotensin II AT_1 receptor blockade in QO_2 of the low Na^+ group may also be an extension of the previously stated premise. In the low Na^+ group, the downstream Na^+ reabsorption was already altered at baseline (Udwan *et al.*, 2017; Frindt *et al.*, 2018) with

increased QO_2 and lower tubular transport efficiency, as presently observed. Indicating that angiotensin II AT_1 receptor is not the only contributor to the overall increased QO_2 in low Na^+ diet. Importantly, the increase of renal oxygen supply maintained cortical PO_2 in the low Na^+ group and increased it in the low Na^+ group to similar levels of baseline normal Na^+ group, restoring the cortico-medulary gradient. The diverse acute effects on renal oxygen metabolism in response to angiotensin II AT_1 receptor blockade between normal and low Na^+ fed animals may also be, to some extent, associated with morphological changes in the outer medulla (Stillman *et al.*, 1994) or with increased angiotensin II availability for activation of AT_2 receptors.

Similar to what was observed during angiotensin II receptor blockade, aldosterone mineralocorticoid receptor blockade did not alter MAP or GFR, when compared with baseline in both diets. However, although RBF and oxygen delivery were not altered in both diets after mineralocorticoid blockade, QO_2 and fractional oxygen extraction were reduced along with significantly increased FE_{Na} in low Na^+ group when compared with respective baseline, with no effect on renal PO_2. Suggesting that aldosterone mineralocorticoid receptor activation is in part responsible for higher low Na^+-induced QO_2 in the kidney. Indeed, aldosterone mineralocorticoid receptor activation in connecting and collecting duct is of major importance for Na^+ conservation during low Na^+ diet as eliminating it will result in Na^+ waste in mice (Ronzaud *et al.*, 2007). Plasma aldosterone increases in Na^+ depleted rats after seven days along with ENaC activity (Frindt *et al.*, 2018). Which is in good agreement with the hypothesis that low Na^+ diet shifts Na^+ reabsorption to less effective sections of the tubule, with higher energy requirements to reabsorb Na^+ resulting in higher QO_2, as presently observed.

High Na^+ is generally associated with increased blood pressure and higher risk for cardiovascular disease (IntersaltCooperativeReseachGroup, 1988; Mente *et al.*, 2014). Accordingly, in hypertensive individuals in which reducing Na^+ intake results in reduced blood pressure, cardiovascular health benefits are achieved (Cook *et al.*, 2007; Adler *et al.*, 2014). However, the debate on how much Na^+ should be removed from the diet and the ideal Na^+ intake is still ongoing (Cook *et al.*, 2020). Indeed, although high Na^+ intake has been demonstrated in numerous studies to have negative cardio-renal effects, drastically reducing Na^+ intake is also somewhat surprisingly associated with worsen outcome (IntersaltCooperativeReseachGroup, 1988; Midgley *et al.*, 1996; Stolarz-Skrzypek *et al.*, 2011; Graudal *et al.*, 2014; Mente *et al.*, 2014; O'Donnell *et al.*, 2014; Adamovich *et al.*,

2017). The association of Na^+ intake and mortality seems to have a U-shaped curve with both low and high Na^+ diets rendering increased cardiovascular events and mortality. Although, it cannot be excluded that the increased risk observed in the low Na^+ diet participants may be influenced by pre-existing comorbidities such as diabetes and history of cardiovascular disease (O'Donnell *et al.*, 2014), which requires further investigation. While the adverse effects of high Na^+ intake seem to be due to blood pressure effects, the increased risk associated with low Na^+ intake was unaffected by adjustment for blood pressure, suggesting blood pressure independent mechanisms (O'Donnell *et al.*, 2014). Hence, a reduction in Na^+ intake has clear beneficial cardiovascular effects when resulting in lower arterial blood pressure, but a profound further reduction of Na^+ intake beyond this point is associated with increased cardiovascular events. In the present study a 90% decrease in Na^+ intake increased kidney QO_2 and induced cortical hypoxia, which could provide an explanation for why in some cases reducing Na^+ intake, without concomitantly reduced arterial blood pressure, is associated with poor outcomes.

In conclusion, the present study demonstrates that low Na^+ intake in normotensive conditions is associated with RAAS activation and increased kidney QO_2 and cortical hypoxia. Furthermore, low Na^+ diet-induced increased QO_2 is mainly associated with increased aldosterone-mediated Na^+ transport. Given the present results, in the absence of high blood pressure, dietary Na^+ restriction needs to be carefully monitored as it may be detrimental for the kidney. Future experiments are necessary to elucidate the mechanisms operating. An in-depth study of the angiotensin (1-7)/Mas receptor axis on oxygen metabolism following low Na^+ diet is highly relevant in this context. Moreover, longitudinal studies to follow-up renal function and PO_2 along with blood pressure during Na^+ reduction in diet will expand our understanding of its effects over time.

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Additional Information

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Competing interests

The authors declare that they have no competing interests.

Author contributions

D.P. and F.P. conceived and designed the work; D.P., C.C., P.P., L.P., J.O. and A.F. conducted the acquisition of data; D.P. performed analysis and D.P., C.C. and M. P-F interpretation of data for the work; D.P. and C.C drafted the manuscript; D.P., C.C., P.P., A.F., L.P., M.P-F., J.O. and F.P. revised and approved the final version.

Funding

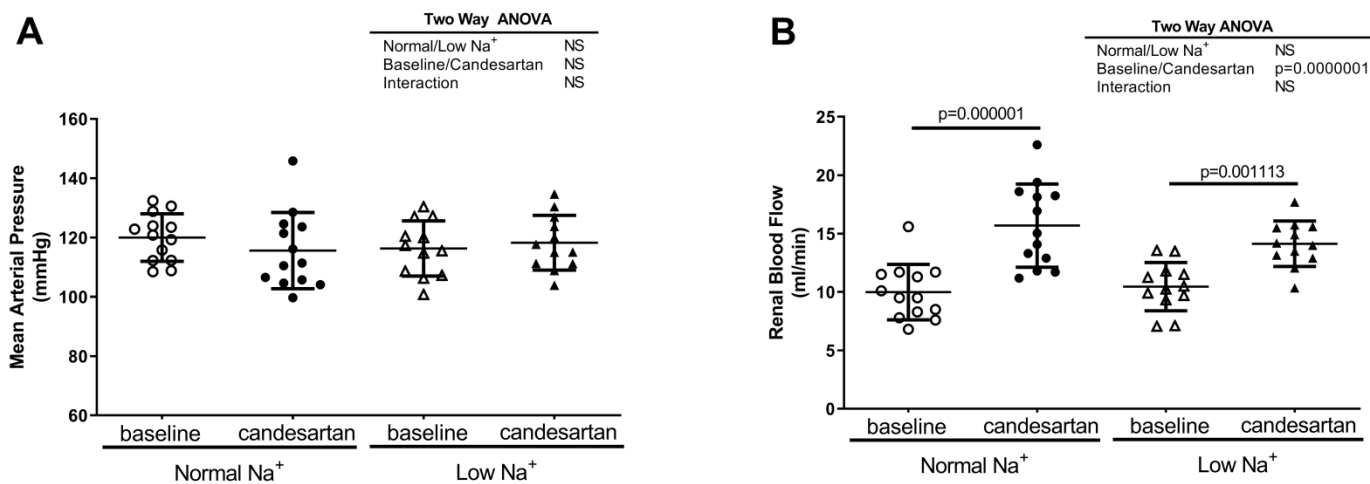**Figure 1.** Schematic representation of the experimental protocol.

Figure 2. Mean arterial pressure (A) and renal blood flow (B) in healthy animals fed normal (n=13) or low (n=12) Na$^+$ diet, before (baseline) and after angiotensin II AT$_1$ receptor blockade using candesartan (4.2 μg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
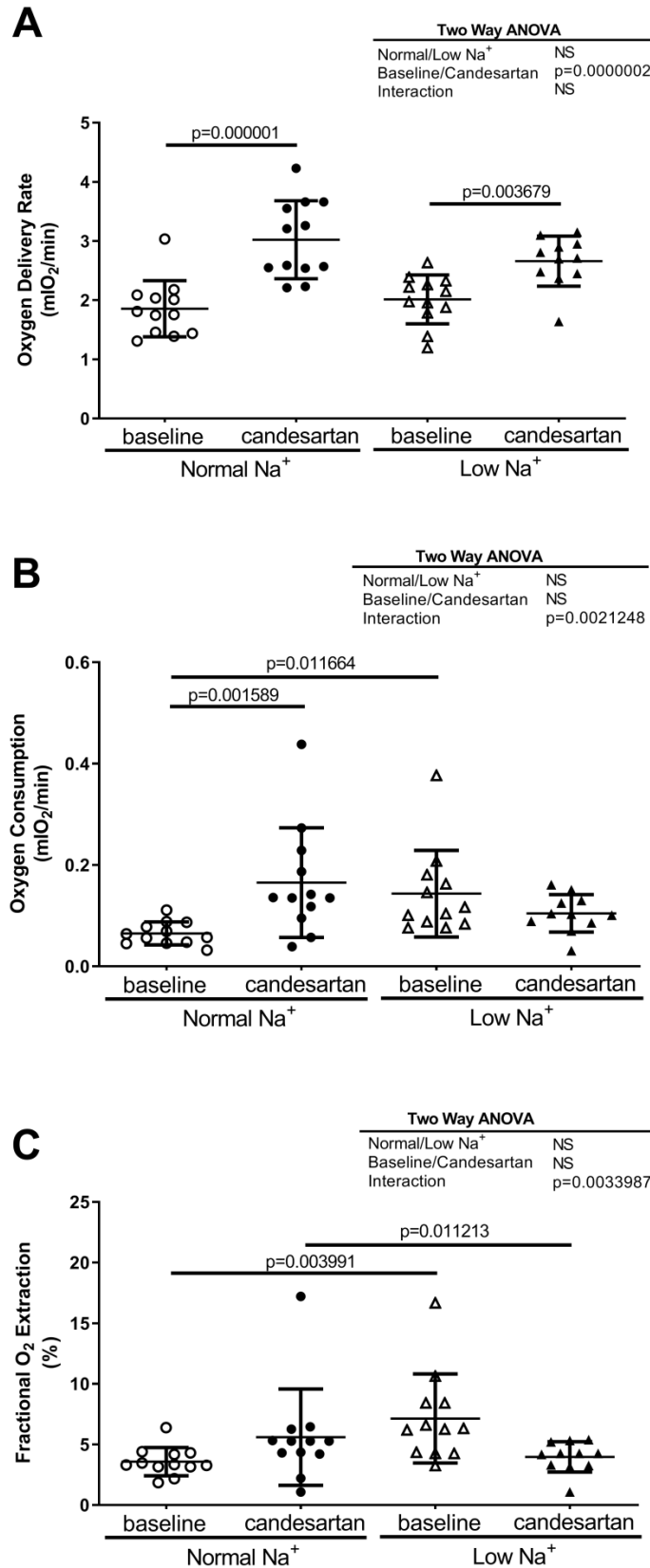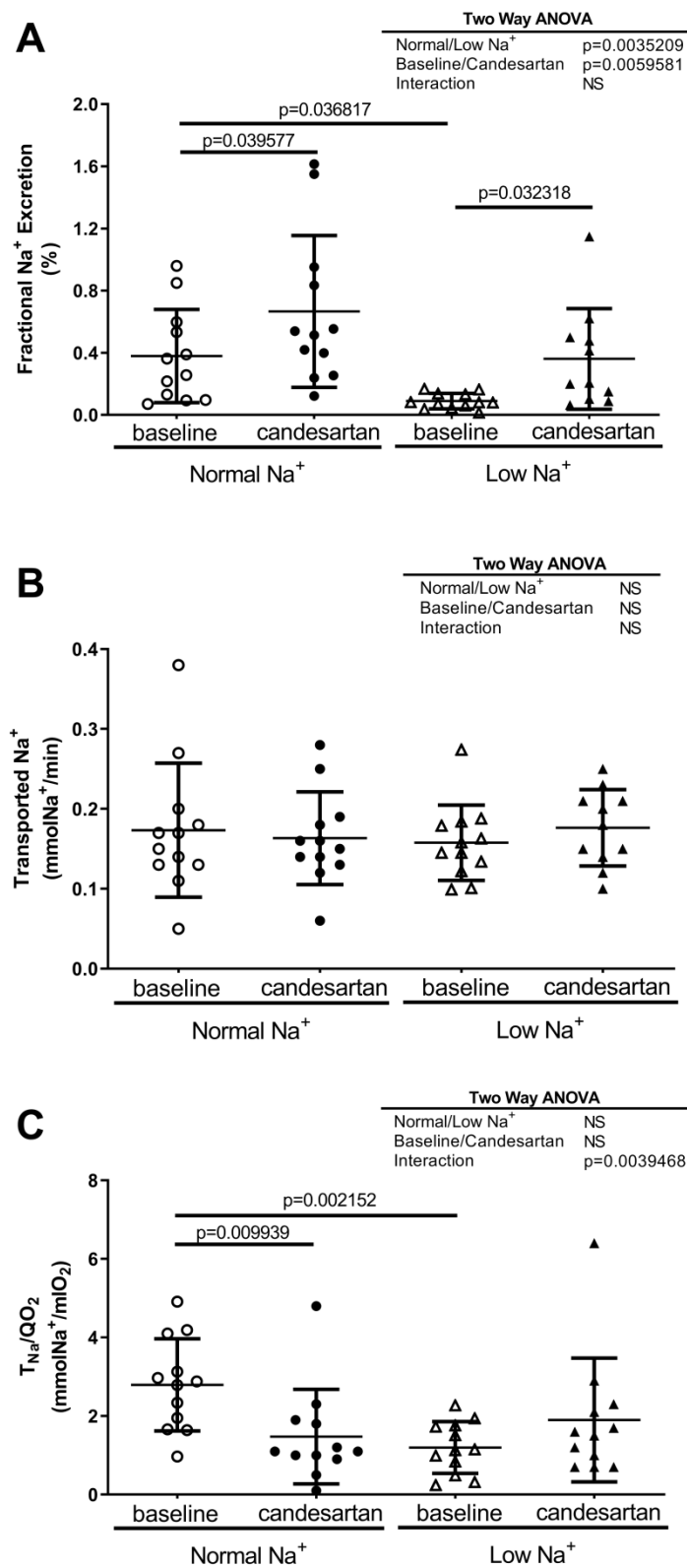
A

Two Way ANOVA

Normal/Low Na$^+$	NS
Baseline/Candesartan	p=0.0000002
Interaction	NS

p=0.000001

p=0.003679

Oxygen Delivery Rate (mlO$_2$/min)

baseline | candesartan | baseline | candesartan
Normal Na$^+$ | Low Na$^+$

B

Two Way ANOVA

Normal/Low Na$^+$	NS
Baseline/Candesartan	NS
Interaction	p=0.0021248

p=0.011664

p=0.001589

Oxygen Consumption (mlO$_2$/min)

baseline | candesartan | baseline | candesartan
Normal Na$^+$ | Low Na$^+$

C

Two Way ANOVA

Normal/Low Na$^+$	NS
Baseline/Candesartan	NS
Interaction	p=0.0033987

p=0.011213

p=0.003991

Fractional O$_2$ Extraction (%)

baseline | candesartan | baseline | candesartan
Normal Na$^+$ | Low Na$^+$

Figure 3. Oxygen delivery rate (A), oxygen consumption (B) and fractional oxygen extraction (D) in healthy animals fed normal (n=12) or low (n=11-12) Na$^+$ diet, before (baseline) and after angiotensin

II AT$_1$ receptor blockade using candesartan (4.2 μg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
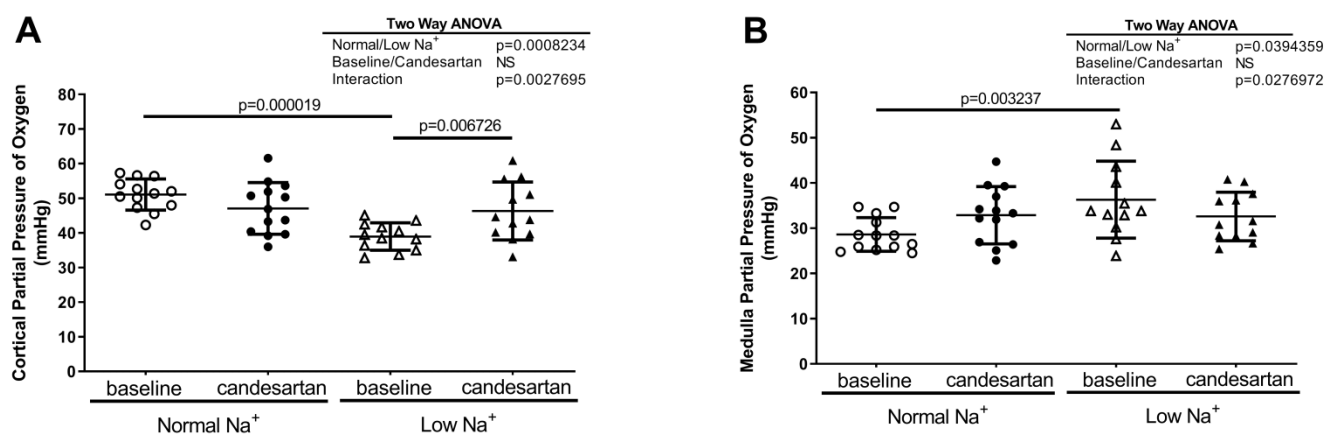
Figure 4. Fractional Na$^+$ excretion (A), transported Na$^+$ (B) and TNa/QO2 (C) in healthy animals fed normal (n=12) or low (n=11-12) Na$^+$ diet, before (baseline) and after angiotensin II AT$_1$ receptor blockade using candesartan (4.2 µg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
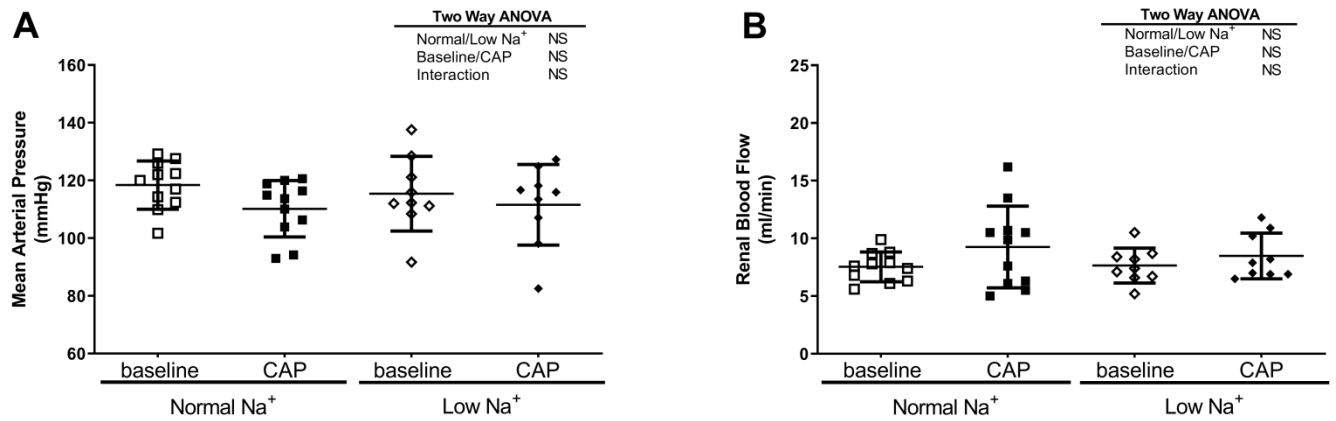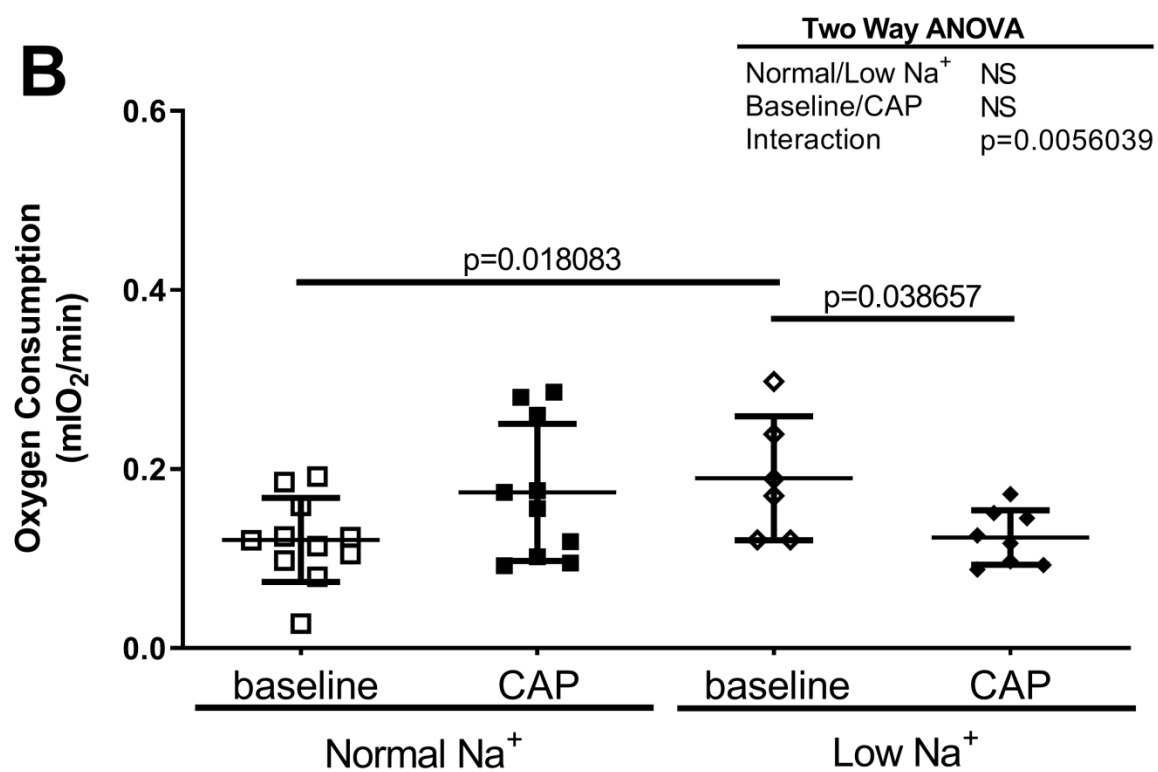

Figure 5. Cortical (A) and medullary (B) partial pressure of oxygen in healthy animals fed normal (n=13) or low (n=12) Na$^+$ diet, before (baseline) and after angiotensin II AT$_1$ receptor blockade using candesartan (4.2 µg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
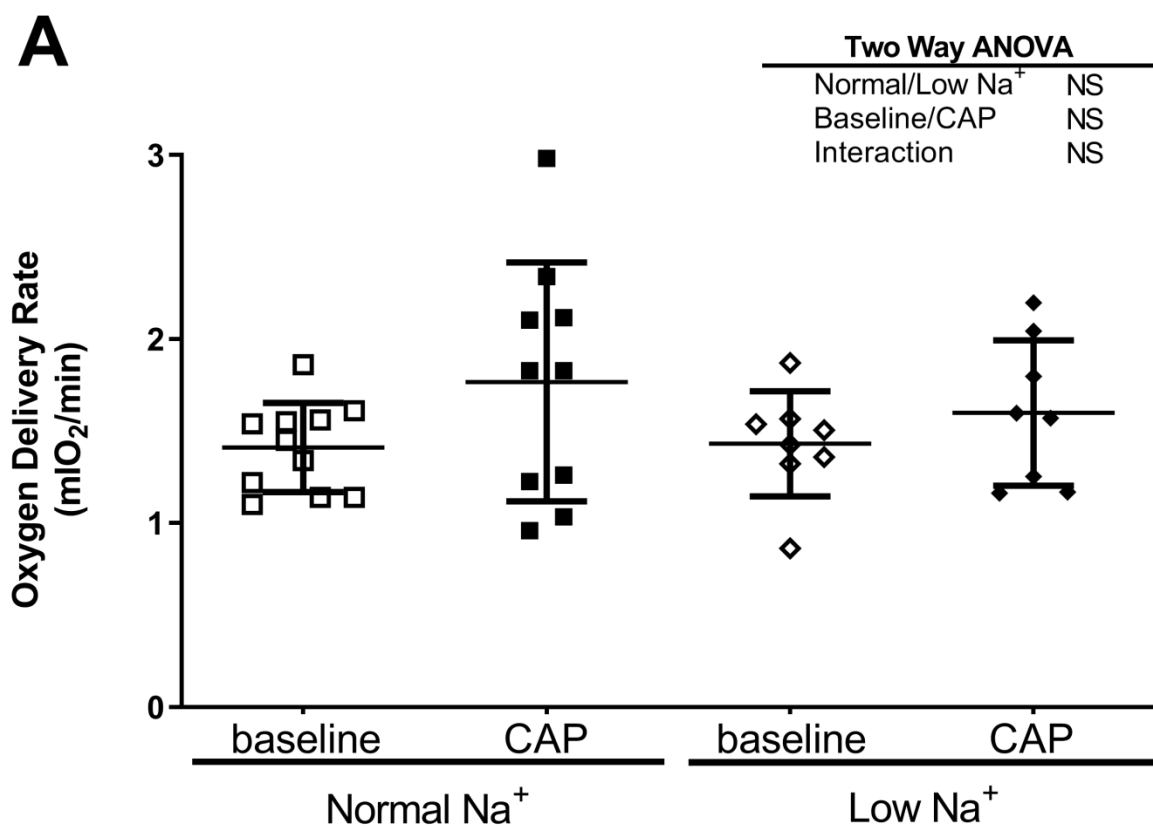
Figure 6. Mean arterial pressure (A) and renal blood flow (B) in healthy animals fed normal (n=11) or low (n=9) Na$^+$ diet, before (baseline) and after aldosterone mineralocorticoid receptor blockade using CAP (20 mg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
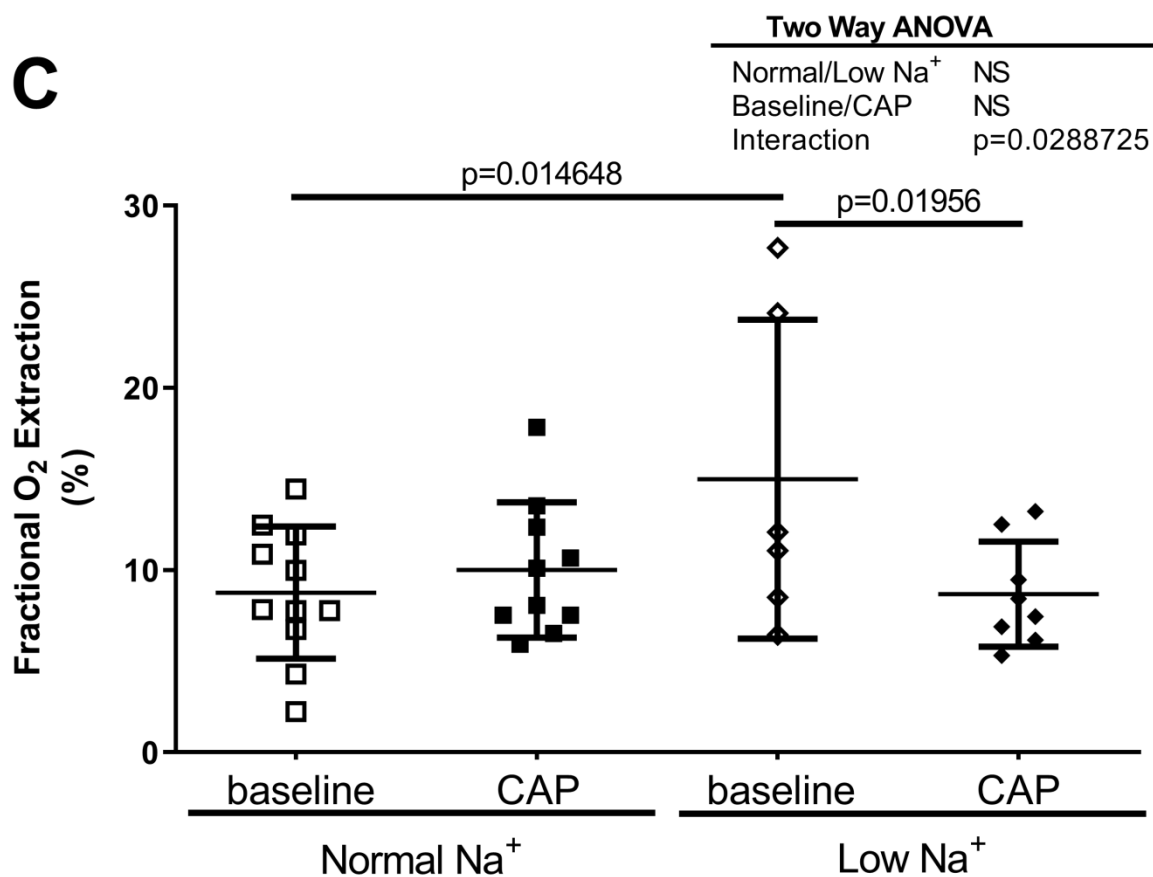
Figure 7. Oxygen delivery rate (A), oxygen consumption (B) and fractional oxygen extraction (D) in healthy animals fed normal (n=10-11) or low (n=6-8) Na$^+$ diet, before (baseline) and after aldosterone mineralocorticoid receptor blockade using CAP (20 mg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
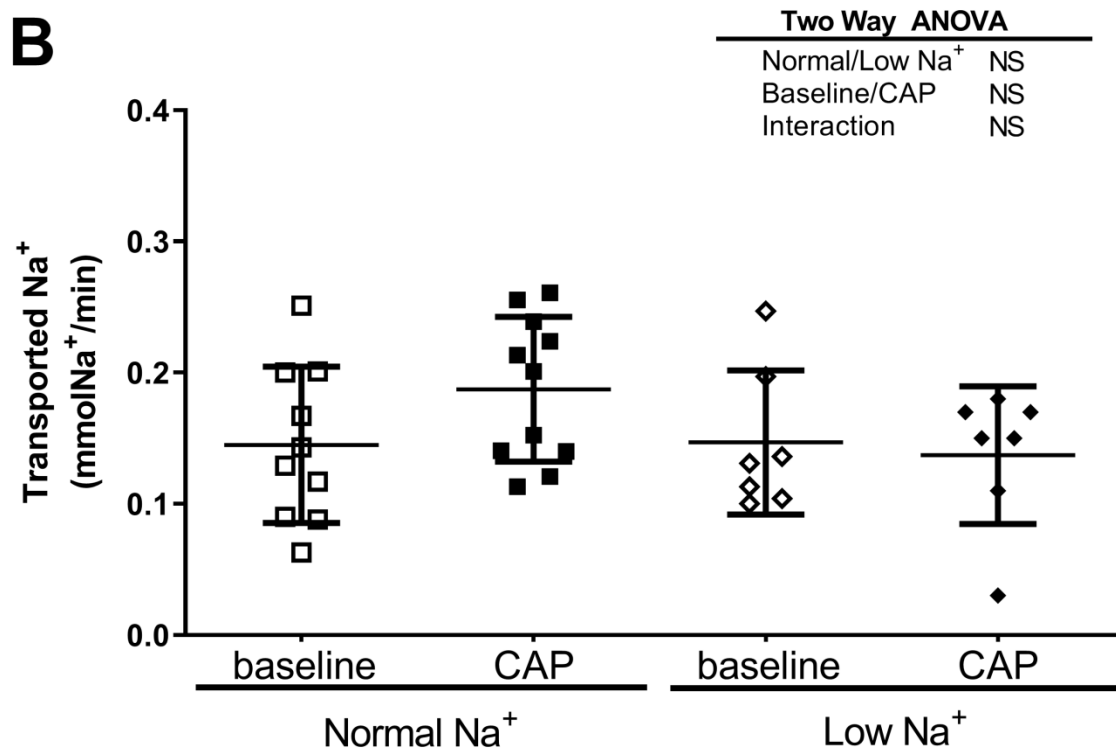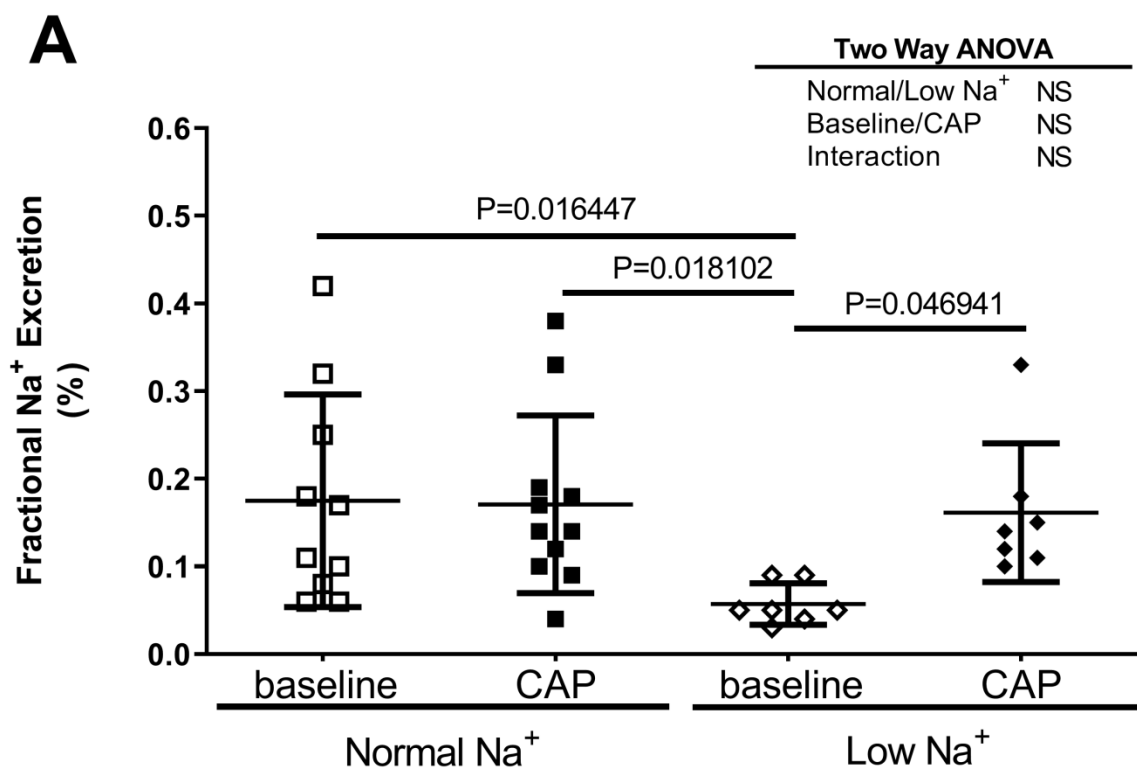
Figure 8. Fractional Na$^+$ excretion (A), transported Na$^+$ (B) and TNa/QO$_2$ (C) in healthy animals fed normal (n=10-11) or low (n=6-7) Na$^+$ diet, before (baseline) and after aldosterone mineralocorticoid receptor blockade using CAP (20 mg/kg). Results expressed as mean ± SD, $p<0.05$ was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
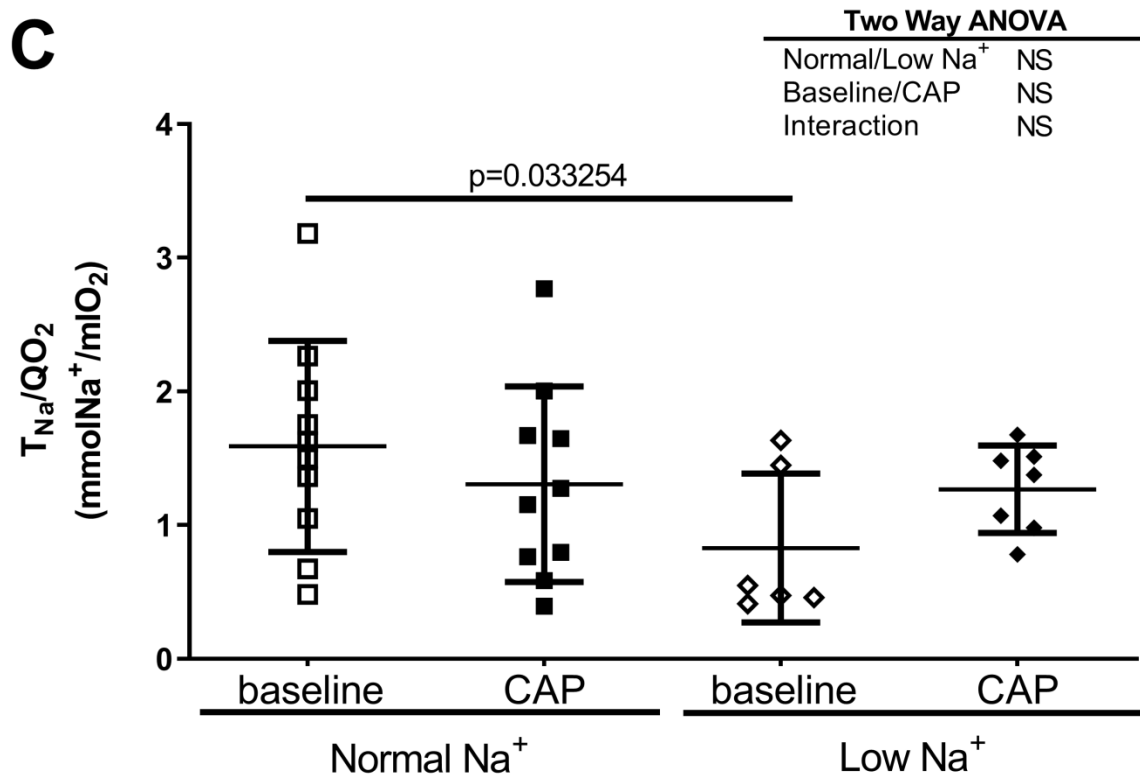
Figure 9. Cortical (A) and medullary (B) partial pressure of oxygen in healthy animals fed normal (n=9-11) or low (n=9) Na$^+$ diet, before (baseline) and after aldosterone mineralocorticoid receptor blockade using CAP (20 mg/kg). Results expressed as mean ± SD, p<0.05 was considered statistically significant. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.
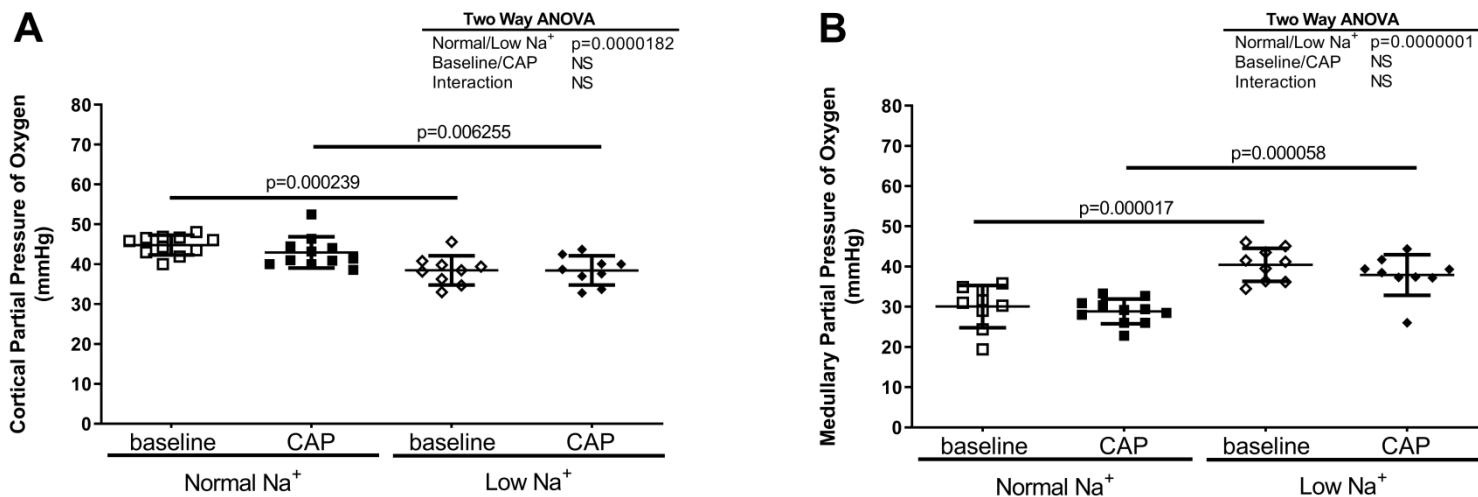
Table 1. RT-PCR primer sequences.

Gene	Sense (5'-3')	Antisense (5'-3')
Angiotensin II AT$_{1A}$ receptor	TCCCTGAGTTAACATATGAGAG	TGTTTTTCTGGGTTGAGTTG
Angiotensin II AT$_{1B}$ receptor	CGGTGCATTTTAAATAGTGTC	AATATGTAATTGTGCCTGCC
β-actin	AAGACCTCTATGCCAACAC	TGATCTTCATGGTGCTAGG

Table 2. Intrarenal tissue angiotensin II concentration (n=5) and relative mRNA expression of angiotensin II AT_{1a} (n=6-9) and AT_{1b} (n=5-6) receptors, in kidney cortex.

	Normal Na^+	Low Na^+
Angiotensin II (fmol/g)	22.0±6.7	32.8±8.0*
AT_{1a} (RT expression/β-actin)	1.07±0.43	0.41±0.14*
AT_{1b} (RT expression/β-actin)	1.08±0.50	2.03±1.12

Results expressed as mean ± SD, * denotes $p < 0.05$ versus normal Na^+. Differences between groups were assessed using unpaired *t* test.

Table 3. Glomerular filtration rate, filtration fraction, urinary electrolyte handling parameters and arterial blood gas status in animals fed normal (n=12-13) or low (n=12) Na^+ diet during baseline and after candesartan.

	Normal Na^+		Low Na^+	
	Baseline	Candesartn	Baseline	Candesartan
GFR (mL/min)	1.3±0.6	1.2±0.4	1.1±0.4	1.3±0.3
FF (%)	23.1±12.1	13.6±5.0*	17.0±9.1	15.3±4.5
FE_K (%)	33.4±12.2	43.6±16.9	22.8±10.6	51.5±14.3*#
Urinary TBARS (pmol/h)	0.18±0.19	0.22±0.27	0.41±0.34	0.81±0.55*#
Urinary protein (mg/h)	2.7±0.6	4.1±1.4*	3.0±0.8	4.5±1.7*

Plasma Na$^+$ (mmol/L)	138.8±1.6	140.2±2.2	140.0±1.7	140.3±2.2
Plasma K$^+$ (mmol/L)	4.6±0.3	4.0±0.3#	5.1±0.8*	4.1±0.2#
Hemoglobin (g/L)	151±8	150±6	151±7	147±7
Hematocrit (%)	45±2	44±2	44±2	43±2
Blood pH	7.40±0.02	7.42±0.02*	7.39±0.03	7.41±0.03#
Blood PO$_2$ (mmHg)	70±5	70±4	74±8	70±6
Blood PCO$_2$ (mmHg)	50±4	46±4*	48±4	44±3#
Plasma TBARS (nmol/L)	4.1±1.3	6.4±7.6	3.1±1.7	3.3±1.7

GFR - glomerular filtration rate; FF - filtration fraction; FE$_K$ - fractional urinary excretion of K$^+$; PO$_2$ – Partial pressure of O$_2$; PCO$_2$ – Partial pressure of CO$_2$. Results expressed as mean ± SD, * denotes $p < 0.05$ versus baseline normal Na$^+$; # denotes $p < 0.05$ versus baseline low Na$^+$. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.

Table 4. Glomerular filtration rate, filtration fraction, urinary electrolyte handling parameters and arterial blood gas status in animals fed normal or low Na^+ diet during baseline and after CAP.

	Normal Na^+		Low Na^+	
	Baseline	CAP	Baseline	CAP
GFR (mL/min)	1.2±0.4	1.4±0.4	1.0±0.4	1.0±0.4
FF (%)	28.8±10.1	29.8±12.7	24.8±6.7	20.5±6.9
FE_K (%)	18.7±14.8	35.3±31.3	15.4±13.0	44.2±28.9*#
Urinary TBARS (pmol/h)	0.35±0.17	0.61±0.32	0.65±0.47	0.78±0.59*
Urinary protein (mg/h)	2.21±0.97	2.14±0.91	2.54±0.71	2.27±0.75
Plasma Na^+ (mmol/L)	137.8±0.8	138.7±2.6	137.4±1.8	138.3±1.7
Plasma K^+ (mmol/L)	4.4±0.3	4.5±0.3	4.9±0.4*	4.8±0.5
Hemoglobin (g/L)	148±13	145±9	154±11	147±12
Hematocrit (%)	44±4	43±3	45±3	43±4
Blood pH	7.40±0.03	7.42±0.03	7.40±0.02	7.41±0.01
Blood PO_2 (mmHg)	66±4	70±6	69±4	71±7*
Blood PCO_2 (mmHg)	53±4	49±2*	49±4*	46±2*
Plasma TBARS (nmol/L)	2.64±0.74	2.14±0.55	2.28±0.32	2.21±0.44

GFR - glomerular filtration rate; FF - filtration fraction; FE_k - fractional urinary excretion of K^+; PO_2 – Partial pressure of O_2; PCO_2 – Partial pressure of CO_2. Results expressed as mean ± SD, * denotes $p<0.05$ versus baseline normal Na^+; # denotes $p<0.05$ versus baseline low Na^+. Differences between groups were assessed using two way ANOVA followed by uncorrected Fisher's LSD test.