



# Renal denervation attenuates hypertension and renal dysfunction in a model of cardiovascular and renal disease, which is associated with reduced NADPH and xanthine oxidase activity



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## ABSTRACT

Oxidative stress is considered a central pathophysiological event in cardiovascular disease, including hypertension. Early age reduction in renal mass is associated with hypertension and oxidative stress in later life, which is aggravated by increased salt intake. The aim of the present study was to examine if renal sympathetic denervation can exert blood pressure lowering effects in uninephrectomized (UNX) rats (3-week old) fed with high salt (HS, 4%; w/w) diet for 4 weeks. Moreover, we investigated if renal denervation is associated with changes in NADPH and xanthine oxidase-derived reactive oxygen species.

Rats with UNX + HS had reduced renal function, elevated systolic and diastolic arterial pressures, which was accompanied by increased heart weight, and cardiac superoxide production compared to sham operated Controls. UNX + HS was also associated with higher expression and activity of NADPH and xanthine oxidase in the kidney. Renal denervation in rats with UNX + HS attenuated the development of hypertension and cardiac hypertrophy, but also improved glomerular filtration rate and reduced proteinuria. Mechanistically, renal denervation was associated with lower expression and activity of both NADPH oxidase and xanthine oxidase in the kidney, but also reduced superoxide production in the heart.

In conclusion, our study shows for the first time that renal denervation has anti-hypertensive, cardio- and reno-protective effects in the UNX + HS model, which can be associated with decreased NADPH oxidase- and xanthine oxidase-derived reactive oxygen species (i.e., superoxide and hydrogen peroxide) in the kidney.

## 1. Introduction

Previous studies have shown that reduction of the total nephron number by uninephrectomy (UNX), after the completion of nephrogenesis, combined with high salt (HS) intake can lead to the development of renal and cardiovascular disease in animals and humans [1–5]. Mechanistically, the progression of hypertension in this setting has been associated with oxidative stress and nitric oxide deficiency in the renal cortex, and pharmacological interventions limiting reactive oxygen species (ROS) have been shown to have therapeutic effects [6,7].

NADPH oxidase and xanthine oxidase are two major enzymes capable of producing large amounts of ROS in the kidney, and their over-activation has been linked to development or progression of cardiovascular disease including hypertension [8–11]. Increased sympathetic nerve activity and noradrenaline-mediated signaling activates renal NADPH oxidase and vice versa, thus creating a vicious cycle with detrimental effects in the cardiovascular system [12–14]. As far the interplay between xanthine oxidase and sympathetic nerves is concerned, there are currently few studies showing that xanthine oxidase-derived ROS may promote sympathetic innervation or conversely that sympathetic stimulation can activate xanthine oxidase [15,16].

**Abbreviations:** HS, high salt; UNX, uninephrectomy; eGFR, estimated glomerular filtration rate; NADPH, nicotinamide adenine dinucleotide phosphate; RDN, renal denervation; ROS, reactive oxygen species; TXB<sub>2</sub>, 11-dehydro-thromboxane B<sub>2</sub>; 8,12-iso-iPF<sub>2a</sub>-VI, 12-iso-5(R), 6E, 14Z-prostaglandin F<sub>2a</sub>; 5-iPF<sub>2a</sub>-VI, 5-prostaglandin F<sub>2a</sub>-VI; ADMA, asymmetrical dimethylarginine; SDMA, symmetrical dimethylarginine; MDA, malondialdehyde; O<sub>2</sub><sup>•-</sup>, superoxide; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; phox, phagocytic oxidase; NOX, NADPH oxidase; XOR, xanthine oxidoreductase; TBP, TATA-binding protein; Crea, creatinine; bpm, beats per minute

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Considering the important role of the sympathetic nervous system in the regulation of cardiovascular and renal functions [17], this has been an important target for the therapeutic interventions. Although there are controversies regarding the therapeutic value of renal sympathetic denervation (RDN) in patients with resistant hypertension [18,19], there are numerous experimental studies demonstrating anti-hypertensive and cardioprotective effects, as well as renoprotection [20,21]. However, the underlying mechanism and the importance of renal sympathetic nerves as modulators of ROS producing enzymes is not fully understood [18].

Therefore, in the present study we aimed to investigate the hypothesis that RDN may attenuate hypertension and modulate the major ROS-producing enzymes NADPH and xanthine oxidase, by using a clinically relevant model of cardiovascular and renal disease (*i.e.*, combination of uninephrectomy and high salt diet).

## 2. Material and methods

### 2.1. Animal protocol

This study was approved by the institutional ethics review board in Stockholm (N139/15). All animal procedures were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or National Institutes of Health guidelines. A detailed description of experimental protocol is given in the Supplement (Fig. S1).

### 2.2. Uninephrectomy and RDN

The surgical procedures were performed as previously described [1,6,7,22]. After surgery, all rats were left to grow with free access to either regular chow or HS diet. The efficiency of RDN was validated by measuring the protein expression of tyrosine hydroxylase (data not shown), which is an established marker of sympathetic nerve activity [23].

### 2.3. Blood pressure and heart rate measurements

Blood pressure and heart rate were measured as previously described [24] by using the Coda High Throughput Non-invasive Tail Monitoring System (Kent Scientific, Torrington, CT, USA). After proper training period to minimize stress due to the restraining procedure, systolic, diastolic, and mean arterial pressure (MAP) together with heart rate were collected over 5 consecutive measurements. Averaged data of all values accepted by the software was used for analysis.

### 2.4. Glomerular filtration, proteinuria and urinary markers associated with oxidative stress

Rats were housed individually in metabolism cages for 12 h and water was given *ad libitum*. Urine production was measured gravimetrically and subsequently stored at  $-80^{\circ}\text{C}$  for later analysis. Estimated glomerular filtration rate (eGFR) was measured by using creatinine clearance in matched urine and plasma samples. Urine protein content was measured with the Bradford assay (Protein Assay Dye Reagent Concentrate, Biorad) in undiluted urine samples centrifuged at 1000g, 15 min,  $4^{\circ}\text{C}$ . Protein concentration was normalized by the total urine volume and the animals' body weight. Urinary markers (11-dehydro TXB<sub>2</sub>, 8,12-iso-iPF2a-VI, 5-iPF2a-VI) were measured by liquid chromatography tandem mass spectrometry (LC-MS) [7] and normalized by urine creatinine.

### 2.5. Plasma markers associated with oxidative stress

Plasma asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and N-monomethylarginine (MNMA) were

measured by HPLC as previously described [7,25]. Spectrophotometric (Absorbance at 532 nm) analysis of thiobarbituric acid-reactive substances was used to measure content of malondialdehyde (MDA) [26,27].

### 2.6. NADPH oxidase and xanthine oxidase activity

NADPH or xanthine oxidase-mediated superoxide ( $\text{O}_2^{\cdot-}$ ) production was measured in renal tissue by lucigenin-based chemiluminescence [9,24]. Cardiac  $\text{O}_2^{\cdot-}$  production was measured in lysed left ventricle by lucigenin-based chemiluminescence with NADPH as a substrate [24]. NADPH or xanthine oxidase-mediated hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production was measured by an Amplex Red mediated-fluorescence method [28]. Relevant modifications of our previously published methods are given in the supplement.

### 2.7. mRNA expression

mRNA expression of NADPH oxidase subunits and of xanthine oxidase was measured by SYBR Green based Real Time PCR [22,24]. All measured genes were normalized by the expression of TATA-binding protein (TBP) using the  $\Delta\Delta\text{Ct}$  method, and expressed as relative changes compared to Controls. Information about the primers sequences is given in the supplement.

### 2.8. Protein expression

Protein expression of NADPH oxidase subunits and xanthine oxidase was measured by western blot [28,29]. All measured proteins were normalized by the expression of  $\beta$ -actin, and expressed as relative changes compared to Controls. Information about the primary antibodies and all the blots are provided as supplement (Table S1 & Fig. S3).

### 2.9. Statistical analysis

Statistical analysis was performed by using one-way ANOVA followed by appropriate posthoc test for comparisons among three or more groups. Single comparisons between normally distributed parameters between two groups were made by using unpaired Student's *t*-test as appropriate. All statistical calculations were made by using Graphpad Prism 6 (La Jolla, CA, USA). Statistical significance was denoted as  $p < 0.05$  and all the values are presented as Mean  $\pm$  SE.

## 3. Results and discussion

At the end of the experimental protocol (*i.e.*, time of termination) there were no differences in body weight among the groups (Supplemental Information).

### 3.1. RDN attenuates hypertension and cardiovascular complications

We have previously shown that UNX + HS, after the completion of nephrogenesis, leads to elevated blood pressure in later life in both rats and mice [1,6,7,30]. The present study supports previous findings, and show that UNX + HS is associated with elevated systolic, diastolic and mean arterial pressure (Fig. 1A-C), compared with Control rats, but without any effect on heart rate (Fig. 1D). In addition, UNX + HS was linked with cardiovascular abnormalities, as manifested by cardiac hypertrophy and increased  $\text{O}_2^{\cdot-}$  production in the cardiac tissue (Fig. 1E-F). Importantly, RDN in the UNX + HS group was associated with similar blood pressure, heart weight and cardiac  $\text{O}_2^{\cdot-}$  production as that observed in Control rats, and had no effect on heart rate (Fig. 1A-F). We have recently demonstrated that RDN is able to attenuate hypertension, cardiac hypertrophy and oxidative stress in a model of renal disease (*i.e.*, experimentally induced hydronephrosis) [22]. In agreement with previous findings in normal healthy animals,

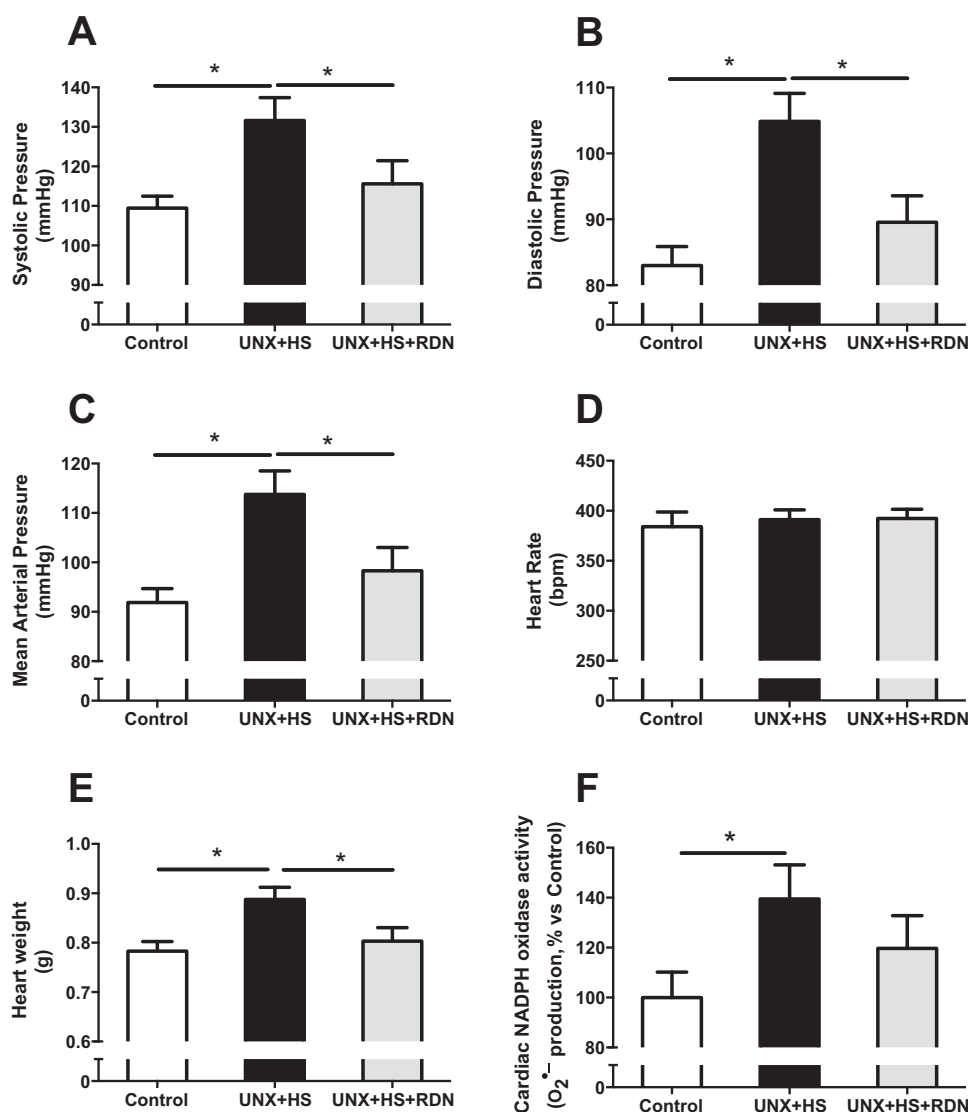


Fig. 1. Systolic (A), diastolic (B), mean arterial pressure (C) and heart rate (D) in Control, UNX + HS and UNX + HS + RDN rats. Cardiac hypertrophy estimated as the total heart weight (E) and cardiac  $O_2^{\cdot-}$  production (F).  $n = 7-10/\text{group}$ ,  $*p < 0.05$  vs. indicated group.

we did not observe any significant effects of RDN alone on cardiovascular parameters, *i.e.*, blood pressure, heart rate, heart weight (Fig. S5A-C). However, this is the first study demonstrating protective effects of RDN in rats with UNX + HS, which a clinically relevant model of compromised kidney function and cardiovascular disease.

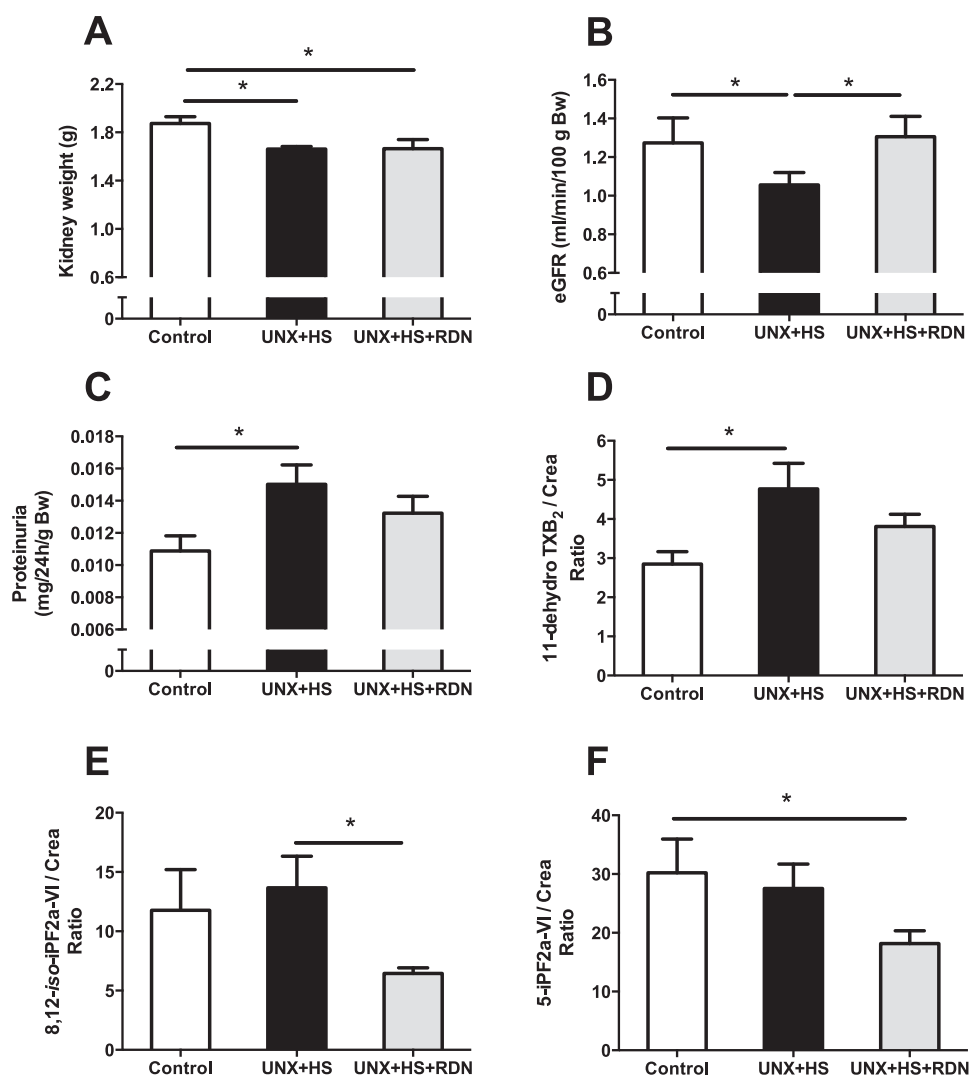
### 3.2. RDN improves kidney function, reduces proteinuria and oxidative stress

UNX + HS rats had reduced total kidney weight, which was not affected by RDN (Fig. 2A). In agreement with previous findings [1], renal function, as determined by eGFR, was reduced in UNX + HS rats and accompanied by proteinuria and higher urinary 11-dehydro TXB<sub>2</sub> levels (Fig. 2B-D). 11-dehydro TXB<sub>2</sub> is positively associated with the risk of cardiovascular events, stroke and mortality in humans [31,32], thus elevated 11-dehydro TXB<sub>2</sub> / creatinine ratio in the UNX + HS group could be indicative of cardiovascular pathologies. Importantly, RDN prevented all abovementioned pathologies associated with UNX + HS (Fig. 2B-D), and also lowered the oxidative stress markers 8,12-iso-iPF2a-VI and 5-iPF2a-VI (Fig. 2E-F). Previous studies, monitoring animals for a longer period of time, showed increased plasma MDA, ADMA and SDMA levels following UNX + HS, but this was not observed in this study using a shorter duration with UNX + HS (Fig. S4A-D). This finding suggests a vicious cycle with UNX + HS-induced renal and cardiovascular abnormalities progressing over time. RDN alone had no

significant effects on estimated renal function or kidney weight ( $1.87 \pm 0.05$  vs.  $1.80 \pm 0.06$  g;  $P > 0.05$ ) in the control rats (Fig. S5E) (Fig. 3).

### 3.3. RDN attenuates expression and activity of NADPH and xanthine oxidase

Our previous findings suggested that increased renal NADPH oxidase-derived  $O_2^{\cdot-}$  production is a causative factor for the development of hypertension in the UNX + HS model [6]. However, there is lack of knowledge regarding the specific NADPH oxidase subunits, and the contribution of xanthine oxidase and potential changes in  $H_2O_2$  production. Our findings show that UNX + HS led to significant elevation of mRNA expression of p22phox (Fig. 4A) and NOX4 (Fig. S2B) as well as xanthine oxidase (Fig. 4C), which were all normalized by RDN. In addition, UNX + HS animals had higher protein expression of p22phox (Fig. 4B) and p47phox (Fig. S2E) as well as xanthine oxidase (Fig. 4D). At the protein level, RDN normalized the expression of p22phox and xanthine oxidase (Fig. 4B, D) and suppressed also p67phox (Fig. S2F). Since both NADPH oxidase and xanthine oxidase are capable of producing large amounts of  $O_2^{\cdot-}$  and  $H_2O_2$ , we also evaluated the production of these oxidative molecules. As shown in Fig. 3, UNX + HS rats presented significantly higher NADPH oxidase-derived  $O_2^{\cdot-}$  and  $H_2O_2$  production (Fig. 3A,B), and increased xanthine oxidase-derived



**Fig. 2.** Total kidney weight (A), kidney function determined as eGFR (B), proteinuria (C) and urinary markers of oxidative stress, *i.e.*, 11-dehydro TXB<sub>2</sub>/Creatinine (D), 8,12-iso-iPF2a-VI/Creatinine (E) and 5-iPF2a-VI/Creatinine Ratio (F) in Control, UNX + HS and UNX + HS + RDN rats. n = 7–10/group for (A), n = 9–10/group for (B), n = 5–8/group for (C–E), \*p < 0.05 vs. indicated group.

H<sub>2</sub>O<sub>2</sub> levels (Fig. 3C). Finally, RDN alone had no significant effects on cardiac or renal NADPH oxidase and xanthine oxidase activities in the control rats (Fig. S5D,F,G,H).

We have previously shown that tempol, which acts as a superoxide dismutase and also as a multifunctional antioxidant [33], is able to attenuate hypertension in the UNX + HS animals, indicating that superoxide and oxidative stress is a causative factor that leads to the progression of hypertension in this model [6]. In a subset of rats we included *in vivo* treatment with tempol in their drinking water, which protected the UNX + HS rats from developing cardiac hypertrophy and NADPH or xanthine oxidase-mediated oxidative stress (Fig. S6). Importantly, the UNX + HS + RDN treated rats that also received tempol in their drinking water did not differ significantly from the UNX + HS treated animals (Fig. S6). Although we cannot be sure that the reduced NADPH and xanthine oxidase activity observed here is the only causative factor leading to the cardiac and renal protection exerted by RDN, our data from tempol treated rats indicate that attenuation of oxidative stress is a possible mechanism, contributing to the observed protective effects following RDN. However, the presence of other simultaneous protective mechanisms mediated by RDN *e.g.*, attenuated activity of the renin-angiotensin-aldosterone system and reduced renal water and sodium reabsorption, as well as modulation of central sympathetic nerve activity, cardiac angiogenesis and immune cell function cannot be excluded [21,34,35].

The prevalence of hypertension is increasing worldwide, and is one of the main risk factors for developing adverse renal and cardiovascular

complications. Emerging evidence suggests that increased sympathetic nerve activity can promote or accelerate the development of hypertension and cardiac hypertrophy, and increase the risk of adverse complications [36]. Several experimental and clinical studies have demonstrated favorable effects of renal denervation (RDN) in hypertension and renal disease [20,21]. However, findings obtained from patients with drug-resistant hypertension (SYMPPLICITY HTN 1–3) have started a global debate regarding the therapeutic value of RDN in patients with resistant hypertension, but also other chronic disorders [21,37,38]. Further evaluation in rigorously designed clinical trials are needed, together with additional experimental studies to investigate the series of events, and also to further characterize the proposed interaction between sympathetic nerves and NADPH oxidase and xanthine oxidase regulation in renal and cardiovascular disease.

In conclusion, reduced renal mass combined with a high salt intake (UNX + HS) is associated with cardiac and renal abnormalities in later life, which is linked with increased oxidative stress. RDN prevents the development of hypertension, cardiac hypertrophy, and renal dysfunction and is associated with an attenuation of NADPH oxidase and xanthine oxidase-mediated O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> production in the kidney.

#### Authors' contributions

M.P. and M.C. designed experiments, performed experiments and wrote the paper. P.F., Z.Z. and C.G. conceived or designed aspects of the research work, acquired data, and played important role in interpreting

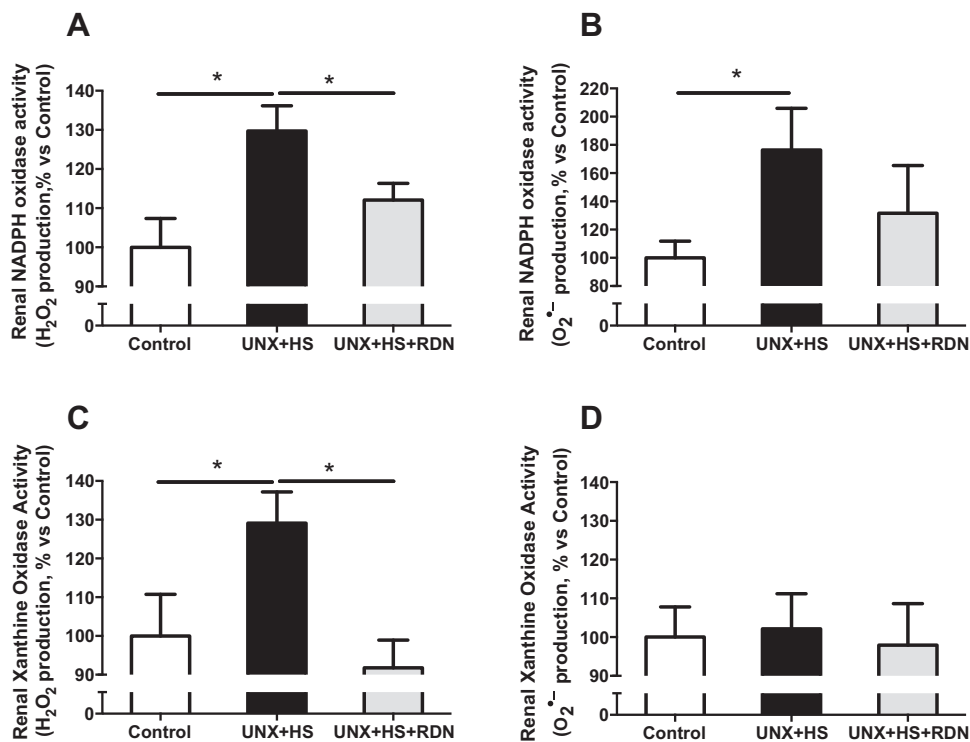


Fig. 3. Renal NADPH oxidase-mediated H<sub>2</sub>O<sub>2</sub> (A) and O<sub>2</sub><sup>-</sup> (B) production, as well as renal xanthine oxidase-mediated H<sub>2</sub>O<sub>2</sub> (C) and O<sub>2</sub><sup>-</sup> (D) production in Control, UNX + HS and UNX + HS + RDN rats. n = 6–10/group, \*p < 0.05 vs. indicated group.

the results. C.E.W. and A.G.P. offered insight into the design and data analysis, and edited the manuscript. M.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Conflict of interest**

The authors declare no conflict of interest.

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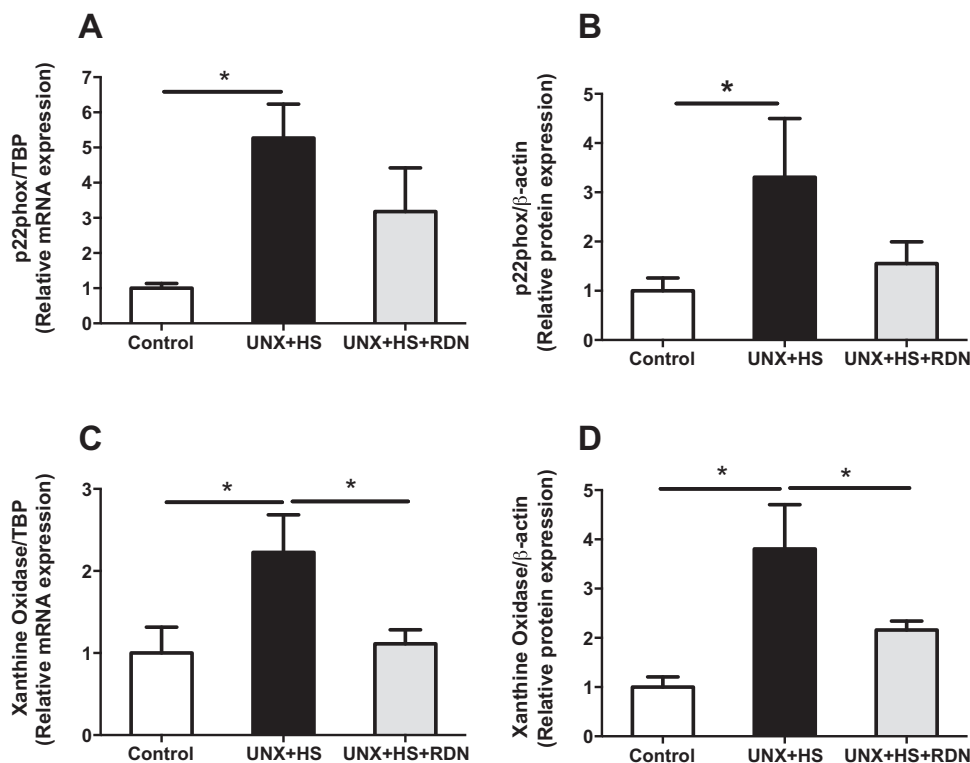


Fig. 4. Relative changes in p22phox mRNA (A) and protein (B) expression, as well as relative changes in xanthine oxidase mRNA (C) and protein (D) expression in kidneys from Control, UNX + HS and UNX + HS + RDN rats. n = 6–10/group for (A & C), n = 5–8/group for (B & D), \*p < 0.05 vs. indicated group.



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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2017.06.013>.

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