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Abstract

The activation of sympathetic nervous system plays a critical role in the development of hypertension. The input from afferent renal nerves may affect central sympathetic outflow; however, its contribution to the development of hypertension remains unclear. We investigated the role of afferent renal nerves in acute and chronic blood pressure regulation using normotensive Wistar-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP). Acute chemical stimulation of afferent renal nerves elicited larger increases in blood pressure and renal sympathetic nerve activity in young 9-week-old SHRSP compared to WKY. Selective afferent renal denervation (ARDN) and conventional total renal denervation (TRDN) ablating both afferent and efferent nerves in young SHRSP revealed that only TRDN, but not ARDN, chronically attenuated blood pressure elevation. ARDN did not affect plasma renin activity or plasma angiotensin II levels, whereas TRDN decreased both. Neither TRDN nor ARDN affected central sympathetic outflow and systemic sympathetic activity determined by neuronal activity in the parvocellular region of hypothalamic paraventricular nucleus and rostral ventrolateral medulla and by plasma and urinary norepinephrine levels, respectively. Renal injury was not apparent in young SHRSP compared with WKY, suggesting that renal afferent input might not be activated in young SHRSP. In

conclusion, the chronic input from afferent renal nerves does not contribute to the development of hypertension in SHRSP despite the increased blood pressure response to the acute stimulation of afferent renal nerves. Efferent renal nerves may be involved in the development of hypertension via activation of the renin-angiotensin system in SHRSP.

Keywords

Afferent renal nerves, Hypertension, Renal denervation, Renin-angiotensin system, Sympathetic nervous system

Introduction

The activation of sympathetic nervous system plays a critical role in the development and progression of hypertension [1,2]. Renal nerves are importantly involved in regulating both the blood pressure and fluid volume through sympathetic output to the kidney and affecting the sympathetic outflow from the brain [3]. The renal nerves consist of sympathetic efferent nerves and sensory afferent nerves. Activation of sympathetic efferent nerves contributes to an increase in renin release, sodium reabsorption, and renal vascular resistance, leading to hypertension [4]. Afferent renal nerves relay the information from kidneys to the central nervous system, including the paraventricular nucleus (PVN) of the hypothalamus [5]. Acute stimulation of the afferent renal nerves can increase central sympathetic outflow [6, 7]; however, the chronic contribution of afferent renal nerve signals to the regulation of sympathetic activity and blood pressure remains unclear.

The activation of PVN neurons within the hypothalamus is a hallmark of the axis of renal afferents-brain-sympathetic outflow. Acute stimulation of the afferent renal nerves activates PVN neurons and increases sympathetic activity and blood pressure, and the lesion of PVN abolishes the afferent stimulation-induced sympathoexcitation and blood pressure elevation [6]. The PVN heavily projects to the rostral ventrolateral

medulla (RVLM), which is a principal center of sympathetic drive, and the activation of afferent renal nerves modulates RVLM-projecting PVN neurons [8]. Therefore, both PVN and RVLM are important nuclei for central sympathetic regulation via the renal afferent nerve signals.

In this study, we hypothesized that afferent renal nerve signals contribute to the development of hypertension as a result of sympathoexcitation. We investigated whether the acute stimulation of afferent renal nerves increases blood pressure and sympathetic tone greater in stroke-prone spontaneously hypertensive rats (SHRSP), which is an established model of essential hypertension with sympathoexcitation [9–11], compared to normotensive control Wistar-Kyoto rats (WKY). Furthermore, the present study assessed the contribution of afferent renal nerve signals to the long-term blood pressure elevation and sympathetic excitation in SHRSP, using a novel method of chronic selective ablation of afferent renal nerves. We evaluated the activities of PVN and RVLM neurons to elucidate the effects of chronic afferent renal denervation on the central sympathetic regulation.

Methods

Animals

All animal experiments were approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences and conducted in accordance with the Guidelines for Animal Experiments of the Kyushu University. Male SHRSP and WKY were purchased from SLC Japan (Hamamatsu, Japan). The animals were housed in a room with controlled temperature (23°C ± 1°C) and a 12-h light/dark cycle, and were fed a standard rat chow with drinking water ad libitum.

Protocol 1: Comparison of the response to acute stimulation of afferent renal nerve between WKY and SHRSP

Recording of renal sympathetic nerve activity (RSNA) and measurements of blood pressure and heart rate were performed under anesthesia with injection of urethane (80 mg/kg ip) and α-chloralose (500 mg/kg ip) in both WKY and SHRSP at 9 weeks of age. After intubation, rats were ventilated mechanically with oxygen-enriched gas [12]. PE-50 tubes were inserted from the left femoral artery and left femoral vein to monitor blood pressure continuously and facilitate intravenous administration, respectively. During the procedure, 50 μl/min of normal saline was infused. A triple lumen catheter

was inserted into the right urinary duct by the retroperitoneal approach to ensure injection into renal pelvis, withdraw urine and administered fluid, and monitor the intrapelvic pressure [13]. The left renal nerve was isolated gently by the retroperitoneal approach, placed on a silver electrode, and fixed with silicone gels (Kwik-Sil; World Precision Instruments, Sarasota, FL, USA). To quantify RSNA, preamplified nerve signals were band-pass filtered at 100–2000 Hz, and the electrical signal from the electrode was amplified and recorded using Labchart (ADInstruments). Intrapelvic 10 μM capsaicin, which was reported to elicit approximately 50% of the maximum afferent renal nerve activity, was used to stimulate the afferent renal nerves [14]. Capsaicin solution was injected at flow rate of 50 μl/min for 3min. The peak values of mean blood pressure, heart rate, and integrated RSNA after the capsaicin intrapelvic injection were compared to the baseline values. At the end of the experiment, a bolus injection of a ganglionic blocker hexamethonium bromide (60 mg/kg) was given to define background RSNA noise. The value of RSNA was calculated by subtracting the background noise from the value of recorded signals.

Protocol 2: Chronic effect of selective afferent renal denervation and total renal denervation in SHRSP

General procedure

A radiotelemetry transmitter was implanted via the left femoral artery under anesthesia with 2.5% isoflurane in SHRSP at 8 weeks of age to measure the rats' blood pressure and heart rate in conscious state. One week after implantation (9 weeks of age), measurements of blood pressure and heart rate were started (Day 0). At day 1, rats were randomly divided to three groups: selective afferent renal denervation (ARDN), total renal denervation (TRDN), and sham surgery (Sham). Blood pressure and heart rate were measured for 20 s every 10 min. From day 15 to 16, rats were placed in a metabolic cage to measure their urine volume, water and food intake, and urine norepinephrine excretion. At day 17, rats were sacrificed. Blood samples were collected from the inferior vena cava immediately after anesthesia induction with 2.5% isoflurane. After blood collection, rats were euthanized with pentobarbital sodium (150 mg/kg, ip). Normal saline was perfused and the kidneys were extracted. After extraction, 10% neutral buffered formaldehyde was perfused for fixation, and the rats' brain was extracted and immersed in 10% neutral buffered formaldehyde for immunohistochemical analysis. Plasma renin activity, plasma angiotensin II concentration, and plasma norepinephrine concentration were examined. Quantitative real time PCR for renin was performed using renal cortex. The number of c-Fos positive

neurons in PVN and RVLM, which is a marker of central sympathetic outflow, was assessed by immunohistochemistry. The specific methods of plasma assay, quantitative real time PCR, and immunohistochemistry of brain c-Fos are provided in Supplementary Information.

Procedures of renal denervation

The 9-week-old SHRSP underwent TRDN, ARDN, or sham surgery under anesthesia with 2%–2.5% isoflurane. Ketoprofen (5 mg/kg sc) and gentamicin sulfate (2.5 mg/kg IM) were administered prior to surgery. A midline abdominal incision was made, and the visceral organs were externalized to expose the left kidney. A hole was made in the peritoneal membrane to expose the renal artery and vein. To perform TRDN, the fat surrounding the renal artery, vein, and fascia was dissected. All visible renal nerves were stripped. After dissection of the visible renal nerves, 10% phenol in ethanol was painted around the renal artery, and the same procedure was performed again on the right side. To perform ARDN, after making a hole in the peritoneal membrane, a small piece of gauze soaked in a capsaicin solution (33 mM in 5% ethanol, 5% Tween 80, and 90% normal saline) was wrapped around the renal artery and vein for 15 min after isolation with a small piece of parafilm [15]. After a 15-min treatment, the gauze was

removed and the area was dried, and the same procedure was performed again on the right side. For sham surgery, the visceral organs were externalized to expose the kidneys, holes were made, and a 15-min waiting time without wrapping was set. After TRDN, ARDN, or sham treatment, the visceral organs were replaced and the abdominal muscles and skin were closed separately with 3–0 silk sutures.

Validation of renal denervation by Western blot and immunohistochemistry

Western blot for calcitonin gene-related peptide (CGRP), which is contained in sensory nerves, and tyrosine hydroxylase (TH), which is a marker of efferent sympathetic nerves, using the protein extraction from the kidney pelvic wall was performed to confirm ARDN and TRDN. The protein samples were loaded onto the 12% SDS-PAGE gel for electrophoresis and then transferred onto nitrocellulose membranes with a pore size of 0.2 μm. The membranes were incubated with 5% skim milk for 1 h for blocking, and were subsequently incubated with primary antibody for CGRP (sc-57053, Santa Cruz Biotechnologym Inc., Heidelberg, Germany) and TH (ab112, Abcam, Cambridge, MA, USA) at 4℃ overnight. A signal enhancer (HIKARI, Nakarai Tesque, Kyoto, Japan) was used to detect the signal of CGRP. After washing, the membranes were incubated with secondary antibodies (7076S and 7074S, Cell Signaling Technology, Danvers, MA,

USA) for 1 h. The expression of CGRP and TH was calculated as the ratio of CGRP and TH band intensity to the intensity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-32233, Santa Cruz Biotechnologym Inc., Heidelberg, Germany).

Renal pelvis sections from a few rats were analyzed by immunohistochemistry. The specific method of immunohistochemistry is provided in Supplementary Information.

Protocol 3: Evaluation and comparison of kidney injury in WKY and SHRSP

Measurement of urinary protein excretion and serum creatinine and histological assessment of kidney injury

Urine collection for 24 h with a metabolic cage was performed to measure urine volume and urinary protein concentration in WKY and SHRSP at 9 weeks of age. After urine collection, the rats were euthanized with pentobarbital sodium (150 mg/kg, IP), and the rats' blood and kidneys were collected for serum creatinine measurement and histological assessment, respectively. The specific methods of quantitative analysis of urinary protein and histological assessment of kidney injury are provided in Supplementary Information.

Statistical analysis

All the results are presented as the mean ± standard error of the mean. The data were analyzed using the t-test or ANOVA followed by the Tukey test. Blood pressure was analyzed using a 2-way repeated measured ANOVA followed by the Tukey's multiple comparison if time by treatment effect was statistically significant. Statistical significance was accepted for $P < 0.05$. Prism 8 (GraphPad Software Corp, San Diego, CA, USA) was used to perform the data analysis.

Results

Response of blood pressure and RSNA to acute chemical stimulation of the afferent renal nerves in WKY and SHRSP

Acute chemical stimulation of afferent renal nerves was performed to determine whether the input from afferent renal nerves could differently contribute to the modulation of sympathetic nerve activity and blood pressure in SHRSP compared to WKY. Figure 1a, b shows the representative recordings of blood pressure, heart rate, and the contralateral RSNA in response to the afferent nerve stimulation by intrapelvic capsaicin infusion. The elevation of mean blood pressure by chemical stimulation of afferent renal nerves was greater in SHRSP than in WKY (11.0 ± 1.1 mmHg *vs*. 4.9 ± 1.2 mmHg, $P = 0.002$) at 9weeks of age (Fig. 1c). The increase in RSNA due to chemical afferent stimulation also tended to be greater in SHRSP than in WKY (19.4 ± 4.9% *vs.* 9.0 ± 1.9%, $P = 0.071$) (Fig. 1e). The changes in heart rate did not differ significantly between SHRSP and WKY (Fig. 1d).

Validation of renal denervation

We validated our technique on renal denervation using Western blotting and immunohistochemistry. The results of Western blotting for CGRP, a marker of afferent

renal nerves, indicated that the expression of CGRP was significantly decreased in both ARDN and TRDN compared to Sham (Fig. 2a, b). The degree of decrease of CGRP was similar in both ARDN and TRDN. The expression of TH, a marker of efferent renal nerves, was significantly decreased only in TRDN compared to Sham, while the expression levels of TH in ARDN were similar to those of Sham (Fig. 2a, c). Figure 2d, e shows the representative immunohistochemical images of the renal pelvis for CGRP and TH, respectively. ARDN abolished the CGRP-positive neurons but preserved TH-positive neurons. In contrast, TRDN abolished both the CGRP- and TH-positive neurons. These results confirmed that the ARDN procedure could successfully denervate only the afferent renal nerves, while the TRDN procedure could successfully denervate both afferent and efferent renal nerves.

Effect of renal denervation on blood pressure elevation

Figure 3a shows the mean blood pressure measured by radiotelemetry transmitters. At 9 weeks of age, the mean blood pressure at baseline (Day 0) was comparable among all three groups (Sham 143.9 ± 1.9 mmHg, ARDN 142.0 ± 2.3 mmHg, TRDN 142.7 ± 1.0 mmHg). The mean blood pressure elevated to 155.6 ± 1.8 mmHg at Day 14 (11 weeks of age) in Sham. In ARDN, the mean blood pressure elevated to similar levels to Sham

(157.8 ± 2.7 mmHg at Day 14). In TRDN, the blood pressure elevation was attenuated, and the mean blood pressure was lower than that in Sham from Day 11 to Day 14 ($P < 0.05$ *vs*. Sham). Heart rate was not significantly different among the three groups throughout the study period (Fig. 3b).

Effect of renal denervation on the renin-angiotensin system and sodium-water balance

Plasma renin activity was significantly decreased in TRDN compared to Sham and ARDN (Sham 22.0 ± 1.8 ng/ml/h, ARDN 20.0 ± 1.3 ng/ml/h, TRDN 10.4 ± 1.6 ng/ml/h; $P < 0.001$ for TRDN *vs*. Sham, $P < 0.001$ for TRDN *vs*. ARDN) (Fig. 4a). Plasma angiotensin II levels were significantly decreased in TRDN compared to Sham, and tended to be lower than ARDN (Sham 39.1 ± 3.6 pg/ml, ARDN 33.4 ± 2.6 pg/ml, TRDN 23.5 ± 2.6 pg/ml; $P = 0.002$ for TRDN *vs*. Sham, $P = 0.066$ for TRDN *vs*. ARDN) (Fig. 4b). Renin mRNA expression in the renal cortex was comparable in all three groups (Fig. 4c). There were no significant differences in sodium balance (Sham 0.27 ± 0.08 mmol/24 h, ARDN 0.10 ± 0.07 mmol/24 h, TRDN 0.19 ± 0.11 mmol/24 h) and water balance (Sham 21.6 ± 1.1 ml/24 h, ARDN 21.3 ± 1.3 ml/24 h, TRDN 23.7 ± 0.9 ml/24 h) among the three groups. These results indicate that contrary to ARDN,

TRDN affected the systemic renin-angiotensin system; however, ARDN and TRDN had no significant effect on the regulation of sodium-water balance.

Effect of renal denervation on the systemic and central sympathetic nervous system activity

Systemic sympathetic nerve activity was determined by plasma norepinephrine concentration and 24-h urinary norepinephrine excretion, and neither method revealed any differences among the three groups (Fig. 5a, b). In addition, the number of c-Fos-positive activated neurons in the parvocellular region of PVN, which contains pre-sympathetic neurons projecting to RVLM, and RVLM, a principal center of sympathetic drive, were investigated. The numbers of c-Fos-positive neurons in the parvocellular region of PVN and RVLM were also similar in all groups (Fig. 5c–e). These results demonstrate that neither ARDN nor TRDN could significantly affect the activity of sympathetic nervous system in SHRSP during the developing phase of hypertension.

Comparison of renal injury between WKY and SHRSP

Renal injury was evaluated by urinary protein excretion, serum creatinine, and

histological analysis at 9 weeks of age in WKY and SHRSP. The urinary protein excretion for 24 h and serum creatinine were comparable between WKY and SHRSP (Fig. 6a, b). Glomerular sclerosis and interstitial fibrosis were also comparable between WKY and SHRSP (Fig. 6c, d). These findings suggest that renal injury was not apparent in 9-week-old SHRSP.

Discussion

The acute stimulation of afferent renal nerves can increase sympathetic activity [6, 7]; however, the role of afferent renal nerves in the pathophysiology of hypertension remains unclear. In this study, we investigated the role of afferent renal nerves in acute and chronic blood pressure regulation using WKY and SHRSP. Our present study showed that acute chemical stimulation of afferent renal nerves elicited larger increases of blood pressure and RSNA in young 9-week-old SHRSP compared to WKY. Consequently, we performed ARDN as well as TRDN to investigate the contribution of afferent renal input to the development of hypertension in young SHRSP, and showed that only TRDN, but not ARDN, could chronically attenuate blood pressure elevation. ARDN did not affect plasma renin activity or plasma angiotensin II levels, whereas TRDN decreased both. Neither TRDN nor ARDN affected the central sympathetic outflow and systemic sympathetic nerve activity determined by the number of c-Fos positive activated neurons in the parvocellular region of PVN and RVLM and by plasma norepinephrine concentration and 24 h urinary norepinephrine excretion, respectively. These results suggest that the afferent renal input does not chronically affect the central sympathetic outflow nor does it contribute to the development of hypertension. Furthermore, the signals of efferent renal nerves may be involved in the

pathophysiology of hypertension partly through an increase in the activity of systemic renin-angiotensin system. Lastly, we showed that renal injury was not apparent in young SHRSP compared with WKY. As the activity of afferent renal nerves can be increased by renal injury [3, 16], the renal afferent input might not be activated in young SHRSP without renal injury, which could explain why ARDN did not attenuate blood pressure elevation.

To our knowledge, this is the first study to elucidate the contribution of afferent and efferent renal nerves to blood pressure elevation separately using ARDN and TRDN techniques in SHRSP. A previous study indicated a decrease in blood pressure at 7 days after TRDN, using the conventional afferent and efferent renal nerve denervation in SHRSP under anesthesia [17]. Another study also showed the suppression of blood pressure elevation by TRDN in conscious SHRSP with 8% salt-loading [18]. We performed selective ARDN, which has been recently developed and proved superior to the classical dorsal rhizotomy by disrupting the afferent nerves not only from the kidneys but also from other organs, including osmosensitive and sodium-sensitive hepatoportal afferent nerves [3, 15, 19]. Western blotting and immunohistochemistry confirmed the success of the ARDN method (Fig. 2). Our findings revealed that selective ARDN did not attenuate sympathoexcitation and blood pressure elevation in

SHRSP, as opposed to TRDN that could significantly mitigate blood pressure elevation (Figs. 3 and 5), which are consistent with previous studies [17, 18]. Therefore, our study indicates that the input from afferent renal nerves in young SHRSP did not chronically contribute to the regulation of the sympathetic nerve activity and blood pressure, at least by itself, and that the effect of TRDN on blood pressure might be largely due to the denervation of efferent renal nerves.

Renal efferent sympathetic activity is critically involved in renin secretion from juxtaglomerular cells [20]. Although neither TRDN nor ARDN decreased central and systemic sympathetic activity, TRDN would have decreased the renal efferent sympathetic activity. Therefore, the present study considered that contrary to ARDN, TRDN could decrease the parameters of systemic renin-angiotensin system, such as plasma renin activity and plasma angiotensin II concentration (Fig. 4a, b). In the TRDN group, daily water and sodium balance was not significantly different compared to Sham group despite the decreased activity of systemic renin-angiotensin system, which is consistent to a previous study using SHRSP [17]. Consequently, a possible mechanism that could explain the association between the decreased activity of the renin-angiotensin system and the attenuation of blood pressure elevation is the decreased action of circulating angiotensin II on the vessels. Angiotensin II contributes

to vasoconstriction mainly through the angiotensin type 1 receptor (AT1R), causing hypertension [21,22]. Additionally, vascular remodeling is induced through AT1R in angiotensin II infusion rats [23] and spontaneously hypertensive rats (SHR) [24]. Therefore, our data together with the results of previous studies suggest that contrary to ARDN, TRDN could attenuate blood pressure elevation probably due to the inhibition of vasoconstriction and vascular remodeling via the decreased activity of systemic renin-angiotensin system, which might be caused by efferent renal denervation in SHRSP. Unlike plasma renin activity, the expression of renin mRNA in renal cortex was not altered by TRDN (Fig. 4c), perhaps because renin synthesis is mainly controlled by the cyclic adenosine monophosphate-binding protein (CREB), which is different compared to renin secretion [20]. It still remains controversial whether renal renin mRNA expression is decreased by TRDN or not [25]; thus, further studies are needed to explore this issue.

Our initial hypothesis was that the input from afferent renal nerves contributes to the development of hypertension through sympathetic activation in SHRSP. Our acute experiment process confirmed this hypothesis and showed that chemical stimulation of afferent renal nerves could elicit a greater increase in blood pressure and RSNA in young SHRSP compared to WKY. However, selective ARDN in the chronic experiment

was not found to attenuate sympathoexcitation and blood pressure elevation in SHRSP. A potential explanation for the results of the chronic experiment could be that the afferent renal input was not enhanced sufficiently enough to increase the central sympathetic activity and blood pressure at least during this study period (9–11 weeks of age). Increase in the afferent renal nerve activity is associated with reduced renal perfusion and renal injury [3, 14, 16]. Our data showed that there were no significant differences in the urinary protein excretion, serum creatinine, and histological glomerular sclerosis and renal fibrosis between young WKY and SHRSP, indicating that renal injury was not apparent in young SHRSP. These findings may indirectly suggest that afferent renal nerve activity was comparable between WKY and SHRSP, whereas we did not directly record afferent renal nerve activity. The afferent renal nerve activity induced by acute chemical stimulation should be significantly higher than that occurring chronically under physiological conditions. Such acute excitation of afferent renal nerves might cause the different responses of sympathetic activity and blood pressure between WKY and SHRSP, probably due to differences in excitability within the hypothalamic PVN, which receives the renal afferent input. In normal rats, capsaicin-induced acute chemical stimulation of afferent renal nerves caused sympathoexcitation and blood pressure elevation, and these capsaicin-induced responses

were significantly inhibited by prior microinjection of losartan, an AT1R antagonist, into the PVN [26]. Neural excitation of the PVN neurons via AT1R activation causes sympathoexcitation [27]. The PVN AT1R expression and the PVN AT1R-mediated sympathoexcitation are increased in SHR compared to WKY [28]. Together with these previous findings, the increased excitability of PVN neurons via AT1R might cause greater responses of blood pressure and sympathetic activity to the acute excitation of afferent renal nerves in SHRSP compared to WKY.

Since the publishing of the novel selective ARDN method, the contribution of afferent renal nerves to the development of hypertension has been elucidated in some animal models [14–16, 29–32]. The contribution of afferent and efferent renal nerves to hypertension does not seem to be uniform among the experimental models [33]. Therefore, the degree of contribution of afferent renal nerves to long-term blood pressure regulation seems to depend on the underlying pathophysiology elevating blood pressure. For example, in 5/6 nephrectomized rats, a model of chronic kidney disease, the activity of afferent renal nerves might be increased unlike SHRSP; therefore, ARDN attenuated the blood pressure elevation with a concurrent decrease in systemic sympathetic nerve activity [30, 33]. As consistent with this result of basic research, renal denervation was effective in hypertensive patients with chronic kidney disease

[34]. The efficacy of renal denervation has been controversial in hypertensive patients and the responders to renal denervation should be identified [35–39]. It is crucial to consider the contribution of afferent and efferent renal nerves for understanding the pathophysiology of hypertension profoundly and applying renal denervation as a therapy for hypertension in humans more effectively.

Our study has some limitations. First, the contribution of afferent and efferent renal nerves was assumed by comparing the effects of ARDN and TRDN, in accordance with the methodologies used in previous studies [31, 32, 40]. Although, selective efferent renal denervation was not performed due to technical difficulties, it might be needed theoretically to enhance the validity of our findings. Second, we used young 9-week-old SHRSP because our interest was whether the input from afferent renal nerves would contribute to the development of hypertension. Although our results showed that kidney injury was not apparent in young SHRSP, which adhered to the findings of a previous report, kidney injury becomes detectable in older SHRSP [41]. Thus, the contribution of afferent renal nerves to blood pressure control in older SHRSP with kidney injury remains unclear. Finally, this study did not investigate the effects of ARDN and TRDN on sympathetic nerve activity or blood pressure in WKY. It can be speculated that ARDN may not decrease blood pressure due to the lack of kidney injury,

and that TRDN may not affect systemic renin-angiotensin system due to lack of hyperactivity of renal efferent sympathetic nerve activity in WKY [42, 43]. Further experiments comparing WKY and SHRSP are necessary to elucidate the mechanism of essential hypertension.

In conclusion, the chronic input from afferent renal nerves does not contribute to the development of hypertension in SHRSP, although the blood pressure response to acute input from the stimulated afferent renal nerves is enhanced. The efferent renal nerves may be greatly involved in the development of hypertension via activation of the renin-angiotensin system in SHRSP.

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Conflicts of interest

K.S. reports grants from Daiichi Sankyo, Nippon Boehringer Ingelheim, and Otsuka Medical Devices. H.T. reports grants and/or personal fees from Daiichi Sankyo, Novartis Pharma, Otsuka Pharmaceutical, Pfizer Japan, Mitsubishi Tanabe Pharma, Teijin Pharma, Nippon Boehringer Ingelheim, AstraZeneca, Ono Pharmaceutical, Kowa,

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Figure legends

Figure 1

The response of blood pressure and renal sympathetic nerve activity to acute chemical stimulation of the afferent renal nerves in WKY and SHRSP. **a, b** Representative recordings of blood pressure (BP), heart rate, and integrated renal sympathetic nerve activity (RSNA) in WKY (**a**) and SHRSP (**b**). The peak responses of mean BP (**c**), heart rate (**d**), and RSNA (**e**) to afferent nerve stimulation by intrapelvic capsaicin infusion; n = 7 for each group; **P* < 0.005 as indicated.

Figure 2

Validation of renal denervation by Western blotting and immunohistochemistry. Representative Western blot images (**a**) and relative expression of CGRP (**b**) and TH (**c**). Representative immunohistochemical images for CGRP (**d**) and TH (**e**) in the renal pelvis. Green, CGRP, and TH. Blue, DAPI. Scale bars: 50 μm. Sham, n = 11; ARDN, n = 10; TRDN, n = 12 in Western blot assay. **P* < 0.001 as indicated.

Figure 3

The chronic effect of renal denervation on blood pressure and heart rate. **a, b** Mean

blood pressure (**a**) and heart rate (**b**) after ARDN, TRDN, or Sham operation. *$P < 0.05$ *vs.* sham, †$P < 0.05$ *vs.* ARDN.

Figure 4

The effect of renal denervation on the renin-angiotensin system. **a** Plasma renin activity. **b** Plasma angiotensin II concentration. **c** mRNA expression in renal cortex. Sham, n = 11; ARDN, n = 10; TRDN, n = 12; *$P < 0.005$ *vs.* sham, †$P < 0.005$ *vs.* ARDN.

Figure 5

The effect of renal denervation on the systemic and central sympathetic nervous system activity. **a** Plasma norepinephrine levels. **b** 24-h urinary norepinephrine excretion. **c** Representative immunohistochemical images for c-Fos-positive neurons in the parvocellular region of PVN and RVLM. The number of c-Fos-positive neurons in the parvocellular region of PVN (**d**) and RVLM (**e**). Scale bars: 200 μm. Sham, n = 11; ARDN, n = 10; TRDN, n = 12 in the analysis of plasma norepinephrine levels and 24-h urinary norepinephrine excretion; n = 6 for each group in the immunohistochemistry of c-Fos.

Figure 6

Comparison of renal injury between WKY and SHRSP. **a** 24-h urinary protein excretion. **b** Serum creatinine levels. Histological analysis of glomerular sclerosis (PAS staining, **c**) and interstitial fibrosis (Sirius Red staining, **d**). Scale bars: 200 μm; n = 7 for each group.

Supplementary Information

Supplemental Methods

Assay for plasma norepinephrine, plasma renin activity, and plasma angiotensin II concentration

Plasma norepinephrine concentration was measured by high performance liquid chromatography, while plasma renin activity was measured by enzyme immunoassay and plasma angiotensin II concentration was measured by radio immunoassay. All assays are commercially available from SRL, Inc., Japan.

Quantitative real time PCR

Renin mRNA expression was quantified by quantitative real time PCR. The renal cortex was isolated and immersed into an RNA stabilization solution (RNAlater Solution, ThermoFisher Scientific, Rockford, IL, USA) for one night and frozen at −80°C until further procedure. Reverse transcription was performed with ReverTra Ace qPCR RT Master Mix (FSQ-201, TOYOBO Co., Ltd., Osaka, Japan) followed by quantitative PCR with THUNDERBIRD SYBR qPCR Mix (QPS-201, TOYOBO Co., Ltd.). The renin mRNA levels were normalized to those of 18S rRNA as the endogenous control. The sequence of primers was as follows: renin (forward, CAGGAACGATGACCTGTGCAT; reverse, CAGTGGGTGGTGGGATGTC) and 18S rRNA (forward, AAGTTTCAGCACATCCTGCGAGTA; reverse, TTGGTGAGGTCAATGTCTGCTTTC).

Immunohistochemistry of brain for c-Fos

The excised brains were immersed into 10% neutral buffered formaldehyde for 24 h and then into 30% sucrose. The rats' brains were embedded in the OCT compound (Tissue-Tek OCT compound; Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and sliced coronary at 30 μm on a

cryostat for free-float staining. The sections, including paraventricular nucleus and rostral ventral lateral medulla, were incubated with 0.2% Triton X-100 and 3% bovine serum albumin in phosphate buffered saline (PBS) as blocking reagent for 60 min. The sections were subsequently incubated with primary antibody for c-Fos (RPCA-c-Fos, EnCor Biotechnology Inc., Alachua, FL, USA) diluted with 0.1% Triton X-100 and 1% bovine serum albumin in PBS at room temperature for 1.5 h. After washing, the sections were incubated with secondary antibody (ab150083, Abcam, Cambridge, MA, USA) diluted in PBS for 1 h. After washing, the sections were mounted on slides with a fluorescence-preserving mounting medium. Signals indicating c-Fos were detected and depicted by a Nikon fluorescence microscope equipped with a NIKON A1 confocal laser microscope.

Immunohistochemical analysis of renal pelvis

Slices containing renal hilus were immersed in 10% neutral buffered formaldehyde for at least 24 h and subsequently in 30% sucrose solution for at least 24 h, and were then frozen in OCT compound and cut at a thickness of 30 μm using a cryostat for free-floating staining. The sections were incubated with 0.2% Triton X-100 and 3% bovine serum albumin in PBS for 1 h for blocking, and were subsequently incubated with primary antibody for calcitonin gene-related peptide (CGRP) (BML-CA1134-0100, ENZO Life sciences, Inc., Farmingdale, NY, USA) and tyrosine hydroxylase (TH) (ab112, Abcam, Cambridge, MA, USA) diluted with 0.1% Triton X-100 and 1% bovine serum albumin in PBS at 4℃ overnight. After washing, the sections were incubated with secondary antibody (ab150077, Abcam, Cambridge, MA, USA) diluted in PBS for 1 h. After washing, the sections were mounted on slides with a fluorescence-preserving mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector, CA, USA) for nuclear counter staining. Signals indicating CGRP or TH and DAPI were detected and depicted by a Nikon fluorescence microscope equipped with a NIKON A1 confocal laser microscope.

Measurement of urinary protein excretion

Urine was centrifuged with 3,000 × g for 5 minutes, and supernatants were collected and diluted 20-fold with PBS. BCA protein assay was performed using a commercially available kit (Pierce BCA Protein Assay, ThermoFisher Scientific, Rockford, IL, USA).

Histological assessment of kidney injury

The harvested kidneys were immersed in 10% neutral buffered formaldehyde for at least 24 h, embedded in paraffin, and cut at a thickness of 4 μm. Glomerular sclerosis was graded semiquantitatively by glomerular sclerosis scores in each glomerulus using PAS staining. Glomerular sclerosis scores were calculated using a scale from 0 to 4 (0, no glomerular sclerosis; 1, segmental glomerular sclerosis in < 25% of the glomeruli; 2, segmental glomerular sclerosis in 25%–50% of the glomeruli; 3, segmental glomerular sclerosis in 50%–75% of the glomeruli; and 4, segmental glomerular sclerosis in ≥75% of the glomeruli) [1]. The scores of randomly selected 50 glomeruli per animal were then averaged. Tubulointerstitial fibrosis was calculated using a Sirius Red stain as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total renal cortical area.

Reference

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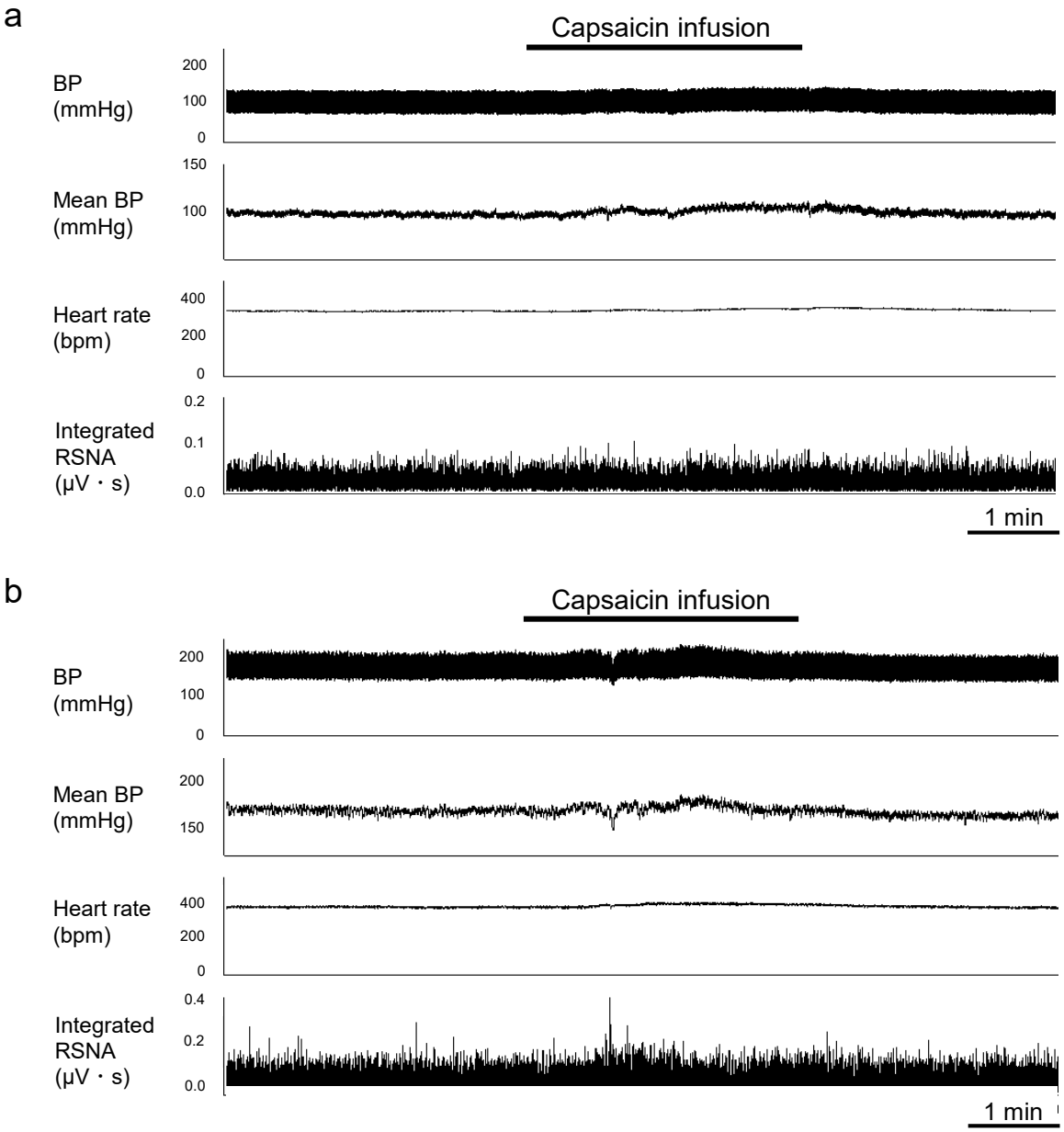
Figure 1


a
Capsaicin infusion
BP (mmHg)
200
100
0
Mean BP (mmHg)
150
100
Heart rate (bpm)
400
200
0
Integrated RSNA (μV・s)
0.2
0.1
0.0
1 min
b
Capsaicin infusion
BP (mmHg)
200
100
0
Mean BP (mmHg)
200
150
Heart rate (bpm)
400
200
0
Integrated RSNA (μV・s)
0.4
0.2
0.0
1 min

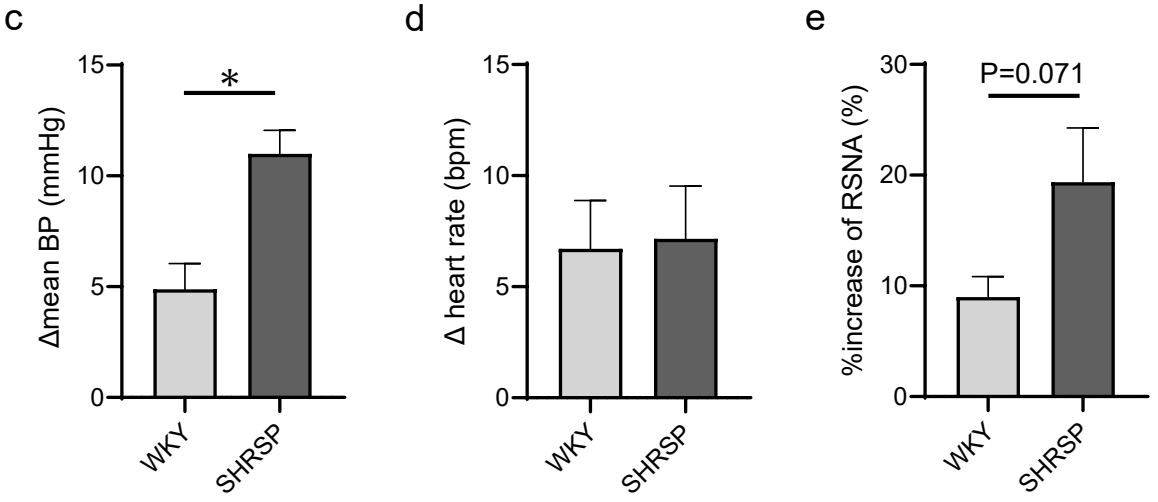

c
Δmean BP (mmHg)
15
10
5
0
*
WKY
SHRSP
d
Δ heart rate (bpm)
15
10
5
0
WKY
SHRSP
e
%increase of RSNA (%)
30
20
10
0
P=0.071
WKY
SHRSP

Figure 2

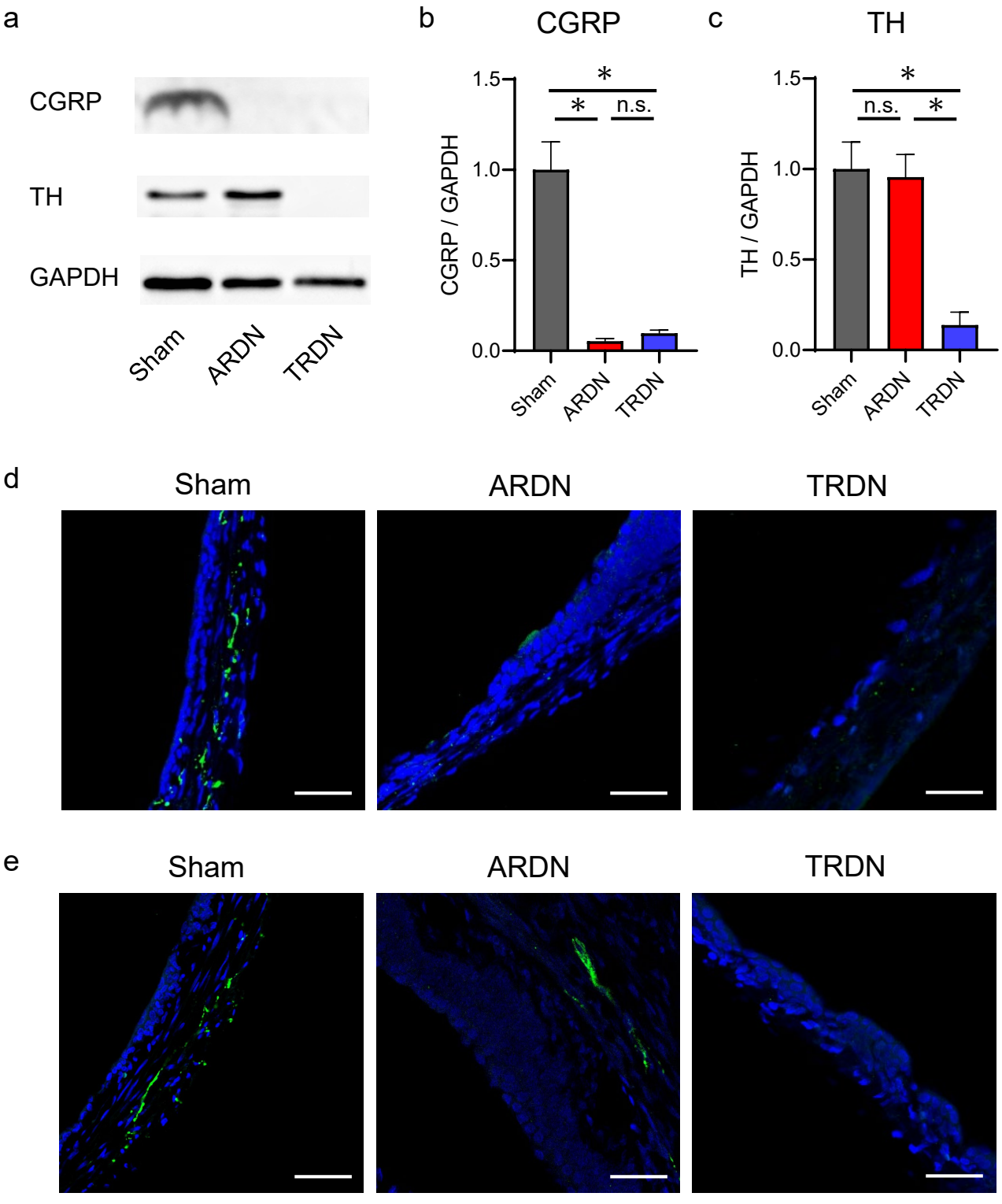

a
CGRP
TH
GAPDH
Sham
ARDN
TRDN
b
CGRP
CGRP / GAPDH
1.5
1.0
0.5
0.0
*
*
n.s.
Sham
ARDN
TRDN
c
TH
TH / GAPDH
1.5
1.0
0.5
0.0
*
n.s.
*
Sham
ARDN
TRDN
d
Sham
ARDN
TRDN
e
Sham
ARDN
TRDN

Figure 3

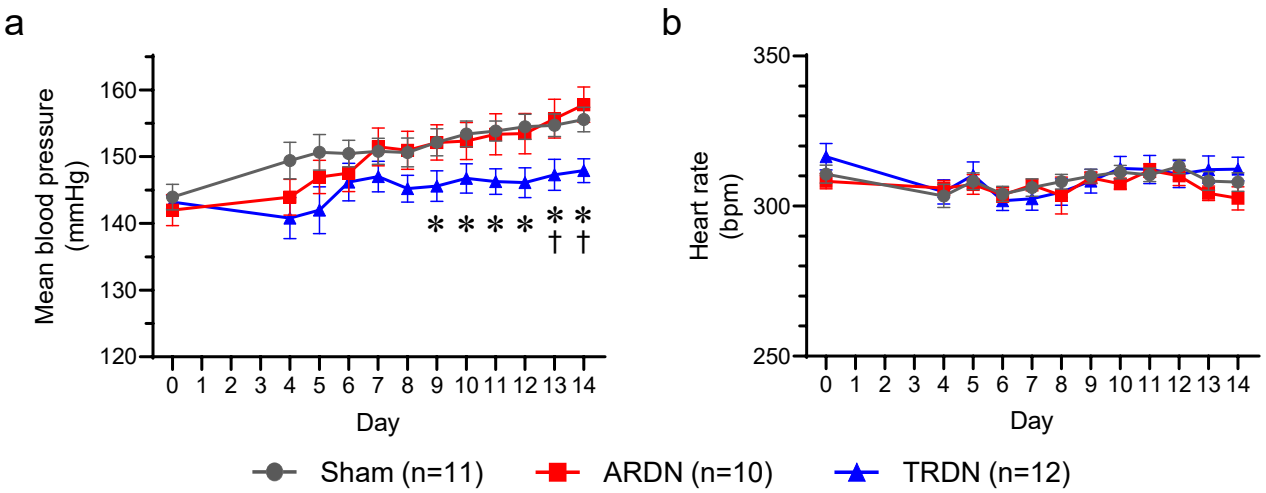

a
Mean blood pressure (mmHg)
160
150
140
130
120
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day
* * * * * *
† †
b
Heart rate (bpm)
350
300
250
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day
Sham (n=11)
ARDN (n=10)
TRDN (n=12)

Figure 4

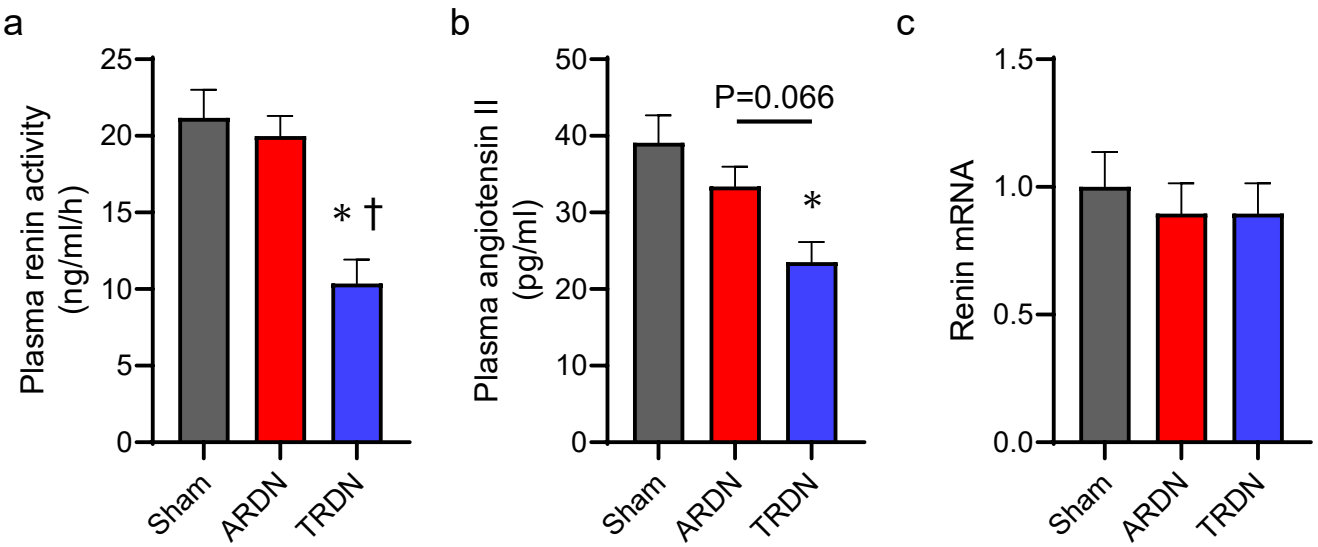

a
Plasma renin activity (ng/ml/h)
25
20
15
10
5
0
* †
Sham
ARDN
TRDN
b
Plasma angiotensin II (pg/ml)
50
40
30
20
10
0
P=0.066
*
Sham
ARDN
TRDN
c
Renin mRNA
1.5
1.0
0.5
0.0
Sham
ARDN
TRDN

Figure 5

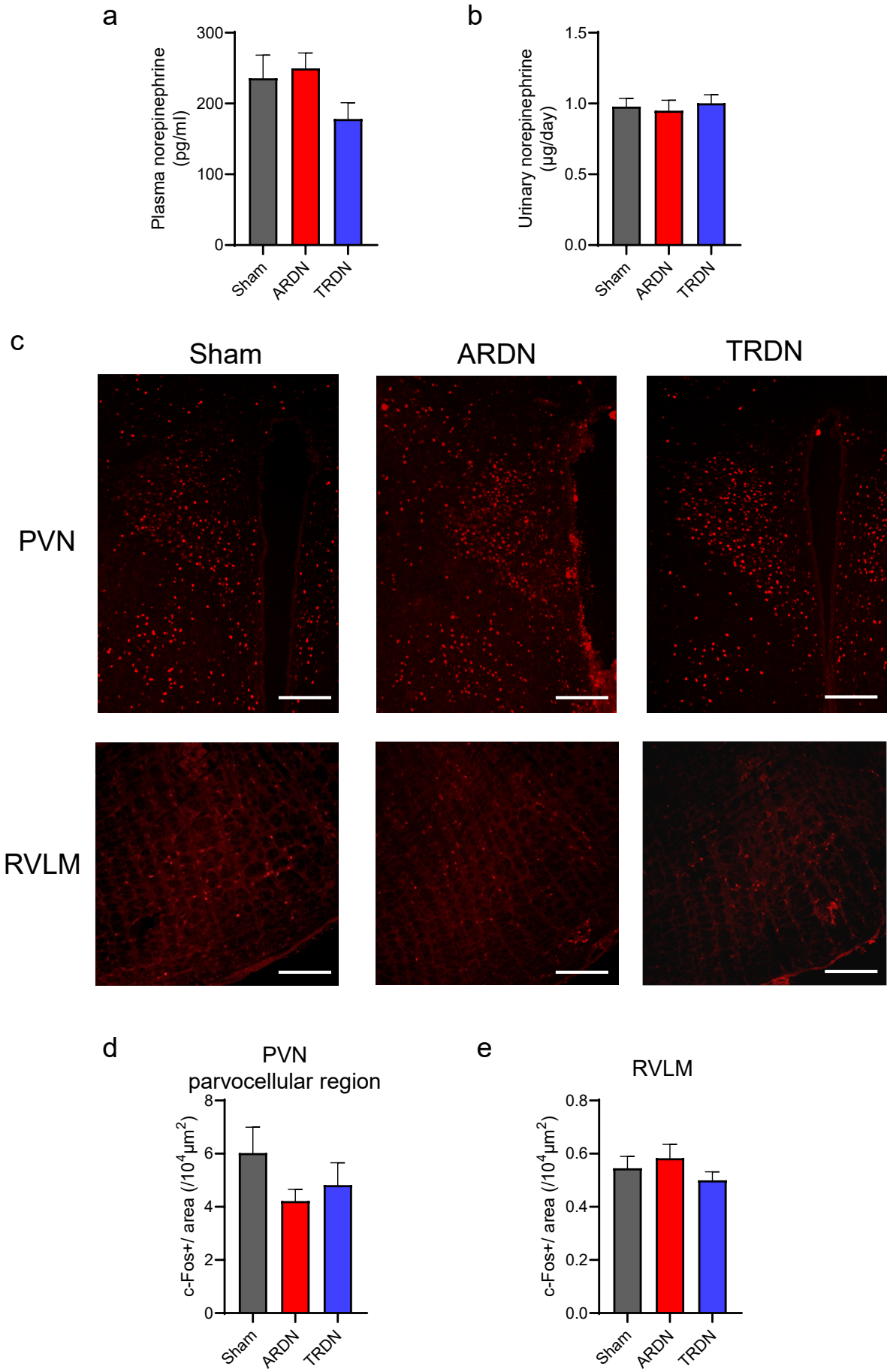
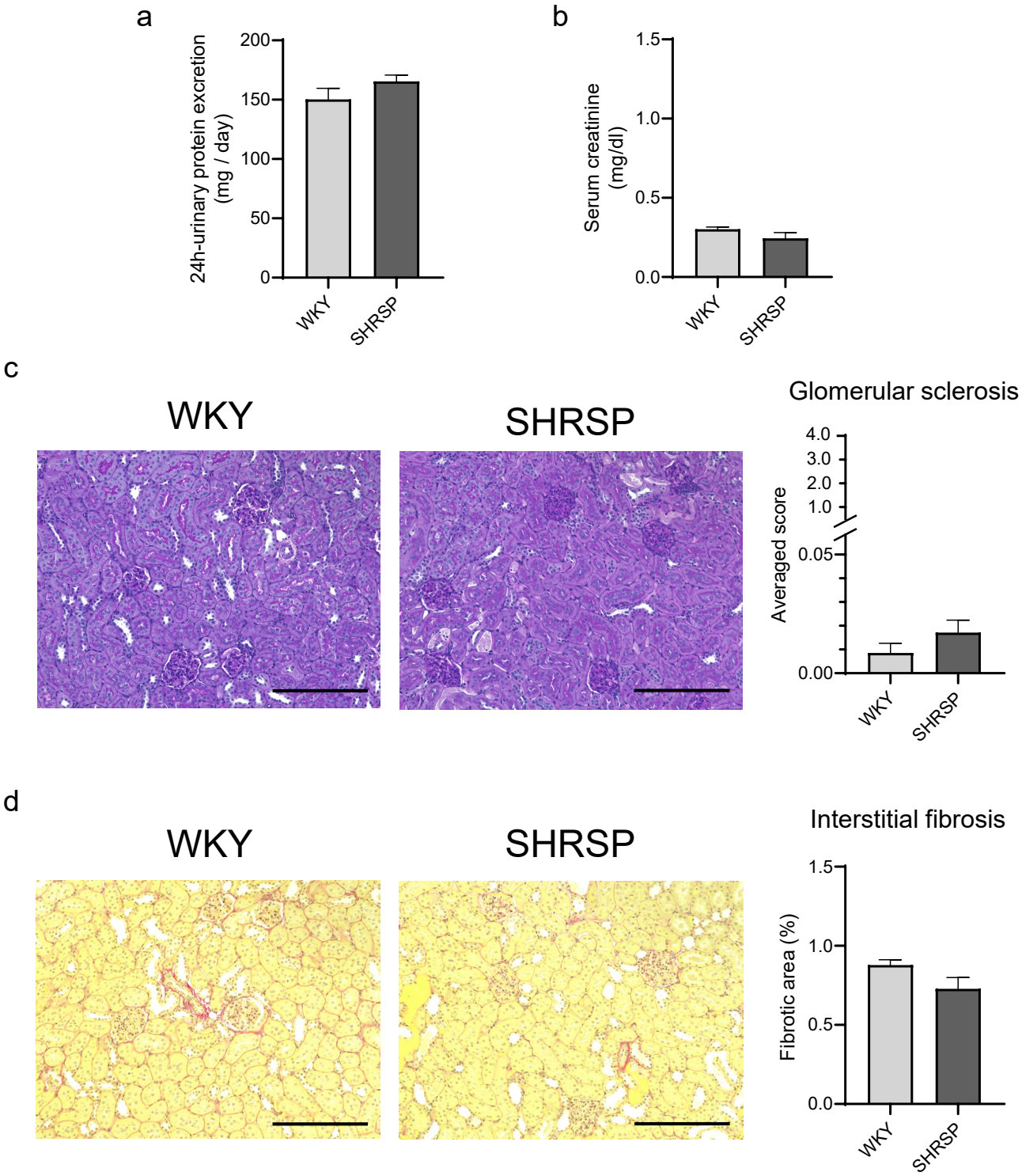

a
Plasma norepinephrine (pg/ml)
300
200
100
0
Sham
ARDN
TRDN
b
Urinary norepinephrine (μg/day)
1.5
1.0
0.5
0.0
Sham
ARDN
TRDN
c
Sham
ARDN
TRDN
PVN
RVLM
d
PVN
parvocellular region
c-Fos+/ area (/10^{4}μm^2)
8
6
4
2
0
Sham
ARDN
TRDN
e
RVLM
c-Fos+/ area (/10^{4}μm^2)
0.8
0.6
0.4
0.2
0.0
Sham
ARDN
TRDN

Figure 6


a
24h-urinary protein excretion (mg / day)
200
150
100
50
0
WKY
SHRSP
b
Serum creatinine (mg/dl)
1.5
1.0
0.5
0.0
WKY
SHRSP
c
WKY
SHRSP
Glomerular sclerosis
Averaged score
4.0
3.0
2.0
1.0
0.05
0.00
WKY
SHRSP
d
WKY
SHRSP
Interstitial fibrosis
Fibrotic area (%)
1.5
1.0
0.5
0.0
WKY
SHRSP