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NOX4/H_2O_2/mTORC1 pathway in salt-induced hypertension and kidney injury

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Abstract

We have reported that a high-salt (4.0% NaCl) dietary intake activates mTORC1 and inhibition of this pathway with rapamycin blunts the chronic phase of salt-induced hypertension and renal injury in Dahl salt-sensitive (SS) rats. In SS rats, high-salt intake is known to increase the renal production of H_2O_2 by NOX4, the most abundant NOX isoform in the kidney, and the global knockout of NOX4 blunts salt-sensitivity in these rats. Here, we explored the hypothesis that elevations of H_2O_2 by NOX4 in high-salt fed SS rat stimulate mTORC1 for the full development of salt-induced hypertension and renal injury. Our *in vitro* studies found that H_2O_2 activates mTORC1 independent of PI3K/AKT and AMPK pathways. To determine the *in vivo* relevance of NOX4/H_2O_2/mTORC1 in the salt-induced hypertension, SS-*Nox4* knockout ($SS^{Nox4-/-}$) rats were daily administrated with vehicle/rapamycin fed a high-salt diet for 21 days. Rapamycin treatment of $SS^{Nox4-/-}$ rats had shown no augmented effect on the salt-induced hypertension nor upon indices of renal injury. Significant reductions of renal T lymphocyte and macrophage together with inhibition of cell proliferation were observed in rapamycin treated rats suggesting a role of mTORC1 independent of NOX4 in the proliferation of immune cell. Given the direct activation of mTORC1 by H_2O_2 and absence of any further protection from salt-induced hypertension in rapamycin treated $SS^{Nox4-/-}$ rats, we conclude that NOX4-H_2O_2 is a major upstream activator of mTORC1 that contributes importantly to salt-induced hypertension and renal injury in the SS rat model.

Summary

A novel role for the NOX4-H_2O_2 has been revealed *in vivo* showing it serves as an upstream regulator of mTORC1 signaling and this pathway is a downstream determinant of salt-induced hypertension and renal injury in a clinically relevant model of hypertension.

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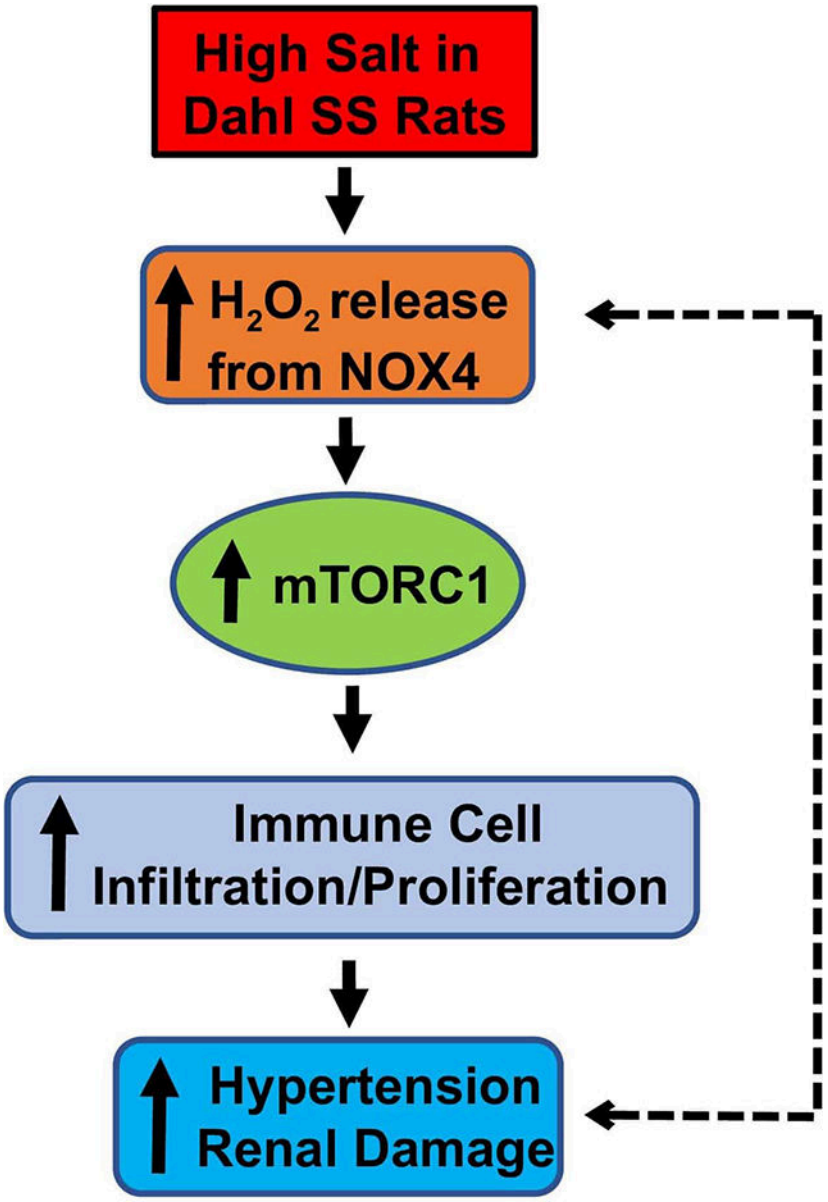
Conflicts of Interest/Disclosures

None.

Graphical Abstract



Keywords

NOX4; Dahl S rats; H_2O_2; mTORC1; salt-sensitive

Introduction

The mTOR (mammalian target of rapamycin) is a highly conserved serine/threonine kinase which senses extracellular and intracellular signals involved in regulation of cell proliferation/growth by growth factors (insulin or insulin-like growth factors), nutrients (amino acids or glucose), and reactive oxygen species (ROS)[1]. mTOR forms two

structurally and functionally distinct multiprotein complexes, mTORC1 and mTORC2, which correspond to two major branches within the overall signaling network. The activity of both complexes has been associated with elevation of ROS including hydrogen peroxide (H_2O_2) in several disease conditions[2, 3]. We found that chronic inhibition of mTORC1 with rapamycin blunted salt-induced hypertension[4] but suppression of both complexes (mTORC1/2) with PP242 inhibitor completely abolished salt-induced hypertension in SS rats[5]. The connecting link between H_2O_2 and mTOR signaling in blood pressure salt-sensitivity is not yet fully understood.

NADPH oxidase 4 (*Nox4*) is the most abundant Nox isoform in the kidney and the major source of H_2O_2[6] but its role in cardiovascular and related renal injury is not well understood. There is growing evidence indicating the role of this gene in many other pathophysiological conditions. For example, transforming growth factor beta induces the upregulation of *Nox4* in human aortic smooth muscle cells [7] and is also a major contributor to oxidative stress in diabetic nephropathy[8]. In type 1 diabetes, the activation of mTOR enhances oxidative stress via upregulation of *Nox4* and *Nox1* and NADPH oxidase activity[9]. *Nox4* dependent activation of mTORC2 via AMPK is observed in idiopathic pulmonary hypertension[10].

We have found that endogenously produced H_2O_2 from NOX4 in the kidney contributes to salt-induced hypertension in SS rats[11, 12]. Also, that mTORC1 is elevated in the kidney of SS fed a high-salt diet and Sprague Dawley rats receiving a non-hypertensive H_2O_2 infusion in the renal interstitial space for 3 days[4]. Together, these observations suggested that mTORC1 activation is signaled by elevations of H_2O_2. However, the molecular mechanism by which mTORC1 is activated by high-salt feeding in SS rats and by H_2O_2 in particular is largely unknown and is the focus of the present study.

Methods Summary

All supporting data used for this study are available within the article and in the online-only Data Supplement. *In vitro* studies were performed in normal rat kidney epithelial (NRK-52E) cell line. Experiments were performed with male SS$^{Nox4-/-}$ rats[11]. Rapamycin (I.P., 1.5 mg/kg/day) was chronically administered to 10 weeks old SS$^{Nox4-/-}$ rats fed a low-salt (0.4% NaCl) diet for 4 day and 21 day after switching to a high-salt (4.0% NaCl) diet. As we have described[11], radiotelemetry catheters and transmitters were surgically implanted for recording 24 h/day arterial pressure. On the final day of the 4.0% NaCl diet period, rats were placed in a metabolic cage for a 24 h urine collection. Immunohistochemistry, Western blot, renal tubular injury and glomerular score were performed as we have described[11, 13, 14]. Detailed experimental methods and description of antibodies are available in the online-only Data Supplement.

Statistical Methods

Data are presented as mean values ± standard error. A two-way analysis of variance (ANOVA) for repeated measures test were used for blood pressure analysis. For multiple groups, statistical significance was analyzed by one-way ANOVA (Tukey's post hoc

multiple comparison test). A paired Student's t test was used to compare the effect of treatments on body weight. $P<0.05$ was considered significant.

Results

H_2O_2 activated mTORC1 independent of PI3K/AKT pathway *in vitro*.

Figure 1A illustrates the canonical activation of mTORC1 pathway. In this study, we examined the effect of H_2O_2 on this pathway using a normal rat kidney epithelial (NRK-52E) cell line in the presence or absence of pharmacological inhibitors, LY294002 (PI3K inhibitor) and rapamycin (mTORC1 inhibitor). As shown in Figure 1B&C and Figure S1A, 1 μM and 5 μM of H_2O_2 showed significant increases of mTORC1 activity measured by functional markers S6K1^{T389}/S6K1, pS6$^{S235/236}$/S6, and 4E-BP1$^{T37/46}$/4E-BP1. Higher dose of H_2O_2 (100 μM) exerted significant inhibition of pS6$^{S235/236}$/S6 and 4E-BP1$^{T37/46}$/4E-BP1. As seen in the Figure 1D and Figure S1B, activation of mTORC1 by H_2O_2 was found to be independent of the suppression of the protein level of negative regulators PTEN and TSC2. The level of NOX4 protein and pAMPK^{T172}/AMPK remained unaffected with the increasing mTORC1 activity however suppression of mTORC1 with H_2O_2 at 100 μM significantly activated AMPK (Figure S1C&D). To further explore the mechanism, NRK-52E cells were pretreated with 0.5–20 μM of LY294002 for 30 minutes to inhibit PI3K/AKT signaling. As shown in Figure S1E. 0.5 μM LY294002 abolished PI3K dependent phosphorylation of AKT at the T308 position thereby blocking the PI3K/AKT upstream stimulatory actions on mTORC1 activity. Quantitively, as shown in Figure 1E and Figure S1F, this dose of LY294002 reduced the levels of pS6$^{S235/236}$/S6 and 4E-BP1$^{T37/46}$/4E-BP1 by 70–85 % which was reversed by different doses of H_2O_2 (1–25 μM). As shown in Figure 1F, increasing doses of H_2O_2 failed to reverse the rapamycin specific inhibition of pS6$^{S235/236}$/S6 suggesting that H_2O_2 directly activates mTORC1 independent of the PI3K/AKT and AMPK pathways.

Elimination of NOX4-H_2O_2 in SS rat reduced mTORC1 activity in response to high-salt diet.

The results of *in vitro* studies prompted us to propose a non-canonical activation of mTORC1 by NOX4-H_2O_2 in the kidney of SS rats fed a high-salt diet (Figure 1A). Consistent with our *in vitro* findings, neither the cortical and medullary protein levels of PTEN (the first upstream negative regulator of mTORC1), nor its downstream effector substrate pAKT^{T308}/AKT controlled by PDK1 kinase differed in SS and SS$^{Nox4-/-}$ rats fed a 4.0% NaCl diet for 21 day (Figure 2A&B). Also seen in Figure 2C, TSC2 protein which is a second upstream negative regulator of mTORC1 remained unchanged in these tissues. As expected, pS6K1^{T389}/S6K1 and pS6$^{S235/236}$/S6 which are downstream substrates of mTORC1 were reduced in the cortex and medulla of SS$^{Nox4-/-}$ rats compared to SS rats (Figure 2D&E). These responses are a consequence of the high-salt diet since no significant differences of pS6$^{S235/236}$/S6 and p4E-BP1$^{T37/46}$/4E-BP1 were observed in the renal cortex and medulla of SS and SS$^{Nox4-/-}$ rats maintained on 0.4% NaCl (low-salt) diet since weaning (Figure 2F and Figure S2).

These data indicate that NOX4 produced H_2O_2 is not essential for the maintenance of mTORC1 activity with a low-salt diet intake but played a critical role in the dysregulation

of this pathway with a high-salt diet in SS rats. The most relevant and novel finding of this study is NOX4 generated H_2O_2 serves an upstream regulator of mTORC1 activity in rats maintained on 4.0% NaCl and *in vitro* observation obtained in the NRK-52E cell line.

Rapamycin failed to augment protection from salt-induced hypertension in SS$^{Nox4-/-}$ rats.

We have found that salt-induced hypertension of rapamycin treated SS rats mimics the phenotypes observed in SS$^{Nox4-/-}$[11]. This suggested that NOX4 generated H_2O_2 most likely activated mTORC1 and that reduction of mTORC1 activity with rapamycin produce a similar phenotype as that found in SS$^{Nox4-/-}$ rat fed a high-salt diet. If this hypothesis was true, further inhibition of mTORC1 with rapamycin in SS$^{Nox4-/-}$ in which renal mTORC1 activity was also found to be low would not be expected to augment additional protection from salt-induced hypertension. This indeed was found to be the case in the present studies s summarized in Figure 3A in which no statistical differences of mean arterial pressure (MAP) were observed between rapamycin and vehicle treated rats throughout the low-salt and high-salt diet period. Historical data from our laboratory[4] of vehicle treated SS rats were plotted in the graph to show the effect of elimination of *Nox4* on MAP.

Pressure recordings were also analyzed for effects of rapamycin on diurnal rhythms. As shown in Figure 3B and Figure 3SA&B, rapamycin treated rats exhibited almost complete abolishment of blood pressure diurnal rhythms in MAP, diastolic blood pressure (DBP) and systolic blood pressure (SBP) which were apparent even during the first day of treatment. Vehicle treated rats exhibited no changes in blood pressure diurnal rhythms. Heart rate responses (Figure 3SC) differed from those of blood pressure in that rapamycin had little effect upon heart rate diurnal rhythms but rather reduced heart rate over the initial 8 days of treatment before returning to baseline through the remainder of the study. No vehicle effects upon heart rate were apparent.

Renal hypertrophy which is commonly observed in high-salt fed SS rats remained unchanged as expressed by kidney to body weight ratio (renal hypertrophy index) in rapamycin treated versus vehicle treated SS$^{Nox4-/-}$ rats (Figure 3C). However, as shown in Figure 3D, rapamycin treated SS$^{Nox4-/-}$ rats exhibited nearly no body weight gain over the 21 day of the 4.0% NaCl diet compared to a significant gain of weight which occurred in vehicle treated SS$^{Nox4-/-}$ rats. Average total food intake did not differ significantly in rapamycin and vehicle treated SS$^{Nox4-/-}$ rats during 4.0% NaCl diet period (Figure 3E). Also shown in Figure 3E and Figure 3SF, steady-state levels of Na^+ and K^+ excretion rate on day 21 of 4.0% NaCl did not differ significantly in rapamycin and vehicle treated SS$^{Nox4-/-}$ rats further indicating rats were eating similar amounts of food.

Rapamycin didn't augment reduction of kidney injury in SS$^{Nox4-/-}$.

As summarized in Figure 4A–C and Figure S4A, rapamycin treatment of SS$^{Nox4-/-}$ rats did not result in significant reduction of kidney injury as evidenced by the albumin-to-creatinine ratios (ACR), protein-to-creatinine ratios (PCR), medullary cast positive area, and glomerular injury scores. Similarly as shown in Figure 4D–F and Figure S4B–D, the expression level of renal fibrosis related genes (*Col1a1*, *α-Sma*), the kidney injury marker *Kim-1*, the oxidative stress related genes (p67, *Nox2*) and the biomarker S-nitrotyrosine

remained unaffected in renal cortex and medulla of rapamycin treated SS$^{Nox4-/-}$ compared with vehicle treated SS$^{Nox4-/-}$ rats. This protection from renal injury that we have previously demonstrated with rapamycin treated SS[4] mimicked that observed in the SS$^{Nox4-/-}$ further indicating that NOX4 generated H_2O_2 is a crucial upstream activator of mTORC1 in kidney injury in SS rats.

Rapamycin reduced immune cell infiltration and cell proliferation in the kidney of SS$^{Nox4-/-}$ rats.

As shown in the Figure 5A&B, rapamycin significantly reduced renal infiltration of T lymphocytes (CD3$^+$ cells/mm^2) in the cortex of SS$^{Nox4-/-}$ rats from 80 ± 6 to 41 ± 5 and in the medulla from 107 ± 7 to 28 ± 3 compared to vehicle treatment. Rapamycin also reduced macrophage (CD68$^+$ cells/mm^2) infiltration in the cortex and medulla of SS$^{Nox4-/-}$ rats from 24 ± 4 to 11 ± 2 and from 32 ± 4 to 10 ± 2, respectively. Rapamycin virtually abolished the cell proliferation (Ki67$^+$ cells/mm^2) in the cortex from 132 ± 29 to 3 ± 0 and in the medulla from 157 ± 50 to 6 ± 0 compared to vehicle treated rats. Yet, as shown in Figure 5E&F, the expression of genes such as *Ccl2* and *Mmp9*, involved in immune cell infiltration did not differ significantly in renal cortex and medulla of rapamycin and vehicle treated SS$^{Nox4-/-}$ rats. Together, these data suggest that rapamycin is a potent inhibitor of cell proliferation which in part may be explained by the reduction of immune cell infiltration into the kidney of SS$^{Nox4-/-}$ rats.

Rapamycin reduced mTORC1 activity in SS$^{Nox4-/-}$ rats.

Renal cortical and medullary mTORC1 and mTORC2 activities were determined in vehicle or rapamycin treated SS$^{Nox4-/-}$ rats by Western blot. As shown in Figure 6A&B, rapamycin significantly reduced the cortical mTORC1 activity with the same tendency in the medullary tissue compared with vehicle treated rats. As we found previously[4], rapamycin had no inhibitory effect on the functional marker of mTORC2 activity (pAKT^{S473}/AKT) in cortex and medulla of kidney of these rats as well (Figure 6C&D).

Discussion

H_2O_2 is an upstream regulator of mTORC1 which does not require PI3K/AKT and AMPK pathways.

Previous *in vitro* studies have failed to clarify the effects of oxidative stress and H_2O_2 upon activation of mTORC1[2, 3]. There have been conflicting reports showing that mTOR is alternatively activated or inhibited by oxidative stress, depending on cell type and oxidant level of the cell[15–17]. Stimulation of S6K activity by H_2O_2 treatment was observed using mouse epidermal JB6 cells with responses being dose- and time-dependent[2, 3]. In contrast, H_2O_2 was found to impair mTORC1 activity in a human lung epithelial cell line A549 and normal bovine aortic smooth cells[17]. The results of the present study reconcile these conflicting observations. First, a biphasic response of H_2O_2 was observed in normal rat kidney epithelial cells (Figure 1) where mTORC1 was strongly activated by physiologically relevant concentrations of H_2O_2 but suppressed by supra-physiological concentrations. Second, H_2O_2 was found to directly activate mTORC1 independent of PI3K/AKT and AMPK pathways in this cell type. This observation contradicts the report that H_2O_2

dependent mitogenic signaling in cultured type II pneumocyte cells activates mTOR-kinase via the PI3K/AKT pathway [23], perhaps a cell specific difference.

The *in vivo* effects of endogenously produced H_2O_2 upon mTORC1 activity have not been previously examined. Evidence indicates that the major reactive oxygen species released by NOX4 is H_2O_2 [18]. Studies in our laboratory have demonstrated that intracellular H_2O_2 in medullary thick ascending limb below detectable limits by fluorescence in SS$^{Nox4-/-}$ rats[12]. The SS$^{Nox4-/-}$ rat model was therefore of great utility in examining the relationship between NOX4-H_2O_2 and mTORC1 signaling. Supporting the conclusion that H_2O_2 mediated activation of mTORC1 is independent of the canonical mTOR pathway (Figure 2) which was shown by the absence of differences in levels of PTEN, pAKT^{T308} and TSC2 expression between SS rats (which exhibit high kidney levels of H_2O_2 in response to high-salt intake) and SS$^{Nox4-/-}$ rats. This further indicates that the actions of H_2O_2 are upstream of mTORC1 since downstream effector components such as pS6K1 and pS6 were reduced in the kidneys of the SS$^{Nox4-/-}$ compared to SS rats. It is interesting to note that pS6 and p4E-BP1 (functional markers of mTORC1 activity) were similar in both SS and SS$^{Nox4-/-}$ rats fed a 0.4% NaCl since weaning. This is perhaps to be expected since mTORC1 normally senses and integrates several signaling inputs including insulin/IGF1, amino acids to maintain renal homeostasis[1, 19] but becomes dysregulated during many pathophysiological conditions such as diabetes[20], cancer[21], and hypertension[4, 5 22]. Together, our observations suggest that the basal physiologic levels of mTORC1 activity are maintained independent of NOX4-H_2O_2 for normal renal function but become dysregulated by NOX4 generated H_2O_2 in SS rats fed a high-salt diet.

Rapamycin treatment of SS rats mimics the protection of salt-induced hypertension in SS$^{Nox4-/-}$ rats.

Over the past two decades, significant progress has been made in understanding the complexity of the mTORC1 regulation and its role in various forms of cancer but there has been limited progress in cardiovascular diseases. Rapamycin is a potent and specific inhibitor of mTORC1 and its effects have been studied in two distinct rat models of hypertension the spontaneously hypertensive rat (SHR)[22] and SS rats[4]. In the heart, *Nox4* and mTORC1 activity have been linked by findings that *Nox4* induced cardiac fibrosis and hypertrophy through activation of AKT/mTOR and NFkB signaling pathways in mice[23].

The present study shows that NOX4 derived H_2O_2 is an important determinant of mTORC1 activation and required for full development of salt-induced hypertension and progressive renal injury in SS rats. Rapamycin treatment of SS rats mimics these responses as illustrated in Figure S5A showing that BP of high-salt diet fed SS rats treated with rapamycin was nearly identical to that of SS$^{Nox4-/-}$ rats. The reductions of renal injury in rapamycin treated SS rats to levels similar to the vehicle treated SS$^{Nox4-/-}$ rats indicate a role of NOX4-H_2O_2 in the regulation of other pathways involved in renal injury (Figure S5B&C). Both SS and SS$^{Nox4-/-}$ exhibited an initial rise of MAP of similar magnitude when rapamycin was administered during low-salt control period. The present study does not shed light on the mechanism(s) responsible for this increase but over observations of the effects of rapamycin on BP diurnal rhythms suggest that this compound has central actions on the autonomous

nervous system. The major finding of the present study was that MAP rose to similar levels with high-salt feeding in SS$^{Nox4-/-}$ vehicle and rapamycin treated rats. This supports our conclusion that the reduction of salt-induced hypertension in SS rats with knockout of NOX4 (SS$^{Nox4-/-}$) maybe in large part a consequence of reduced mTORC1 activity.

Inhibition of mTORC1 with rapamycin reduced renal immune cell by inhibiting cell proliferation independent of NOX4.

It is well recognized that in experimental and human hypertension, lymphocytes and macrophages home to regions of renal injury. In SS rats, inflammation contributes to the later phase of hypertension and renal injury[24] and several studies have shown that although suppression of renal inflammation in SS rats does not affect the initial rise in BP during the first 7–10 day after switching to a high-salt diet, the progression to the malignant phase of hypertension is prevented[4, 25]. *Nox4* is recognized to be important in innate[26] and adaptive[27] immunity but has not been previously examined with regard to the inflammatory related renal injury observed in SS rats. We have shown that rapamycin treatment of SS rats which reduced mTORC1 activity was associated with a reduced renal infiltration of T lymphocyte and macrophage and inhibition of cell proliferation[4]. And yet, in SS$^{Nox4-/-}$ rats in which mTORC1 activity is already low compared with rapamycin treated SS rat[4] resulted in yet a further reduction of T lymphocyte and macrophage infiltration vis- a-vis complete inhibition of cell proliferation (Figure 5SD&E) indicating that these rapamycin actions were independent of NOX4–H_2O_2 but dependent on inhibition of cell proliferation. This cannot be explained by a reduction in renal perfusion pressure which was very similar in rapamycin treated SS rats as in SS$^{Nox4-/-}$ rats.

Effect of rapamycin on the expression of known genes involved in salt-induced hypertension in SS rats.

We have reported that the protective effect from salt-induced hypertension exhibited by SS$^{Nox4-/-}$ rats is attributed in part to the parallel reduction of oxidative stress via *Nox2* and its cytosolic subunits p47, p67 and others[11]. There is plethora of data showing that the benefits of rapamycin in many pathologies are related to transcriptional changes in ROS related genes. Increased levels of NOX2 in the heart of adult offspring from diabetic mothers is the result of AKT/mTOR activity and rapamycin treatment reduced NOX2 protein in cardiomyocytes[28]. Rapamycin reduces the expression of *Mmp9* in Glioblastoma cells by modulation of NFkB and PKC-α signaling[29]. Effects of mTORC1 activity on chemokine gene expression of *Ccl2* in macrophages has been reported[30] and of *Col1*[31] and *α-Sma*[32] in fibroblasts derived from urethral scar tissue and nasal polyps, respectively. It is interesting to note that many of these genes have also been implicated to be directly or indirectly involved in BP salt-sensitivity in SS rats[11]. Given these observations, it appears that the effects of rapamycin are similar at both the genomic and functional level to those observed in SS$^{Nox4-/-}$ rats. This would explain why rapamycin did not show any augmentation in the protection from salt-induced hypertension in SS$^{Nox4-/-}$ rats.

Little is known about the link of NOX4-mTORC2 in salt-induced hypertension. Others have found that a NOX4 dependent activation of mTORC2 in pulmonary hypertension[10]. We have reported the combined inhibition of both mTORC1 and mTORC2 with PP242 completely

prevented and reversed salt-induced hypertension in SS rats[5], a far greater response than observed with specific inhibition of mTORC1 alone with rapamycin treatment[4] indicating that mTORC2 contributed importantly to this remarkable protection from salt-induced hypertension in SS rats. However, until a specific inhibitor of mTORC2 is developed, the connection between NOX4 and mTORC2 can only be inferred. We cannot rule out the possibility that a similar NOX4/H_2O_2/mTORC2 cascade maybe operating in SS rats fed a high-salt diet.

In summary, the results of the present study show that the protective effects upon BP and renal injury observed in SS$^{Nox4-/-}$ rats fed a high salt diet are largely a consequence of reduction of NOX4-H_2O_2 stimulated mTORC1 activity. H_2O_2 was found to effectively activate mTORC1 independent of the PI3K-AKT and AMPK pathways. Rapamycin treatment of SS$^{Nox4-/-}$ rats resulted in no further protection of BP and kidney injury than that observed in SS$^{Nox4-/-}$ rats. We conclude that NOX4 driven BP and kidney injury in SS rats fed a high-salt diet is a consequence of H_2O_2 activation of mTORC1 pathway.

Perspectives

High-salt dietary intake stimulates H_2O_2 production in the kidney in part through the activation of NOX4 and through increased activity of the electron transport chain in SS rat. The present study identifies a novel direct pathway whereby elevations of tissue H_2O_2 can stimulate mTORC1 independent of the PI3K/AKT and AMPK pathways. mTORC1 is one of the major pathways contributing to cellular energy sensing and the control of a number of important cellular functions such as mitochondrial biogenesis and function, cellular proliferation, and autophagy. The novel evidence that H_2O_2 can directly target mTORC1 and thereby enhances salt-induced hypertension and renal injury heightens our understanding of redox sensing and the complexity of intracellular signaling involved in the coordination of kidney energy metabolism in the development of salt-sensitive hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What is new?

First study to identify the importance of NOX4 in the regulation of mTORC1 pathway in salt-induced hypertension and kidney injury in SS rats.

First study showing that H_2O_2 can directly activate mTORC1 independent of PI3K-AKT and AMPK pathways.

First study demonstrating that a high-salt diet stimulates NOX4-H_2O_2 which serves as an upstream regulator of mTORC1 activity and its related downstream pathways involved in determining BP salt-sensitivity and kidney injury.

What is relevant?

Evidences of NOX4-H_2O_2 and mTORC1 signaling have been studied separately in the Dahl S model of hypertension. The present study integrated and revealed *in vivo* relevance of NOX4-H_2O_2 in the activation of mTORC1 in salt-induced hypertension and renal injury in SS rats.

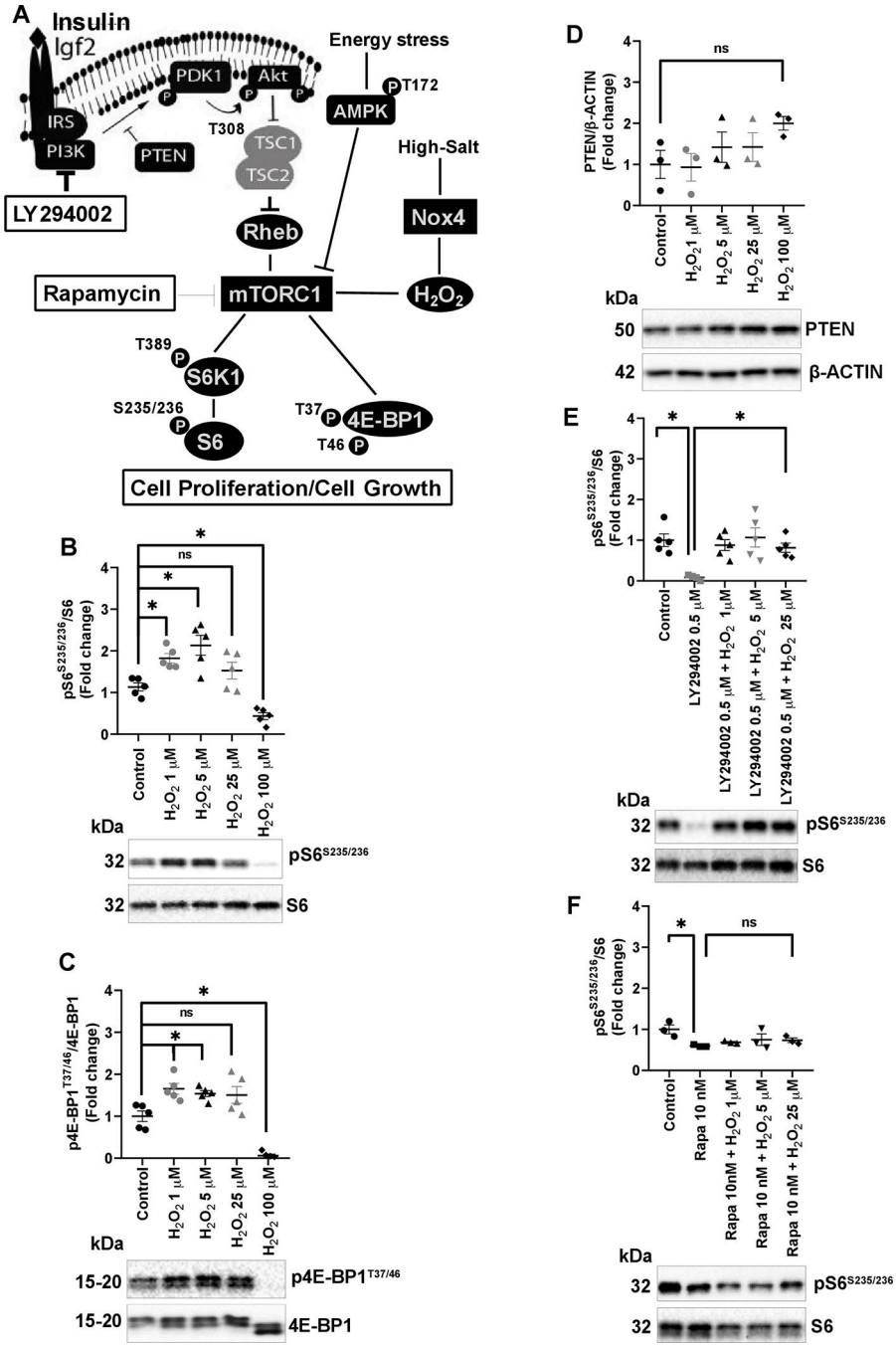

Figure 1:

A, in the canonical mTOR pathway, ligand IGF binds to the receptor tyrosine kinase (RTK), which activates phosphatidylinositol-3-kinase (PI3K) which in turn phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). Phosphatase and tensin homolog (PTEN) dephosphorylate PIP3 acting as a key negative regulator of PI3K signaling. PIP3 activates PDK1 which phosphorylates protein kinase B (PKB/AKT) at T308 thereby phosphorylating the negative regulator tuberous sclerosis complex 1 (TSC1)-TSC2 to activate mTORC1. mTORC1 is rapamycin sensitive

and thought to facilitate many of its downstream effects through S6 kinase 1 (S6K1), ribosomal S6, and translational inhibitor 4E-binding protein 1 (4E-BP1). Energy stress activates AMPK (AMP-activated protein kinase) which represses mTORC1. LY294002 and rapamycin inhibit the PI3K/AKT and mTORC1, respectively. *In vitro* experiment, NRK-52E cells were serum deprived overnight and treated with H_2O_2 for 30 min or pretreated with LY294002 or rapamycin for 30 min before incubating with increasing concentration of H_2O_2 for 30 min. **B-D**, effect of increasing concentrations of H_2O_2 on $pS6^{S235/236}$/S6, $p4E\text{-}BP1^{T37/46}$/4E-BP1, and PTEN/β-ACTIN from three different experiments. **E**, pretreatment with LY294002 inhibited $pS6^{S235/236}$/S6 and addition of H_2O_2 reversed $pS6^{S235/236}$/S6 in pretreated cells. **F.** H_2O_2 failed to reverse $pS6^{S235/236}$/S6 in rapamycin pretreated cells. Quantification of Western blots performed using Image Lab 5.2. Representative Western blots are shown below each graph. Analysis of variance (ANOVA) with Tukey's post hoc test was used for multiple comparisons. * $P<0.05$, ns; non-significant.

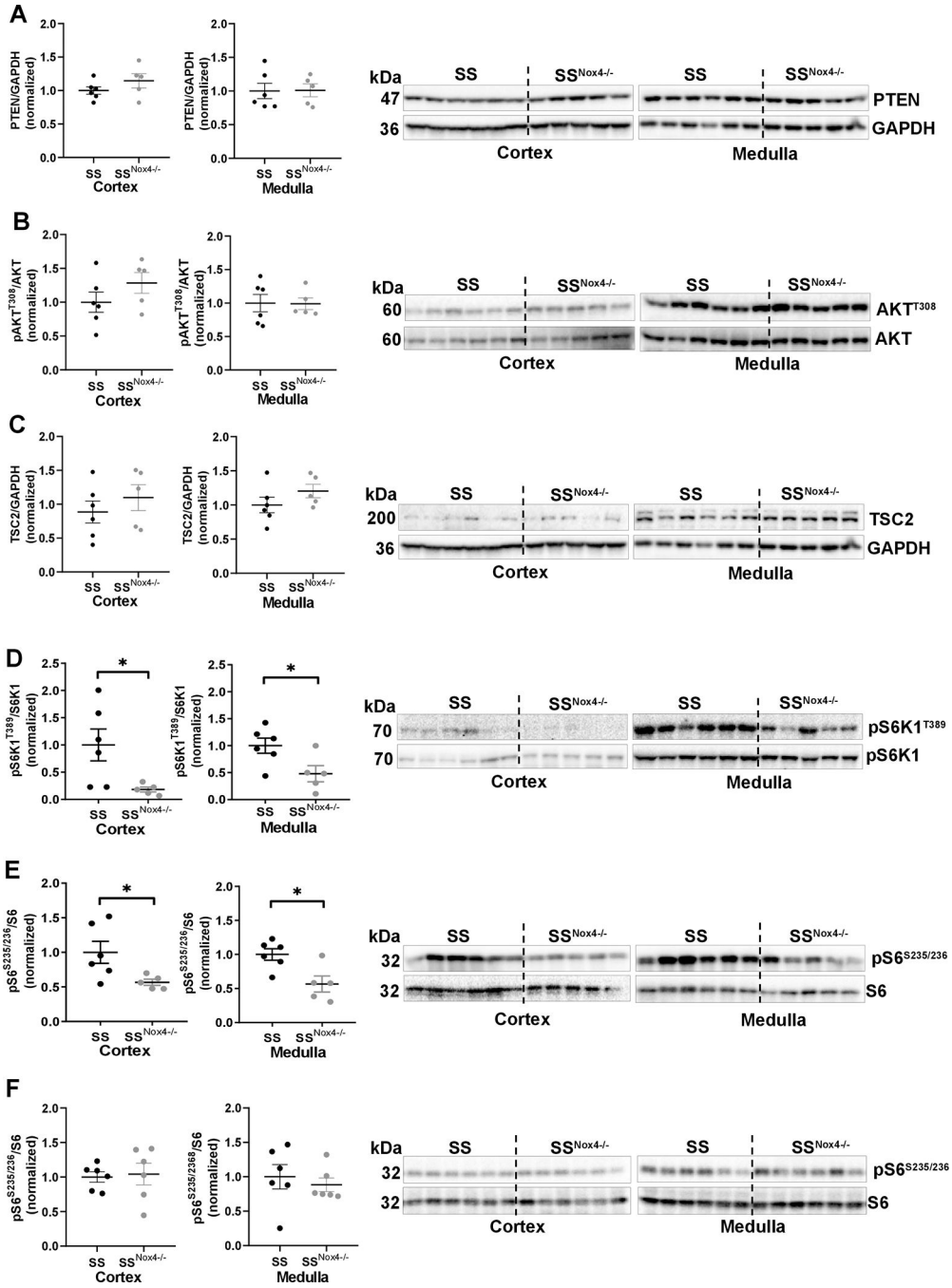

Figure 2:

Western blot of cortical and medullary tissues obtained from SS (n=6) and SS*Nox4*−/− (n=5 or 6) rats fed a 4.0% NaCl (**A-E**) or 0.4% NaCl (**F**) diet for 21 days. Shown here is the membrane staining of PTEN, pAKT^{T308}, AKT, TSC2, pS6K1^{T389}, S6K1, pS6$^{S235/236}$, S6 and GAPDH. Unpaired t test was used to compare the strain difference. * P<0.05.

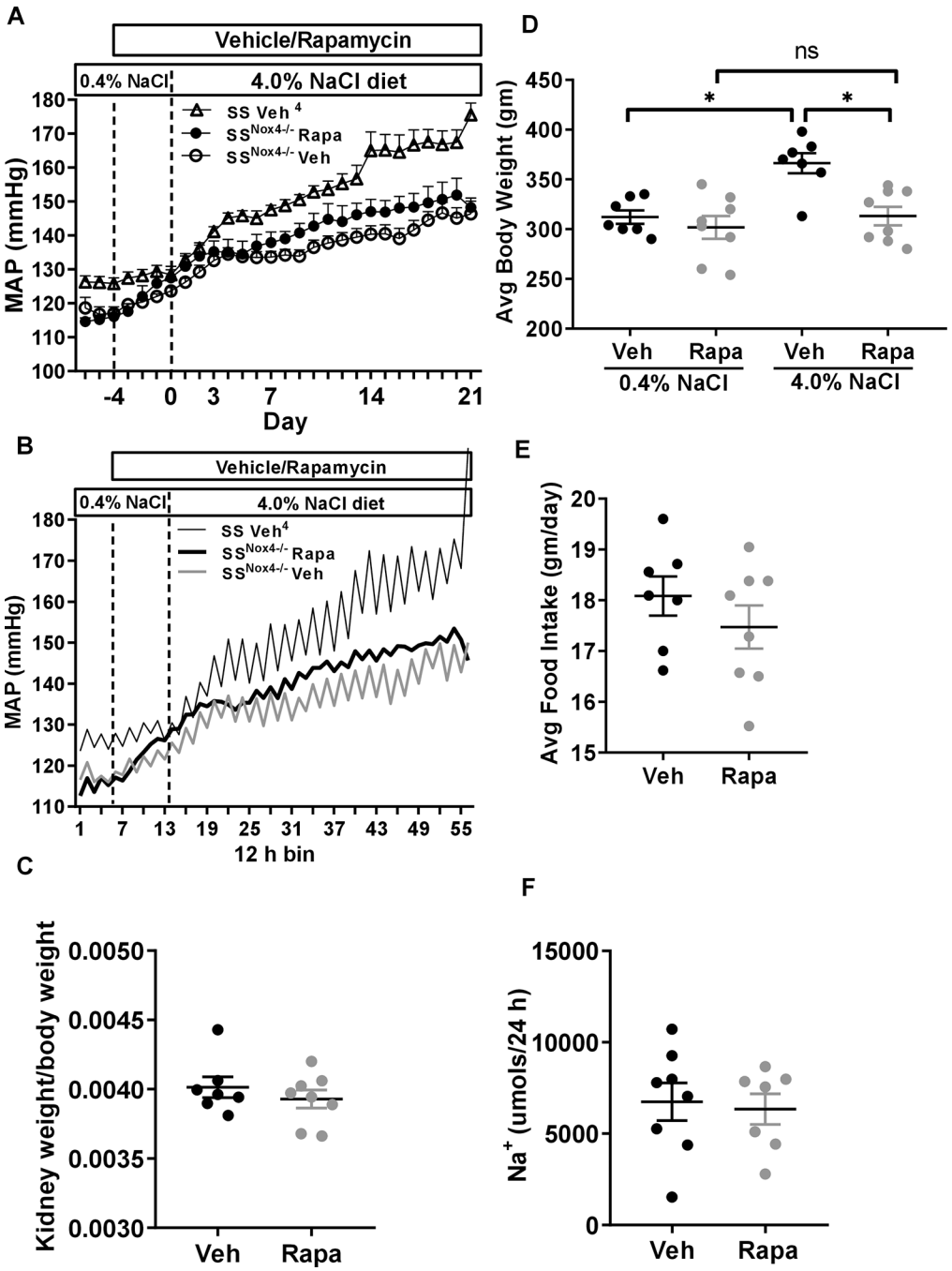

Figure 3:

A, Baseline of mean arterial pressure (MAP) of SS$^{Nox4-/-}$ rat was obtained by measuring three days of continuous arterial pressure followed by four day treatment of vehicle (close circle; n=8) or rapamycin (I.P. 1.5 mg/kg, open circle; n=8) on 0.4% NaCl then switched to 4.0% NaCl diet for 21 day receiving similar treatment. B, Diurnal variation of MAP averaged in 12 h bins to correspond to the day (6am-6pm) and night (6pm- 6am) cycles of these rats is shown. Historical MAP data of vehicle treated SS rats is shown[4]. **C**, hypertrophy index (kidney weight/body weight). **D**, showing average body weight before

and after treatment. **E**, average food intake over the 21 days of 4.0% NaCl diet. **F**, steady-state sodium excretion at day 21 of the 4.0% NaCl diet. A two-way analysis of variance (ANOVA) for repeated measures; Holm-Sidak post hoc was used for arterial pressure. Unpaired t-test was used to compare the effect of treatments. * $P<0.05$ and ns; non-significant.

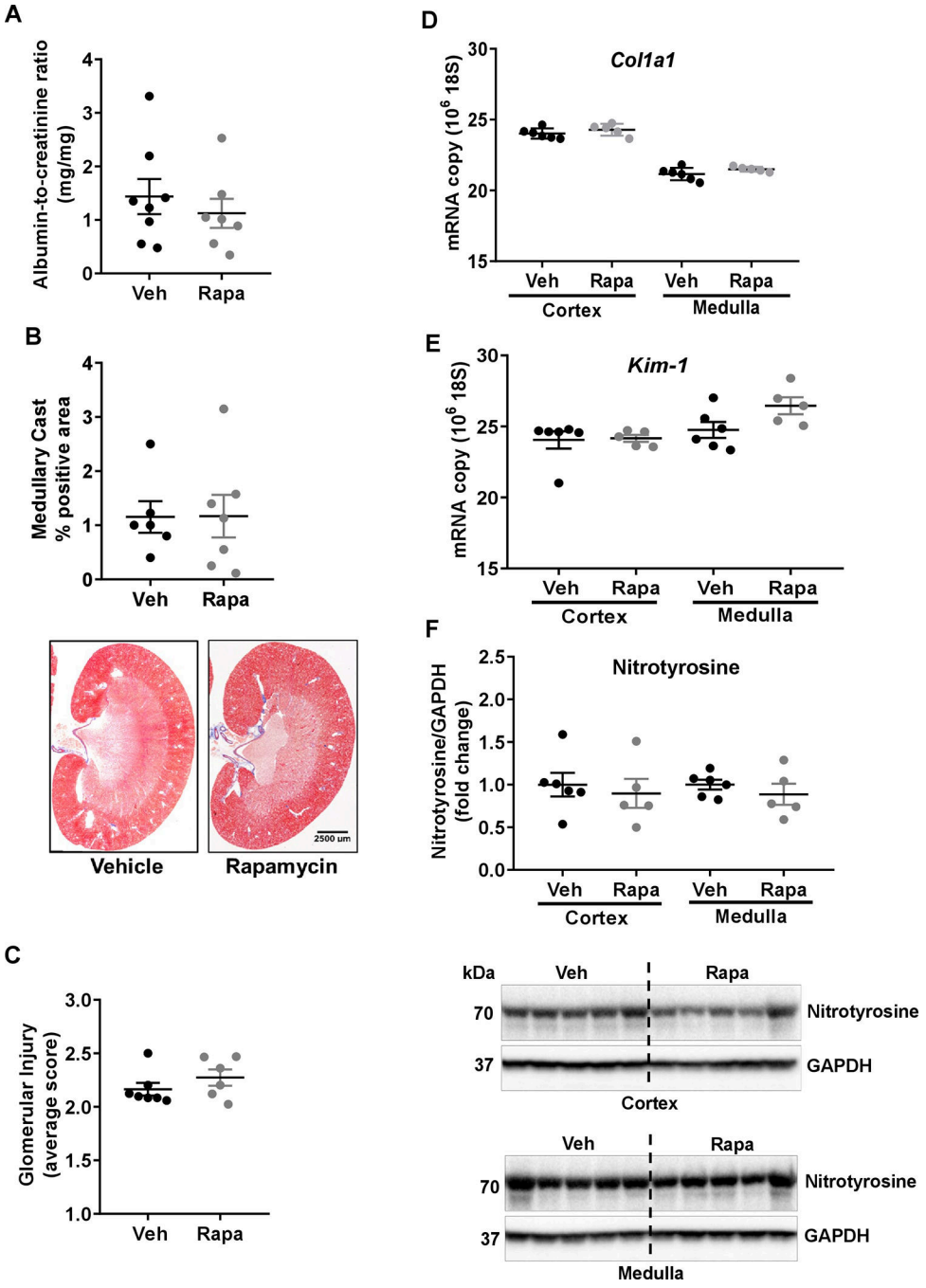

Figure 4:

A, albumin to creatinine was determined in urine samples of SS$^{Nox4-/-}$ rats treated with vehicle (n=7) or rapamycin (n=7) collected for 24 h on the last day of the 4.0 % NaCl. **B**, summary of the outer medullary tubular injury (percentage of tubular cast positive region) and representative sections of trichrome-stained kidney from vehicle (n=6) or rapamycin (n=7) treated rats. **C**. summary of glomerular injury. **D&E**, Quantitative polymerase chain reaction assessment of genes involved in renal fibrosis (*Col1a1*) and renal injury (*Kim-1*) in the cortex and medulla of vehicle (n=6) or rapamycin (n=5) treated SS$^{Nox4-/-}$ rats. **F,**

S-nitrotyrosine expression was determined in cortex and medulla of these rats by Western blot. Representative immunoblots of cortex and medulla are shown. Unpaired t-test was used to compare the effect of treatments. * $P<0.05$.

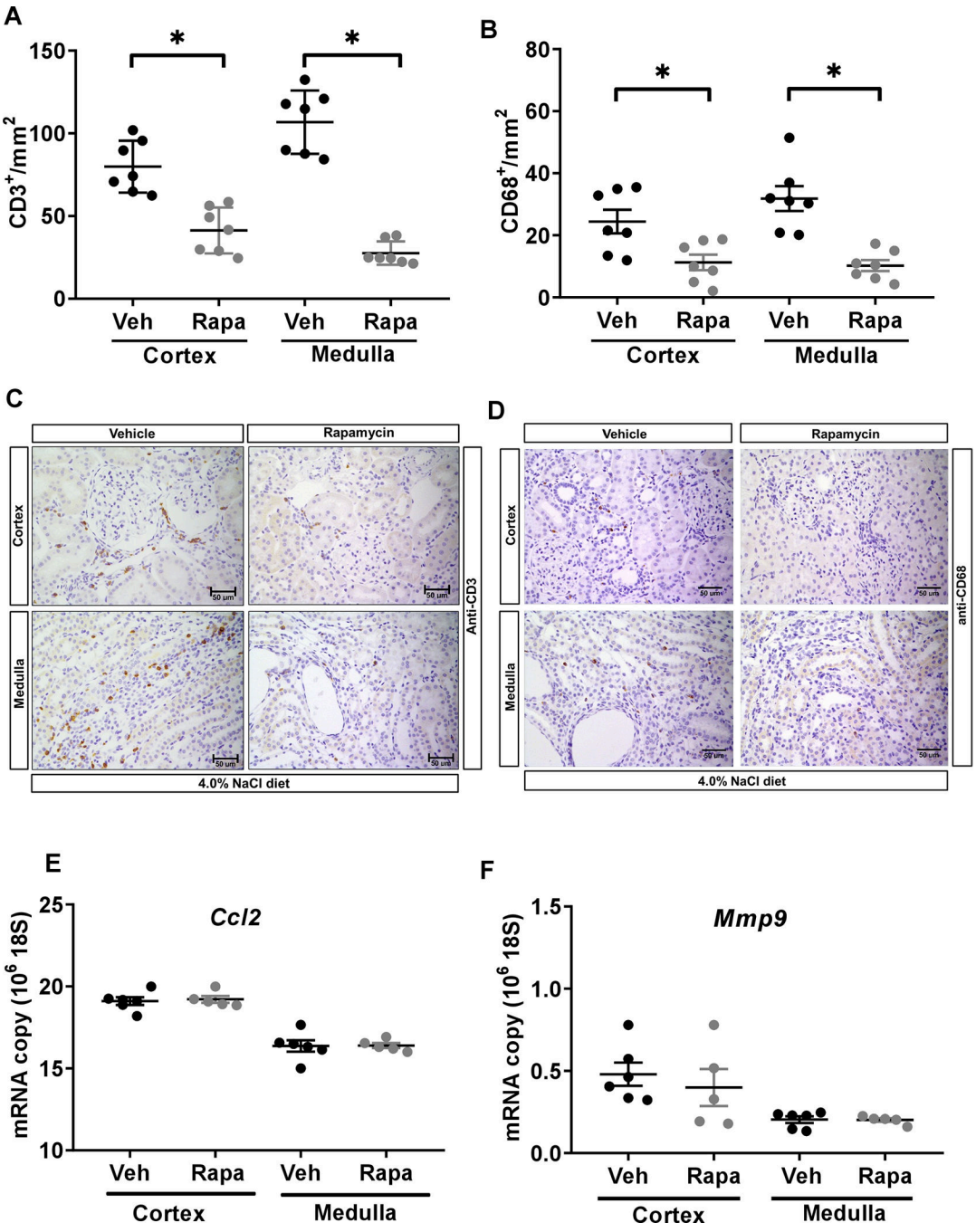

Figure 5:

A-D, quantification of T lymphocyte ($CD3^+$) and macrophage ($CD68^+$) cells per mm^2 of kidneys of vehicle (n=7) or rapamycin (n=7) treated $SS^{Nox4-/-}$ rats. (**A&C**) $CD3^+$ cells/mm^2 and (**B&D**) $CD68^+$ cells/mm^2 in the renal cortex and medulla and representative kidney sections illustrating the immunohistochemical localization of T lymphocyte and macrophage cells in the kidney of these rats. Scale bar 50 μM. **E&F**, Quantitative polymerase chain reaction assessment of *Ccl2* and *Mmp9* in the cortex and medulla of vehicle (n=6) or

rapamycin (n=5) treated SS$^{Nox4-/-}$ rats. Unpaired t-test was used to compare the effect of treatments. * $P<0.05$.

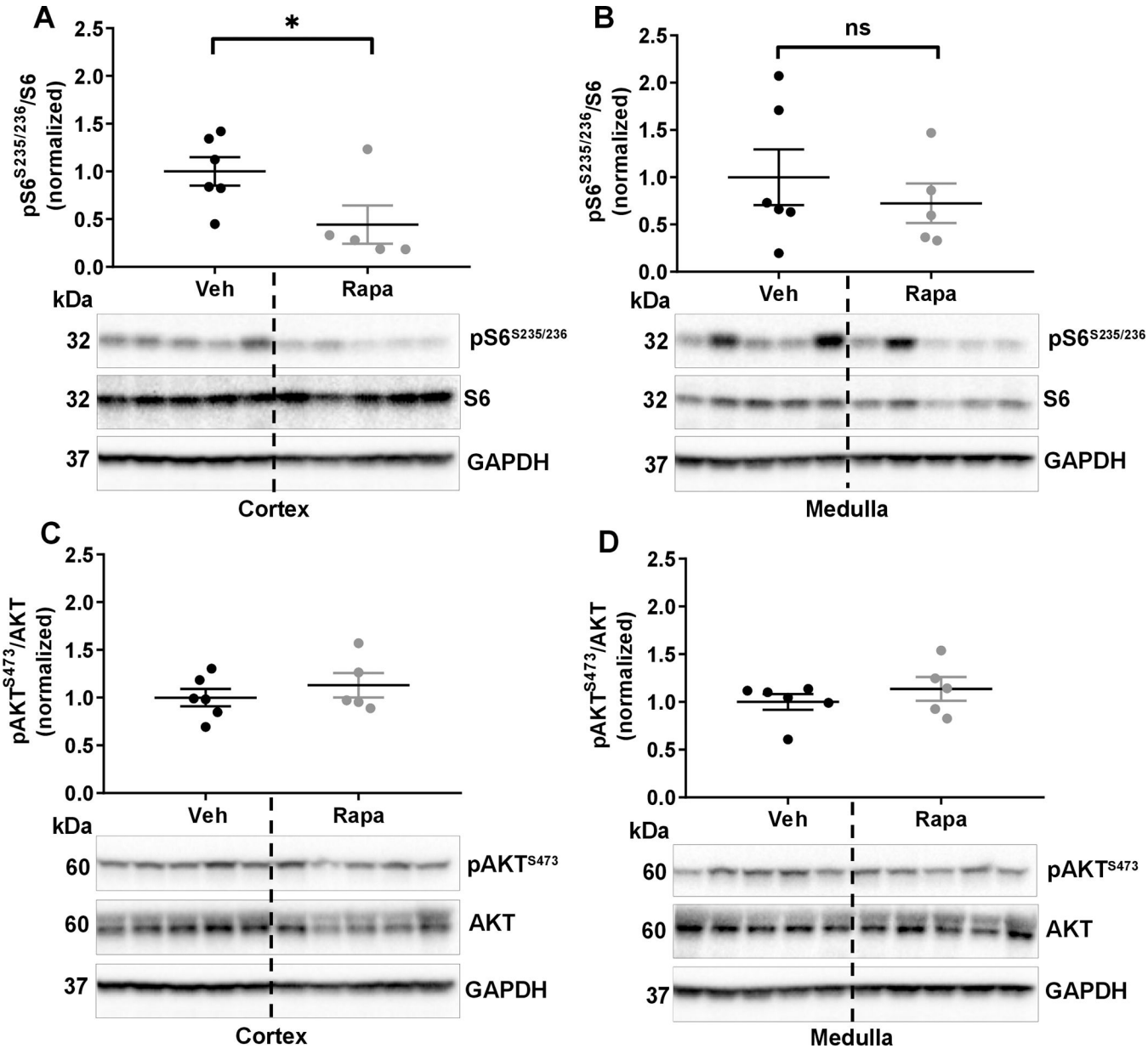

Figure 6:

A&B, $pS6^{S235/236}$ and S6 were measured in the cortex and medulla of $SS^{Nox4-/-}$ rats treated with vehicle (n=6) or rapamycin (n=5) by Western blot. Corresponding representative immunoblots are shown below each graph. **C&D,** measurement and quantification of $pAKT^{S473}$ and AKT and corresponding representative immunoblots are shown below each graph. GAPDH is a loading control. Unpaired t test was used to compare treatments. * $P<0.05$ and ns; non-significant.