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## HIGH SALT ENHANCES ROS AND ANG II CONTRACTIONS OF GLOMERULAR AFFERENT ARTERIOLES FROM MICE WITH REDUCED RENAL MASS

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### Abstract

High salt, angiotensin II (Ang II), and reactive oxygen species (ROS) enhance progression of chronic kidney disease (CKD). We tested the hypothesis that a high salt intake generates specific ROS to enhance Ang II contractions of afferent arterioles from mice with reduced renal mass (RRM). C57BL/6 mice were subjected to surgical RRM or sham operations and received 6% or 0.4% NaCl salt diet for 3 months. Ang II contractions were measured in perfused afferent arterioles and superoxide ( $O_2^{\cdot-}$ ) and  $H_2O_2$  by fluorescence microscopy. RRM enhanced the afferent arteriolar gene expression for p47<sup>phox</sup> and NOX2 and high salt intake in RRM mice enhanced gene expression for AT1 receptors, POLDIP2 and NOX4 and reduced catalase. High salt in mice with RRM enhanced arteriolar  $O_2^{\cdot-}$  and  $H_2O_2$  generation and maximal contractions to Ang II ( $10^{-6}$  mol·L<sup>-1</sup>) that were dependent on  $O_2^{\cdot-}$  since they were prevented by gene deletion of p47<sup>phox</sup> and on  $H_2O_2$  since they were prevented by transgenic smooth muscle cell expression of catalase (tg<sup>CAT-SMC</sup>) and POLDIP2 gene deletion. Three months of tempol normalized arteriolar ROS and Ang II contractions. However, arteriolar contractions to lower concentrations of Ang II ( $10^{-8}$  to  $10^{-11}$  mol·L<sup>-1</sup>) were paradoxically inhibited by  $H_2O_2$  and POLDIP2. In conclusion, both  $O_2^{\cdot-}$  from p47<sup>phox</sup>/NOX2 and  $H_2O_2$  from NOX4/POLDIP2 enhance maximal arteriolar Ang II contractions from RRM mice during high salt but  $H_2O_2$  and NOX4/POLDIP2 reduce the sensitivity to lower concentrations of Ang II by >100-fold. Tempol prevents all of these changes in function.

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CONFLICT OF INTEREST

None

## Keywords

Hydrogen peroxide; superoxide; POLDIP2; p47<sup>phox</sup>; tempol

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## INTRODUCTION

Angiotensin II (Ang II) enhances the progression of acute or chronic kidney disease (CKD) in experimental <sup>1, 2</sup> and clinical <sup>3</sup> studies. Some of the adverse effects of Ang II on progression of CKD may relate to renal vasoconstriction that limits renal blood flow and O<sub>2</sub> delivery <sup>4-7</sup> since hypoxia promotes the progression of CKD <sup>8, 9</sup>. A high salt intake accelerates the progression of CKD in both experimental <sup>2, 8</sup> and clinical studies <sup>10</sup> but paradoxically activates the intrarenal renin-angiotensin system (RAS), despite suppressing systemic renin release <sup>2</sup>, perhaps by enhancing the intrarenal generation of reactive oxygen species (ROS) <sup>8, 11, 12</sup>. ROS can have opposing effects on afferent arteriolar function <sup>13</sup>. Thus, the NOX2 isoform of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated by p47<sup>phox</sup>/p67<sup>phox</sup> to generate superoxide (O<sub>2</sub><sup>-</sup>) that can enhance arteriolar contractility to perfusion pressure <sup>14</sup>. However, the NOX4 isoform is activated by POLDIP2 to generate principally hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) <sup>15</sup> that, at low concentrations, reduces contraction to perfusion pressure <sup>14, 16</sup>. However, the effects of these two ROS in mediating the effects of high salt intake on the renal afferent arteriolar reactivity to Ang II in models of CKD have not been studied. This was the object of the study.

We tested the hypothesis that a high salt diet increases O<sub>2</sub><sup>-</sup> and/or H<sub>2</sub>O<sub>2</sub> in afferent arterioles from mice with reduced renal mass (RRM) and that these regulate contractions to Ang II. To assess the sources of ROS on the afferent arteriolar contractions to Ang II, the effects of a high salt intake in mice with RRM were assessed in p47<sup>phox</sup> or POLDIP2 gene deleted mice or mice transgenic for catalase in vascular smooth muscle cells (Tg<sup>CAT-SMC</sup>). Finally tempol was administered to test the role of prolonged excess ROS and the potential impact of effective antioxidant therapy on Ang II contractility.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Male C57Bl/6 mice aged 2 to 3 months weighing 25 – 31 g (Charles River Inc. Germantown, MD, USA) were randomized to surgical RRM or sham operation (sham) after which both groups were randomized for 3 months to feeding a 0.4g·100g<sup>-1</sup> (normal) or a 6g·100g<sup>-1</sup> (high) NaCl salt diet (Harlan Teklad, CA). Studies of gene expression were undertaken in RRM and sham mice fed 0.4 or 6% salt diets, but subsequent studies of mechanisms were undertaken in the light of these gene expression studies only in mice fed 6% salt. Additional groups of high salt fed C57Bl/6 mice were randomized to drink tempol (2 mmol·L<sup>-1</sup> in water) or vehicle <sup>8</sup>. Further groups of p47<sup>phox</sup><sup>-/-</sup>, <sup>17</sup> Tg<sup>CAT-SMC</sup> <sup>16</sup> and POLDIP2<sup>+/-</sup> <sup>15, 16</sup> mice, and their wild type (+/+) littermates, were fed a high salt diet and randomized to RRM or sham operations. POLDIP2<sup>+/-</sup> mice were used since few POLDIP2<sup>-/-</sup> mice survive <sup>15</sup>.

All procedures conformed to the Guide for Care and Use of Laboratory Animals prepared by The Institute for Laboratory Animal Research. Studies were approved by the Georgetown University Animal Care and Use Committee.

#### **Animal surgery and preparation of afferent arterioles:**

A two-step surgical 5/6 nephrectomy procedure was used to create RRM as described<sup>8</sup>. Sham-operated control mice (sham) were subject to a similar two stage procedure without removal of kidney tissue. Other groups of wild type mice fed a high salt diet were randomized to receive a vehicle (water) or tempol (2 mmol·L<sup>-1</sup>) in the water. After three months, mice (n = 6 – 12 per group) were euthanized and renal afferent arterioles were dissected, mounted and perfused via a pipette whose pressure at its tip was recorded by a calibrated intra-luminal micropipette, as described<sup>17</sup>.

#### **Measurements of afferent arteriolar responses to angiotensin II:**

Graded concentrations of Ang II (10<sup>-12</sup> to 10<sup>-6</sup> mol·l<sup>-1</sup>) were added to the bath for 2 mins and the arteriolar diameter recorded<sup>17</sup>. Arterioles were perfused at 40 mmHg<sup>18</sup>.

#### **ROS measurements in afferent arterioles:**

Incubation of afferent arterioles with paraquat to generate O<sub>2</sub><sup>-</sup> increases the fluorescence ratio of ethidium: dihydroethidium (E:DHE) that is >80% prevented by incubation with PEG-superoxide dismutase (SOD). Therefore, PEG-SOD-inhibitable E: DHE fluorescence was selected as a measure of arteriolar O<sub>2</sub><sup>-</sup>. Further, incubation with H<sub>2</sub>O<sub>2</sub> increases the fluorescence of 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) that is >90% prevented by incubation with PEG-catalase. Therefore, PEG-catalase-inhibitable H<sub>2</sub>DCFDA fluorescence was selected as a measure of H<sub>2</sub>O<sub>2</sub><sup>14</sup>.

#### **Gene expression in afferent arterioles:**

These studies were performed in ~15 afferent arterioles dissected from each mouse, pooled and placed in 2 ml of lysing matrix in a D tube (MP Biochemical) containing QIAzol lysis reagent and homogenized by MP Fast Prep. Total RNA was extracted with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Primers and probes for mouse AT<sub>1</sub>R, AT<sub>2</sub>R, catalase, p47<sup>phox</sup>, NOX2, POLDIP-2, NOX4 and 18s rRNA (ID: Mm03928990\_g1) were used to quantitate the mRNA expressions (Applied Biosystems, Foster City, CA) using RT/PCR primers as previously described<sup>16, 19, 20</sup>.

#### **Statistics:**

Data are expressed as mean ± SEM. A 2 × 2 analyses of variance (ANOVA) was applied to assess the effects of RRM and salt intake, genotype, or tempol and the interaction. Changes were analyzed using nonparametric statistics (GraphPad Prism, GraphPad Software). P < 0.05 was considered statistically significant.

## RESULTS

### 1. High dietary salt enhances afferent arteriolar $O_2^-$ and $H_2O_2$ generation and contractions to high concentrations of Ang II in mice with RRM

Both RRM and high salt enhanced  $O_2^-$  and  $H_2O_2$  generation with  $10^{-6}$  mol·L<sup>-1</sup> Ang II in afferent arterioles (Fig 1A and B). Moreover, a high salt intake exaggerated the effects of RRM to increase  $H_2O_2$  (Fig 1B; positive interaction term). RRM did not change the afferent arteriolar contractions to Ang II in mice fed a normal salt diet (Figure 1C) but enhanced contractions to high concentrations of Ang II ( $10^{-7}$  to  $10^{-6}$  mol·L<sup>-1</sup>) in mice fed high salt diet by > 60% (maximum change in diameter,  $-72 \pm 2\%$  versus  $-45 \pm 2\%$ ;  $P < 0.005$ ).

### 2. RRM and high dietary salt modulate gene expression in afferent arterioles.

Compared to sham, mice with RRM fed a normal salt diet had an increase in afferent arteriolar mRNA expression for p47<sup>phox</sup> and NOX2 and a decrease in NOX4. Mice with RRM fed a high salt diet had an increase on afferent arteriolar mRNA expression for AT<sub>1</sub>R, p47<sup>phox</sup>, POLDIP2, NOX2, and NOX4 but a reduction in mRNA for catalase (Figure 2). Expression of AT2R was unchanged by salt or RRM (data not shown). Therefore, the roles of p47<sup>phox</sup>/NOX2, POLDIP2/NOX4 and catalase in ANGII contractions were explored in mice with RRM fed a high salt diet.

### 3. Enhanced responses of afferent arterioles to high concentrations of Ang II in mice with RRM fed high dietary salt are prevented by metabolism of $O_2^-$ or $H_2O_2$ .

Compared to sham, incubation of arterioles from mice with RRM fed a high salt diet with Ang II increased the generation of  $O_2^-$  (especially at high Ang II concentrations; positive interaction term in Fig 3A) and  $H_2O_2$  (Fig 3C). PEG-SOD and PEG-catalase reduced the contractions to high concentrations of Ang II ( $10^{-7}$  and  $10^{-6}$  mol·L<sup>-1</sup>) in arterioles from mice with RRM fed high dietary salt to levels of sham mice (Figure 3B and 3D) but PEG-catalase enhanced the contractions to lower concentrations of Ang II ( $10^{-11}$  to  $10^{-9}$  mol·L<sup>-1</sup>; Figure 3D).

### 4. Enhanced afferent arteriolar $O_2^-$ and contractions to high concentrations of Ang II in afferent arterioles from mice with RRM fed high dietary salt are prevented in mice with deletion of the p47<sup>phox</sup> gene or by 3 months of oral tempol.

Compared to sham, afferent arterioles from p47<sup>phox</sup> wild type (+/+) mice with RRM fed a high salt diet had increased generation of  $O_2^-$  (Fig 4A) and increased contractions to high concentrations of Ang II ( $10^{-7}$  to  $10^{-6}$  mol·L<sup>-1</sup>) (Fig 4B), similar to C57Bl/6 mice (Fig 1). However, arterioles from littermate p47<sup>phox</sup> knockout (-/-) mice with RRM fed a high salt diet did not generate more  $O_2^-$  (Fig 4A) and did not have stronger contractions with Ang II (Fig 4B) than arterioles from sham mice. These effects of p47<sup>phox</sup> -/- were similar to incubation of arterioles from C57Bl/6 WT mice with PEG-SOD (Fig 3A and B). Indeed, three months of oral tempol (2 mmol·L<sup>-1</sup>) vs vehicle in C57Bl/6 mice with RRM fed a high salt diet prevented the increases in  $O_2^-$  generation (Fig 4C) and in contractions to high concentrations of Ang II (Fig 4D). These results of tempol in mice with RRM fed a high salt diet were similar to mice with RRM fed a normal salt diet (Fig 1C)

## 5. Differential modulation of afferent arteriolar H<sub>2</sub>O<sub>2</sub> and contractions to high and low concentrations of Ang II in mice with RRM fed high dietary salt in Tg<sup>CAT-SMC</sup> and POLDIP2 +/- mouse strains

Incubation of afferent arterioles from mice with RRM from Tg<sup>CAT-SMC</sup> (vs wt) (Fig 5A and B) and POLDIP2 +/- (vs +/+) strains (Fig 5C and D) fed a high a salt diet with high concentrations of Ang II (10<sup>-7</sup> and 10<sup>-6</sup> mol·l<sup>-1</sup>) failed to increase H<sub>2</sub>O<sub>2</sub> generation or contractions, yet both had increased contractions to lower concentrations of Ang II (10<sup>-10</sup> to 10<sup>-9</sup> mol·l<sup>-1</sup>). These effects of Tg<sup>CAT-SMC</sup> and POLDIP2 +/- were similar to arterioles from C57Bl/6 WT mice incubated with PEG-catalase (Fig 3C and D).

## DISCUSSION

The present study confirms that high dietary salt increases O<sub>2</sub><sup>-</sup>, and especially H<sub>2</sub>O<sub>2</sub>, in afferent arterioles from mice with RRM but extends this from ROS generation with perfusion pressure<sup>8, 14, 16</sup> to ROS generation with Ang II.

The new findings, summarized in Figure 6, are that mice with RRM fed a normal or high dietary salt had enhanced mRNA expression in their afferent arterioles for p47<sup>phox</sup> and NOX2. However only mice with RRM fed high dietary salt had enhanced mRNA expression for AT<sub>1</sub>R, POLDIP2 and NOX4 and decreased mRNA expression for catalase. The increased arteriolar expression of p47<sup>phox</sup>/NOX2, was a source of increased O<sub>2</sub><sup>-</sup> since the increased O<sub>2</sub><sup>-</sup> was prevented in p47<sup>phox</sup> -/- mouse arterioles. Moreover, the increased expression of POLDIP2/NOX4 and decreased expression of catalase were sources of increased H<sub>2</sub>O<sub>2</sub> since the increased H<sub>2</sub>O<sub>2</sub> was prevented in POLDIP2 +/- and in Tg<sup>CAT-SMC</sup> mouse arterioles. Both the increased O<sub>2</sub><sup>-</sup> and contractions to maximal concentrations of Ang II in mice with RRM fed a high dietary salt were prevented in arterioles from p47<sup>phox</sup> (-/-) mice, similar to the effects of metabolism of O<sub>2</sub><sup>-</sup> with PEG-SOD. Likewise, both the increased H<sub>2</sub>O<sub>2</sub> and contractions to high concentrations of Ang II in RRM mice fed high dietary salt were prevented in arterioles from tg<sup>CAT-SMC</sup> and POLDIP2 (+/-) mice, similar to the effect of metabolism of H<sub>2</sub>O<sub>2</sub> with PEG-catalase. However, the increased H<sub>2</sub>O<sub>2</sub> in arterioles from mice with RRM fed high dietary salt paradoxically reduced contractions to lower concentrations of Ang II (10<sup>-10</sup> to 10<sup>-9</sup> mol·L<sup>-1</sup>). Indeed, in POLDIP2 +/- mouse arterioles or after metabolism of H<sub>2</sub>O<sub>2</sub>, the sensitivity of the arterioles to Ang II was increased 100 to 1000-fold. Three months of oral tempol prevented the increased generation of O<sub>2</sub><sup>-</sup> and normalized the contractions to Ang II.

The p47<sup>phox</sup>/NOX2 isoform of NADPH generates primarily O<sub>2</sub><sup>-</sup><sup>13, 16, 19</sup>. Therefore, the effects of Ang II to generate increased O<sub>2</sub><sup>-</sup> in arterioles from mice with RRM is consistent with the effect of RRM to enhance p47<sup>phox</sup> and NOX2 gene expression. Moreover, gene deletion of p47<sup>phox</sup> prevented any increase in O<sub>2</sub><sup>-</sup> with Ang II in arterioles from mice with RRM fed a high salt diet.

The POLDIP2/NOX4 isoform of NADPH oxidase generates primarily H<sub>2</sub>O<sub>2</sub><sup>13, 15, 16</sup>. Therefore, the effect of Ang II to generate increased H<sub>2</sub>O<sub>2</sub> in arterioles from mice with RRM fed high dietary salt is consistent with the effect of RRM to increase the arteriolar expression of POLDIP2 and NOX4, and to reduce the expression of catalase. Moreover,

deletion of POLDIP2 or enhanced expression of SMC catalase prevented any increase in  $\text{H}_2\text{O}_2$  with Ang II in arterioles from mice with RRM fed high dietary salt.

There was internal consistency in the results of the effects of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  in this model of RRM and high dietary salt. Thus, both metabolism of  $\text{O}_2^-$  with PEG-SOD or tempol and reduction of  $\text{O}_2^-$  in p47<sup>phox</sup> knockout reduced maximal Ang II contractions. Likewise, both metabolism of  $\text{H}_2\text{O}_2$  with PEG-catalase and reduction of  $\text{H}_2\text{O}_2$  in Tg<sup>CAT-SMC</sup> and POLDIP2 +/- mice enhanced contraction to lower concentrations of Ang II but reduced contractions to high concentrations of Ang II.

The physiological concentrations of Ang II in the renal afferent arteriole are unknown but may be uniquely high since the juxtaglomerular cells of the afferent arteriole are the site of most of the kidney renin production<sup>21</sup>. The concentration of Ang II in the circulation is approximately  $2 \times 10^{-11} \text{ mol} \cdot \text{L}^{-1}$  and is increased by approximately 4-fold in CKD<sup>22</sup> while the Ang II concentrations in the renal parenchyma are higher than in plasma<sup>23</sup> and the renin and Ang II concentrations of renal lymph are significantly higher than plasma reflecting higher levels in renal interstitial fluid<sup>24, 25</sup>. Whereas high dietary salt in rats reduces circulating levels of Ang II, it paradoxically increases renal parenchymal levels of Ang II<sup>2</sup>. The concentrations of Ang II in proximal tubule fluid and efferent arteriolar plasma are  $3 \times 10^{-8}$  and  $10^{-7} \text{ mol} \cdot \text{L}^{-1}$  respectively<sup>26</sup>. Thus, although it is difficult to predict the endogenous concentrations of Ang II in or around afferent arterioles, it is likely to be high and to be enhanced further by a high salt intake and by CKD. It may be within the range of Ang II concentration of  $10^{-11}$  to  $10^{-8} \text{ mol} \cdot \text{L}^{-1}$  that contracted afferent arterioles after metabolism of  $\text{H}_2\text{O}_2$  or in POLDIP2 gene deleted mice, but it may even be within the range of Ang II concentration of  $10^{-7}$  to  $10^{-6} \text{ mol} \cdot \text{L}^{-1}$  that contracted afferent arterioles in this study of mice with RRM fed high dietary salt diet.

It is not clear why three months of RRM, even with a high salt diet, did not lead to severe CKD despite increased systemic and renal levels of ROS<sup>8</sup>. It is possible that the time was not sufficient or that this represents the resistance of this C57Bl/6 mouse strain to develop CKD, or that an addition stressor such as Ang II infusion is required<sup>27</sup>.

High dietary salt intake selectively enhanced the mRNA expression for AT<sub>1</sub>R (but not AT<sub>2</sub>R) in afferent arterioles from mice with RRM despite the report that intrarenal Ang II concentrations are enhanced in this model<sup>2</sup> and should thereby downregulate receptor expression. The present findings are consistent with our earlier report that prolonged Ang II infusion enhanced AT<sub>1</sub>R expression in rabbit afferent arterioles. The enhanced AT<sub>1</sub>R expression in these two models may contribute to the findings of increased contractions to Ang II in this and prior studies<sup>28, 29</sup>. The increased AT<sub>1</sub>R expression may relate to the increased afferent arteriolar ROS since this was increased by high dietary salt in RRM<sup>2</sup> and by Ang II infusion<sup>28, 29</sup> in both these models and ROS can enhance vascular AT<sub>1</sub>R expression<sup>12</sup>. However, mice with RRM fed a normal salt intake also had increased afferent arteriolar  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  (Figure 1A and B) yet AT<sub>1</sub>R expression was not increased (Figure 2A). It is possible that a more augmented increase in ROS produced by RRM and a high salt diet (Figure 1A and B) is required to increase AT<sub>1</sub>R expression. In a similar mouse model of RRM and high salt diet, tempol prevented any increase in AT<sub>1</sub>R expression in the kidneys

and prevented any increase in ROS as indexed by the excretion of 8-isoprostane  $F_{2\alpha}$  or  $H_2O_2$  <sup>8</sup>.

Tempol is a well-established SOD mimetic <sup>30</sup>, but also acts as a catalase mimetic <sup>8, 31, 32</sup>. Tempol abrogated excessive  $O_2^{\cdot-}$  and, in parallel, abrogated excessive Ang II contractions in mice with RRM fed high dietary salt yet it preserved afferent arteriolar myogenic contractions and renal parenchymal  $PO_2$  in similar mouse models <sup>8</sup>. This combination of reduced Ang II contractility but preserved myogenic contractility and renal parenchymal oxygenation could contribute to the beneficial effects of nitroxides such as tempol in preventing progression of CKD <sup>8, 32</sup>.

Prior studies have reported that  $O_2^{\cdot-}$  can enhance Ang II contractions of afferent arterioles <sup>28, 29, 33, 34</sup> consistent with the findings in this study. Low concentration of  $H_2O_2$  ( $10 \mu \text{mol}\cdot\text{L}^{-1}$ ) reduces Ang II contraction of afferent arterioles from mice with renal ischemia-reperfusion injury but higher concentrations of  $H_2O_2$  ( $25 \mu \text{mol}\cdot\text{L}^{-1}$ ) <sup>35</sup> can enhance contractions consistent with the findings in this study. The mechanism of enhanced Ang II contractions with higher concentrations of  $H_2O_2$  has been related to activation of vascular L-type calcium channels <sup>36</sup> and tyrosine kinase <sup>37</sup>. The reports of variable effects of  $H_2O_2$  on Ang II contractions <sup>35–39</sup> may be explained by our finding of biphasic effects of  $H_2O_2$ : reduced contractions to lower concentrations of Ang II yet enhanced contractions to higher concentration of Ang II in mice with RRM fed high dietary salt.

We acknowledge some limitations. First, the BP was not measured. However, in a prior study with this model neither RRM, salt intake nor tempol changed the mean arterial pressure (MAP) measured telemetrically over 3 months <sup>8</sup>. Second, we acknowledge that mice in this RRM model in which the BP is maintained even during high salt intake, and the glomerular filtration rate (GFR) is well maintained despite removal of 5/6 of kidney tissue, show greater ability to adapt than human subject with CKD who typically develop salt-sensitive hypertension and have a more limited ability to maintain their GFR after severe loss of nephrons. <sup>40</sup> Third, the mechanisms whereby  $O_2^{\cdot-}$  and  $H_2O_2$  modulate arteriolar contractility with Ang II were not evaluated. However, these mechanisms have been studied by us previously <sup>14</sup>. Fourth, tempol has effects beyond its well-established role as a superoxide dismutase mimetic, notably acting as a catalase mimetic during long term studies <sup>8</sup> and actions on a host of other ROS <sup>30</sup> that could have contributed to its beneficial effects.

In conclusion, feeding high dietary salt to mice with RRM increased afferent arteriolar  $O_2^{\cdot-}$  from  $p47^{\text{phox}}/\text{NOX2}$  and  $H_2O_2$  from  $\text{POLDIP2}/\text{NOX4}$ , which contributed to enhanced contractions to  $10^{-7}$  to  $10^{-6} \text{mol}\cdot\text{L}^{-1}$  Ang II. In contrast, metabolism of  $H_2O_2$  by catalase or prevention of  $H_2O_2$  generation by  $\text{POLDIP2}$  gene deletion increased the sensitivity of the afferent arterioles to Ang II by 100–1000 fold. Tempol administration prevented excessive ROS generation in arterioles from mice with RRM fed high dietary salt and normalized the Ang II contractions.

## PERSPECTIVE

Mice with CKD fed high dietary salt have increased renal parenchymal ROS and Ang II that elicit reflex increases in renal inflammation and damage<sup>2</sup>. The present findings demonstrate that mice with RRM fed high dietary salt also have enhanced afferent arteriolar ROS and contractile responses to Ang II. Ang II and high dietary salt in normal rats both increase renal ROS<sup>11, 41</sup> that contributes to renal parenchymal hypoxia<sup>6, 7</sup>. These findings suggest that high dietary salt may impair renal function in a damaged kidney<sup>1, 2, 10</sup> by increasing afferent arteriolar contractions to Ang II and reducing renal blood flow and O<sub>2</sub> delivery while increasing renal O<sub>2</sub> demand<sup>4-7</sup> and renal parenchymal hypoxia<sup>8</sup>. Hypoxia can enhance the progression of CKD<sup>42, 43</sup>. This could explain the renal protection provided by renin-angiotensin system blockade or antioxidants and reduced dietary salt in the RRM model<sup>44</sup> or in subjects with CKD<sup>3, 8, 45</sup>. Thus, effective blockade of ROS and/or of Ang II may prevent these adverse effects of high dietary salt on CKD where dietary advice is not effective. However, this remains to be tested.

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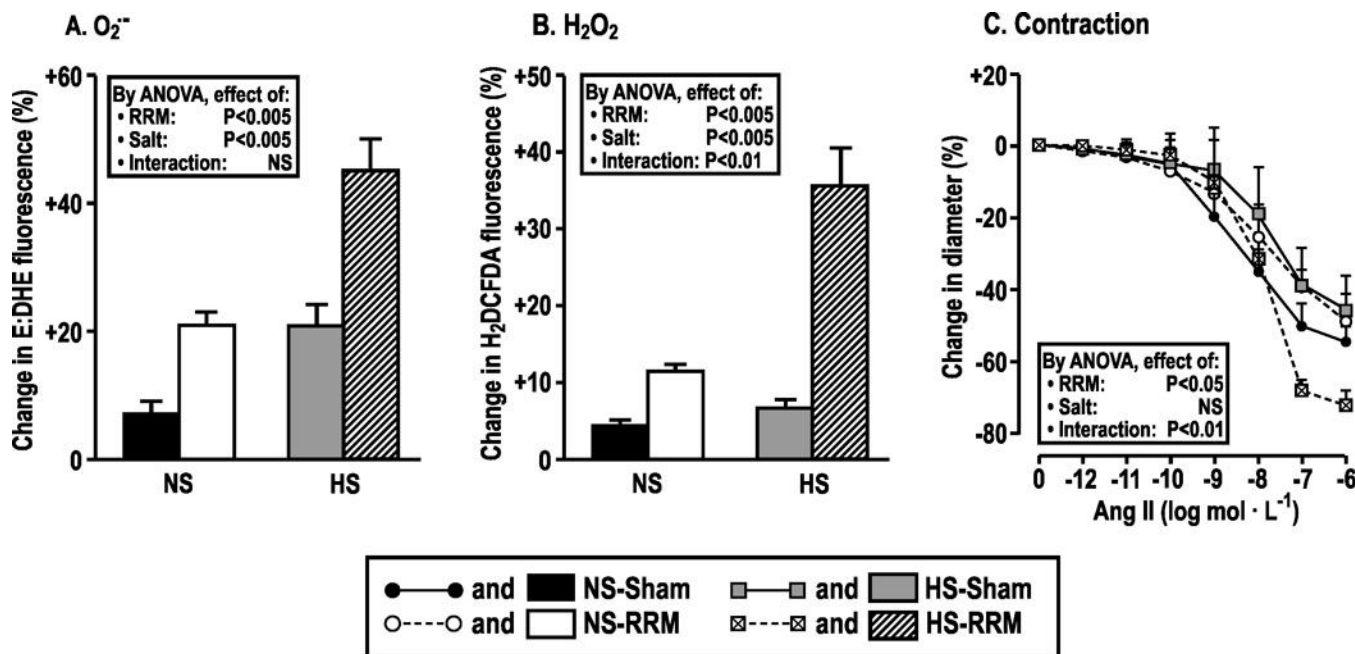
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### NOVELTY AND SIGNIFICANCE

What is New? A high dietary salt intake in a mouse model of CKD transcribes genes that generate  $O_2^{\cdot-}$  and  $H_2O_2$  with Ang II in afferent arterioles. The increased  $H_2O_2$  reduces the sensitivity to Ang II contractions, but  $O_2^{\cdot-}$  and  $H_2O_2$  enhance the responsiveness.

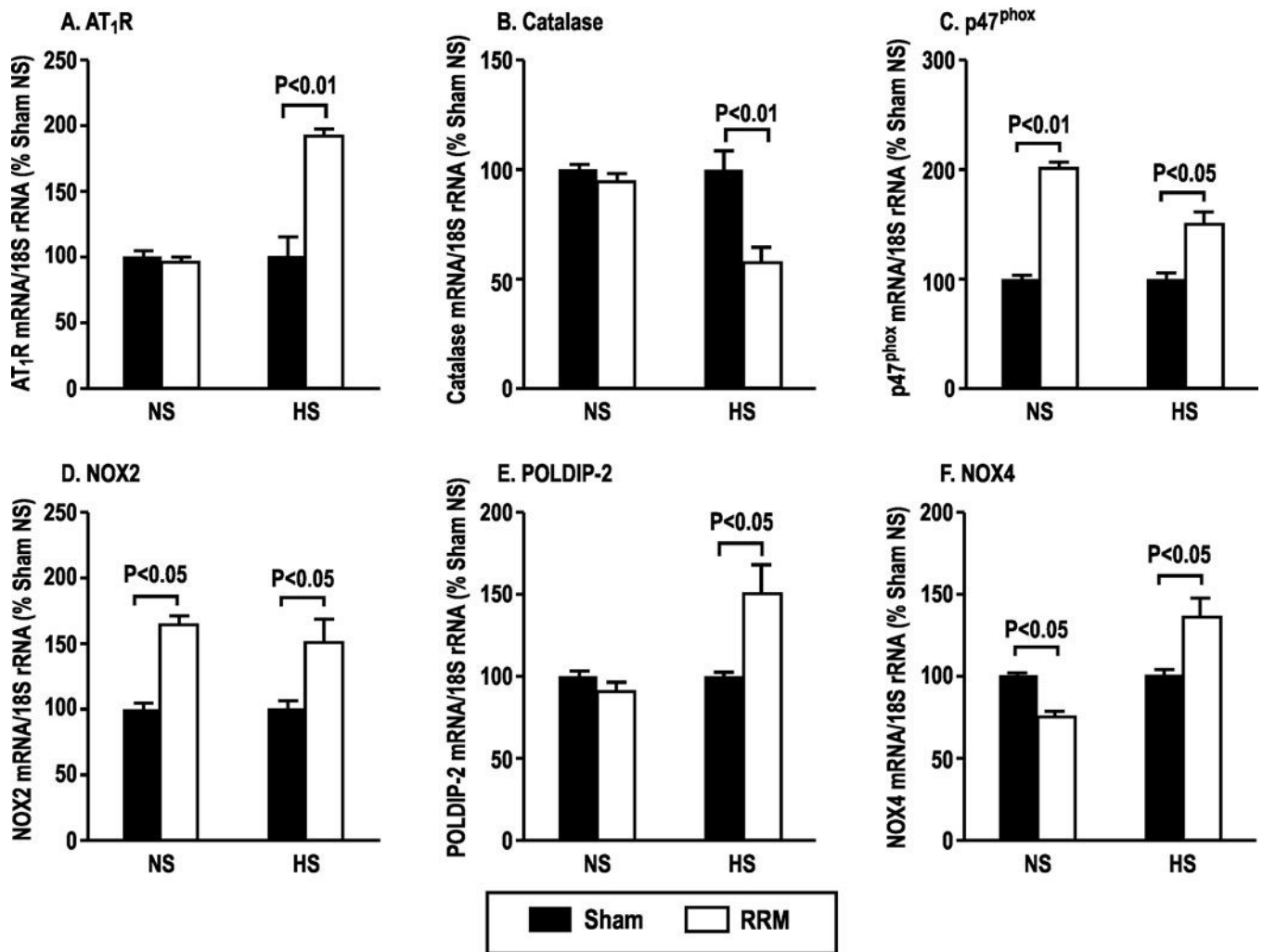
What is Relevant? Progression of CKD in patients or animal models is accelerated by a high salt intake, and is slowed in many patients by drugs that block the renin angiotensin system. Progression of CKD is dependent on reduced renal parenchymal oxygenation. Therefore, enhanced Ang II vasoconstriction of the afferent arterioles of mice with CKD during a high salt intake may contribute to CKD progression by limiting renal blood flow and oxygenation.

Summary: A high dietary salt intake in a mouse model of CKD increases the expression of the NOX2 isoform of NADPH oxidase that generates  $O_2^{\cdot-}$  and the NOX4 isoform that generates  $H_2O_2$  in the kidney's main resistance arteriole both of which modulate the responsiveness of the arteriole to angiotensin II.



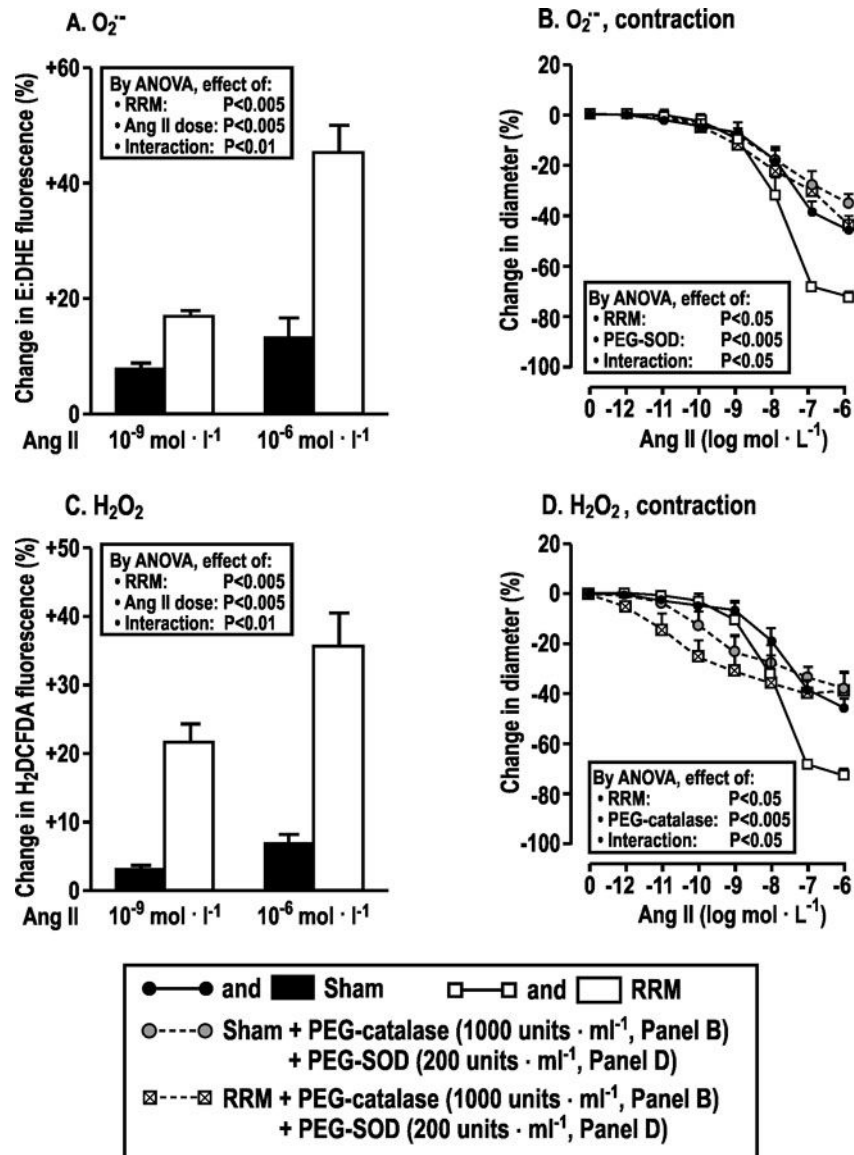
**Figure 1:**

Dietary salt intake increases the ROS production and contractions to angiotensin II in afferent arterioles from mice with RRM. Mean  $\pm$  SEM values ( $n = 5$  to  $6$ ) for O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and contractions to angiotensin II of afferent arterioles from C57Bl/6 WT mice fed normal (NS) or high salt (HS) diets for three months after sham surgery (NS-sham, solid boxes or solid circles with continuous line; HS-sham, gray boxes or gray squares with continuous line) or RRM (NS-RRM, open boxes or open circles with broken line; HS-RRM, slash-hatched boxes or crossed boxes with broken line). Data are shown for changes in ethidium: dihydroethidium (E: DHE) ratio (panel A) and H<sub>2</sub>DCFDA fluorescence (panel B) with angiotensin II ( $10^{-6}$  mol · L<sup>-1</sup>) and changes in diameter in afferent arterioles from sham or RRM mice fed normal or high salt with graded angiotensin II (panel C). Ang II: angiotensin II; E: DHE: ethidium: dihydroethidium; H<sub>2</sub>DCFDA: 2',7'-dichlorodihydrofluorescein diacetate.

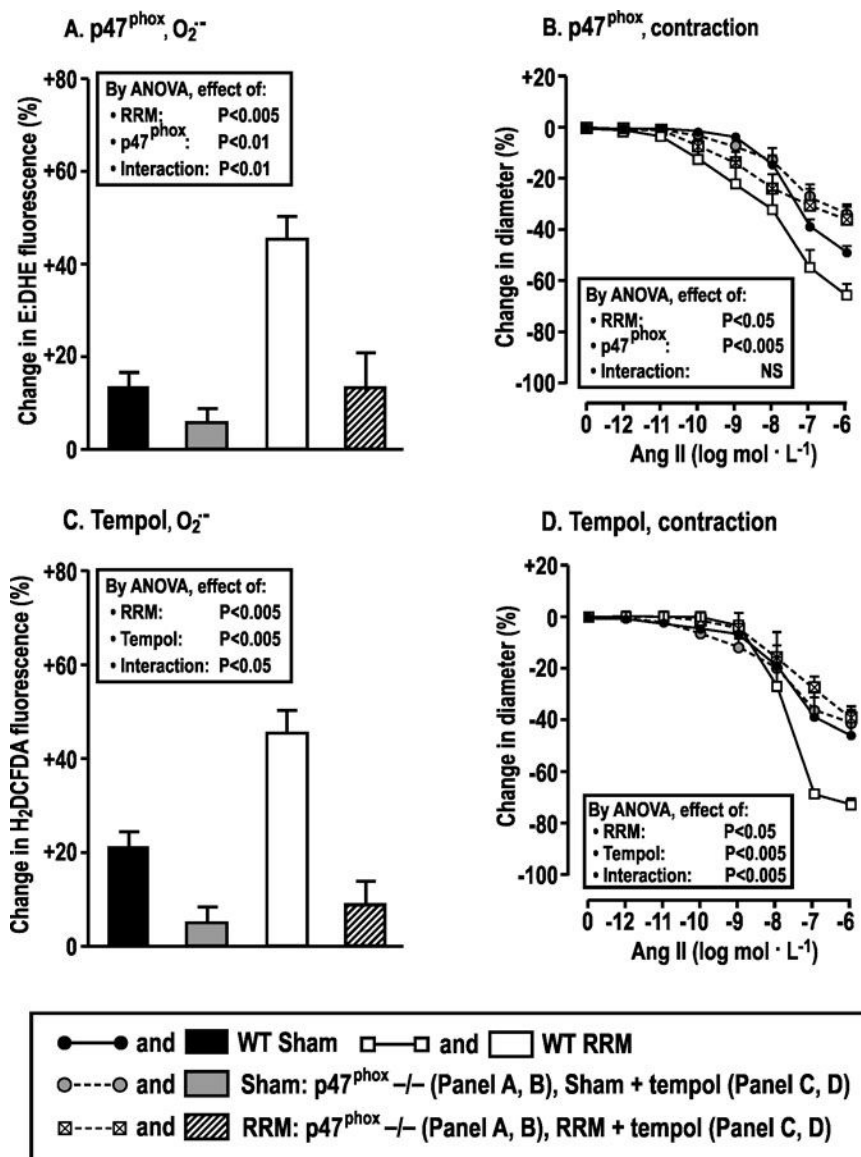


**Figure 2:**

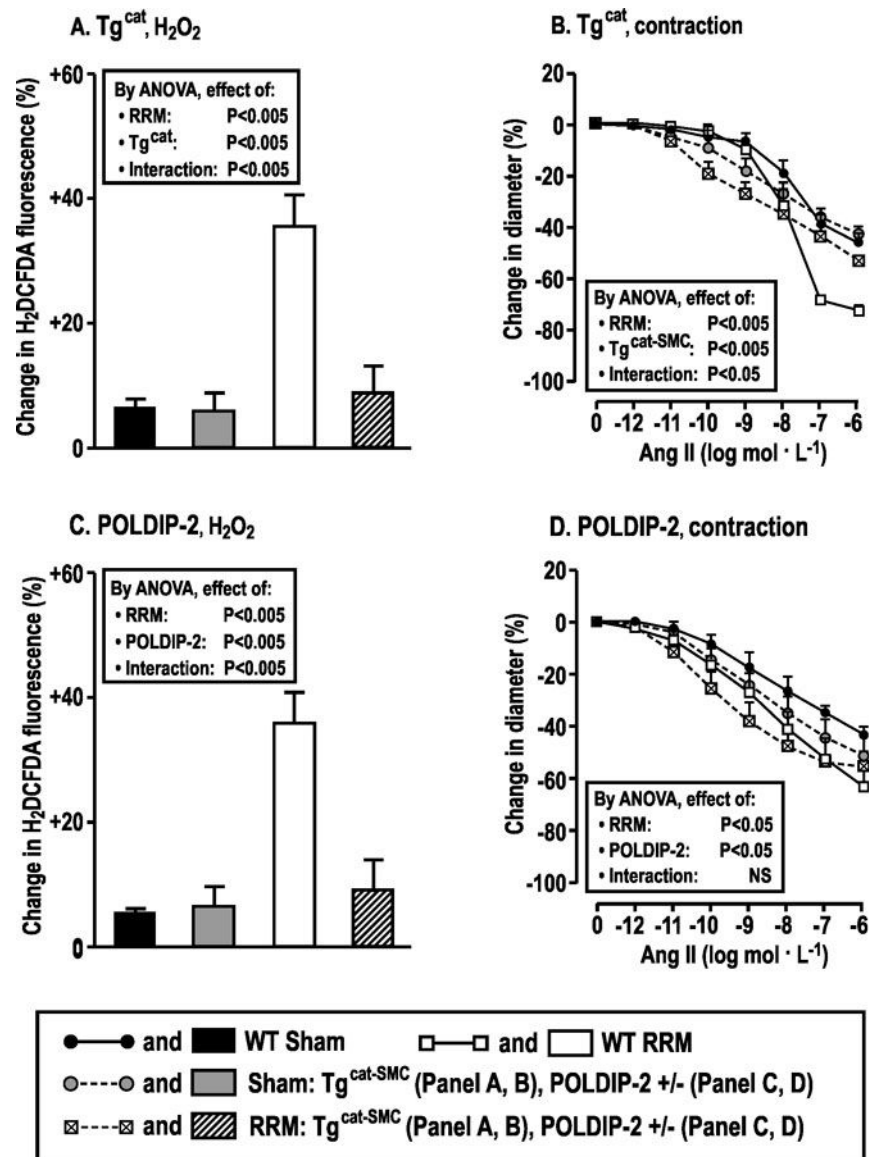
Effects of dietary salt intake and reduced renal mass on gene expression in afferent arterioles. Mean  $\pm$  SEM values ( $n=6$ ) for expression of mRNAs for angiotensin type 1 receptor (AT<sub>1</sub>R), catalase, p47<sup>phox</sup>, neutrophil oxidase 2 (NOX2), POLDIP-2 and NOX4 in afferent arterioles dissected from C57Bl/6 WT mice fed with normal salt (NS) or high salt (HS) diets for three months after sham (solid boxes) and RRM (open boxes) surgeries.



**Figure 3:** Reduced renal mass in mice fed a high salt diet increases the afferent arteriolar  $O_2^{\cdot -}$  and  $H_2O_2$  generation with angiotensin II that contributes to angiotensin II contractions. Mean  $\pm$  SEM values ( $n = 6$ ) for changes in  $O_2^{\cdot -}$ ,  $H_2O_2$  and responses to angiotensin II of afferent arterioles from C57Bl/6 wild type (WT) mice fed a high salt diet for three months after sham surgery (solid boxes, solid circles with continuous line) or RRM (open boxes, open squares with continuous lines) and after addition of PEG-catalase or PEG-SOD to the bath of perfused afferent arterioles from mice after sham (gray filled cycles with broken line) or RRM (crossed boxes with broken lines). Data are shown for changes in ethidium: dihydrithydium (E: DHE) fluorescence (Panel A) or  $H_2$ DCFDA fluorescence (panel C) with  $10^{-9}$  or  $10^{-6} \text{ mol} \cdot \text{L}^{-1}$  of angiotensin II, and changes in diameters (panels B, D) in response to graded angiotensin II.

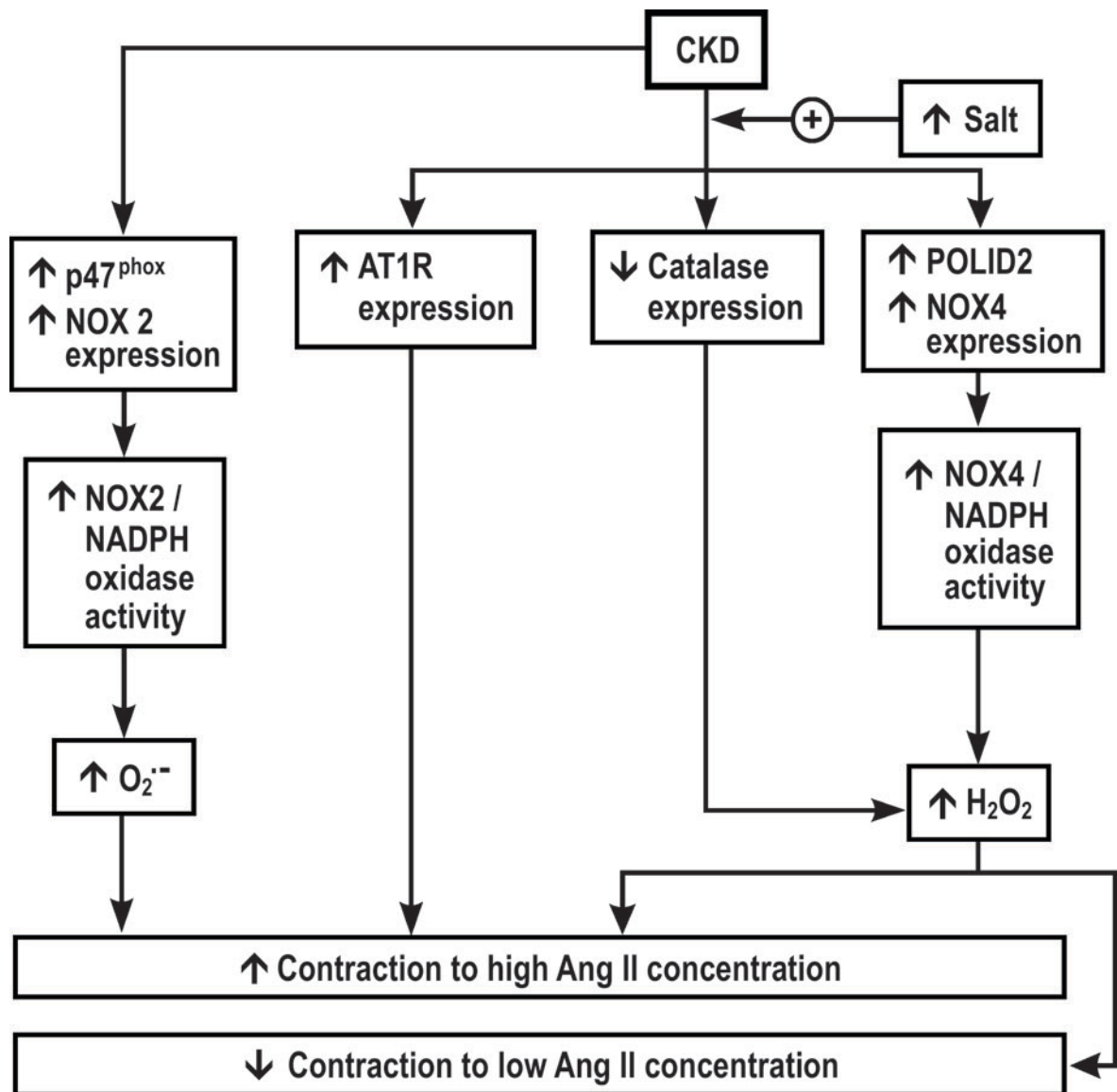


**Figure 4:** Superoxide generated by p47<sup>phox</sup>/NOX2 is required for enhanced contractions to high concentrations of Ang II in arterioles from mice with reduced renal mass fed a high salt diet. Mean  $\pm$  SEM values ( $n = 5 - 7$ ) for changes in O<sub>2</sub><sup>-</sup> and responses to angiotensin II of afferent arterioles from mice fed a high salt diet for three months after sham surgery (p47<sup>phox</sup> +/+ : solid boxes, or solid circles with continuous lines; p47<sup>phox</sup> -/- : gray filled boxes or gray filled circles with broken lines) or RRM (p47<sup>phox</sup> +/+ : open boxes, open squares with continuous lines; p47<sup>phox</sup> -/- : slash hatched boxes, crossed boxes with broken lines) surgeries. In other studies, sham or RRM mice fed a high salt diet were administered tempol (2 mmol·L<sup>-1</sup>) or vehicle for 3 months. Data are shown in panels A and C for changes in ethidium: dihydroethidium (E: DHE) ratio with 10<sup>-6</sup> mol·L<sup>-1</sup> of angiotensin II, and in panels B and D for changes in diameter in response to graded angiotensin II.



**Figure 5:** Hydrogen peroxide generated by POLDIP2/NOX4 modulates contractions to angiotensin II in afferent arterioles from mice with reduced renal mass fed a high salt diet. Mean  $\pm$  SEM values ( $n = 6$ ) for changes in  $H_2O_2$  and responses to angiotensin II of afferent arterioles from WT and transgenic mice over-expressing catalase in smooth muscle cells ( $Tg^{CAT-SMC}$ ) or POLDIP2 $\pm$  mice fed a high salt diet for three months after sham surgery (WT-sham: solid boxes, solid circles with continuous lines; sham  $Tg^{CAT-SMC}$  or sham POLDIP2 $\pm$ : gray filled boxes, gray filled circles with broken lines) or RRM (WT-RRM: open boxes, open squares with continuous lines; RRM  $Tg^{CAT-SMC}$  or RRM POLDIP2 $\pm$ : slash hatched boxes, crossed boxes with broken lines). Data are shown for changes in  $H_2DCFDA$  fluorescence with  $10^{-6} \text{ mol} \cdot \text{L}^{-1}$  of angiotensin II (panel A, C), and changes in diameters (Panel B, D) with graded angiotensin II.





**Figure 6:**

Hypothesis for effects of dietary salt and CKD on angiotensin II contractions of renal afferent arterioles. CKD, chronic kidney disease; AT<sub>1</sub>R, angiotensin type 1 receptor; NOX, neutrophil oxidase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase. The effects of CKD to increase afferent arteriolar expression of AT<sub>1</sub>R, POLDIP2 and NOX4 and to decrease catalase (and hence to increase H<sub>2</sub>O<sub>2</sub>) are enhanced by a high salt diet whereas CKD itself enhances expression of p47<sup>phox</sup> and NOX2 that increase superoxide (O<sub>2</sub><sup>-</sup>). Superoxide, AT<sub>1</sub>R and H<sub>2</sub>O<sub>2</sub> all increase maximal concentrations with angiotensin II whereas H<sub>2</sub>O<sub>2</sub> reduces contractions to lower concentrations of angiotensin II.