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Microinjection of Salusin-β into the Nucleus Tractus Solitarii Inhibits Cardiovascular Function by Suppressing Presympathetic Neurons in Rostral Ventrolateral Medulla in Rats

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Summary

Salusin-β is newly identified bioactive peptide of 20 amino acids, which is widely distributed in hematopoietic system, endocrine system, and the central nervous system (CNS). Although salusin-β extensively expressed in the CNS, the central cardiovascular functions of salusin-β are unclear. Our main objective was to determine the cardiovascular effect of microinjection of salusin-β into the nucleus tractus solitarii (NTS) in anesthetized rats. Bilateral or unilateral microinjection of salusin-β (0.94-94 μg/rat) into the NTS dose-dependently decreased blood pressure and heart rate. Bilateral NTS microinjection of salusin-β (9.4 μg/rat) did not alter baroreflex sensitivity. Prior application of the glutamate receptor antagonist kynurenic acid (0.19 μg/rat, n=9) into the NTS did not alter the salusin-β (9.4 μg/rat) induced hypotension and bradycardia. However, pretreatment with the GABA receptor agonist muscimol (0.5 ng/rat) within the rostral ventrolateral medulla (RVLM) completely abolished the hypotension (−14±5 vs. −3±5 mm Hg, $P<0.05$) and bradycardia (−22±6 vs. −6±5 bpm, $P<0.05$) evoked by intra-NTS salusin-β (9.4 μg/rat). In addition, we found that vagotomy didn't influence the actions of salusin-β (9.4 μg/rat) in the NTS. In conclusion, our present study shows that microinjection of salusin-β into the NTS significantly produces hypotension and bradycardia, presumably by suppressing the activities of presympathetic neurons in the RVLM.

Key words

Salusin • Medulla • Baroreflex • Presympathetic neuron • Rat

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Introduction

Salusin-α and salusin-β are the novel bioactive peptides of 28 and 20 amino acid residues (Shichiri *et al.* 2003). Both of them originate from preprosalusin, an alternative-splicing product of the torsion dystonia-related gene (TOR2A) (Shichiri *et al.* 2003), and are ubiquitously expressed in tissues including the brain (Izumiyama *et al.* 2005, Takenoya *et al.* 2005, Suzuki *et al.* 2007, 2011, Nakayama *et al.* 2009). Previous studies show that salusins have multiple functions in the cardiovascular, endocrine and immune systems (Shichiri *et al.* 2003, Yu *et al.* 2004, Saito *et al.* 2008, Watanabe *et al.* 2008a,b). Intravenous administration of salusin-α or salusin-β in rats produces hypotension, bradycardia, and cardiac dysfunction in anesthetized rats, which is mainly mediated by cholinergic mechanism but not the results of vascular smooth muscles dilations (Shichiri *et al.* 2003, Izumiyama *et al.* 2005). In addition, salusins exhibit mitogenic activities on a variety of cell types, including vascular smooth muscle cells and cardiomyocytes

(Shichiri *et al.* 2003). Besides, salusins help to modulate the intracellular signaling pathways (Shichiri *et al.* 2003) and the formation of foam cells (Watanabe *et al.* 2008a,b, 2011, Nagashima *et al.* 2010). They inhibit cardiac ventricular myocytes L-type calcium currents I(N) (aCa), increase I(to) (Ren *et al.* 2013), but not affect I(N) (a), I(sus) and I(K) (Ren *et al.* 2013). Salusins may be a potential survival factor against myocardial cell death in cardiomyocytes induced by serum deprivation (Yan *et al.* 2006). More recently, it has been shown that both salusin-α and salusin-β are involved in processing of atherosclerosis (Watanabe *et al.* 2008a,b, 2011, Kimoto *et al.* 2010, Nagashima *et al.* 2010, Zhou *et al.* 2012), renal insufficiency (Kimoto *et al.* 2010), thrombosis/bleeding disorders (Koyama 2010) by opposite effects. In the CNS, pre-salusin is expressed abundantly in the hypothalamus and pituitary (Takenoya *et al.* 2005, Suzuki *et al.* 2007). Salusin-β is capable of stimulating the release of arginin-vasopressin (AVP) from the posterior pituitary, suggesting that salusin-β is a potential candidate of neuropeptide (Shichiri *et al.* 2003, Takenoya *et al.* 2005, Wang *et al.* 2006, Saito *et al.* 2008). Although salusin-β is expressed abundantly in the CNS, the effects of salusins on cardiovascular regulation have not been determined. Furthermore, it is not clear if salusins exert their physiological functions in the central cardiovascular regions. The nucleus tractus solitarii (NTS) is the primary site of termination of afferent fibers from arterial baro- and chemoreceptors (Kubo and Kihara 1990, Lawrence and Jarrott 1996). Stimulation of baroreceptor afferents activates excitatory amino acid receptors within the NTS. NTS neuron sends excitatory amino acid projections to the caudal ventrolateral medulla (CVLM), which in turn inhibits rostral ventrolateral medulla (RVLM) neuron *via* a GABAergic inhibitory pathway (Sapru 1996). Although the NTS is an important area for integrating the tonic and reflex control of the cardiovascular activity, and the expression of salusin-β in the medulla had been confirmed in previous study (Nakayama *et al.* 2009, Suzuki *et al.* 2011), the cardiovascular functions of salusin-β are not determined. The present work was designed to determine the effect of exogenous salusin-β on cardiovascular activity at the NTS level.

Material and Methods

General procedure

Male Sprague-Dawley (SD) rats (weighing between 250 to 300 g, provided by Sino-British SIPPR/BK Lab Animal Ltd) were employed in this study. Each animal experimentation was in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, or European Guidelines on Laboratory Animal Care. The methods for animal preparation, microinjection and histological procedures were similar to described previously (Lu *et al.* 2005, 2007, Liu *et al.* 2011, Qiao *et al.* 2011).

Briefly, rats were anesthetized with urethane (1.3 g/kg, i.p.). A catheter was inserted into the right femoral artery and connected to a pressure transducer (P-300B) to measure blood pressure (BP) directly. BP was sequentially measured by signal acquisition and processing system (RM6240, China), and heart rate (HR) was computed from the BP waveforms. Another catheter was inserted into right femoral vein for drug administration. Following tracheotomy, the rats were paralyzed with triethiodide (10 mg/kg initially and 4 mg/kg every 30 min, i.v.) for artificially respiration with oxygen-enriched room air. Adequacy of anesthesia was assessed by monitoring the stability of BP, and anesthetics were supplemented when necessary. The rats were fixed on a stereotaxic frame (MK-8003, China). Part of the occipital bone was removed to expose the dorsal surface of the medulla. Body temperature was maintained at about 37 °C with an infrared heating lamp.

Microinjection procedure

Under the guidance of a stereotaxic apparatus, the multi-barreled micropipette (tip diameter 20-30 μm) was inserted into the NTS (0.5 mm rostral to the calamus scriptorius, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the medulla) or RVLM (2.0-2.5 mm rostral to the calamus scriptorius, 1.8-2.0 mm lateral to the midline, and 2.8-3.2 below the dorsal surface of the medulla). The micropipettes were filled with L-glutamate, salusin-β, the glutamate receptor antagonist kynurenic acid (KYN) or the GABA receptor agonist muscimol. Salusin-β was obtained from Phoenix (USA), and the others were obtained from the (Sigma, St. Louis, MO). KYN was initially dissolved in 10 % sodium hydroxide (NaOH), and diluted with in an artificial cerebrospinal fluid (aCSF) to the final concentration (pH was adjusted to 7.4 with 10 % HCl). The doses of KYN and muscimol were based on the previous studies (Carvalho *et al.* 2003, Schreihofer *et al.* 2005). All drugs were administered into the NTS in a volume of 100 nl, and delivered approximately 10 s. As previously

described (Lu *et al.* 2005, Wang *et al.* 2006), the chemical identification of the NTS and RVLM was based on the depressor or pressor response, respectively, to injection of 0.19 μg/rat of L-glutamate at the beginning of each experiment.

Baroreflex activation

The methods of baroreflex sensitivity determination were based on previous reports (Smyth *et al.* 1969, Fu *et al.* 2006). In brief, rats were anesthetized with urethane (800 mg/kg, ip) and α-chloralose (40 mg/kg, ip). The cardiac baroreflex was evoked using bolus intravenous injections of the α-adrenergic receptor agonist phenylephrine hydrochloride (Sigma, St. Louis, MO). The peak amplitude of the resulting pressor and reflex bradycardia responses evoked by phenylephrine hydrochloride (10 mg/kg) was plotted against each other. Regression lines were obtained by the least-squares method, and the slope of each line was calculated to provide an index of baroreflex sensitivity. The slope of the baroreflex sensitivity curves was determined before and 5 min, 30 min after pharmacological manipulation.

Protocols

Ten groups (n=4-7, Table 1) were designed to test the dose-dependent effects of bilateral or unilateral microinjection of salusin-β (0.94, 9.4 and 94 μg/rat) into the NTS on cardiovascular activity. And baroreflex sensitivity function was also detected after salusin-β (9.4 μg/rat) injected bilateral into the NTS. Six groups (n=4-9, Table 2) were for determining the mechanisms underlying the effects of unilateral microinjection of salusin-β into the NTS on cardiovascular activity by bilateral vagus dissection, pretreatment with glutamate receptor antagonist in the NTS and pretreatment with the GABAA receptor agonist muscimol in the RVLM. To observe the dose-dependent effects of bilateral or unilateral microinjection of salusin-β (0.94, 9.4 and 94 μg/rat) into the NTS of rats, BP and HR responses were continuously monitored for at least 60 min. In 11 rats, baroreflex sensitivity was determined before and 5 and 30 min after bilateral microinjection of salusin-β (9.4 μg/rat) or vehicle (aCSF, 100 nl) into the NTS. In other groups, the cardiovascular response to salusin-β (9.4 μg/rat) was monitored after the non-selective glutamate receptor antagonist KYN (1 nmol, n=9)/vehicle (aCSF, 100 nl, n=4) or the GABA receptor agonist muscimol (0.5 ng/rat, n=7)/vehicle (aCSF, 100 nl, n=4) injected into the RVLM. Finally, in 7 rats, bilateral vagus dissection was performed to observe whether peripheral cholinergic mechanism is involved in mediating the cardiovascular effects of intra-NTS salusin-β (9.4 μg/rat).

Table 1. Baseline values of MAP and HR in experimental groups of dose-dependent effects of bilateral or unilateral microinjection of salusin-β (0.94-94 μg) into the nucleus tractus solitarii (NTS) on the blood pressure and heart rate of rats.

Groups	n	MAP (mm Hg)	HR (bpm)
bilateral microinjection			
aCSF (100 nl)	4	114 ± 2	454 ± 21
salusin-β (0.94 μg/rat)	7	90 ± 3	436 ± 20
salusin-β (9.4 μg/rat)	7	99 ± 6	398 ± 15
salusin-β (94 μg/rat)	7	91 ± 3	459 ± 21
aCSF+baroreflex	4	102 ± 3	397 ± 18
salusin-β (9.4 μg/rat) +baroreflex	7	97 ± 4	417 ± 16
unilateral microinjection			
aCSF (100 nl)	4	101 ± 2	451 ± 12
salusin-β (0.94 μg/rat)	7	93 ± 4	445 ± 28
salusin-β (9.4 μg/rat)	7	96 ± 5	445 ± 13
salusin-β (94 μg/rat)	7	94 ± 5	461 ± 22

Table 2. Baseline values of MAP and HR in experimental groups of prior application with glutamate receptor antagonist kynurenic acid (0.19 μg/rat, n=9) within the NTS, GABA receptor agonist muscimol within the rostral ventrolateral medulla (RVLM) or prior bilateral vagotomy on the BP and HR responses to microinjection of salusin-β (9.4 μg/rat) into the NTS

Factors of prior treatment	n	MAP (mm Hg)	HR (bpm)
aCSF in the NTS+salusin-β (9.4 μg/rat) in the NTS	4	96 ± 5	408 ± 20
KYN in the NTS+salusin-β (9.4 μg/rat) in the NTS	9	93 ± 6	410 ± 10
aCSF in RVLM+salusin-β (9.4 μg/rat) in the NTS	4	105 ± 5	407 ± 20
muscimol in RVLM+salusin-β (9.4 μg/rat) in the NTS	7	97 ± 6	395 ± 17
vagus-intact+salusin-β (9.4 μg/rat)	4	99 ± 6	398 ± 15
vagotomy+salusin-β (9.4 μg/rat) in the NTS	7	106 ± 5	440 ± 15

Histological analysis

The site of microinjection was verified histologically at the end of each experiment by injection of 20 μl of 2 % pontamine sky blue solution in the same location as described previously (Cai *et al.* 2007, Wang *et al.* 2008). The animal was perfused transcardially with 0.9 % NaCl and 10 % phosphate-buffered formalin, and then the brain tissue was stored overnight in 10 % phosphate-buffered formalin, and then transferred to fixative containing 30 % sucrose. The frozen brain tissue was sectioned coronally (50 μm), and the location of drug microinjections was reconstructed from the dye spots by the atlases (Paxinos and Watson 1997). The histological distributions of drug microinjection sites within the medulla oblongata are illustrated in Figure 1.

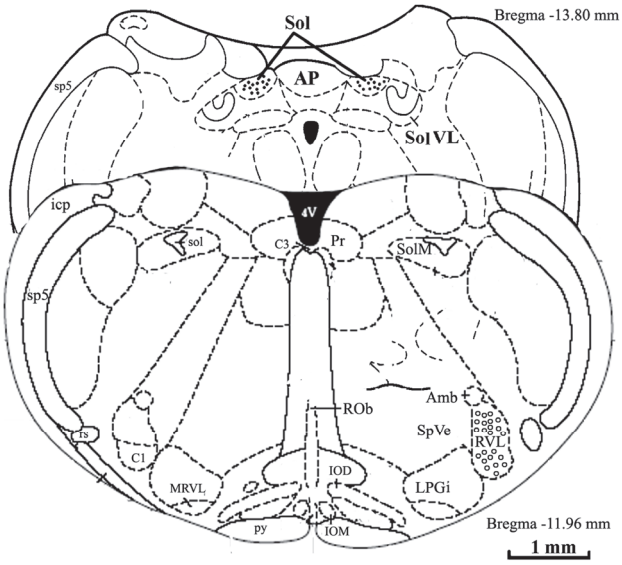


Fig. 1. The injection sites (•) in the nucleus tractus solitarii (NTS) mapped on a standard section through medulla 0.3-0.8 mm rostral to the obex and the injection sites (○) in the rostral ventrolateral medulla (RVLM) mapped on a standard section through medulla 2.5-2.8 mm rostral to the obex. AP: area postrema; sol: solitary tract; SolVL: nucleus of the solitary tract, ventrolateral part; icp: icp inferior cerebellar peduncle (restiform body); ROb raphe obscurus nucleus; Amb: ambiguus nucleus; IOD inferior olive, dorsal nucleus; IOM: inferior olive, medial nucleus; sp5: spinal trigeminal tract; py: pyramidal tract; SpVe: spinal vestibular nucleus; LPGi lateral paragigantocellular nucleus; C1: C1 adrenaline cells; MRVL: medial rostroventrolateral medulla; rs: rubrospinal tract

Statistical analysis

All values are presented as mean ± SD. The magnitudes of the changes in MAP and HR at different time after injections of salusin-β were compared by analysis of unpaired Student's *t*-test. A one way repeated-measures ANOVA followed with the Newman-Keuls test for *post hoc* analysis was used when multiple comparisons were made. A P value of <0.05 was regarded as statistically significant.

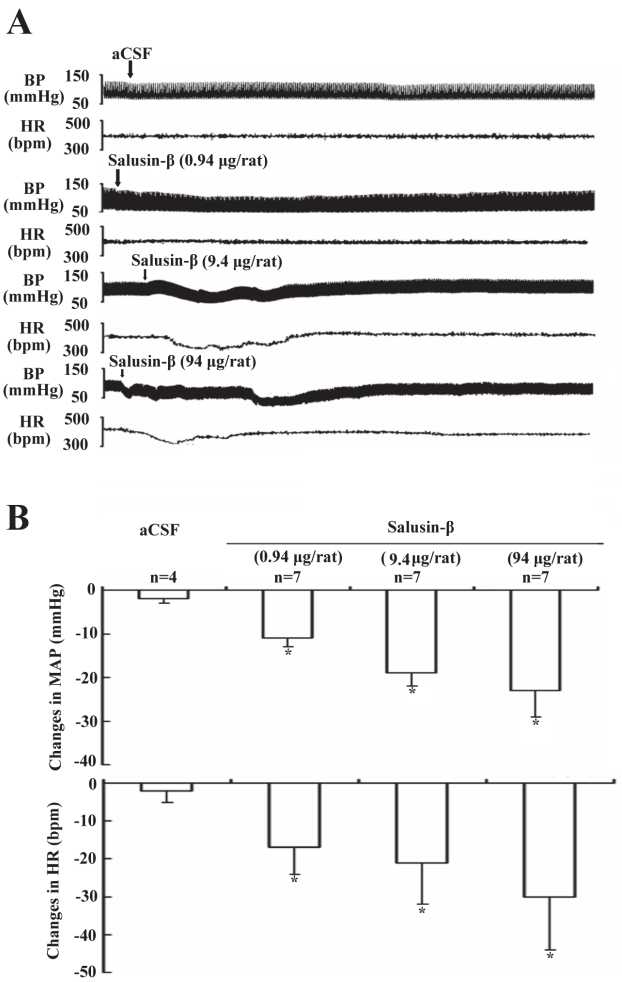


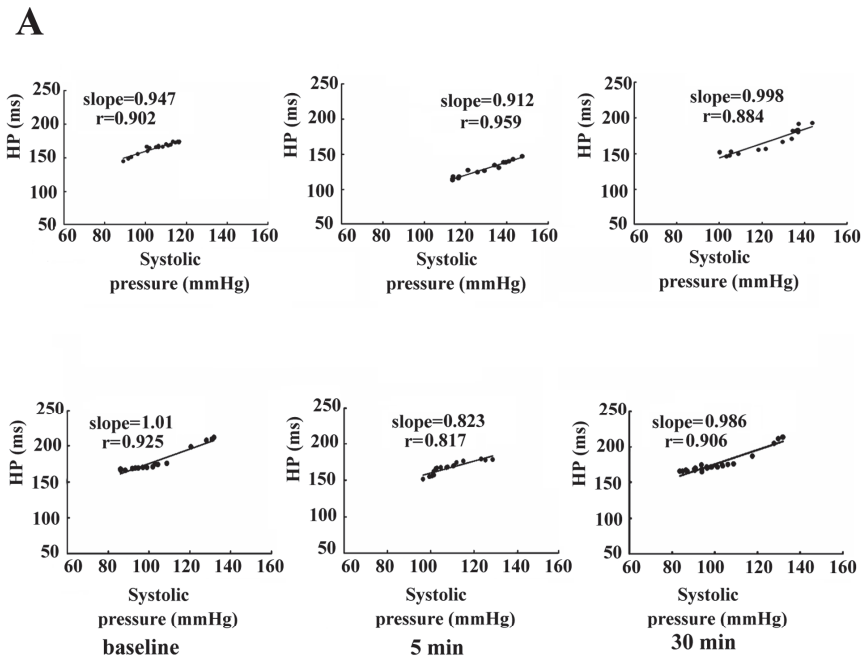
Fig. 2. Effects of bilateral microinjection of salusin-β (0.94, 9.4 and 94 μg/rat) into the nucleus tractus solitarii (NTS) on the blood pressure (BP) and heart rate (HR) in anesthetized rats. **Panel A** showing representative original tracings of the BP and HR response to bilateral microinjection of salusin-β (0.94, 9.4 and 94 μg/rat), or artificial cerebrospinal fluid (aCSF, 100 nl, n=4) into the NTS of rats. **Panel B** showing the changes (mean ± SE) in MAP and HR induced by bilateral microinjection of salusin-β (0.94, 9.4, 94 μg/rat) or artificial cerebrospinal fluid (aCSF, 100 nl, n=4) into the NTS. **P*<0.05 vs. aCSF.

Results

The cardiovascular responses to microinjection of salusin-β into the NTS

Figure 2 shows the representative original tracings of BP and HR response to bilateral microinjection of salusin-β (9.4-94 μg/rat) or aCSF (100 nl) into the NTS. Bilateral microinjection of aCSF (100 nl) didn't alter basal BP and HR. However, microinjection of salusin-β (0.94-94 μg/rat) dose-

dependently produced hypotension (9.4 μg/rat: −11±2 mm Hg; 9.4 μg/rat: −19±3 mm Hg; 94 μg/rat: −23±6 mm Hg vs. aCSF: −2±1 mm Hg, P<0.05) and bradycardia [(0.94 μg/rat: −17±7 bpm (beats per minute); 9.4 μg/rat: −21±11 bpm; 94 μg/rat: −30±14 bpm vs. aCSF: −2±3 bpm, P<0.05)]. The hypotension and bradycardia began at 10 s after injection of salusin-β, at the nadir within 60 s, and returned to baseline within 450 s. The cardiovascular effects of bilateral microinjection of salusin-β (9.4-94 μg/rat) into the NTS are summarized in Figure 2.



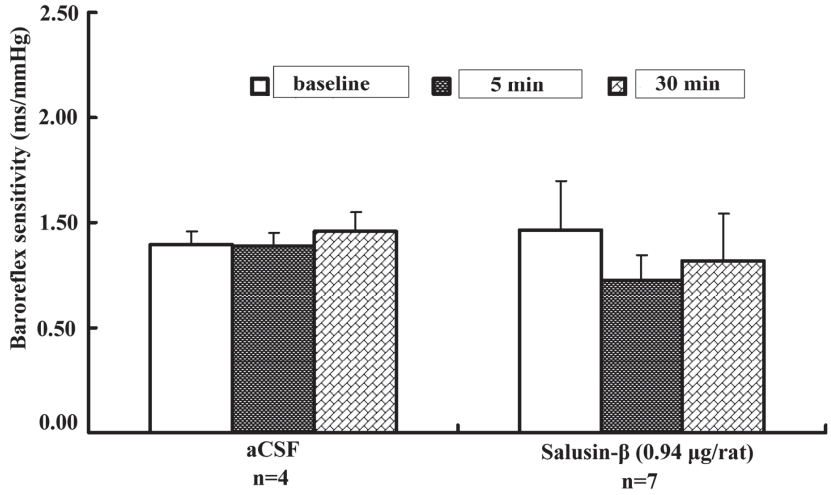


Fig. 3. The effects of bilateral microinjection of salusin-β (9.4 μg/rat for each side, n=7) or vehicle (aCSF, 100 nl for each side, n=4) on BP and HR responses induced by intravenous injection of phenylephrine (10 mg/kg). **Panel A**: The sample traces of phenylephrine-evoked baroreflex before and after 5 min and 30 min of microinjection of salusin-β (9.4 μg/rat) or aCSF (100 nl) into the nucleus tractus solitarii (NTS). HP: heart beat period; Values of slope are the values of baroreflex sensitivity. **Panel B**: Responses of BRSBP before and after 5 min, 30 min of microinjection of salusin-β (9.4 μg/rat) or aCSF (100 nl) into the NTS. □: baseline; ▩: 5 min after microinjection of 9.4 μg/rat of salusin-β or aCSF (100 nl) into the NTS; ⊠: 30 min after microinjection of salusin-β (9.4 μg/rat) or aCSF (100 nl) into the NTS

Figure 3 shows the effects of bilateral microinjection of salusin-β (9.4 μg/rat for each side, n=7) or vehicle (aCSF, 100 nl for each side, n=4) on BP and HR responses induced by intravenous injection of phenylephrine (10 mg/kg). Bilateral injection of salusin-β (9.4 μg/rat for each side, n=7) into the NTS didn't significantly alter BRS_{HP} (baroreflex heart period, before: 0.97±0.23; 5 min after microinjection: 0.73±0.12 ms/mm Hg; 30 min after microinjection: 0.82±0.23, *P*>0.05). Similarly, bilateral microinjection of aCSF (100 nl for each side, n=4) also didn't influence the BTS_{HP} of rats (before microinjection: 0.92±0.03; 5 min after microinjection: 0.86±0.01 ms/mm Hg; 30 min after microinjection: 0.96±0.09, *P*>0.05, Fig. 3).

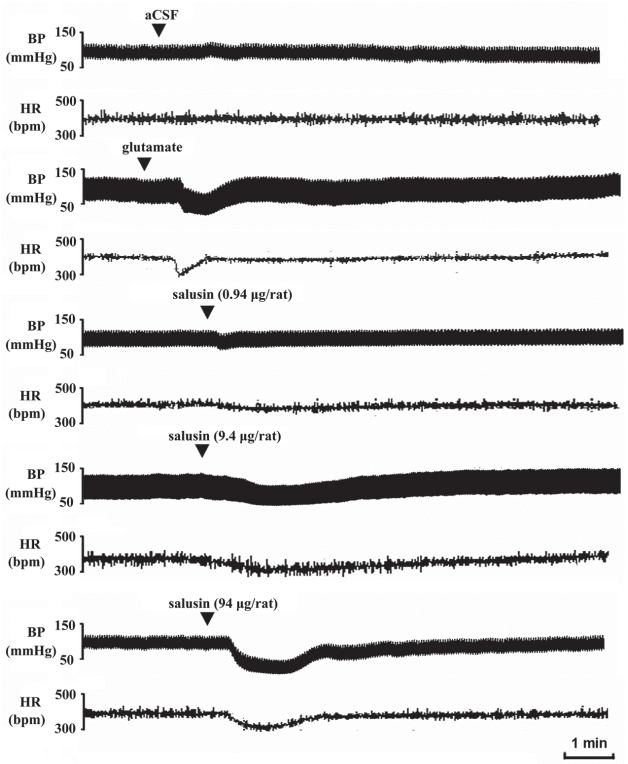


Fig. 4. Representative original tracings of the blood pressure response (BP) to unilateral microinjection of salusin-β (0.94, 9.4 and 94 μg/rat), L-glutamate (0.8 μg/rat, n=7) or artificial cerebrospinal fluid (aCSF, 100 nl, n=4) into the NTS of rats.

Pretreatment with the glutamate receptor antagonist KYN on the cardiovascular responses of intra-NTS salusin-β

Figure 4 presents the representative original tracings of BP and HR response of unilateral microinjection of salusin-β (9.4-94 μg/rat) into the NTS. Intra-NTS injection of aCSF produced no significant influences in the basal MAP or HR of rats. However, microinjection of salusin-β into the NTS produced a dose-dependently hypotension (9.4 μg/rat: −8±1 mm Hg; 9.4 μg/rat: −13±5 mm Hg; 94 μg/rat: −17±3 mm Hg, $P<0.05$) and bradycardia (9.4 μg/rat: −12±4 bpm; 94 μg/rat: −17±6 bpm) compared to control (−1±2 bpm). The hypotension and bradycardia evoked by application of salusin-β reached the nadir within 30 s, and returned to baseline within 180 s. The cardiovascular responses of unilateral microinjection of aCSF or salusin-β (0.94, 9.4, or 94 μg/rat) into the NTS were summarized in Figure 5.

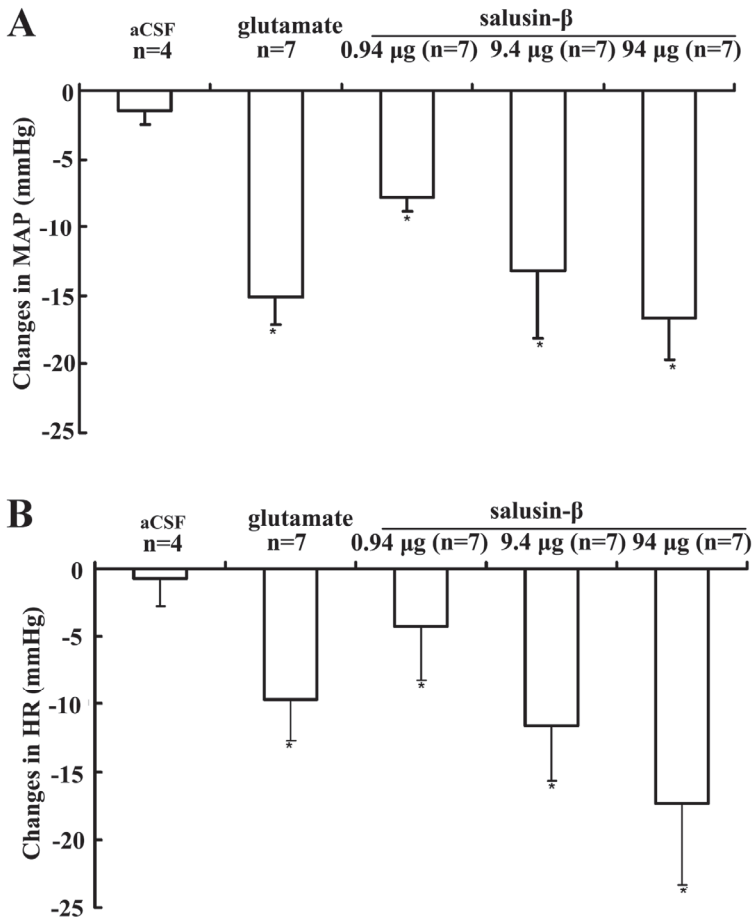


Fig. 5. The magnitude of changes (mean ± SD) in MAP (**Panel A**) and HR (**Panel B**) evoked by unilateral microinjection of salusin-β (0.94, 9.4, 94 μg/rat), L-glutamate (0.8 μg/rat, n=7) or artificial cerebrospinal fluid (aCSF, 100 nl, n=4) into the NTS. *$P<0.05$ vs. aCSF

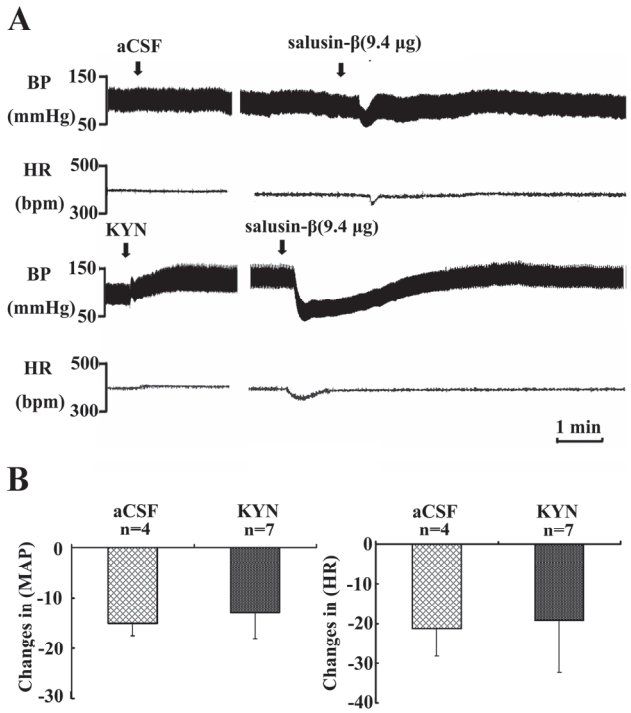


Fig. 6. The effects of prior application of glutamate receptor antagonist kynurenic acid (KYN, 0.19 μg/rat) within the nucleus tractus solitarii (NTS) on the blood pressure (BP) or heart rate (HR) responses of intra-NTS salusin-β (9.4 μg/rat). **Panel A**: The representative original tracings of the effects of prior application of artificial cerebrospinal fluid (aCSF, 100 nl) or glutamate receptor antagonist KYN (0.19 μg/rat) into the NTS on the BP responses of intra-NTS salusin-β (9.4 μg/rat). **Panel B**: The effects of pretreatments with vehicle (aCSF, 100 nl, n=4) or glutamate receptor antagonist KYN (0.19 μg/rat) on the BP (left) and HR (right) responses of intra-NTS salusin-β (9.4 μg/rat). $P<0.05$ vs. aCSF

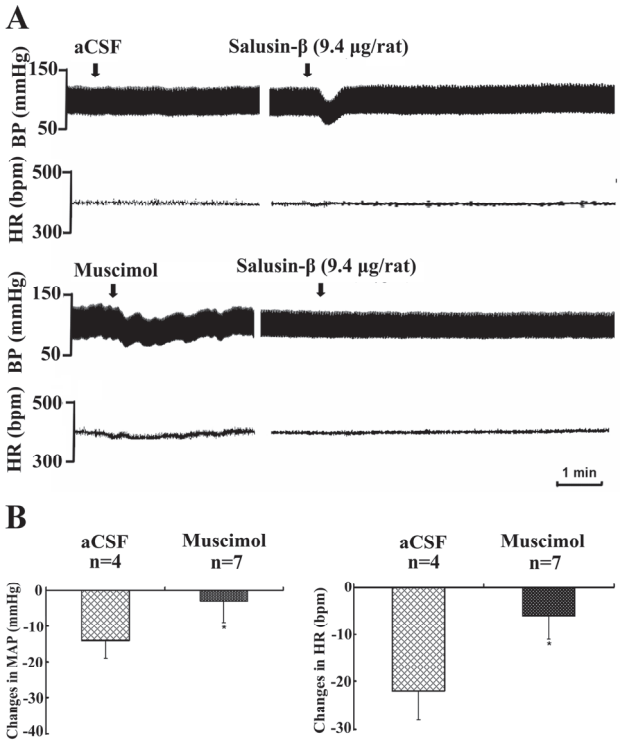


Fig. 7. The effects of prior application of γ-aminobutyric acid (GABA) A receptor agonist muscimol (0.5 ng) within the rostral ventrolateral medulla (RVLM) on the blood pressure (BP) or heart rate (HR) responses of intra-NTS salusin-β (9.4 μg/rat). **Panel A** showing the representative original tracing showing the effects of prior application of aCSF (100 nl) or the GABA A receptor agonist muscimol (0.5 ng) into the rostral ventrolateral medulla (RVLM) on the BP responses of intra-NTS salusin-β (9.4 μg/rat). **Panel B** showing the effects of pretreatments with GABA A receptor agonist muscimol (0.5 ng, n=4) or aCSF (100 nl, n=7) within the RVLM on the BP and HR responses of intra-NTS salusin-β (9.4 μg/rat).

Figure 6 presents the representative original tracings of BP and HR response to intra-NTS salusin-β (9.4 μg/rat) after pretreatment with KYN (0.19 μg/rat, n=9) or vehicle (aCSF, 100 nl, n=4). Prior injection of aCSF altered the salusin-β-induced decrease in BP (from 95±12 to 80±11 mm Hg, $P<0.05$) and HR (from 404±18 to 382±24 bpm, $P<0.05$). Injection of KYN significantly increased BP (from 93±6 mm Hg to 103±6 mm Hg, $P<0.05$) and HR (from 410±10 bpm to 420±14 bpm, $P<0.05$). However, pretreatment with KYN did not affect the BP (pretreatment with aCSF: −15±2 mm Hg vs. pretreatment with KYN: −13±5 mm Hg, $P>0.05$) and HR (pretreatment with aCSF: −21±5 bpm vs. pretreatment with KYN: −19±4 bpm, $P>0.05$) responses evoked by salusin-β in the NTS. Figure 6 summarized the effects of pretreatment with KYN in the RVLM on the cardiovascular responses to intra-NTS salusin-β.

The effect of prior microinjection of the GABA receptor agonist muscimol into the RVLM on the BP and HR responses to microinjection of salusin-β (9.4 μg/rat) into the NTS

Figure 7 Panel A presents the representative original tracings of BP and HR response to intra-NTS salusin-β (9.4 μg/rat) after pretreatment with muscimol (0.5 ng/rat, n=7) injected into the RVLM. Microinjection of the GABA receptor agonist muscimol into the RVLM produced a significant decrease in BP (from 97±6 to 84±14 mm Hg, $P<0.05$) and HR (from 395±17 to 364±20 bpm, $P<0.05$). Notable, pretreatment with muscimol within the RVLM completely abolished the BP (pretreatment with aCSF: −14±5 mm Hg vs. pretreatment with muscimol: −3±5 mm Hg, $P<0.05$) and HR (pretreatment with aCSF: −22±6 bpm vs. pretreatment with muscimol: −6±5 bpm, $P<0.05$) responses induced by application of salusin-β in the NTS. Microinjection of vehicle (aCSF, 100 μl) into the RVLM did not alter the

basal BP (from 105±5 mm Hg to 106±4 mm Hg, $P>0.05$) and HR (from 407±20 bpm to 415±19 bpm, $P>0.05$). Pretreatment with aCSF within the RVLM also did not affect the BP (from 105±5 to 91±11 mm Hg, $P<0.05$) and HR (from 412±16 to 390±24 bpm, $P<0.05$) responses to microinjection of salusin-β into the NTS. The cardiovascular responses of prior application of GABA receptor agonist muscimol into RVLM on the effects of intra-NTS salusin-β were summarized in Figure 7 Panel B.

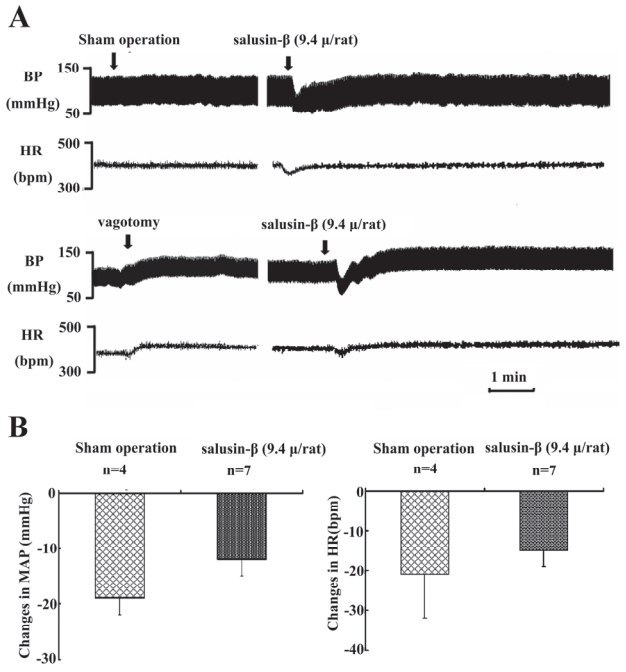


Fig. 8. The influences of bilateral vagotomy on the blood pressure (BP) and heart rate (HR) responses of bilateral microinjection of salusin-β (9.4 μg/rat). **Panel A**: representative tracings showing the effects of sham operation or bilateral vagotomy on the BP and HR responses of bilateral microinjection of salusin-β (9.4 μg/rat). **Panel B**: bar showing the effects of bilateral sham operation or vagotomy on the mean arterial pressure and HR responses of bilateral microinjection of salusin-β (9.4 μg/rat).

The effects of bilateral vagotomy on the cardiovascular functions of intra-NTS salusin-β (9.4 μg/rat)

Figure 8 Panel A presents representative tracings of BP and HR response to bilateral microinjection of salusin-β (9.4 μg/rat) into the NTS after bilateral vagotomy. Bilateral vagotomy increased MAP and HR significantly. However, bilateral vagotomy didn't alter the hypotension (sham: −19±3 mm Hg vs. vagotomy: −12±3 mm Hg, $P>0.05$) and bradycardia (sham: −21±11 bpm vs. vagotomy: −15±4 bpm, $P>0.05$) induced by bilateral microinjection of salusin-β (9.4 μg/rat) into the NTS. The effects of vagotomy on the cardiovascular functions of intra-NTS salusin-β (9.4 μg/rat) were summarized in Figure 8 Panel B.

Discussion

In our present study, our most important findings are: (1) intra-NTS application of salusin-β produces a dose-dependent hypotension and bradycardia in anesthetized rats; and (2) the hypotension and bradycardia induced by intra-NTS salusin-β might be resulted from the suppression of presympathetic neurons in the RVLM.

Salusins are not only considered as a novel bioactive peptide involving in hypotension, mitogenic activities and intracellular signaling pathways etc, but also characterized as a novel candidate of neuropeptide because (1) salusin-β stimulates the secretion of AVP from rat neurohypophysis *in vitro* (Shichiri *et al.* 2003, Saito *et al.* 2008); (2) salusin-β coexists with AVP in the hypothalamo-neurohypophyseal system of the rat under normal (Takenoya *et al.* 2005). Although multiple physiological functions of salusin-β have been identified, its receptor is not clear. Previous study shows that human salusin-β is a surrogate ligand of the mouse MrgA1 (mas-related G protein-coupled receptors), however it could not activate human MrgA1 (Wang *et al.* 2006). Because the exact receptors and its post-receptor signaling pathways are not clear, it is difficult to elucidate the mechanisms of cardiovascular roles of salusin-β.

As the first projection site of afferent fibers from arterial baroreceptors and chemoreceptors, the NTS plays an important role in the integration of cardiovascular autonomic and visceral regulation (Guyenet *et al.* 1987, Lin *et al.* 1999, Machado 2001). At the same time, as the primary site of termination of afferent fibers from arterial baro- and chemoreceptors in medullary reticular formation (Kubo and Kihara 1990, Lawrence and Jarrott 1996), various transmitters or active peptides produce regulative actions on the sensitivity of baro- or chemoreflex in the NTS level (Lo *et al.* 1997, Lin *et al.* 2008). Hence, it is reasonable for us to hypothesize that salusin-β probably produce regulative actions in the NTS. In our present study, we found that bilateral microinjection of salusin-β (0.94-94 μg/rat) into the NTS produced very similar dose-dependent hypotension and bradycardia effects as L-glutamate within the NTS, leading us to suggest that the salusin-β might influence glutamatergic synapses factors. However, pretreatment

with the non-selective glutamate receptor antagonist KYN could not decrease the BP responses induced by intra-NTS salusin-β, indicating that intra-NTS salusin-βmight not influence cardiovascular functions by alter glutamatergic synapses factors. Besides, salusin-β didn't influence baroreflex sensitivity at the NTS level as that of the activation of glutamate receptors (Kubo and Kihara 1991), indirectly proving that salusin-β within the NTS probably is independent with glutamate system. Nevertheless, present results suggest that salusin-β is an excitatory agent and might produce cardiovascular functions within the NTS by its own signal pathway. Previous reports suggest that human salusin-β activates the mouse mas-like G protein-coupled receptor but not human mas-like G protein-coupled receptors (Wang *et al.* 2006). The mas-like G protein-coupled receptor is probably not receptor of salusin-β. The signal pathway mechanism of salusin-β need to be further determined.

However, all studies shown above could not explain the exact mechanism of hypotension and bradycardia of intra-NTS salusin-β. The activation of excitatory amino acid receptors within the NTS are involved in the inhibition of presympathetic neurons in the RVLM (Guyenet *et al.* 1987, Jhamandas and Harris 1992). Presympathetic neurons in the RVLM project to sympathetic preganglionic neurons in the spinal cord and provide the major drive for sympathetic vasomotor tone (Chan and Sawchenko 1998, Schreihofer and Guyenet 2002). We hypothesized that the hypotension and bradycardia of intra-NTS salusin-β probably originated from the inhibition of presympathetic neurons in the RVLM. To test this hypothesis, we unilaterally applied of muscimol into the RVLM to selectively suppress GABA receptors in the RVLM. It has been reported that microinjection of muscimol into the RVLM could silence most activities of presympathetic neurons (Schreihofer *et al.* 2005). Our study showed that the hypotension of intra-NTS salusin-β was completely abolished by prior application of muscimol within the RVLM, indicating that the hypotension of intra-NTS salusin-β is the results of inhibition of the presympathetic neurons within the RVLM. Besides, although Shichiri *et al.* (2003) and Izumiyama *et al.* (2005) have demonstrated that the hypotension, bradycardia and cardiac dysfunction by an bolus intravenous injection of salusin-α or salusin-β in rats are probably mediated by cholinergic mechanism, the cardiovascular functions of salusin-β within the NTS might not be mediated by peripheral cholinergic mechanisms because the bilateral vagotomy in our study didn't effectively abolish the hypotension and bradycardia of intra-NTS salusin-β. Our results demonstrated that the cardiovascular functions of intra-NTS salusin-β were not related with peripheral cholinergic mechanism.

Based on above observations, we proposed that salusin-β might directly excite the NTS neurons. Excitation of the NTS neurons would suppress the activities of presympathetic neurons in the RVLM probably *via* the CVLM pathway, which in turn produces inhibitory effects on the cardiovascular functions in rats.

Conflict of Interest

There is no conflict of interest.

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