# Nox4

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The NADPH oxidase Nox4 is a hydrogen peroxide (H2O2)-producing enzyme, with the highest expression in the kidney. As the kidney is involved in volume and blood pressure control through sodium handling, we set out to determine the impact of a low sodium diet on these parameters in WT and Nox4-/- mice. Nox4 expression in the murine kidney was restricted to the proximal tubule. Nevertheless, low-sodium-induced weight loss and sodium sparing function was similar in WT and Nox4-/- mice, disputing an important function of renal Nox4 in sodium handling. In contrast, a low sodium diet resulted in a reduction in systolic blood pressure in Nox4-/- as compared to WT mice. This was associated with a selectively lower pressure to heart-rate ratio, as well as heart to body weight ratio. In general, a low sodium diet leads to activation of sympathetic tone and the renin angiotensin system, which subsequently increases peripheral resistance. Our observations suggest that the control by this system is attenuated in Nox4-/- mice, resulting in lower blood pressure in response to low sodium.

NADPH oxidase 4 proximal tubule cells reactive oxygen species

## 1. Introduction

Reactive oxygen species (ROS) and oxidative stress have been implicated in kidney disease [1]. Numerous ROS generator systems are present in the kidney and have been linked to renal pathologies. An important ROS generator system is the Nox family of NADPH oxidases (Noxes), which are expressed in the kidney and may contribute to ROS-dependent pathologies [2][3][4][5].

The individual cells of the kidney exhibit a cell-specific expression pattern of the different Nox homologues, as well as a differential response to Nox enzyme-inducing and -activating stimuli. In models of angiotensin II infusion and a high salt diet, the renal expression of Nox2 and its accessory subunits is increased [3] and the hypertension induced in response to these stimuli has been, at least in part, attributed to an increased renal production of superoxide anions and/or hydrogen peroxide [6][7][8]. Despite a growing body of literature, the specific functions of most NADPH oxidases in the kidney are insufficiently understood. This aspect is particularly true for the NADPH oxidase Nox4. In fact, Nox4 was initially identified in the kidney. At the protein level, the kidney has, by far, the highest Nox4 expression. Conversely, in all other organs Nox4 is hardly detectable; the kidney yields a strong and specific signal for Nox4 by Western blot [9]. Different to all other Nox enzymes, Nox4 is constitutively active and is only dependent on p22phox [10], not on cytosolic subunits. Moreover, Nox4, like the Duox-NADPH oxidases, directly produces $H_2O_2$, due to its ability to trap $O_2^{\bullet-}$ in a pocket of its E-loop [11]. $H_2O_2$ is a relatively long-lived ROS, which can exert a signaling function through direct reaction with cysteines [12] and the metal centers of enzymes [13]. Despite numerous publications [14][15][16], there is currently no consensus regarding the role of Nox4

in renal disease, as interpretations vary according to the loss of function strategy (inhibitor, knockdown, knockout, dominant negative enzyme) or model system (cell culture, mouse model, rat model) [17][18][19][20][21]. In models of diabetes and renal fibrosis, knockout of Nox4 did not result in renal protection [9][22]. In contrast, in hypertension and kidney injury in a Dahl salt-sensitive (SS) rat model, knockout of Nox4 attenuates blood pressure increase in response to a high salt diet (4%) [23]. Given that the sodium-sparing function of the kidney is essential to body water conservation and, thus, blood pressure maintenance through plasma volume control, the function of Nox4 in this context becomes an important question. We therefore hypothesized that Nox4 contributes to renal sodium handling, and studied this aspect using a low sodium diet in wild-type (WT) and Nox4-/- mice.

## 2. Nox4 Expression Is Restricted to Proximal Tubule Cells

To begin, we determined which cells of the kidney express Nox4. For this, we used high-resolution in situ hybridization (RNAscope®) to visualize Nox4 mRNA, which has been reported to strongly correlate with the protein level of Nox4 [24]. A custom-designed probe and chromogenic staining (Brown kit) confirmed that Nox4 mRNA expression is restricted to the renal cortex (**Figure 1**A). No staining was detected in the kidneys of Nox4-/- mice. To specifically identify the Nox4 mRNA expressing cell, we combined RNAscope® with immunofluorescence using megalin and aquaporin-2 as markers for proximal tubule and collecting duct cells, respectively. Nox4 staining was restricted to megalin-positive cells (**Figure 1**B); also in addition, no staining was observed in the glomeruli (visualized by its distinct morphology). These results demonstrated that Nox4 expression in the kidney is restricted to the proximal tubule cells.

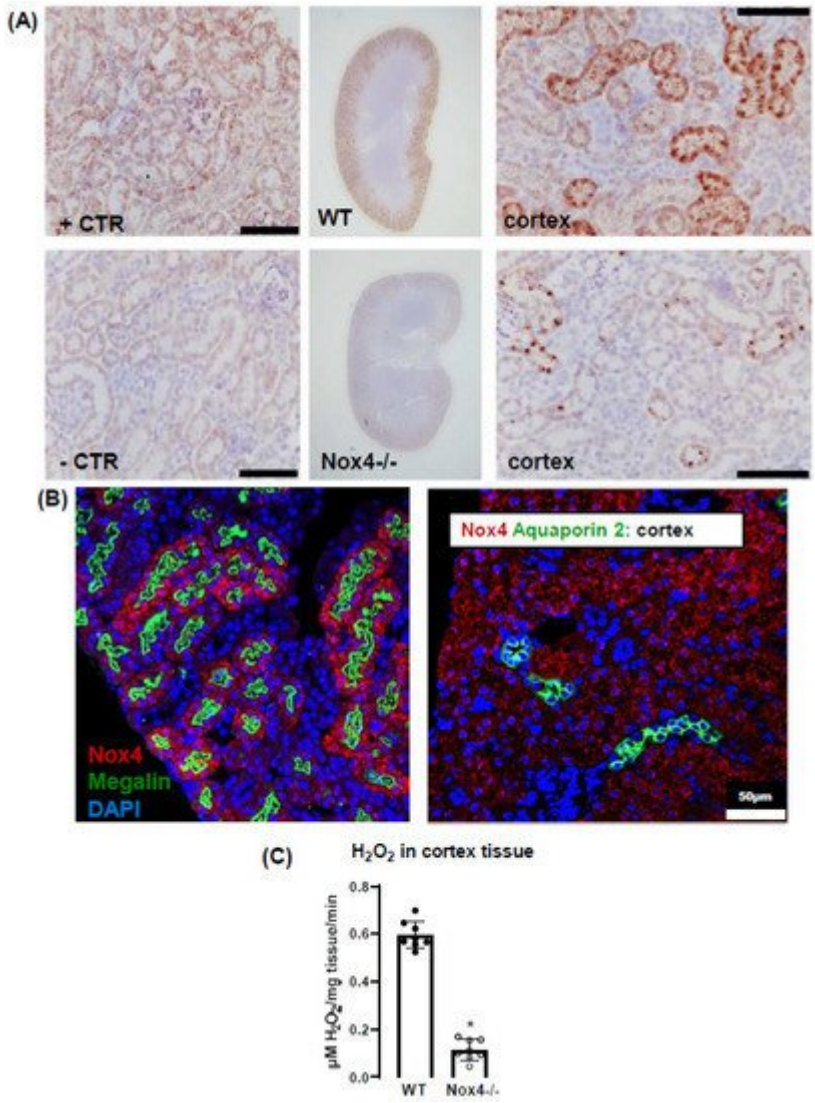



**Figure 1.** Nox4 is expressed in the proximal tubule cells and produces $H_2O_2$. (**A**): RNAscope in kidney. Left panel: +CTR: peptidylprolyl isomerase (**B**), −CTR: *B. subtilis* dihydrodipicolinate reductase. Middle: staining of Nox4 in cortex and medulla of WT and Nox4-/- mice. Right panel: higher magnification. Scale bar: 20 µm. B: RNAscope combined to IF shows Nox4 expression at the proximal tubule and not at the collecting tubule. (**C**): $H_2O_2$ measurement from renal tissue using Amplex red. * $p < 0.05$. $n = 8$ mice for each group.

## 3. Nox4 Contributes to $H_2O_2$ Production of the Renal Cortex

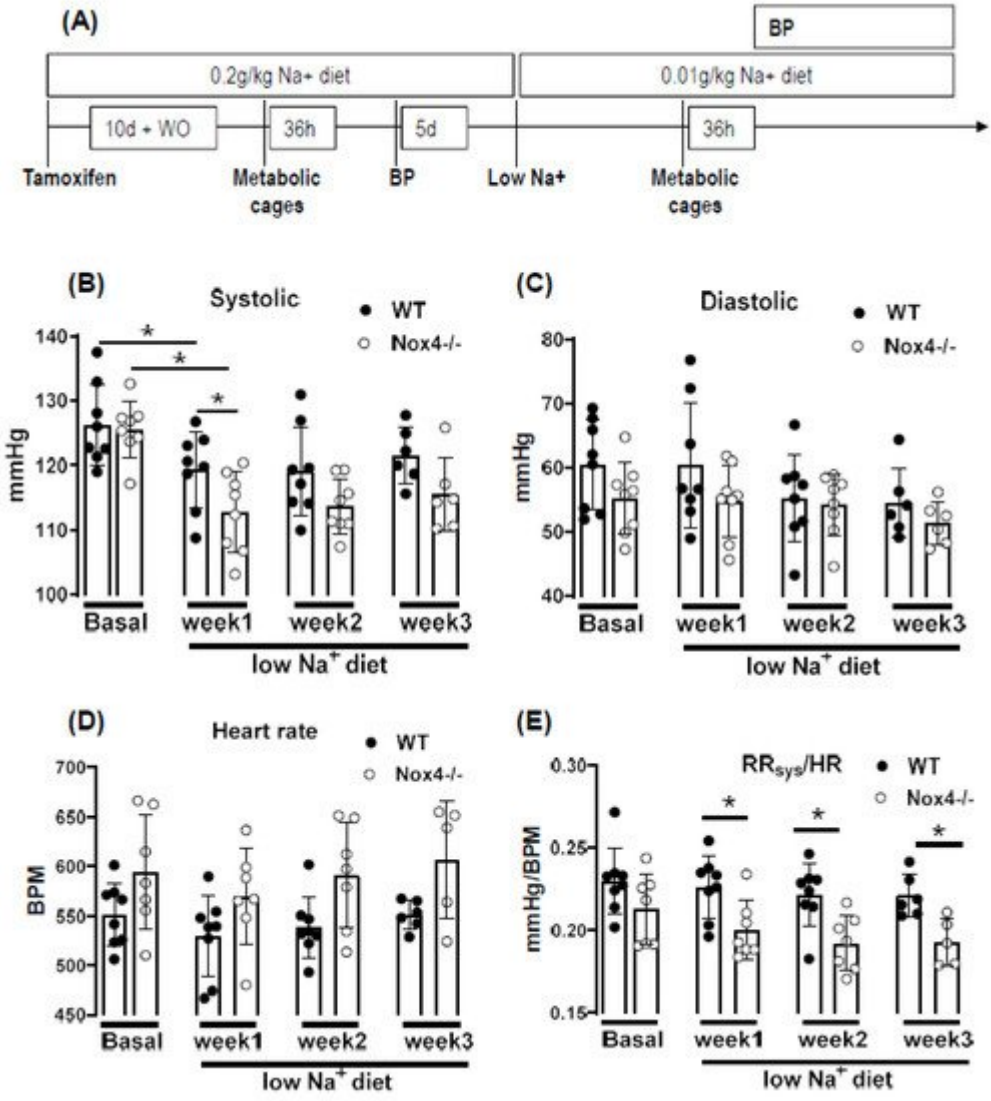
Next, we measured $H_2O_2$ as a readout for an active Nox4 enzyme. For this, we utilized Amplex red® with HRP as a sensitive method for $H_2O_2$.

Freshly dissected kidneys from WT and Nox4-/- mice were separated into cortex and medulla and minced in HT (Hepes Tyrode) buffer and incubated with Amplex red®/HRP solution. Renal cortex of Nox4-/- showed a significantly lower (16% of WT mice) release of $H_2O_2$, demonstrating that Nox4 is the source of $H_2O_2$ in the murine renal cortex (**Figure 1**C).

## 4. Knockout of Nox4 Lowers Blood Pressure and Cardiac Mass in Response to Low Sodium Diet

The proximal tubule is the renal site of mass absorption. More than 65% of the water, a high proportion of bicarbonate and phosphate, and basically all amino acids, glucose, as well as numerous vitamins and trace elements, are recycled at this site. Almost all transport processes in the proximal tubule are coupled, directly or indirectly, to sodium reabsorption. The fine-tuning of sodium excretion occurs in the late distal tubule and collecting duct. A previous publication on the role of Nox4 in salt-induced hypertension in Dahl rats [25] suggests that Nox4 has an impact on sodium handling and, thus, total body water content and plasma volume.

To study this aspect, mice were challenged with a low sodium diet: body weight, blood pressure and sodium excretion were first studied on regular chow (0.2 g/kg sodium), and subsequently on a low sodium diet (0.01 g/kg) applied for up to three weeks with prior tamoxifen-mediated knockout of Nox4 (**Figure 2**A).



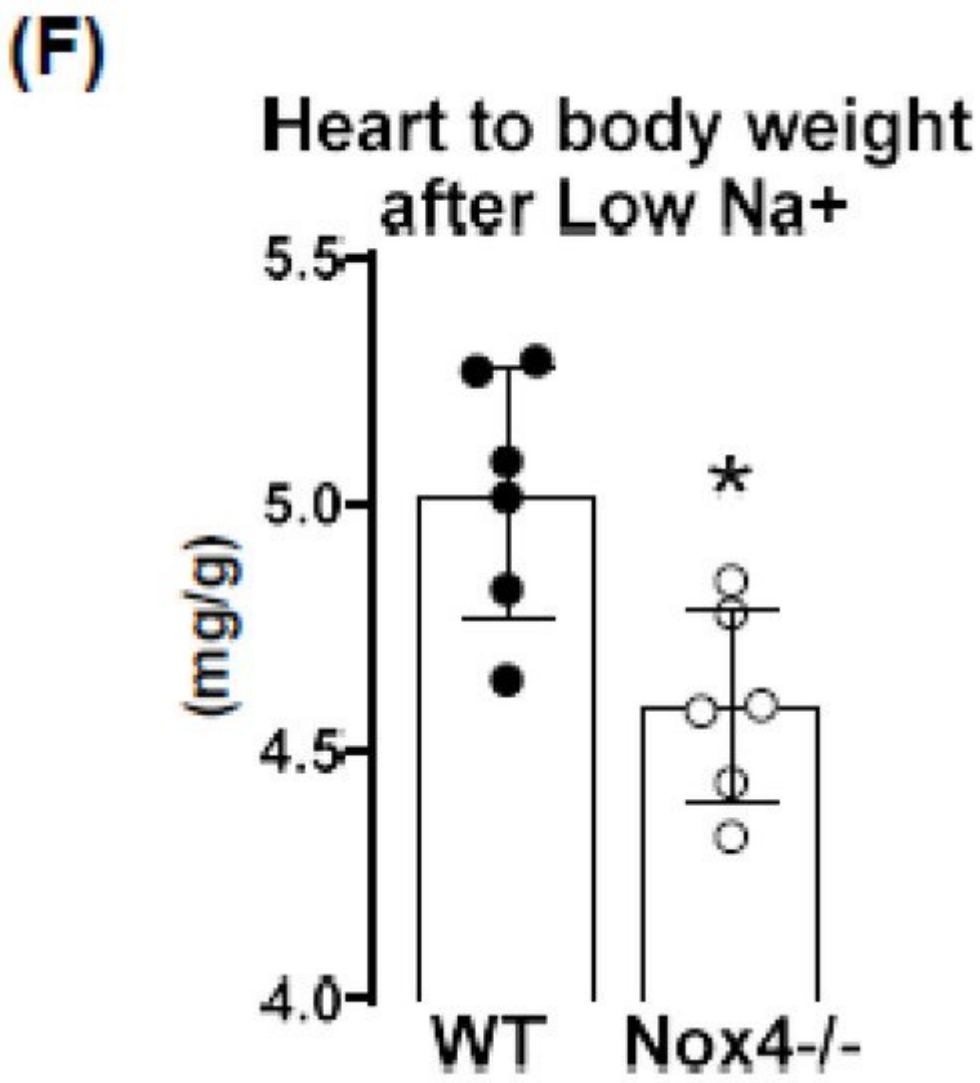



**Figure 2.** Experimental setup and cardiovascular parameters. (**A**): experimental design. (**B**): systolic blood pressure. (**C**): diastolic. (**D**): heart rate. (**E**): ratio of systolic blood pressure to heart rate. (**F**): ratio of heart to body weight. * $p < 0.05$, non-parametric test, Mann–Whitney. $n \geq 6$.

Under normal chow, the blood pressure and heart rate of WT and Nox4-/- mice were similar. A reduction in sodium intake slightly lowered systolic blood pressure in both strains; this effect was more pronounced in Nox4-/- than in WT mice (only significant at week 1 of low Na+). There was also a trend towards a higher heart rate in Nox4-/- vs. WT mice (**Figure 2**B–D). The values do not reach significance at all timepoints, potentially due to insufficient group size or high variability in tail cuff measurements. The combination of lower blood pressure and increased heart rate might indicate that peripheral resistance or plasma volume are different between WT and Nox4-/- mice. Indeed, whereas there was no difference under normal chow, when exposed to a low sodium diet, the ratio of systolic blood pressure to heart rate was significantly lower in Nox4-/- compared to WT mice (**Figure 2**E). Lower peripheral resistance or volume should attenuate cardiac load (after and preload, respectively), thus resulting in small hearts. Indeed, at the end of the 3-week diet, the heart to body weight ratio was lower in Nox4-/- as compared to WT mice, whereas the body weight was similar (**Figure 2**F).

## 5. Discussion

In this study, we report that a low sodium diet results in an acute reduction in systolic blood pressure and a prolonged reduction in peripheral resistance in Nox4-/- mice. Despite the high expression of Nox4 in the kidney, this effect was unrelated to salt and water intake and renal sodium handling. Sodium restriction resulted in weight loss and renal sodium sparing, and this effect was similar between WT and Nox4-/- mice. Collectively, these data support the previous report of Nox4 in renal blood pressure control [25] but exclude a direct effect on sodium handling as an underlying mechanism.

In the present study, we observe a high expression of Nox4 in the proximal renal tubule. Our data are in line with the single-cell sequencing atlas of the murine kidney [26], where Nox4 is expressed in the cluster of proximal tubule cells and a novel cluster that is also positive for megalin. In addition, in the human kidney, Nox4 appears to localize to the proximal tubule [18]. Despite these data on expression, very little is known concerning the function of Nox4 in the kidney. The location in the proximal tubule makes studying the function of Nox4 difficult, given that the physiological function of these cells depends on directionality, which is challenging in model systems of isolated cultured cells. Our past data indicated that Nox4 expression in the kidney is highest under quiescent conditions in healthy animals, whereas inflammation and diseases such as diabetes decreased Nox4 [9]. The finding that renal disease reduces Nox4 expression has, meanwhile, been recapitulated by others [18][27] and suggests that Nox4 is a marker for healthy, differentiated, intact renal tissue. This behavior also has significance for renal cell culture models: the isolation of proximal tubule cells leads to a rapid loss in Nox4. Therefore, renal proximal tubule cell lines are alternatives. The opossum kidney OK cell line (from *Didelphis virginiana*) is a broadly used model to study ion transport and membrane trafficking mechanisms in the proximal tubule. Transcriptomics of these cells, compared with mammalian proximal tubule cells, however, reveal that Nox4 expression is also lost in this cultured cell line [28].

Therefore, the search for a function of Nox4 has to rely on in vivo data. Given that the transport processes in the proximal tubule are largely sodium-coupled, studying sodium handling and its indirect consequence, plasma volume and blood pressure, can be seen as first approaches to dissect the function of Nox4 in the kidney. The proximal tubule reabsorbs two-thirds of filtered Na+ [29] and, consequently, is essential in sodium homeostasis. It is also important to note that, despite this behavior, the contribution of this renal segment to volume- and blood-pressure-control is, at least, controversial. From the knowledge available to date, the transport processes in this renal segment are not well controlled, potentially with the exception of phosphate reabsorption, which is inhibited by parathormone. Tubular-glomerular feedback (TGF), which controls proximal tubular urine flux, has a sensor in the distal tubule and affects glomerular filtration rate. To date, TGF has not been linked to Nox4; comparatively, it is associated with nitric oxide and adenosine [30]. The second system that is relevant in conjunction with low sodium is the renin–angiotensin system (RAS) because a low sodium diet increases RAS activity by volume depletion and subsequent sympathetic nerve activation. The combination of increased sympathetic tone and RAS activation usually compensates for the effect of hypovolemia on blood pressure: cardiac output, renal water retention and peripheral resistance all increase. The fact that the blood pressure/heart rate ratio of Nox4 mice on a low sodium diet was significantly lower than that in wild-type mice, suggests that neither peripheral resistance nor volume

retention can be adequately increased after deletion of Nox4. As body weight and sodium excretion were similar between WT and Nox4-/- mice, differences in peripheral resistance should be considered.

Interestingly, cerebral knockdown of Nox4 resulted in attenuated sympathico-excitation in response to cardiac damage [31]. Whether Nox4 impacts directly on peripheral resistance is unclear. It has been suggested that SERCA is oxidized by Nox4 in the heart and endothelium [32][33] and also in TRP-channels [34][35][36][37], which contribute to the control of resting calcium, and are targets of Nox4. Moreover, Nox4 has been linked to smooth muscle cell differentiation and, thus, contractility [38][39][40][41]. Collectively, these observations provide support for a role of Nox4 in vascular tone control. Additional studies will be needed to substantiate this assumption.

Is there any evidence for a direct function of reactive oxygen species for renal hypertension? Superoxide anions lead to reduced medullary blood flow and increased sodium retention and, thus, hypertension [7], as shown by in vivo treatment with a superoxide dismutase mimetic. Moreover, superoxide inhibits proximal tubule fluid reabsorption in spontaneously hypertensive rats [42]. Renal hemodynamic and excretory functions, such as urine flow, sodium excretion and glomerular filtration were increased in hypertensive rats infused with the superoxide scavenger tempol without altering arterial pressure [8]. In contrast, infusion of $H_2O_2$ directly into the renal medulla increases mean arterial pressure [6]. Nox4 has been linked to renal hypertension and sodium retention in Dahl salt-sensitive rats, where volume expansion is considered to be the main cause of salt-sensitive hypertension [43]. Our results corroborate the findings in the Dahl salt-sensitive rats; however, we cannot link the effect of Nox4 to $Na^+$ homeostasis because $Na^+$ excretion and clearance were similar in WT and Nox4-/- mice. On the other hand, the effects might have been so subtle and transient that our study was not sensitive enough to detect them.

The current study has several limitations. Blood pressure was measured by tail cuff technology, which is less accurate than telemetry. Moreover, we only estimated cardiac output and peripheral resistance from the blood pressure to heart rate ratio. A true determination of cardiac output and peripheral resistance would have required indicator injection dilution methodology. Moreover, metabolic cages impose a considerable amount of stress on mice. Food and fluid intake in the cages is low, which is also documented in the present study by the substantial weight loss. A possible alternative would have been clearance measurements using radioactive isotopes, but this technology was not available to us.

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