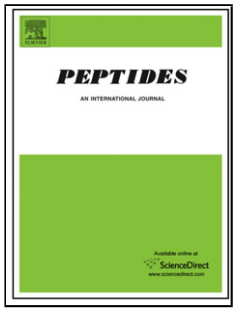

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Superoxide anions modulate the performance of apelin in the paraventricular nucleus on sympathetic activity and blood pressure in spontaneously hypertensive rats

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Highlights

- Apelin microinjection into PVN increases blood pressure and sympathetic output.
- Apelin exerts more pronounced effects in spontaneously hypertensive rats than WKY rats.
- Superoxide anion scavengers, or NAD(P)H oxidase inhibitor attenuated apelin effects.
- Superoxide dismutase inhibitor potentiated the effects of apelin.
- Superoxide anion is involved in apelin–mediated processes in PVN.

Abstract

The present study was designed to determine how apelin in paraventricular nucleus (PVN) modulates the renal sympathetic nerve activity (RSNA), arterial blood pressure (ABP), mean arterial pressure (MAP), and heart rate (HR), and whether superoxide anions regulate the performance of PVN apelin in spontaneously hypertensive rats (SHRs). Acute experiment was carried out with 13-week-old male Wistar-Kyoto rats (WKY) and SHRs under anaesthesia. RSNA, ABP, MAP and HR after PVN microinjection were measured. Apelin microinjection into PVN increased RSNA, ABP, MAP and HR in WKY rats and SHRs, more obviously in SHRs. APJ antagonist F13A decreased the RSNA, ABP, MAP and HR in SHRs, and inhibited the effects of apelin. Apelin and APJ mRNA levels were higher in the PVN in SHRs. PVN microinjection of superoxide anion scavengers tempol and tiron, or NAD(P)H oxidase inhibitor apocynin, decreased the RSNA, ABP, MAP and HR in SHRs, and inhibited the effects of apelin, but the superoxide dismutase (SOD) inhibitor diethyldithiocarbamic acid (DETC) potentiated the effects of apelin. NAD(P)H oxidase activity and superoxide anion levels in PVN were

increased by apelin, but decreased by APJ antagonist F13A. The apelin-induced increases in NAD(P)H oxidase activity and superoxide anion level were abolished by pre-treatment with F13A. These results indicate that apelin in PVN increases the sympathetic outflow and blood pressure via activating APJ receptor. The enhanced activity of endogenous apelin and APJ receptor in PVN contributes to sympathetic activation in hypertension, and the superoxide anion is involved in these apelin–mediated processes in PVN.

Keywords paraventricular nucleus, apelin, renal sympathetic nerve activity, blood pressure, superoxide anion

Introduction

Sympathetic activity intensifies in patients with hypertension [1], and spontaneously hypertensive rats (SHRs) [2]. Paraventricular nucleus (PVN) controls cardiovascular activity and sympathetic outflow via its projections to the rostral ventrolateral medulla (RVLM) and the intermediolateral column of the spinal cord [3, 4]. Studies have proven the involvement of PVN in the pathogenesis of hypertension and organ damage [5, 6], and the regulation of PVN in sympathoexcitatory reflexes, such as adipose afferent reflex [7], cardiac sympathetic afferent reflex [8] and the baroreflex [9].

Apelin is a peptide hormone secreted by adipose tissues [10], and widely found in cerebral [11], pulmonary [12], and cardiovascular tissues [13]. As an endogenous ligand of the orphan G protein-coupled receptor APJ [14], apelin co-works with APJ involved in cardiovascular diseases, obesity, diabetes and cancer [10, 15]. The expression of apelin increases in many pathological processes, such as cardiovascular and metabolic disorders [16]. Apelin and APJ are mainly expressed in the PVN of human, mouse, or rat brain [16]. The microinjection of apelin into PVN significantly increases in the sympathetic nerve activity innervating BAT [17],

and heart rate (HR) [18]. Bilateral RVLM microinjection of apelin significantly increased arterial blood pressure (ABP), a process that was blocked by pre-treatment with the apelin receptor antagonist F13A in normotensive rats [19]. Plasma apelin levels were significantly lower in high normal blood pressure (BP) subjects compared with normal or optimal BP subjects [20].

Superoxide anions regulate sympathetic activity and blood pressure in the brain. Microinjection of either a superoxide anion scavenger or an NAD(P)H oxidase inhibitor into the RVLM attenuates the sympathetic activity [21]. Superoxide anions in the PVN mediate cardiac sympathetic afferent reflex in rats with insulin resistance [22]. Sympathoexcitation increases with the level of NAD(P)H oxidase-derived reactive oxygen species (ROS) in the hypothalamus, a process that elevates the arterial pressure in DOCA-salt hypertensive rats [23]. However, it is unknown whether superoxide anions regulate the effects of apelin on sympathetic activity and blood pressure (BP) in hypertension in the PVN. The present study examines whether superoxide anions in the PVN modulate the effects of apelin on increasing the RSNA, BP, and heart rate (HR) in SHRs.

Materials and Methods

Animals

Experiments were carried out using 13-week-old male normotensive Wistar-Kyoto (WKY) rats and SHRs (Vital River Biological Co., Ltd, Beijing, China). All procedures were approved by the Experimental Animal Care and Use Committee of Xuzhou Medical University, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). The rats were kept in a temperature-controlled room in a

12 h light–dark cycle with free access to standard chow and tap water.

Chemicals

Apelin (3, 30, or 300 pmol) and F13A (2 nmol) were purchased from Phoenix Pharmaceuticals (CA, USA). Superoxide anion scavengers tempol (4-hydroxy-2,2,6,6 –tetramethylpiperidine 1-oxyl, 20 nmol) and tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, 10 nmol), SOD inhibitor diethyldithiocarbamic acid (DETC, 10 nmol), NAD(P)H oxidase inhibitor apocynin (APO, 1 nmol), NAD(P)H, and lucigenin were obtained from Sigma Chemical Co. (MO, USA). The chemicals were dissolved in artificial cerebrospinal fluid (CSF, Sigma, MO, USA) except APO, which was dissolved in artificial CSF containing 1% dimethyl sulfoxide (DMSO, Sigma, MO, USA).

Acute Experiment

The rats were anesthetized with urethane (800 mg/kg, i.p.) and α-chloralose (40 mg/kg, i.p.). Urethane and α-chloralose were dissolved in normal saline (Nanjing Biochannel Biotechnology Co., Ltd., Nanjing China). Status of anesthesia was assessed by testing the corneal reflexes and the paw withdrawal response to a noxious pinch. Supplemental anesthetic agents (at one-tenth of the initial dose) were administered intravenously during the experiment to maintain an appropriate level of anesthesia. Rats were ventilated with room air using a rodent ventilator (Harved Apparatus Inc., MA, USA). The right carotid artery was cannulated and connected to a pressure transducer (ADInstruments, Australia) for continuously recording arterial blood pressure (ABP), mean arterial pressure (MAP) and heart rate (HR).

Recoding of renal sympathetic nerve activity

A retroperitoneal incision was made, and then the left renal sympathetic nerve was isolated.

The renal nerve was cut distally to eliminate its afferent activity. The nerve was placed on a pair of silver electrodes and immersed in warm mineral oil. The raw and the integrated renal sympathetic nerve activity (RSNA) were simultaneously recorded on a PowerLab data acquisition system (AD Instruments, Australia). The RSNA was amplified with an AC/DC differential amplifier (Warner Instruments, CT, USA) at a low-frequency cutoff at 30 Hz and a high-frequency cutoff at 3000 Hz. The amplified and filtered signals were integrated at a time constant of 1.0 s. At the end of each experiment, the background noise was determined after sectioning of the central end of the nerve and the related value was subtracted from the integrated values of RSNA.

PVN microinjections

The rats were placed in a stereotaxic frame (Stoelting, IL, USA). The PVN co-ordinates were 1.8 mm caudal to bregma, 0.4 mm lateral to the mid-line and 7.9 mm ventral to the dorsal surface according to Paxinos & Watson's rat atlas. The bilateral PVN microinjections were completed within1 min (50 nL at each side). At the end of the experiment, the same volume of Evans Blue (2%) was injected into the microinjection site for microscopic histological identification. The rats with microinjection sites outside the PVN were excluded from data analysis. In addition, rats were excluded from analysis if the distance between the center point of the microinjection and the boundary of the PVN was shorter than 0.15 mm.

PVN samples preparation

The rats were killed with an overdose of pentobarbital (100 mg/kg, I.V.). The brains were removed and immediately frozen in liquid nitrogen and stored at −70 ℃ until they were used. Coronal sections of the brain were made with a cryostat microtome (Leica, Germany) at the

PVN level. The thickness of PVN sections was 450 μm and the PVN areas were punched out with a 15-gauge needle (inner diameter 1.5 mm).

Measurement of apelin and APJ mRNA with RT-PCR

Apelin-13 and APJ mRNA in the PVN were measured using a real-time fluorescence quantitative PCR system (Roche, Basel, Switzerland). In brief, the total RNA in samples was extracted with TRIzol (Ambion, TX, USA). The extracted RNA was converted cDNA with reverse transcription using random primers in a total volume of 10 μL according to the instructions of the PrimeScript™ RT Master Mix (Takara, China). All cDNA were stored at − 70 °C before used. Apelin-13 and APJ mRNA was determined with SYBR Green I fluorescence. All samples were amplified in triplicates for 45 cycles in a 96-well plate. The relative gene expression was determined by calculating the values of Δcycle threshold (ΔCt) as a relative quantity to the endogenous control. The rat primer pairs were listed as follows. Apelin-13: 5'-GGCTAGAAGAAGGCAACATGC-3'(forward), 5'-CCGCTGTCTGCGAAATTTC-30'(reverse); APJ: 5'-CCACCTGGTGAAGACTCTCTACA-3'(forward), 5'-CTGACGTAACTGATGCAGGTG-30'(reverse).

Measurement of NAD(P)H oxidase activity

The NAD(P)H oxidase activity in the PVN was measured by enhanced lucigenin chemiluminescence. Briefly, NAD(P)H (100 μM) was added to the media as a substrate to react with NAD(P)H oxidase and generate superoxide anions. The light emission produced by the reaction of lucigenin (5 μM) with superoxide anions was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 min. The values represented the NAD(P)H oxidase activity and were expressed as the mean light units (MLU) per minute per milligram of

protein.

Measurement of superoxide anions

Superoxide anion level in the PVN was determined by lucigenin-derived chemiluminescence. Briefly, the reaction with superoxide anions was initiated by adding dark-adapted lucigenin (5μM) to each sample to cause photon emission, which was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 min. The values representing the superoxide anions level were expressed as the MLU per minute per milligram of protein.

Statistical analyses

Data presented as the mean ± standard error of the mean (SEM) were analyzed using GraphPad Prism 4.0 (GraphPad software Inc., CA, USA). One-way or two-way ANOVA was performed, followed by Bonferroni test for *post hoc* analysis when multiple comparisons were made. A two-tailed P-value <0.05 was considered statistically significant.

Results

Effects of different doses of apelin

PVN microinjection of apelin increased the RSNA, systolic blood pressure (SBP), diastolic blood pressure (DBP), MAP, and HR in both WKY rats and SHRs in a dose-dependent manner. Three doses of apelin (3, 30, 300 pmol) increased RNSA, SBP, DBP, MAP, and HR in PVN of SHRs, and only moderate (30 pmol) and high (300 pmol) doses of apelin augmented RNSA, SBP, DBP, MAP, and HR in WKY rats. Apelin (3 pmol) showed no significant effect on RSNA, SBP, DBP, MAP, and HR in WKY rats compared with artificial CSF. Apelin treatment caused larger increases in RSNA, SBP, DBP, MAP, and HR in SHRs than in WKY rats (Figure 1).

Effects of APJ antagonist F13A

Microinjection of F13A (an APJ antagonist, 2 nmol) into PVN significantly reduced the RSNA, SBP, DBP, MAP, and HR in SHRs, but not in WKY rats (Figure 2A). The increases of RSNA and MAP induced by microinjection of apelin (300 pmol) into the PVN were abolished by the pre-treatment with F13A (Figure 2B).

Expression of apelin and APJ

The level of mRNA of apelin in PVN in SHRs was higher than that in WKY rats. APJ m RNA expression in PVN was also higher in SHRs than in WKY rats. Apelin and APJ mRNA levels in PVN were 5.6 and 3.7 times higher in SHRs than in WKY rats, respectively (Figure 3).

Effects of tempol, tiron, APO, and DETC

PVN microinjection of superoxides anion scaverger tempol (20 nmol) or tiron (10 nmol) reduced the RSAN, SBP, DBP, MAP, and HR both in WKY rats and SHRs. Tempol or tiron treatment decreased RSNA, SBP, DBP, MAP, and HR more obviously in SHRs than in WKY rats. Pre-treatment with tempol or tiron inhibited apelin (300 pmol) to increase RSNA, SBP, DBP, MAP, and HR. NAD(P)H oxidase inhibitor APO (1 nmol) reduced the RSAN, SBP, DBP, MAP, and HR both in WKY rats and SHRs, more obviously in SHRs. Pre-treatment with APO inhibited apelin to increase RSNA, SBP, DBP, MAP, and HR both in WKY rats and SHRs. DETC (10 nmol), a SOD inhibitor, increased RSNA, SBP, DBP, MAP, and HR both in WKY rats and SHRs, more obviously in SHRs. DETC pre-treatment potentiated the effect of apelin both in the WKY rats and SHRs, more obviously in SHRs (Figure 4).

NAD(P)H oxidase activity and superoxide anions level

NAD(P)H oxidase activity and superoxide anions levels in PVN were higher in SHRs than in WKY rats. NAD(P)H oxidase activity and superoxide anion levels were increased by apelin

microinjection into PVN, but reduced by APJ antagonist F13A both in WKY rats and SHRs.. The increases of NAD(P)H oxidase activity and superoxide anions level induced by apelin administration were larger in SHRs than in WKY rats. F13A pre-treatment weakened the ability of apelin in increasing NAD(P)H oxidase activity and superoxide anions level in PVN both in WKY rats and SHRs (Figure 5).

Discussion

An endogenous ligand of the orphan G protein-coupled receptor APJ, apelin is initially isolated from bovine stomach extracts [24]. Excessive sympathetic output contributes to hypertension and even organ damage [25, 26]. Suppressing sympathetic activation is considered to be an antihypertensive strategy [5]. Apelin significantly increases the activity of sympathetic nerves that control the blood pressure in the brain [14, 19, 27]. The present study showed that APJ receptor activation with apelin in the PVN increased sympathetic outflow and blood pressure. Activating endogenous apelin and APJ receptor contribute to sympathetic hyperactivity in SHRs. Superoxide anion is involved in apelin-mediated effect in the PVN.

In the present study, microinjection apelin into PVN increased the RSNA, SBP, DBP, MAP, and HR in both WKY rats and SHRs in a dose-dependent manner; apelin treatment increased RSNA, SBP, DBP, MAP, and HR more obviously in SHRs than in WKY rats, which is supported by a previous finding that apelin and APJ in the PVN triggered hypertension via activating sympathetic nerves and releasing arginine vasopressin in SHRs [14]. Our recent study has shown that APJ receptor antagonist F13A in PVN reduced the RSNA, SBP, DBP, MAP, and HR in the SHRs, but not in WKY rats. Microinjecting APJ receptor antagonist F13A into PVN blocked apelin-mediated effects, including the increases of RSNA, SBP, DBP, MAP,

and HR in both SHRs and WKY rats. Apelin and APJ expression levels in PVN were higher in SHR than in WKY rats. These results suggest that exogenous apelin can increase RSNA, blood pressure, and HR in PVN, a process mediated by APJ receptor, and that apelin is more active in SHRs than in WKY rats. Apelin and APJ receptor activity in the PVN gets enhanced in the case of hypertensive rats.

Superoxide anion can modulate sympathetic activity and blood pressure [28]. Scavenging the superoxide anions weakens the sympathetic nerve activity [21]. Long-term oral administration of tempol attenuates ventricular dysfunction and sympathetic activity in heart failure rats [29]. Tiron and tempol are stable membrane-permeable superoxide anion scavengers that mimic SOD to scavenge superoxide anions [30, 31]. In the present study, PVN microinjection of superoxide anion temol and tiron reduced the RSNA, SBP, DBP, MAP, and HR in WKY rats. The results demonstrated that superoxide anion in PVN was involved in the tonic control of sympathetic activity and blood pressure in the normal state. We found that temol and tiron in PVN caused greater decreases in RSNA, SBP, DBP, MAP, and HR in SHRs than in WKY rats. SOD inhibitor DETC increased the RSNA, SBP, DBP, MAP, and HR in both SHRs and WKY rats, and the DETC–mediated effect on the RSNA, SBP, DBP, MAP, and HR was more evident in hypertensive rats than in the normotensive rats. Furthermore, superoxide anion level in PVN was higher in SHRs than in WKY rats. The results showed that superoxide anion is involved in sympathetic activation and hypertension.

Apelin directly increases neuronal activity via stimulating NAD(P)H oxidase-derived superoxide in the RVLM [32]. Apelin attenuates cisplatin-induced cardiotoxicity through inhibiting ROS-mediated DNA damage, and regulating mitogen-activated protein kinase and

protein kinase B pathways in H9C2 cells [33]. The results of the present study showed that PVN microinjection of SOD inhibitor DETC potentiated, but the superoxide anion scavenger (either tempol or tiron) inhibited the effects of apelin on the RSNA, SBP, DBP, MAP, and HR. In addition, PVN microinjection of apelin increased, but F13A decreased the level of superoxide anions in the PVN. These results indicate that superoxide anions are involved in the performance of apelin in PVN.

Superoxide anions can be produced by several intracellular substances, including cytochrome, cyclooxygenases, xanthine oxidase, P450, lipoxygenases, and NAD(P)H oxidases [34]. NAD(P)H oxidase-derived ROS in PVN mediates the effect of angiotensin II on RSNA and MAP in rats [35, 36], and regulates neuronal functions [37]. In our present study, pre-treatment with NAD(P)H oxidase inhibitor APO weakened the ability of apelin in increasing RSNA, SBP, DBP, MAP, and HR. NAD(P)H oxidase activity in PVN was increased by apelin, but decreased by APJ receptor antagonist F13A. Pre-treatment with F13A abolished the effect of apelin in increasing the NAD(P)H oxidase activity and superoxide anion level. These results indicate that the catalysis of NAD(P)H oxidase generates superoxide anions that modulate the effect of apelin in the PVN.

SHR had higher levels of the subunit of NAD(P)H oxidase (gp91phox), mRNA expressions of NOX-2 and NOX-4, and ROS in the PVN [38-40]. Chronic inhibition of ROS in the PVN restores the balance of neurotransmitters and cytokines in the PVN, thereby attenuating hypertensive response and sympathetic activity [38]. We found that NAD(P)H oxidase activity and superoxide anions levels in PVN were higher in SHRs than in WKY rats. According to these findings, increased oxidative stress may be caused by sympathetic enhancement induced by apelin

in PVN of SHRs

Serum apelin level is significantly lower in hypertensive patients with left ventricular hypertrophy (LVH) compared with those without LVH [41]. Compared to normal or optimal blood pressure (BP) subjects, apelin levels were significantly lower in high normal BP subjects. Lower apelin plasma levels in high normal BP subjects compared to normal or optimal BP individuals could partially explain the higher cardiovascular risk of the high normal BP group [20]. Apelin/APJ system may be a novel therapeutic target for pharmacological intervention in treating hypertension. Adenoviral vectors containing human superoxide dismutase 1 (SOD1) microinjected into the PVN of SHRs. Significant depressor effects were observed from weeks 1 to 4 after SOD1 gene transfer in SHR [42]. Furthermore, the human SOD1 overexpression in the PVN prevented the increases in left ventricular end-diastolic pressure and volume, and the decreases in ejection fraction and peak velocities of contraction in myocardial infarction rats. Superoxide anion in PVN is a novel therapeutic target for treating cardiovascular diseases.

In conclusion, apelin in PVN increases sympathetic output and blood pressure via activating APJ receptor. The increased endogenous apelin and APJ receptor activity in PVN enhance the sympathetic activation in hypertension. Superoxide anions in PVN can reinforce the responses of RSNA, BP, and HR to apelin in PVN of hypertensive rats. NAD(P)H oxidase in PVN is a major source of superoxide anions that modulate the effects of apelin.

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Figure legends

Figure 1. Effects of paraventricular nucleus (PVN) microinjection of artificial cerebrospinal fluid (CSF) or 3 doses of apelin (3, 30, 300 pmol) on renal sympathetic renal activity (RSNA), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). Values are mean ± SE. *P<0.05 vs. CSF; #P<0.05 vs. WKY. n=6 for each group.

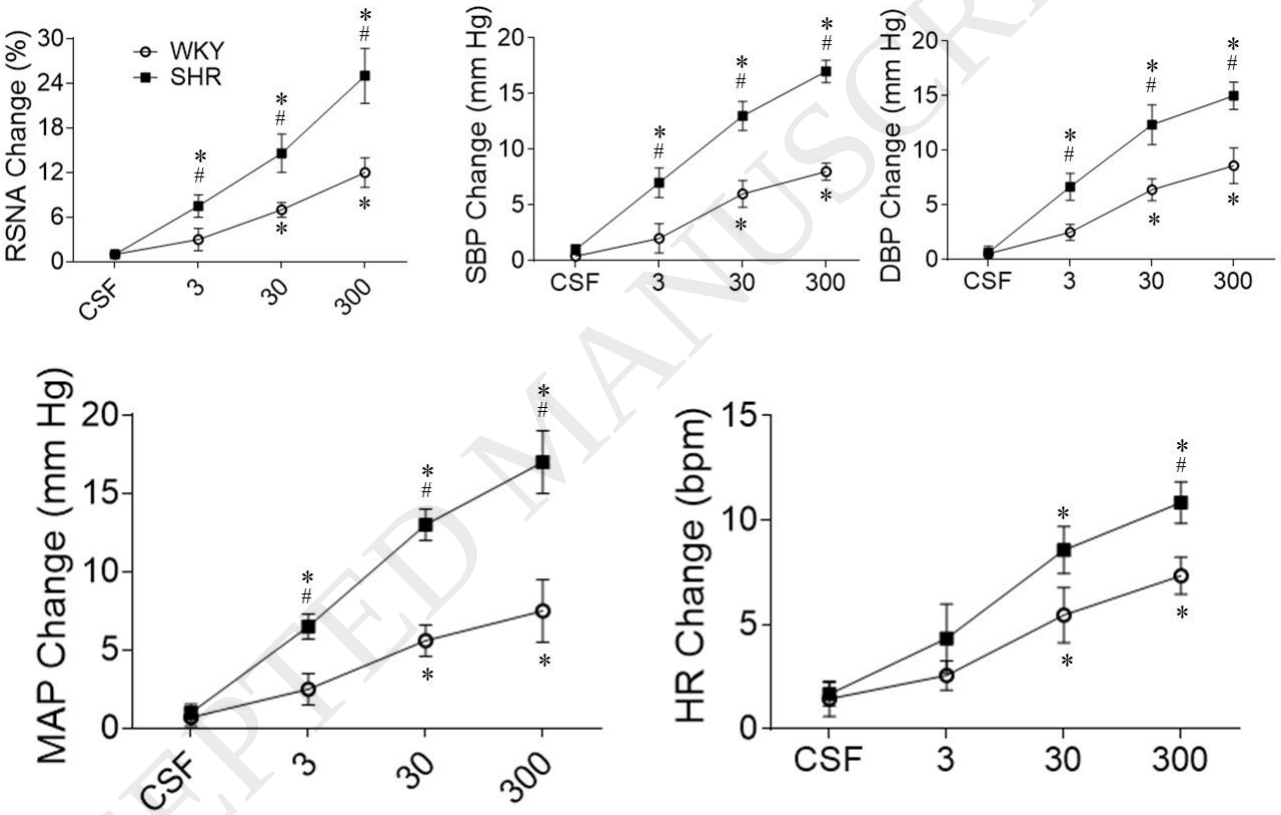

Figure 2. Effects of paraventricular nucleus (PVN) microinjection of artificial cerebrospinal fluid (CSF) or apelin (300 pmol) receotor APJ antagonist F13A (2 nmol) on renal sympathetic renal activity (RSNA) , systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). Values are mean ± SE. *P<0.05 vs. CSF; #P<0.05 vs. WKY; &P P<0.05 vs. apelin. n=6 for each group.

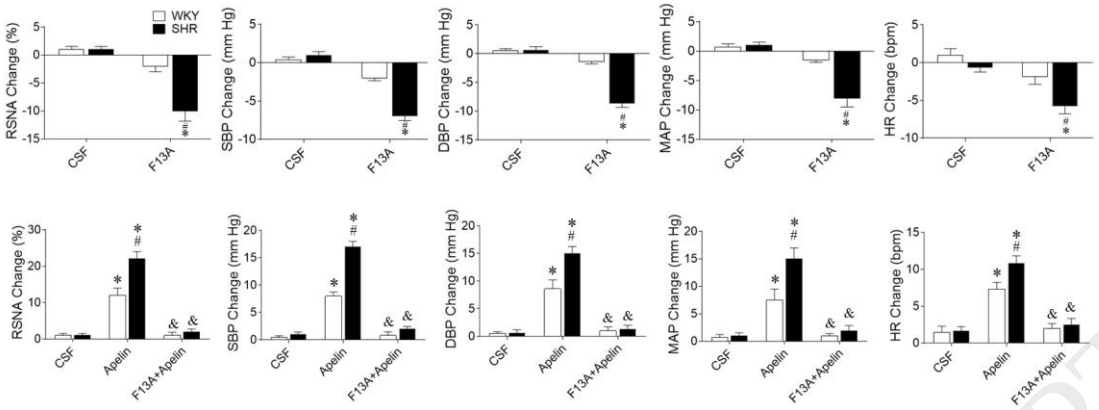

Figure 3. Expression of apelin and apelin receptor APJ in paraventricular nucleus (PVN) in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). Values are mean ± SE. *P<0.05 vs. WKY. n=6 for each group.

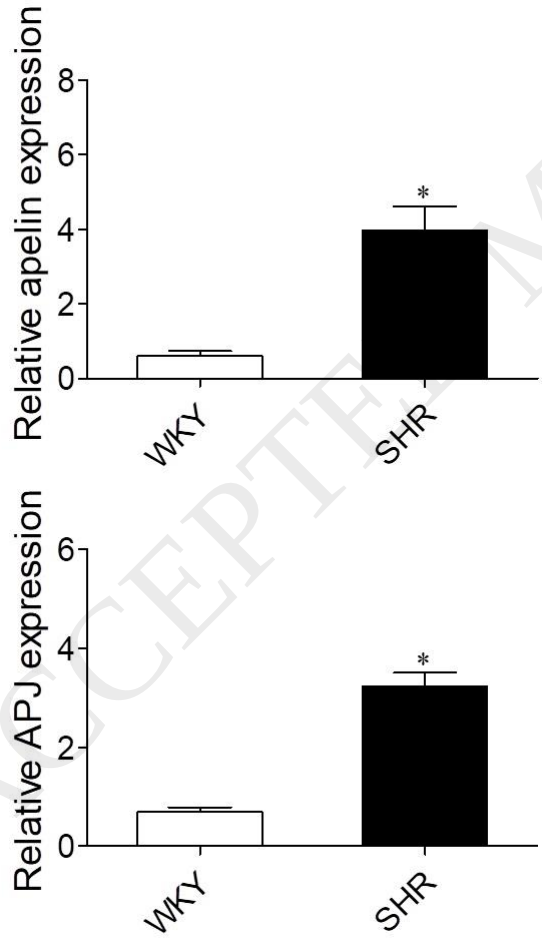

Figure 4. Effects of paraventricular nucleus (PVN) microinjection of artificial cerebrospinal fluid (CSF), superoxide anion scavengers tempol (20 nmol) or tiron (10 nmol), NAD(P)H

oxidase inhibitor apocynin (APO, 1 nmol), and Superoxide dismutase (SOD) inhibitor diethyldithiocarbamic acid (DETC, 10 nmol) on renal sympathetic renal activity (RSNA) , systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). *P<0.05 vs. CSF; #P<0.05 vs. WKY; &P P<0.05 vs. apelin (300 pmol). n=6 for each group.

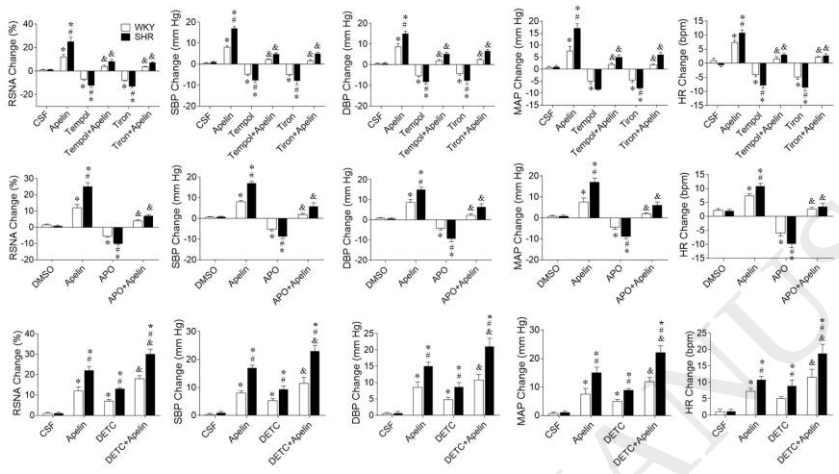

Figure 5. Levels of NAD(P)H oxidase activity and superoxide anions in paraventricular nucleus (PVN) in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). Artificial cerebrospinal fluid (CSF), apelin (300 pmol), F13A (2 nmol), or apelin+F13A were microinjected into the PVN. The NAD(P)H oxidase activity in the PVN was measured by enhanced lucigenin chemiluminescence, and superoxide anions level in the PVN was determined by lucigenin-derived chemiluminescence. *P<0.05 vs. CSF; #P<0.05 vs. WKY; &P P<0.05 vs. apelin. n=6 for each group.