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3 **Anti-hypertensive effect of radiofrequency renal denervation in spontaneously**  
4 **hypertensive rats.**

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14

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**Abstract**

**Aims:** We aimed to investigate the anti-hypertensive effect of radiofrequency (RF) renal denervation (RDN) in an animal model of hypertension.

**Materials and Methods:** RF energy was delivered with opening abdomen to bilateral renal arteries through a 2Fr catheter in 8 spontaneously hypertensive rats (SHR) and 8 Wistar-Kyoto rats (WKY). Sham operation was performed in other 8 SHR and 8 WKY. Blood pressure (BP), heart rate (HR), and urinary norepinephrine excretion were followed up for 3 months. Plasma and renal tissue concentrations of norepinephrine and plasma renin activity were measured 3 months after the procedure. The RDN was confirmed by a decrease in renal tissue norepinephrine.

**Key findings:** RF-RDN restrained a spontaneous rise in systolic BP ( $46\pm 12\%$  increase from  $158\pm 8$  to  $230\pm 14$  mmHg vs.  $21\pm 18\%$  increase from  $165\pm 9$  to  $197\pm 20$  mmHg,  $p=0.01$ ) and diastolic BP ( $55\pm 27\%$  increase from  $117\pm 9$  to  $179\pm 23$  mmHg vs.  $28\pm 13\%$  increase from  $120\pm 7$  to  $154\pm 13$  mmHg,  $p=0.04$ ) in SHR; however, WKY were not affected. Although there were no changes in HR and systemic norepinephrine, the renal tissue norepinephrine was decreased by RF-RDN in both SHR ( $302\pm 41$  vs.  $159\pm 44$  ng/g kidney,  $p<0.01$ ) and WKY ( $203\pm 33$  vs.  $145\pm 26$  ng/g kidney,  $p=0.01$ ). Plasma renin activity was reduced by the RF-RDN only in SHR ( $35.3\pm 9.5$  vs.  $21.4\pm 8.6$  ng/mL/hr,  $p<0.01$ ).

**Significance:** RF-RDN demonstrated an anti-hypertensive effect with a reduction of renal tissue norepinephrine and plasma renin activity in SHR.

**Key words:** Renal Denervation; Resistant Hypertension; SHR; Norepinephrine; Renin; Endothelin

## Introduction

Hypertension is one of the most important modifiable risk factors for cardiovascular morbidity and mortality (Lewington et al. 2002; Staessen et al. 2003; Hisham et al. 2013; Zambon et al. 2014). A billion people in the world have hypertension and the incidence is predicted to increase by 60% in 2025 (Kearney et al. 2005). Furthermore, approximately 13% of patients with hypertension remains above target blood pressure (BP) despite concurrent use of three or more anti-hypertensive drugs of different classes including diuretics; namely, the resistant hypertension (Kumbhani et al. 2013).

Renal efferent sympathetic and afferent sensory nerves, which adjacently surround the renal arterial wall, play a crucial role in the development and maintenance of hypertension (DiBona and Esler 2010; Kopp et al. 2011; DiBona 2013). Actually, a radical surgical sympathetic denervation had been demonstrated an anti-hypertensive effect; however, it was associated with high perioperative morbidity and mortality and long-term complications (Morrissey et al. 1953; Smithwick and Thompson 1953; Evelyn et al. 1960). Recently, a radiofrequency renal denervation (RF-RDN) using a catheter-based technique has been featured as an effective and less invasive approach for the resistant hypertension (Krum et al. 2009; Esler et al. 2012). The mechanisms of RF-RDN have been thoroughly investigated; however, they are not so clear yet. Furthermore, the responders and adequate procedural endpoint of RF-RDN have been uncertain.

In animal models of hypertension, the anti-hypertensive effect of surgical RDN and its procedural techniques consisting of cutting renal nerves and swabbing phenol have been well established (Kline et al 1980; Winternitz et al 1980; Janssen et al 1989;

1 Girchev et al. 2006; Lohmeier et al. 2012; Rafiq et al. 2012; Katayama et al. 2013). The  
2 RF-RDN studies in animals, however, have been limited to normotensive animals such  
3 as swine and canine (Rippy et al. 2011; Steigerwald et al. 2012; Chinushi et al. 2013).  
4 There is no report concerning the effects of RF-RDN on BP in hypertensive animals such  
5 as spontaneously hypertensive rats (SHR). Furthermore, technical details of RF-RDN  
6 in small animals such as rats have not been reported. Because the RF-RDN is applied  
7 to the patients with resistant hypertension, it seems to be quite important to reveal the  
8 precise effects of RF-RDN on BP in SHR.

9         Therefore, this study aimed to investigate the anti-hypertensive effect of RF-RDN  
10 in a rat model of hypertension, SHR.

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## Material and Methods

### Animals

Male SHR and their normotensive controls, Wistar-Kyoto rats (WKY) were purchased from Japan Charles River (Kanagawa, Japan) at 8 weeks of age. All rats were housed in an animal facility with a 12-hour light/dark cycle. They received standard chow (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water ad libitum. All experimental procedures were performed in accordance with institutional guidelines for animal research approved by the Experimental Animal Committee at University of Tsukuba.

### Experimental Protocol

To evaluate the anti-hypertensive effect of RF-RDN, all rats were followed up for 3 months after treatments: bilateral RF-RDN (SHR-RDN, n=8; WKY-RDN, n=8) and sham operation (SHR-Sham, n=8; WKY-Sham, n=8). The RF-RDN and sham operation were performed at 12 weeks of age, as described below. Blood pressure (BP) and heart rate (HR) were recorded at baseline (10 weeks of age) and every month after the treatments. The 24-hour urinary samples were collected at baseline (10 weeks of age), 1 month, and 3 months after the treatments. After the 3-month follow-up period, arterial blood was obtained by cardiac puncture under anesthetization with pentobarbital sodium; the plasma was collected for measurement of norepinephrine and renin activity by centrifugation and stored at  $-80^{\circ}\text{C}$  until analysis. Renal arteries were immediately excised and fixed with 4% paraformaldehyde for histological examination of renal nerves. Kidneys were frozen for measurement of renal tissue norepinephrine to verify completion of the RF-RDN. Biochemistry of the renal tissue, blood, and urine were performed at SRL Inc. (Tokyo, Japan).

## 1 **Radiofrequency Renal Denervation and Sham Operation**

2 Rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). A ventral  
3 incision was made in the midline. The bilateral renal arteries were exposed by blunt  
4 dissection. A 2Fr ablation catheter (Ensemble, Japan Lifeline Co., Tokyo, Japan) was  
5 placed on a renal artery. RF energy was delivered from a distal tip of the catheter under  
6 temperature control mode targeting 60 °C with maximum output of 3 W. As a dispersive  
7 electrode, a rectangular electro-conductive plate (60 cm<sup>2</sup>) was put on the shaved and  
8 depilated back of rats. The abdominal cavity was irrigated with saline during each RF  
9 application (30 sec). Local impedance and its decrease after the RF application were  
10 obtained at the distal tip of the catheter. The RF application was interrupted before  
11 reaching 30 sec under the following conditions: a change in local impedance exceeding 60  
12  $\Omega$  and a rise in local temperature exceeding 60 °C. The RF application was repeated until  
13 the number of RF application with duration of more than 20 sec reached three times for  
14 each renal artery. As a sham operation, the renal arteries were exposed and the local  
15 impedance was obtained in the same way as the RF-RDN; however, the RF energy was  
16 not delivered.

## 17 **Blood Pressure, Heart Rate, and Urine Samples**

18 Before recordings of BP and HR, rats were pre-heated in a chamber at 35 °C for 10  
19 min, then placed in plastic restrainers. A cuff with a pneumatic pulse sensor was  
20 attached to the proximal tail. BP and HR were recorded on a Model BP-98A (Softron  
21 Co., Ltd., Tokyo, Japan) with heating; the records were averaged from five consecutive  
22 readings obtained from each rat. After the recordings of BP and HR, all rats were housed  
23 in metabolic cages (CT-10S type II; CLEA Japan, Inc., Tokyo, Japan) to collect urinary

1 samples for 24 hours to measure urinary norepinephrine excretion. The urine sample  
2 was collected in a bottle containing 6 N hydrochloric acid.

### 3 **Renal Tissue Norepinephrine**

4 The frozen kidneys were homogenized in ice-cold 0.3 N perchloric acid. The  
5 homogenate was centrifuged at 18,600 g for 20 min (Avanti HP-25; Beckman Coulter,  
6 Inc., CA, USA); the supernatant was stored at  $-80^{\circ}\text{C}$  until analysis. The renal tissue  
7 norepinephrine concentration was determined by high performance liquid  
8 chromatography (Nakashima et al. 1996). The renal tissue norepinephrine (ng/g kidney)  
9 was calculated as follows: renal tissue norepinephrine concentration (ng/mL)  $\times$   
10 homogenate volume (mL) / kidney weight (g).

### 11 **Histological Examination**

12 Renal arteries were fixed with 4% paraformaldehyde, embedded in paraffin,  
13 sectioned into 4- $\mu\text{m}$ -thick slices, and stained with Masson's trichrome protocol for  
14 evaluation of the renal nerves that adjacently surround the renal arterial wall. Images  
15 were obtained by a digital microscopy (Biozero BZ-8000, Keyence, Chicago, IL).

### 16 **Statistical Analysis**

17 All data were expressed as mean $\pm$ standard deviation. Experimental groups were  
18 compared by one-way analysis of variance followed by the Tukey's test for multiple  
19 comparisons. Differences were considered statistically significant with  $p < 0.05$ . Analysis  
20 was performed using IBM SPSS version 21.0 software (IBM Co., Armonk, NY, USA).

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## Results

### Procedural Data

The RF-RDN was performed to a similar extent in the WKY-RDN and SHR-RDN (Table 1). There were no differences in mean RF power and total number, duration, and energy of RF application. The mean decrease in local impedance after each RF application was also comparable between the two groups. The local impedance was not different among the 4 groups (WKY-Sham, WKY-RDN, SHR-Sham, and SHR-RDN).

### Effects of RF-RDN on Blood Pressure

The RF-RDN for SHR significantly restrained a spontaneous rise in systolic and diastolic BP after the 3 months follow-up (Figure 1A and 1B). The SHR-RDN demonstrated a lower spontaneous rise in systolic BP than the SHR-Sham (21±18% increase from 165±9 to 197±20 mmHg vs. 46±12% increase from 158±8 to 230±14 mmHg,  $p=0.01$ ). The SHR-RDN also demonstrated a lower spontaneous rise in diastolic BP than the SHR-Sham (28±13% increase from 120±7 to 154±13 mmHg vs. 55±27% increase from 117±9 to 179±23 mmHg,  $p=0.04$ ).

The BP of WKY, however, was not affected by the RF-RDN after the 3 months follow-up (Figure 1A and 1B). A change in systolic BP was not different between the WKY-RDN and WKY-Sham (6±18% increase from 120±8 to 126±15 mmHg vs. 8±8% increase from 116±3 to 124±10 mmHg,  $p=0.99$ ) as well as a change in diastolic BP (6±19% increase from 90±7 to 95±12 mmHg vs. 7±14% increase from 88±5 to 94±11 mmHg,  $p=1.00$ ).

### Effects of RF-RDN on Heart Rate

HR was not affected by the RF-RDN in the WKY and SHR during the 3-month follow-up period (Figure 2). The HR was not different between the SHR-RDN and



1 SHR-Sham at baseline ( $350\pm 46$  vs.  $360\pm 41$  bpm,  $p=0.96$ ), 1 month ( $349\pm 19$  vs.  $381\pm 42$   
2 bpm,  $p=0.10$ ), 2 months ( $362\pm 11$  vs.  $350\pm 31$  bpm,  $p=0.88$ ), and 3 months ( $364\pm 49$  vs.  
3  $376\pm 51$  bpm,  $p=0.97$ ), or between the WKY-RDN and Sham WKY at baseline ( $355\pm 30$  vs.  
4  $365\pm 48$  bpm,  $p=0.97$ ), 1 month ( $346\pm 20$  vs.  $338\pm 21$  ng/day,  $p=0.94$ ), 2 months ( $366\pm 46$  vs.  
5  $345\pm 34$  ng/day,  $p=0.60$ ), and 3 months ( $372\pm 64$  vs.  $342\pm 35$  ng/day,  $p=0.64$ ).

### 6 **Effects of RF-RDN on Urine Output**

7 Urine output for 24 hours was not affected by the RF-RDN in the WKY and SHR  
8 during the 3-month follow-up period (Figure 3). The 24-hour urine output was not  
9 different between the SHR-RDN and SHR-Sham at baseline ( $7.4\pm 1.6$  vs.  $7.1\pm 1.1$  mL/day,  
10  $p=0.99$ ), 1 month ( $7.7\pm 1.5$  vs.  $7.6\pm 1.8$  mL/day,  $p=1.00$ ), and 3 months ( $8.4\pm 1.4$  vs.  $7.8\pm 1.8$   
11 mL/day,  $p=0.97$ ), or between the WKY-RDN and WKY-Sham at baseline ( $13.3\pm 2.4$  vs.  
12  $13.4\pm 3.7$  mL/day,  $p=1.00$ ), 1 month ( $15.5\pm 3.9$  vs.  $15.5\pm 2.6$  mL/day,  $p=1.00$ ), and 3 months  
13 ( $15.9\pm 4.0$  vs.  $14.7\pm 3.4$  mL/day,  $p=0.84$ ).

14 However, there was a difference in 24-hour urine output between the SHR and  
15 WKY. The SHR-Sham demonstrated a lower 24-hour urine output than the WKY-Sham  
16 at baseline ( $7.1\pm 1.1$  vs.  $13.4\pm 3.7$  mL/day,  $p<0.01$ ), 1 month ( $7.6\pm 1.8$  vs.  $15.5\pm 2.6$  mL/day,  
17  $p<0.01$ ), and 3 months ( $7.8\pm 1.8$  vs.  $14.7\pm 3.4$  mL/day,  $p<0.01$ ). The SHR-RDN also  
18 demonstrated a lower 24-hour urine output than the WKY-RDN at baseline ( $7.4\pm 1.6$  vs.  
19  $13.3\pm 2.4$  mL/day,  $p<0.01$ ), 1 month ( $7.7\pm 1.5$  vs.  $15.5\pm 3.9$  mL/day,  $p<0.01$ ), and 3 months  
20 ( $8.4\pm 1.4$  vs.  $15.9\pm 4.0$  mL/day,  $p<0.01$ ).

### 21 **Effects of RF-RDN on Urinary Norepinephrine**

22 The 24-hour urinary norepinephrine excretion was not affected by the RF-RDN in  
23 the WKY and SHR during the 3-month follow-up period (Figure 4). The 24-hour urinary  
24 norepinephrine excretion was not different between the SHR-RDN and SHR-Sham at

1 baseline ( $605 \pm 123$  vs.  $655 \pm 81$  ng/day,  $p=1.00$ ), 1 month ( $773 \pm 102$  vs.  $860 \pm 116$  ng/day,  
2  $p=0.86$ ), and 3 months ( $1103 \pm 182$  vs.  $1121 \pm 133$  ng/day,  $p=0.84$ ), or between the  
3 WKY-RDN and WKY-Sham at baseline ( $401 \pm 50$  vs.  $368 \pm 58$  ng/day,  $p=0.86$ ), 1 month  
4 ( $499 \pm 99$  vs.  $544 \pm 97$  ng/day,  $p=0.83$ ), and 3 months ( $654 \pm 75$  vs.  $636 \pm 56$  ng/day,  $p=0.99$ ).

5 However, there was a difference in 24-hour urinary norepinephrine excretion  
6 between the SHR and WKY. The SHR-Sham demonstrated a higher 24-hour urinary  
7 norepinephrine excretion than the WKY-Sham at baseline ( $655 \pm 81$  vs.  $368 \pm 58$  ng/day,  
8  $p<0.01$ ), 1 month ( $860 \pm 116$  vs.  $544 \pm 97$  ng/day,  $p<0.01$ ), and 3 months ( $1121 \pm 133$  vs.  
9  $636 \pm 56$  ng/day,  $p<0.01$ ). The SHR-RDN also demonstrated a higher 24-hour urinary  
10 norepinephrine excretion than the WKY-RDN at baseline ( $605 \pm 123$  vs.  $401 \pm 50$  ng/day,  
11  $p<0.01$ ), 1 month ( $773 \pm 102$  vs.  $499 \pm 99$  ng/day,  $p<0.01$ ), and 3 months ( $1103 \pm 182$  vs.  
12  $654 \pm 75$  ng/day,  $p<0.01$ ).

### 13 **Effects of RF-RDN on Plasma Norepinephrine**

14 The plasma level of norepinephrine was not affected by the RF-RDN in the WKY  
15 ( $650 \pm 193$  vs.  $618 \pm 176$  pg/mL,  $p=0.99$ ) and SHR ( $1281 \pm 208$  vs.  $1174 \pm 196$  pg/mL,  $p=0.67$ ;  
16 Figure 5). However, there was a difference in plasma norepinephrine between SHR and  
17 WKY. The SHR-Sham demonstrated a higher plasma norepinephrine than the  
18 WKY-Sham ( $1281 \pm 208$  vs.  $651 \pm 193$  pg/mL,  $p<0.01$ ). The SHR-RDN also demonstrated a  
19 higher plasma norepinephrine than the WKY-RDN ( $1174 \pm 196$  vs.  $618 \pm 176$  pg/mL,  
20  $p<0.01$ ).

### 21 **Effects of RF-RDN on Plasma Renin Activity**

22 Plasma renin activity was significantly decreased by the RF-RDN in SHR  
23 ( $35.3 \pm 9.5$  vs.  $21.4 \pm 8.6$  ng/mL/hr,  $p<0.01$ ); however, there was no difference between the  
24 WKY-Sham and WKY-RDN ( $24.5 \pm 5.1$  vs.  $25.0 \pm 4.5$  ng/mL/hr,  $p=1.00$ ; Figure 6). The

1 SHR-Sham demonstrated a higher plasma renin activity than the WKY-Sham ( $35.3 \pm 9.5$   
2 vs.  $24.5 \pm 5.1$  ng/mL/hr,  $p=0.03$ ); however, there was no difference between the SHR-RDN  
3 and WKY-RDN ( $21.4 \pm 8.6$  vs.  $25.0 \pm 4.5$  ng/mL/hr,  $p=0.77$ ).

#### 4 **Effects of RF-RDN on Renal Tissue Norepinephrine**

5 Renal tissue norepinephrine was significantly decreased by the RF-RDN in both  
6 WKY ( $203 \pm 33$  vs.  $145 \pm 26$  ng/g kidney,  $p=0.01$ ) and SHR ( $302 \pm 41$  vs.  $159 \pm 44$  ng/g kidney,  
7  $p<0.01$ ; Figure 7). The SHR-Sham demonstrated higher renal norepinephrine content  
8 than the WKY-Sham ( $302 \pm 41$  vs.  $203 \pm 33$  ng/g kidney,  $p<0.01$ ); however, there was no  
9 difference between the SHR-RDN and WKY-RDN ( $159 \pm 44$  vs.  $145 \pm 26$  ng/g kidney,  
10  $p=0.84$ ).

#### 11 **Histological Changes in Renal Nerve Bundle**

12 Renal nerve bundle was surrounded by a thin fibrotic connective tissue sheath in  
13 the SHR-Sham. By contrast, the structures of renal nerve bundle and fibrotic sheath  
14 were broken in the SHR-RDN (Figure 8). Similar changes in the renal nerve structures  
15 were also observed between the WKY-Sham and WKY-RDN.

16

## Discussion

This is the first report of an anti-hypertensive effect of RF-RDN in an animal model of hypertension. Although a surgical RDN has demonstrated anti-hypertensive effects in animal models of hypertension for more than 30 years ago (Kline et al 1980; Winternitz et al 1980; Janssen et al 1989), the previous studies of RF-RDN in animals have been limited to normotensive animals such as swine and canine (Rippy et al. 2011; Steigerwald et al. 2012; Chinushi et al. 2013). The RF-RDN in human study demonstrated the antihypertensive effect as well as surgical RDN (Krum et al. 2009; Esler et al. 2012). However, the RF-RDN study in animal models of hypertension has been lacked. We here demonstrated firstly the effects of RF-RDN on hypertension in SHR.

### Major Findings

The major findings of this study were following: (1) RF-RDN significantly lowered BP only in SHR; (2) RF-RDN significantly decreased renal tissue norepinephrine with histological disruption to the renal nerve bundle; (3) systemic (urinary and plasma) norepinephrine was higher in SHR than WKY; (4) the systemic norepinephrine was not affected by RF-RDN; (5) RF-RDN significantly reduced plasma renin activity only in SHR; (6) urine output was lower in SHR than WKY; and (7) the RF-RDN did not affect the urine output and HR.

### Anti-hypertensive Effect of RF-RDN

The renin-angiotensin system plays an important role in the regulation of BP (Griendling et al. 1993; Unger et al. 2011). Thus, we examined the effect of RF-RDN on plasma renin activity. The RF-RDN significantly reduced plasma renin activity in SHR. This finding is consistent with previous reports of surgical RDN (Lohmeier et al. 2012;

1 Katayama et al. 2013). However, we also found that the RF-RDN did not reduce plasma  
2 renin activity in WKY. In addition, the significant reduction of BP was demonstrated  
3 only in SHR. Therefore, the anti-hypertensive effect of RF-RDN in SHR might be  
4 associated with the reduction of plasma renin activity.

5 Renin release from juxtaglomerular granular cells (JGC) is evoked by (1) an  
6 increase in efferent renal sympathetic nerve activity via beta 1 adrenergic receptor at  
7 JGC, (2) a decrease in perfusion pressure inside the afferent renal arteriole detected by  
8 JGC, (3) a decrease in NaCl concentration, usually owing to a decrease in glomerular  
9 filtration ratio (GFR), at the macula densa in the distal tubule (DiBona and Esler 2010;  
10 Kurtz 2011). The GFR is reduced by a constriction of afferent renal arterioles according  
11 to an increase in efferent renal sympathetic nerve activity (Fleming et al. 1992; Chen  
12 and Fleming 1993). These regulation systems with renin and sympathetic nerve well  
13 explain a mechanism of hypertension and anti-hypertensive effect of RF-RDN in SHR.

14 The SHR-sham demonstrated higher norepinephrine levels in urine, plasma, and  
15 renal tissue than the WKY-sham. These findings indicated the enhanced renal and  
16 systemic sympathetic activity in SHR. The enhanced renal sympathetic activity directly  
17 evokes the renin release from JGC. The SHR-sham also demonstrated lower urine  
18 output than the WKY-sham. This can be explained by a reduction in GFR due to a  
19 constriction of renal afferent arteriole evoked by enhanced renal sympathetic activity,  
20 leading to further renin release from JGC. Increased renin secretion produces  
21 angiotensin II, which increases circulating blood volume through increasing sodium  
22 reabsorption by aldosterone and induces contraction of small arteries. Moreover,  
23 angiotensin II drives systemic sympathetic activity through direct activation of central  
24 sympathetic neuron. Taken together, activated renin-angiotensin system and

1 sympathetic hyperactivity creates a vicious cycle, resulting to accelerating hypertension  
2 (Griendling et al. 1993; Unger et al. 2011). The RF-RDN could restrain the spontaneous  
3 BP rise in the SHR by interrupting the vicious cycle between activated sympathetic  
4 nerve system and renin-angiotensin system. The BP could fall until the increased GFR  
5 returned to a former state to maintain the fluid homeostasis.

6 As for the depressor effects of the renin-angiotensin system inhibitors in SHRs,  
7 Susic et al reported that a chronic treatment with AT1 receptor antagonist losartan  
8 showed 39 mmHg reduction in the systolic BP of SHRs fed normal-salt diet (Susic D et  
9 al. 2011). In the present our study, the RF-RDN showed a comparable reduction in the  
10 systolic BP (33 mmHg) of SHRs fed normal-salt diet to the chronic treatment with  
11 losartan. The depressor effects of RF-RDN on SHRs fed high-salt diet, however, need a  
12 further study. Moreover, a further study on the combination therapy of the RF-RDN and  
13 AT1 blocker vs. single therapy of each alone in SHRs fed normal-salt diet and high-salt  
14 diet would be interesting and important, because the depressor effects of the AT1  
15 receptor blocker in SHRs were reported to be different between in normal-salt diet and  
16 in high-salt diet (Susic D et al. 2011).

17 The afferent renal sensory nerve plays an important role in the regulation of  
18 systemic sympathetic activity by modulating the central nervous system (Kopp et al.  
19 2011; Chinushi et al. 2013). Therefore, RF-RDN is expected to attenuate not only renal  
20 sympathetic activity but also systemic sympathetic activity and its regulating HR.  
21 Indeed, clinical trials of RF-RDN have shown a significant reduction of HR by RF-RDN  
22 (Krum et al. 2009). The possible attenuation of systemic sympathetic activity and HR,  
23 however, was not demonstrated by RF-RDN in both the SHR and WKY despite the  
24 significant attenuation of renal sympathetic activity. These findings were consistent

1 with the previous reports of surgical RDN for animal models (Lohmeier et al. 2012;  
2 Katayama et al. 2013). Furthermore, selective afferent RDN by a dorsal rhizotomy  
3 significantly decreased BP in uni-nephrectomized SHR (Janssen et al. 1989). HR,  
4 plasma norepinephrine concentrations, and responses to hexamethonium were not  
5 affected by this procedure. However, it significantly increased responses in HR to  
6 phenylephrine but not to nitroprusside. Therefore, afferent renal nerves seem to have  
7 less impact on the development and maintenance of hypertension in SHR; however, it  
8 may contribute to the mechanisms that alter sympathetic function and baroreceptor  
9 reflex sensitivity during the development of hypertension.

10       The issue of selecting responders to RDN is problematic and controversial. Our  
11 data might suggest that the RF-RDN is also effective for hypertension associated with  
12 obesity and chronic kidney disease, which also demonstrates high renin and enhanced  
13 systemic sympathetic activity. Obesity and chronic kidney disease as well as high  
14 plasma renin activity and high systemic norepinephrine might be useful indicators for  
15 screening the responders to RF-RDN (Lohmeier et al. 2012; Katayama et al. 2013;  
16 Kiuchi et al. 2013; Petras et al. 2013). On the other hand, bilateral surgical RDN delayed  
17 the onset and development of hypertension with a significant increase in urinary sodium  
18 excretion in 7-week-old SHR but not in 18-week-old SHR (Winternitz et al. 1980).  
19 Therefore, they concluded that the renal sympathetic nerves contributed to the  
20 development of hypertension during early stage in the SHR in part by causing enhanced  
21 sodium retention; however, the renal nerves did not play a significant role in the  
22 maintenance of increased BP in established hypertension. Early intervention by RDN  
23 might inhibit or delay the development of drug-resistant hypertension.

#### 24 **Technique of RF-RDN**

1           An adequate RF-RDN protocol remains a major issue, although a surgical RDN  
2 has an established protocol of cutting nerves with phenol swabbing (Girchev et al. 2006;  
3 Lohmeier et al. 2012; Rafiq et al. 2012; Katayama et al. 2013). The RF-RDN protocol  
4 should provide both safety and efficacy.

5           In this study, the RF-RDN did not cause complications such as a pop phenomenon  
6 resulting in a rupture of renal artery. A rapid increase in the local temperature above the  
7 boiling point can vaporize blood and surrounding saline, causing a mini-explosion and  
8 audible pop. Evaporation may occur intramurally, leading to gas bubble formation  
9 within the renal arterial wall. With continued energy application, this bubble expands  
10 and erupts through the weakest path, cleaving the renal artery (Juneja et al. 2001).  
11 Indeed, we had frequently experienced this pop phenomenon in our preliminary  
12 experiments of RF-RDN in SHR and WKY under the following conditions: an absence of  
13 temperature control mode and maximum output of 4W or greater. The pop  
14 phenomenon, however, had never occurred under the temperature control mode  
15 targeting 60 °C with maximum output of 3 W.

16           This study demonstrated an anti-hypertensive effect of RF-RDN with a  
17 significant decrease in renal tissue norepinephrine. Currently, the decrease in renal  
18 tissue norepinephrine remains the gold standard of RDN in animal studies (Nakashima  
19 et al. 1996; DiBona 2013). The measurement of renal tissue norepinephrine, however, is  
20 not immediate and is not clinically applicable. We found that the local impedance  
21 decreased approximately 40  $\Omega$  after each RF application, which might provide a useful  
22 readout for the effective RF-RDN, similar to an autonomic response to electrical  
23 stimulation of renal nerves (Chinushi et al. 2013). In addition, the RF-RDN might be  
24 effective when the RF application is repeated until a number of RF applications with



1 duration of more than 20 sec reaches three times for each renal artery as described in the  
2 methods section.

3 The RF-RDN procedure exhibited excellent safety under the temperature control  
4 mode with a limited maximum power output. Repeated RF applications guided by a  
5 decrease in local impedance and sufficient RF duration might be required for the  
6 effective RF-RDN.

### 7 **Limitations**

8 The mechanisms of regulating BP include not only the sympathetic nerve system  
9 and renin-angiotensin system but also the carotid baroreflex system (Lohmeier et al.  
10 2012), renal sodium handling (Katayama et al. 2013), reactive oxygen species (Hubens  
11 et al. 2013), and endothelin (Girchev et al. 2006; Weber et al. 2009; Dhaun et al. 2011;  
12 Moorhouse et al. 2013). However, this study did not address potential mechanisms other  
13 than the sympathetic nerve system and renin-angiotensin system. Therefore, further  
14 studies are required to clarify the precise mechanisms responsible for the  
15 anti-hypertensive effect of RF-RDN in SHR.

16 Direct recording of renal nerve activity was not performed in this study.  
17 Individual recording of renal afferent and efferent nerves was reported; however, it  
18 required cutting renal nerves (Xie and Wang 2009; Kopp et al. 2011). Continuous  
19 recording of renal nerve was also reported; however, the maximum recording duration  
20 did not reach 3 months (Fujisawa et al. 2011). Furthermore, detachment of renal nerve  
21 for recording its activity might hurt the renal nerve itself. To compare the long-term  
22 effect of RF-RDN with sham operation, this study did not take the risk of renal nerve  
23 injury by the direct recording of renal nerve activity.

1           The RF energy in this study was applied to renal arteries from the external side,  
2 which is opposite to the clinical setting (Krum et al. 2009; Esler et al. 2012). This might  
3 strengthen the effects of RF-RDN on renal nerves, because the renal nerves lie adjacent  
4 to the outer side of renal arterial wall (Rippy et al. 2011; Steigerwald et al. 2012). The  
5 endovascular RF-RDN may require more radical RF energy application to assure the  
6 anti-hypertensive effect. However, the RF-RDN has never been validated in animal  
7 models of hypertension regardless of its energy application side (internally or externally).  
8 This study takes a first step to clarify the mechanism of reducing BP by RF-RDN and to  
9 establish the adequate protocol of RF-RDN, demonstrating the anti-hypertensive effects  
10 of RF-RDN for the first time in animal models of hypertension. Therefore, although the  
11 endovascular RF-RDN should also be investigated in animal models of hypertension in a  
12 future, this study provides a valuable insight into the novel treatment option for  
13 hypertension.

14           Although the RF-RDN restrained a spontaneous rise in BP, it failed to completely  
15 normalize the elevated BP in SHR. Our findings were in accordance with the Kline's  
16 report that bilateral surgical RDN significantly delayed but did not completely inhibit  
17 the development of hypertension in SHR (Kline et al. 1980). Although the renal tissue  
18 norepinephrine was significantly decreased after the RDN, it partially recovered  
19 suggesting the renal reinnervation. Thus, the RF-RDN may not provide the complete  
20 resolution of resistant hypertension, indicating the need for hybrid therapy. Recently, a  
21 selective endothelin-receptor antagonist demonstrated an additional anti-hypertensive  
22 effect in resistant hypertension (Weber et al. 2009; Dhaun et al. 2011; Miyauchi and  
23 Goto 2013, Moorhouse et al. 2013;). A hybrid therapy by combining the RF-RDN with

1 new drugs such as selective endothelin-receptor antagonists might provide some hope of  
2 cure for the resistant hypertension.

### 3 **Conclusions**

4 This study provides an experimental evidence for an anti-hypertensive effect of  
5 RF-RDN for the first time in an animal model of hypertension. The RF-RDN restrained a  
6 spontaneous BP rise in SHR despite the systemic sympathetic hyperactivity. The  
7 anti-hypertensive effect of RF-RDN seemed to be mediated by a reduction of enhanced  
8 plasma renin activity. Technical aspects of RF-RDN were also examined in detail to  
9 provide safety and efficacy for replication. This study provides a novel insight into the  
10 use of RF-RDN, which is a clinically expanding treatment for the resistant hypertension.

### 11 **Conflict of interest**

12 The authors declare that there are no conflicts of interest.

13

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- 3

**Figure legends**

**Figure 1. Effects of radiofrequency renal denervation on systolic blood pressure (A) and diastolic blood pressure (B).** Values are expressed as mean±standard deviation (n=8 in each group). Asterisk indicates statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not significant; WKY-Sham, sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected to radiofrequency renal denervation; SHR-Sham, sham-operated spontaneously hypertensive rats; SHR-RDN, SHR subjected to radiofrequency renal denervation.

**Figure 2. Effects of radiofrequency renal denervation on heart rate.** Values are expressed as mean±standard deviation (n=8 in each group). Asterisk indicates statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not significant; WKY-Sham, sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected to radiofrequency renal denervation; SHR-Sham, sham-operated spontaneously hypertensive rats; SHR-RDN, SHR subjected to radiofrequency renal denervation.

**Figure 3. Effects of radiofrequency renal denervation on urine output for 24 hours.** Values are expressed as mean±standard deviation (n=8 in each group). Asterisk indicates statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not significant; WKY-Sham, sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected to radiofrequency renal denervation; SHR-Sham, sham-operated spontaneously hypertensive rats; SHR-RDN, SHR subjected to radiofrequency renal denervation.

1 **Figure 4. Effects of radiofrequency renal denervation on urinary norepinephrine**

2 **excretion.** Values are expressed as mean±standard deviation (n=8 in each group).

3 Asterisk indicates statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not

4 significant; WKY-Sham, sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected

5 to radiofrequency renal denervation; SHR-Sham, sham-operated spontaneously

6 hypertensive rats; SHR-RDN, SHR subjected to radiofrequency renal denervation.

7

8 **Figure 5. Effects of radiofrequency renal denervation on plasma norepinephrine.** Values

9 are expressed as mean±standard deviation (n=8 in each group). Asterisk indicates

10 statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not significant; WKY-Sham,

11 sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected to radiofrequency renal

12 denervation; SHR-Sham, sham-operated spontaneously hypertensive rats; SHR-RDN,

13 SHR subjected to radiofrequency renal denervation. SHRs are also compared with

14 WKYs; the statistical significance is indicated by the sharp ( $p<0.05$  vs. WKY-Sham) and

15 dagger ( $p<0.05$  vs. WKY-RDN).

16

17 **Figure 6. Effects of radiofrequency renal denervation on plasma renin activity.** Values

18 are expressed as mean±standard deviation (n=8 in each group). Asterisk indicates

19 statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not significant; WKY-Sham,

20 sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected to radiofrequency renal

21 denervation; SHR-Sham, sham-operated spontaneously hypertensive rats; SHR-RDN,

22 SHR subjected to radiofrequency renal denervation. SHRs are also compared with

23 WKYs; the statistical significance is indicated by the sharp ( $p<0.05$  vs. WKY-Sham) and

24 dagger ( $p<0.05$  vs. WKY-RDN).

1

2 **Figure 7. Effects of radiofrequency renal denervation on renal tissue norepinephrine.**

3 Values are expressed as mean±standard deviation (n=8 in each group). Asterisk

4 indicates statistical significance ( $p<0.05$ ) vs. sham operated rats; N.S., not significant;

5 WKY-Sham, sham-operated Wistar-Kyoto rats; WKY-RDN, WKY subjected to

6 radiofrequency renal denervation; SHR-Sham, sham-operated spontaneously

7 hypertensive rats; SHR-RDN, SHR subjected to radiofrequency renal denervation. SHRs

8 are also compared with WKYs; the statistical significance is indicated by the sharp

9 ( $p<0.05$  vs. WKY-Sham) and dagger ( $p<0.05$  vs. WKY-RDN).

10

11 **Figure 8. Histological changes in renal nerve bundle by radiofrequency renal**12 **denervation.** Horizontal bar indicates a scale of 100  $\mu\text{m}$ ; SHR-Sham, sham-operated

13 spontaneously hypertensive rats; SHR-RDN, SHR subjected to radio frequency renal

14 denervation.

Table 1. Procedural data of the radiofrequency renal denervation

Parameters of radiofrequency application	WKY		SHR		P-value
	Sham (n=8)	RDN (n=8)	Sham (n=8)	RDN (n=8)	
Mean Power, W	-	2.4 ± 0.7	-	2.5 ± 0.6	0.71
Total number, time	-	8 ± 1	-	8 ± 1	0.84
Total duration, sec	-	246 ± 79	-	215 ± 58	0.38
Total energy, J	-	514 ± 176	-	421 ± 54	0.19
Mean local impedance, Ω	233 ± 35	220 ± 36	249 ± 18	237 ± 17	0.24
Mean decrease in local impedance, Ω	-	40 ± 11	-	44 ± 15	0.45

Data are expressed as mean ± standard deviation. WKY indicates Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; Sham, sham operation; RDN, radiofrequency renal denervation.

Figure 1A. Systolic blood pressure (mmHg)

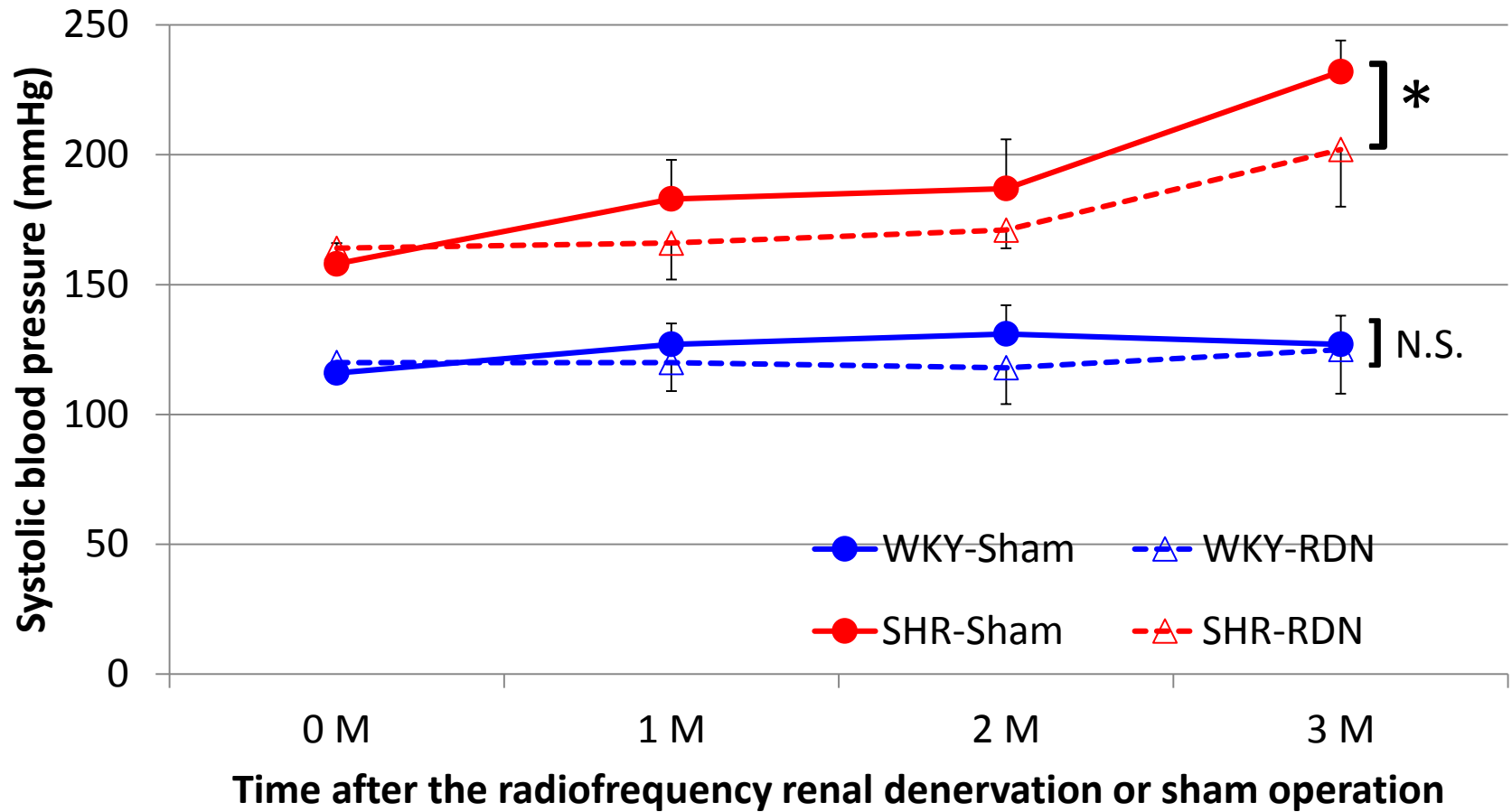


Figure 1B. Diastolic blood pressure (mmHg)

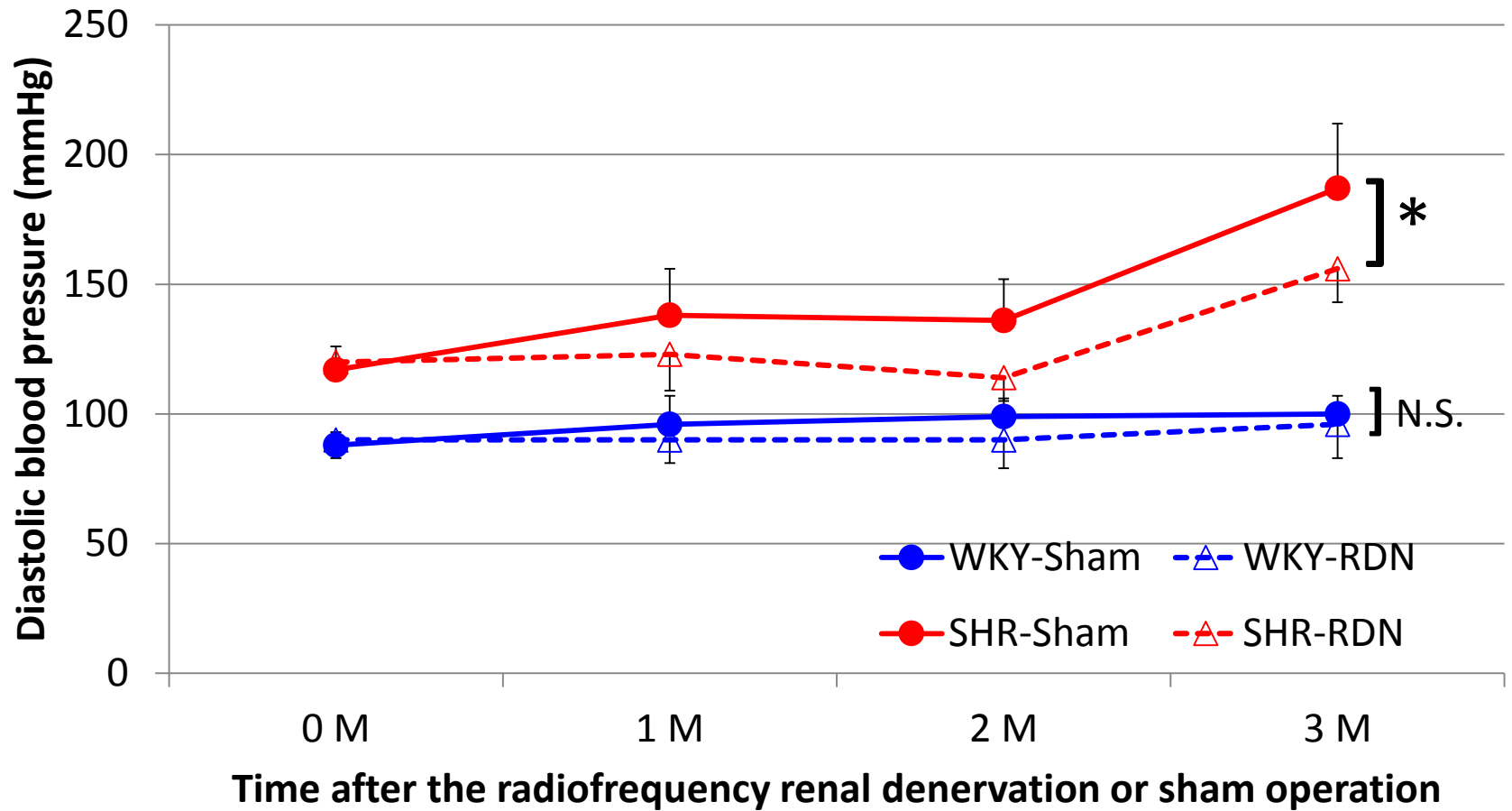


Figure 2. Heart Rate (bpm)

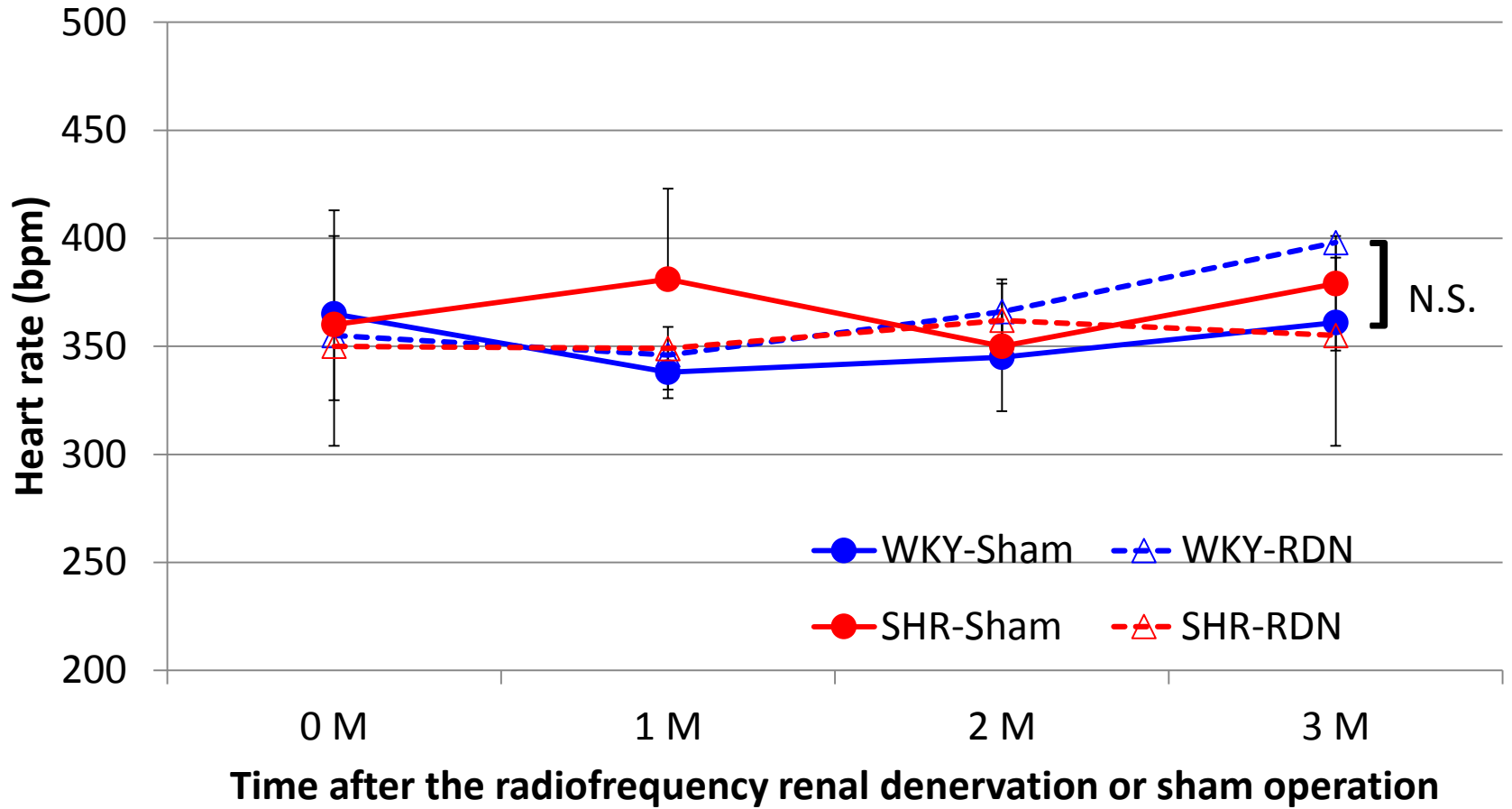




Figure 3. Urine output (mL/day)

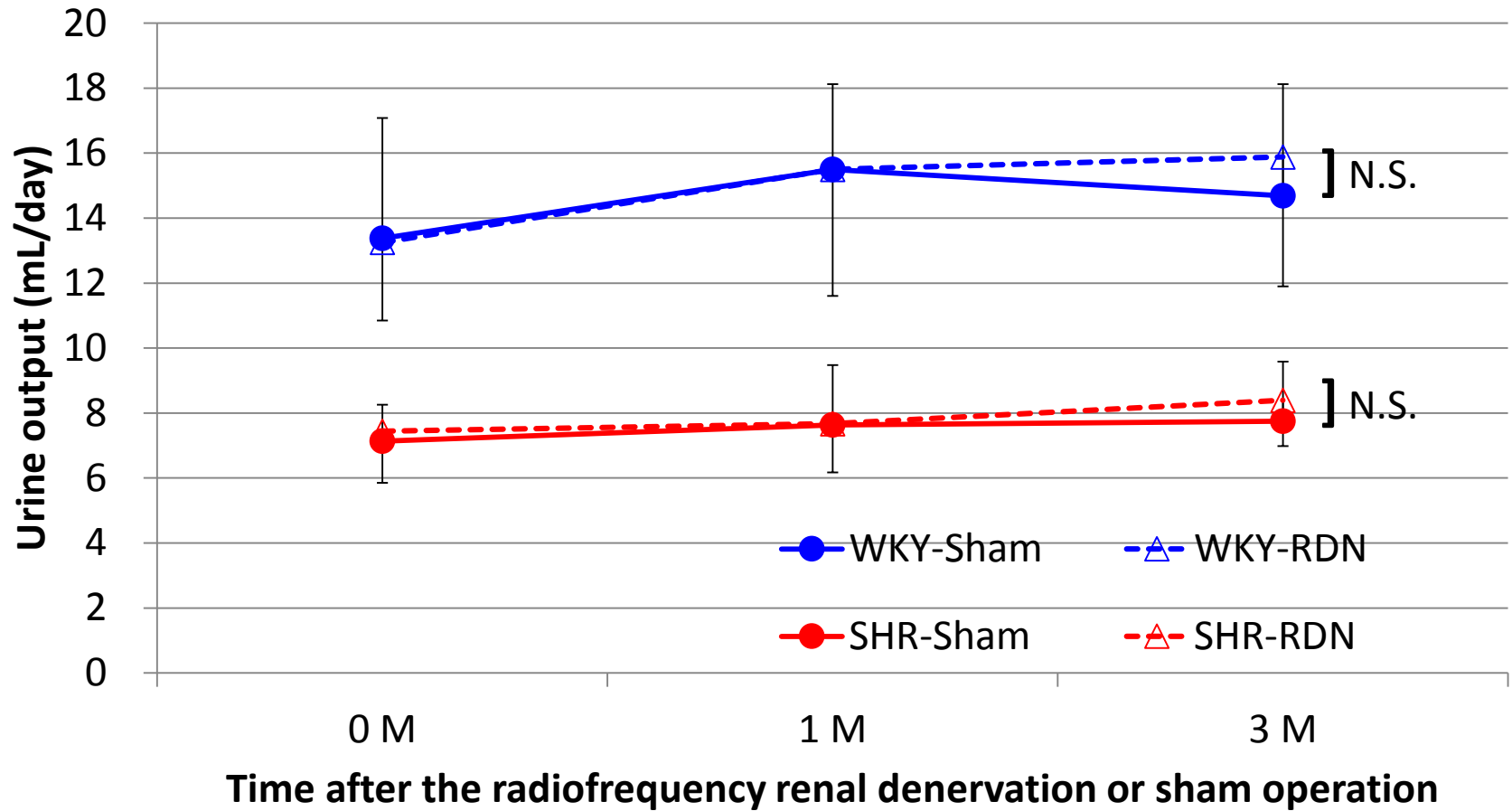


Figure 4. Urinary norepinephrine excretion (ng/day)

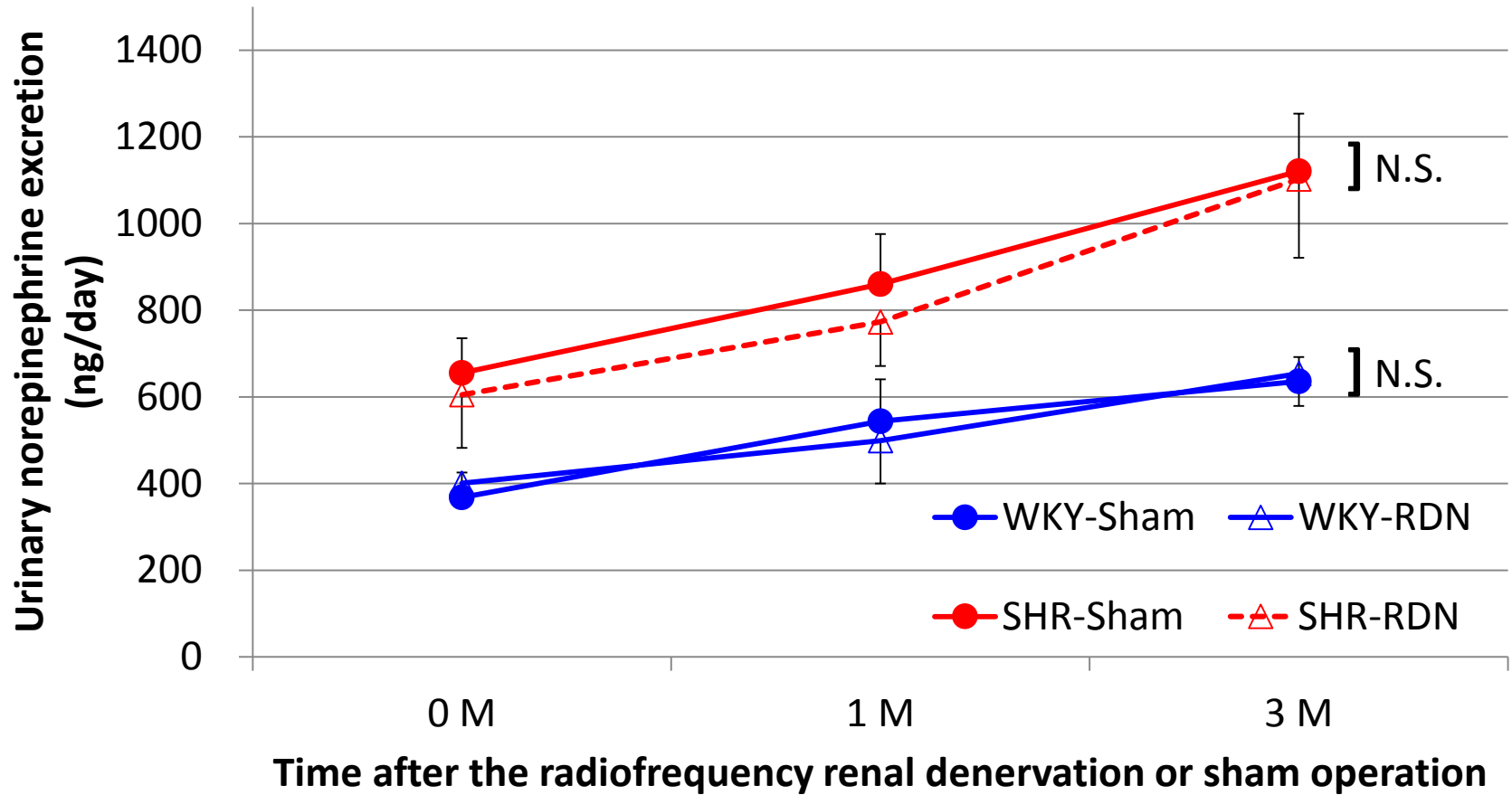


Figure 5. Plasma norepinephrine (pg/mL)

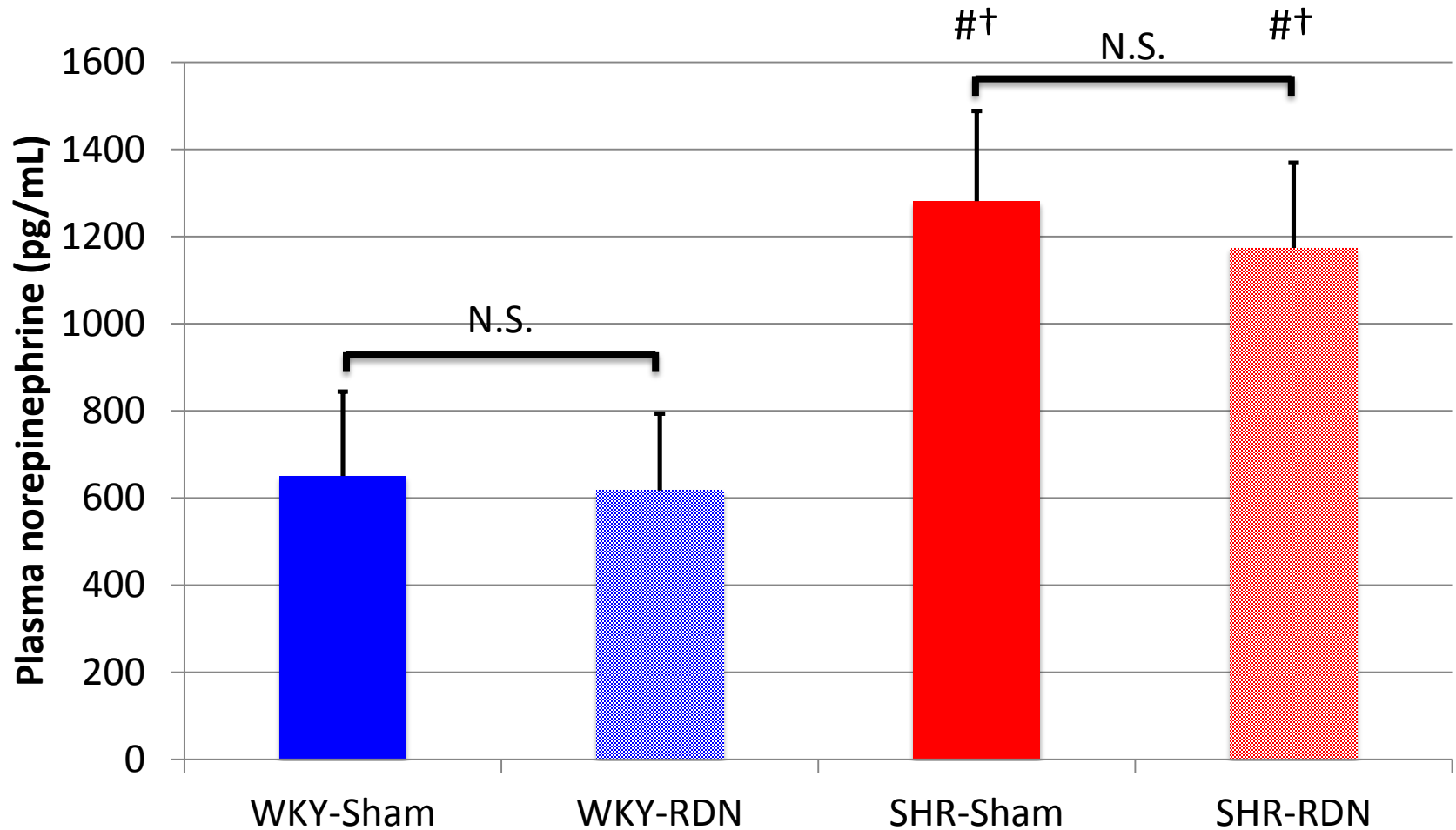


Figure 6. Plasma renin activity (ng/mL/hr)

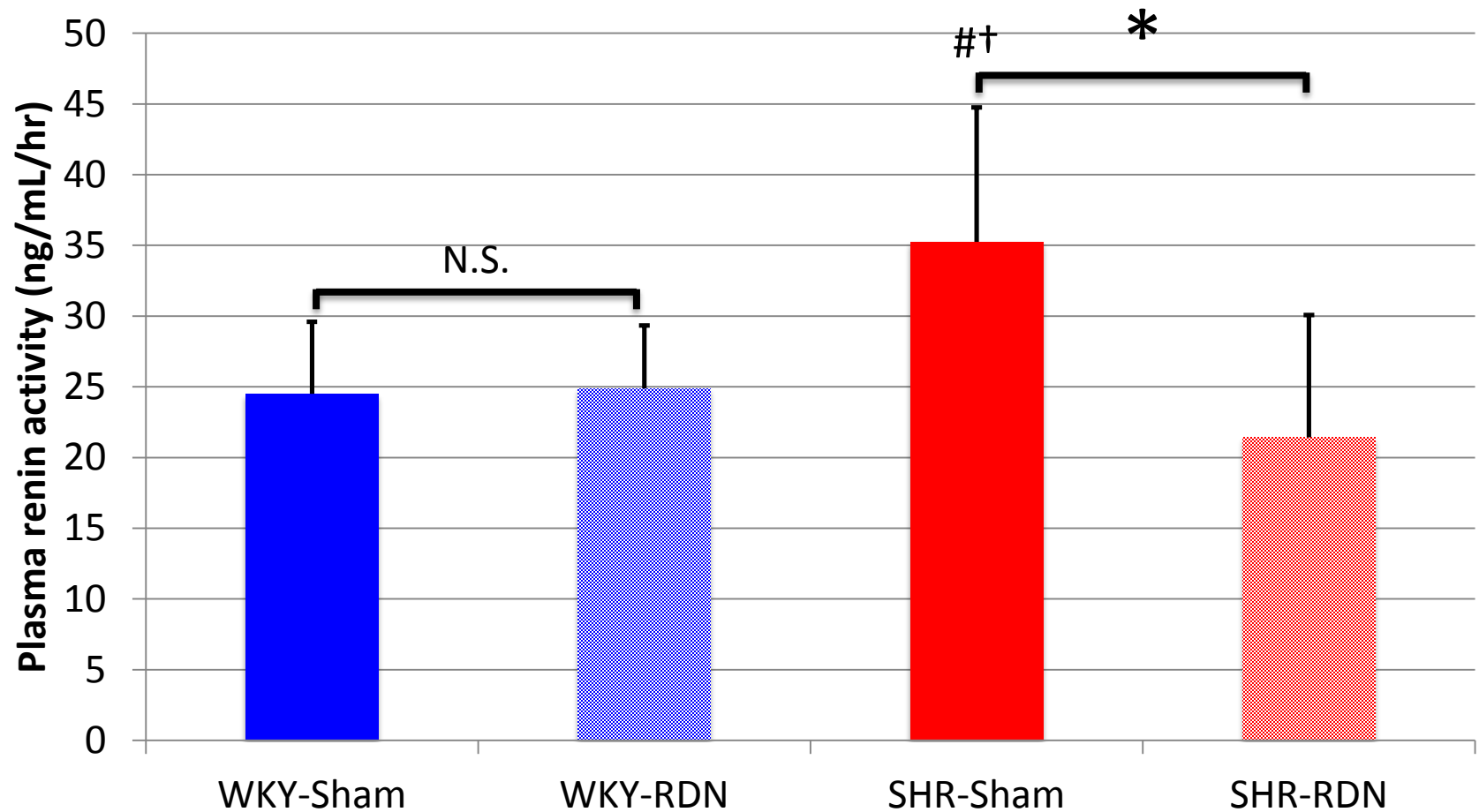
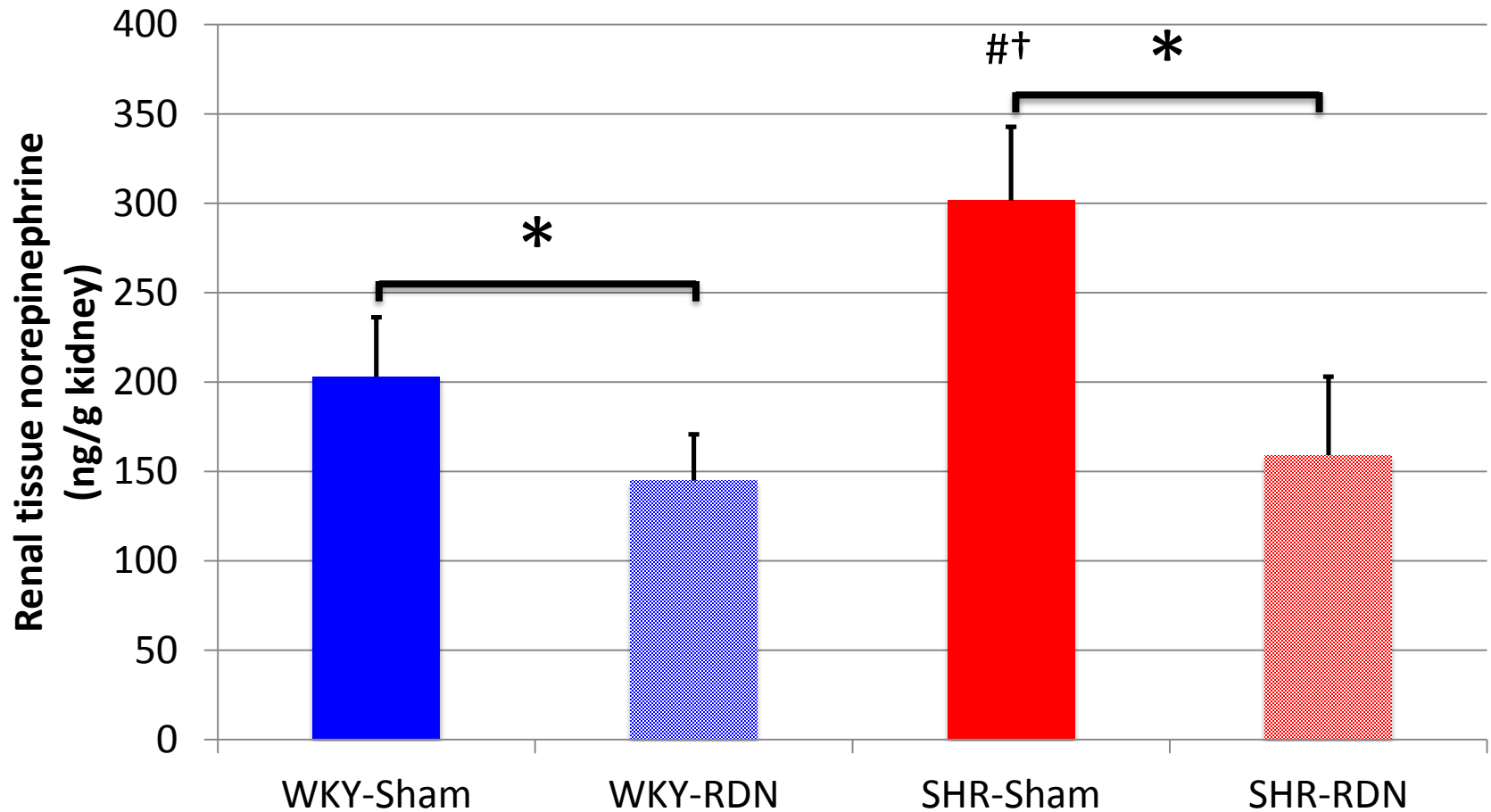
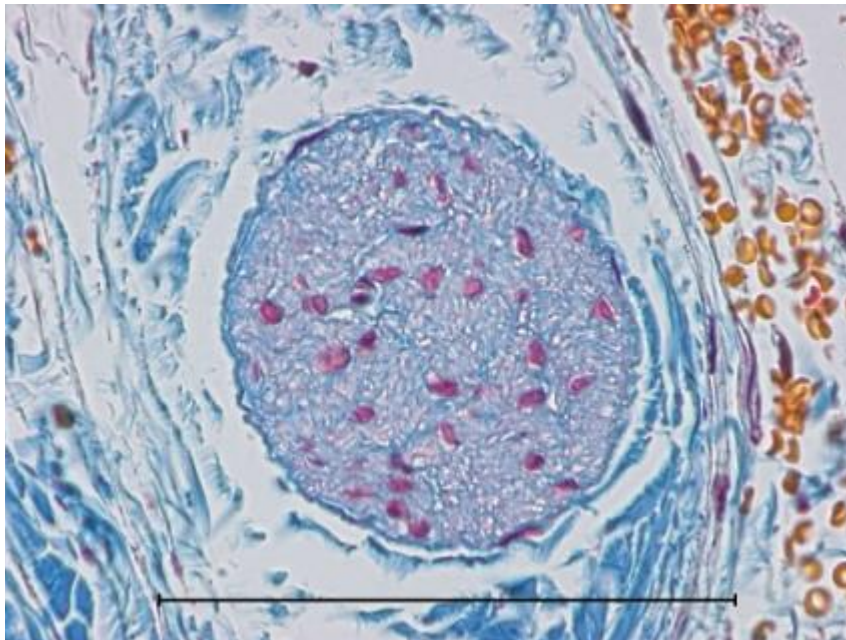


Figure 7. Renal tissue norepinephrine (ng/g kidney)



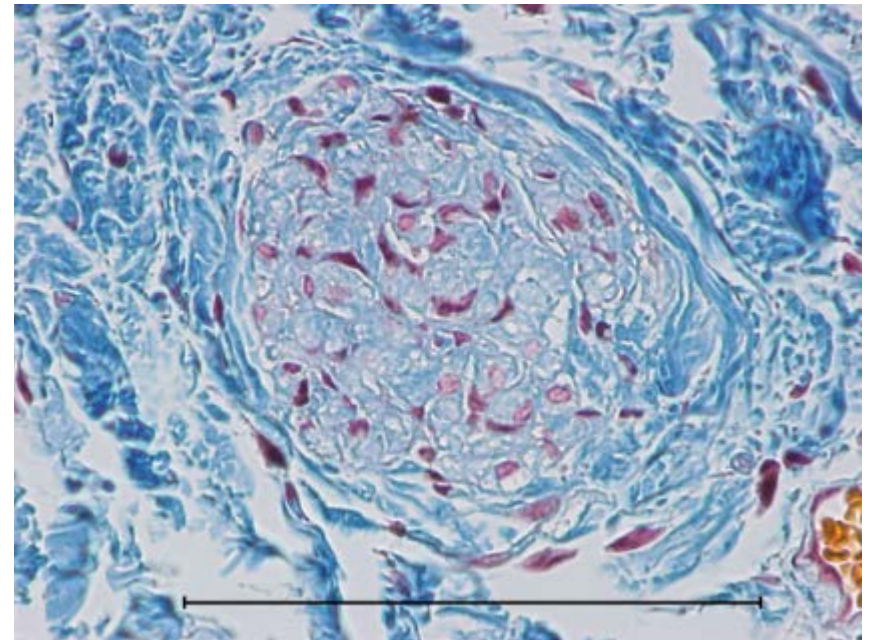
## Figure 8. Renal Nerve Bundle

A. SHR-Sham



100  $\mu\text{m}$

B. SHR-RDN



100  $\mu\text{m}$