Effects of anserine on the renal sympathetic nerve and blood pressure in urethane-anesthetized rats

(Short title)
Cardiovascular effect of anserine in rats

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(Abstract)

Previous studies have demonstrated that central injection of L-carnosine (β-alynyl-L-histidine), dipeptide synthesized in mammalian muscles, affects renal sympathetic nerve activity (RSNA) and blood pressure (BP) in anesthetized rats. In the present study, using urethane-anesthetized rats, we examined the dose-dependent effects of intravenous (IV) injection of various doses of anserine, dipeptide of similar structure to L-carnosine, on RSNA, BP and heart rate (HR). We found that injection of a low dose of anserine (1 μg) suppressed RSNA, BP and HR significantly. Conversely, a high dose (1000 μg) of anserine elevated RSNA, BP and HR significantly. Pretreatment with intralateral cerebral ventricular (LCV) injection of thioperamide, a histaminergic H3-receptor antagonist, eliminated the effects of a low dose of anserine on RSNA, BP and HR. LCV injection of diphenhydramine, a histaminergic H1-receptor antagonist, abolished the effects of a high dose of anserine on RSNA, BP and HR. These findings suggest that anserine affects RSNA, BP and HR in a dose-dependent manner, and that the histaminergic nerve may be involved in the dose-different effects of anserine in rats.

(Introduction)

Chicken soup is known worldwide for its health benefits. In Asian countries, essence of chicken muscles is used as a traditional health food for recovery from physical fatigue, mental stress, and cardiovascular disease (Lo et al. 2005, Nagai et al. 1996, Matsumura et al. 2002). In fact, long-term supplementation of chicken muscle essence has been shown to attenuate the development of hypertension in spontaneous hypertensive rats (Matsumura et al. 2002). However, the active ingredient in the chicken muscle essence is unknown. Previous studies have shown that this essence is abundant in dipeptides, anserine, and L-carnosine (Drummond 1917, Sato et al. 2008). The structure of anserine (β-alanyl-1-N-methyl-histidine), which is synthesized and released into the blood by the skeletal muscles of vertebrates, is similar to that of L-carnosine (β-alynyl-L-histidine). The concentration of L-carnosine in the skeletal muscles of hypertensive rats is markedly low (Johnson and Hammer 1992). Moreover, our recent observations of neural suppression or elevation of sympathetic nerve activity on supplying the kidney and brown adipose tissue with low or high doses of L-carnosine, respectively, indicates that L-carnosine affects the autonomic nerves, and changes the blood pressure (BP) and body temperature in a dose-dependent manner (Tanida et al. 2005, Tanida et al. 2007a). However, whether the administration of various doses of anserine change the BP and renal sympathetic nerve activity (RSNA) which plays an important role in BP regulation (Morgan 1995), is still unclear.

Because it was found that the degradation enzyme of L-carnosine is localized in the

hypothalamic region of rat brain (Otani et al. 2005), it is suggested that L-carnosine functions as a cardiovascular regulator via the central nervous system. Central neural histamine is localized in the histaminergic tuberomammillary nucleus (TMN), a site of origin for histamine neurons (Watanabe et al. 1984), and is involved in the regulation of cardiovascular functions via histaminergic receptors (Tanida et al. 2007b). Our previous finding that a lateral cerebral ventricular (LCV) preinjection of thioperamide (an antagonist of histaminergic H3-receptor) inhibited the effects of a low dose of histamine on RSNA and BP, and that a LCV preinjection of diphenhydramine (an antagonist of histaminergic H1-receptor) abolished the accelerating effects of a high dose of histamine on RSNA and BP suggest that a central histaminergic neurotransmission might be involved in the regulation of cardiovascular functions via histaminergic receptors (Tanida et al. 2007b). L-histidine, a component of both L-carnosine and anserine, is converted to histamine in the TMN. In fact, we previously showed that the suppressing or elevating effects of L-carnosine on RSNA and BP were eliminated by pretreatment with thioperamide or diphenhydramine, respectively, which indicates a possible role of histaminergic receptors in L-carnosine activity (Tanida et al. 2005). Thus, it is hypothesized that the effects of anserine on RSNA and BP may also be mediated by the central histamine receptors.

The present study assessed the effects of peripheral administration of various amounts of anserine on RSNA and BP, and evaluated the effects of histaminergic blockers (H3- or H1-receptor antagonist) on changes in the cardiovascular function of urethane-anesthetized

rats.

(Materials & Methods)

Animals Male Wistar rats, weighing 280-330 g were used. Rats were housed in a room maintained at 24 ± 1°C and illuminated for 12 h (08:00 to 20:00) everyday. Food and water were freely available. Rats were adapted to the environment for at least 1 week before the experiment. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

General animal preparation General preparation was performed as described previously (Tanida *et al*. 2005). Briefly, on the experimental day, food was removed 4-6 h before surgery. Anesthesia was induced with an intraperitoneal (IP) injection of 1g/kg urethane, a polyethylene catheter was inserted into the left femoral vein for IV-injections, and another catheter was inserted into the left femoral artery for BP determination. The rat was then fixed in a stereotaxic apparatus after a tracheal cannulation. The body temperature was maintained at 37.0-37.5°C using a heating pad and a thermometer inserted into the rectum. We evaluated the adequacy of the depth of anesthesia by checking every half-hour throughout experimental procedure whether rapid variation of BP (±5 mmHg) and HR (±10%) would be caused by paw pinch. So, when any one of these responses was found, a supplemental urethane (0.1-0.2 g/kg) was given with an intraperitoneal injection. The depth of anesthesia was maintained under certain conditions during experimental period. Using a dissecting microscope, the left renal

nerve was exposed retroperitoneally through a left flank incision. The distal end of the nerve was ligated, and then hooked up with a pair of silver wire electrodes for recording the efferent RSNA. The recording electrodes were immersed in a pool of liquid paraffin oil to prevent dehydration and for electrical insulation. The rat was allowed to stabilize for 30-60 min after being placed on the recording electrodes.

Electrical changes in RSNA was amplified 2000-5000 times with a band path of 100 to 1000 kHz, and monitored by an oscilloscope. Raw data of the nerve activity was converted to standard pulses by a window discriminator, which separated discharge from electrical background noise which remained post mortem. Both the discharge rates and the neurogram were sampled with a Power-Lab analog-to-digital converter for recording and data analysis on a computer. The catheter in the left femoral artery was connected to a BP transducer (DX-100, Nihon Kohden, Japan), and the output signal of the transducer was amplified in a BP amplifier (AP641G, Nihon Kohden, Japan). Two needle electrodes were placed under the skin at the right arm and left leg to record an electrocardiogram (ECG). The ECG signal was amplified with a bioelectric amplifier (AB-620G, Nihon Kohden, Japan). The BP and ECG were monitored with an oscilloscope, sampled with the Power-Lab, and stored on a hard disk for off-line analysis to calculate mean arterial pressure (MAP) and HR.

Intracerebroventricular cannulation At least 1 week before the experiment, a brain cannula made of polyethylene tubing (PE-10; Clay Adams, Parsippany, NJ) was inserted into the left lateral cerebral ventricle (A-P, 1.5 mm caudal to the bregma,; L, 2.0 mm lateral to the

midline; V, 3.0 mm below the skull surface) under pentobarbital anesthesia (35 mg/kg, IP) as previously described (Yamamoto *et al*. 1988). The cannula implanted into the brain was securely fixed by dental cement and synthetic resin. When injections were given to rats, the microsyringe for injection was directly connected to the cannula.

Experimental protocol Baseline measurements of RSNA, MAP, and HR were made for 5 min just before IV injections of anserine (0.1, 1, 10, 100 and 1000 μg/0.1ml saline) or saline (0.1ml). In regard to injection doses, those of this study are determined by referring previous observation measuring the plasma levels of anserine (about 50 μg/ml) in male Wistar rats (Al-Bekairi 1989). After the injection, these parameters were recorded for 60 min. Effects of thioperamide maleate [20 μg/10 μl artificial cerebrospinal fluid (aCSF); LCV], a histaminergic H3-antagonist, or diphenhydramine hydrochloride (5 μg/10 μl aCSF; LCV), a histaminergic H1-antagonist, on anserine-induce effects on RSNA, BP and HR were examined. These antagonists were centrally administered 15 min prior to IV injection of anserine. We previously observed that thioperamide or diphenhydramine alone did not affect RSNA, MAP or HR (Tanida *et al*. 2006). At the end of the experiment, hexamethonium chloride was intravenously administered (10 mg/kg) to ensure that the recording was made from post-ganglionic efferent sympathetic nerve activity.

Data Analyses The RSNA, MAP, and HR measured during each 5 min period after injections of anserine or saline were evaluated by digital signal processing and statistical analyses. All data were expressed as means ± SEM. Because of the inter-individual variability

in the pre-injection state, percent change from the baseline was also calculated for RSNA. Absolute value changes from the baseline were calculated for MAP and HR. Two-way ANOVA was applied to compare group responses of the RSNA, MAP and HR. To perform statistical analysis of the dose response to anserine, ANOVA, followed by multiple comparisons using Dunnett's multiple range tests, was used. $P < 0.05$ was considered statistically significant.

(Results)

Sample recordings of RSNA and BP before and throughout a 60 min period following IV injection of saline or anserine are presented in Fig. 1A-B. Saline injection did not affect either RSNA or MAP. However, both were suppressed by IV injection of 1 μg of anserine, and both were elevated by IV injection of 1000 μg of anserine. Following injection of 1 μg of anserine, RSNA, MAP and HR are suppressed gradually (Fig. 1C-E), with the greatest level of suppression occurring at 20 - 60 min. Suppressive response of RSNA maintained after a peak. The lowest levels attained were with 61.5 ± 7.3% (RSNA), -8.3 ± 4.2mmHg (MAP) and -18 ± 9 beats/min (HR). Following injection of 1000μg of anserine, RSNA, MAP and HR are elevated depending on a tendency to each acceleration (Fig. 1C-E), with maxima occurring at 25- 60 min. The highest levels attained were 179.8 ± 22.4% (RSNA), 8.1 ± 2.5mmHg (MAP) and 27 ± 12 beats/min (HR). In contrast, injection of saline did not cause a significant alteration in the levels of RSNA, MAP and HR. The significance of the differences between

values from 5-60 min, was analyzed as a group by ANOVA. The following comparisons were made: 1. RSNA: Saline vs. anserine (1 μg), $P<0.01$ ($F=30.5$); saline vs. anserine (1000 μg), $P<0.01$ ($F=12.8$); 2. MAP: Saline vs. anserine (1 μg), $P<0.05$ ($F=8.9$); saline vs. anserine (1000 μg), $P<0.05$ ($F=6.6$). 2. HR: Saline vs. anserine (1 μg), $P<0.05$ ($F=5.5$); saline vs. anserine (1000 μg), $P<0.05$ ($F=6.0$). Absolute basal (0 min) RSNA, MAP and HR values for the experiments shown in Fig. 1 are summarized in Table 1. Differences in respective basal values were not statistically significant (Mann-Whitney U test).

60 min following IV injection of lower dose of anserine (0.1μg), the levels of RSNA, MAP and HR decreased (Fig. 2A-C). The maximum suppressive responses occurred following injection of 1 μg of anserine. In constrast, at 60 min, the higher dose of anserine (1000 μg) had significantly increased RSNA, MAP and HR. Absolute basal (0 min) RSNA, MAP and HR values for the experiments shown in Fig. 2 are summarized in Table 1. Differences in respective basal values were not statistically significant (Mann-Whitney U test).

To clarify role of histaminergic receptors in anserine action, in this study, responses of RSNA, MAP and HR to IV injections of anserine were compared between aCSF-control group and groups given injection of thioperamide or diphenhydramine. RSNA and MAP were found to be significantly reduced by 1 μg of anserine and elevated by IV-injection of 1000 μg of anserine in aCSF pre-treated rats (Fig. 3A, B). HR was significantly elevated by IV-injection of 1000 μg of anserine in aCSF pre-treated rats (Fig. 3C). Pre-treatment with thioperamide or diphenhydramine eliminated the effects of IV injection of low- or high-doses of anserine,

respectively for LCV experimental groups (Fig. 3A-C). The significance of the differences between values from 5-60 min analyzed as a group by ANOVA, is as follows: 1. RSNA: aCSF-saline vs. aCSF-anserine (1 μg), $P<0.01$ ($F=17.7$); aCSF-saline vs. aCSF-anserine (1000 μg), $P<0.05$ ($F=8.6$); aCSF-anserine (1 μg) vs. thiop-anserine (1 μg), $P<0.01$ ($F=14.1$); aCSF-anserine (1000 μg) vs. diphen-anserine (1000 μg), $P<0.05$ ($F=4.6$). 2. MAP: aCSF-saline vs. aCSF-anserine (1 μg), $P<0.005$ ($F=63.7$); aCSF-saline vs. aCSF-anserine (1000 μg), $P<0.05$ ($F=4.2$); aCSF-anserine (1 μg) vs. thiop-anserine (1 μg), $P<0.005$ ($F=40.9$); aCSF-anserine (1000 μg) vs. diphen-anserine (1000 μg), $P<0.05$ ($F=6.6$). HR: aCSF-saline vs. aCSF-anserine (1 μg), N.S. $P=0.05<0.1$ ($F=2.8$); aCSF-saline vs. aCSF-anserine (1000 μg), $P<0.05$ ($F=6.2$); aCSF-anserine (1 μg) vs. thiop-anserine (1 μg), $P<0.05$ ($F=8.4$); aCSF-anserine (1000 μg) vs. diphen-anserine (1000 μg), $P<0.05$ ($F=3.1$). Absolute basal (0 min) RSNA, MAP and HR values for the experiments shown in Figs. 3 are summarized in Table 1. Differences in respective basal values were not statistically significant (Mann-Whitney U test).

(Discussion)

Several dipeptides released from mammalian skeletal muscle into the blood are thought to be beneficial to health. Our previous studies revealed that peripheral injection of L-carnosine showed bioactive effects, e.g., it affects autonomic nerves, and regulates BP, blood glucose concentrations, and body temperature (Tanida et al. 2005, Tanida et al. 2007a, Yamano et al.

2001). However, the effects of anserine and other dipeptides on autonomic nerve and cardiovascular functions have not been studied, in vivo, until now. Present study showed that intravenous injection of a low dose of anserine reduced RSNA, BP, and HR in anesthetized rats (Fig. 1). In addition, a high dose of anserine accelerated the RSNA, BP, and HR in anesthetized rats. This novel finding indicates that anserine induces autonomic and cardiovascular responses in a dose-dependent manner, which is similar to the effects of L-carnosine (Tanida et al. 2005). Furthermore, because the autonomic and cardiovascular systems are modulated by the brain, the central mechanism responsible for the action of anserine was next investigated. It was found that LCV treatment with central histamine receptor blockers such as thioperamide or diphenhydramine significantly eliminated the changes in RSNA, BP, and HR due to low or high doses of anserine (Fig. 3). Thus, the data suggests that anserine might act in the brain, and affect the sympathetic and cardiovascular functions via the central pathway of histaminergic neurotransmission.

In the present study, the biphasic effects of anserine on RSNA, BP, and HR lasted for a long time after anserine injection (Fig. 1). It is generally accepted that anserine released from skeletal muscle into the blood is hydrolyzed by carnosinase, resulting in L-histidine and β-alanine (Lenny 1976, Pegova et al. 2000). Because L-histidine is taken in by histaminergic neurons in the brain for the synthesis and release of histamine, it is thought that the long-lasting effects of anserine might be induced via the central histaminergic mechanism. Our observations that LCV injections of various doses of histamine result in long-term changes in

RSNA and BP (Tanida et al. 2007b), and that the biphasic effects of anserine on sympathetic and cardiovascular responses are abolished by pretreatment with histaminergic antagonists (Fig. 3) strongly support the theory that long-lasting effects of anserine might be induced via the central histaminergic mechanism.

In the present study, LCV preinjection of the histamine antagonist thioperamide eliminated the suppressive effects of 1 μg anserine on RSNA and BP, and preinjection of the histamine antagonist diphenhydramine eliminated the elevating effects of 1000 μg anserine (Fig. 3). These findings suggest that the suppressive and elevating effects of anserine on RSNA and BP might be mediated by histamine H3- and H1-receptors, respectively. With regards to signal transduction in the synapse of neurons containing histamine, the presynaptic H3-receptor mediates the autoinhibition of histamine release from the histaminergic neurons to the synaptic clefts. Affinity of the H3-receptor for histamine is much higher than the affinities of the postsynaptic histaminergic H1- and H2-receptors (Arrang et al. 1983). Therefore, a small amount of histamine suppresses histamine release from the presynaptic histaminergic neurons via the H3-receptor. However, a large amount of histamine transmits histaminergic neural signals via the H1-receptor. In fact, our previous study showed that the cardiovascular effects of the central injection of low- or high-dose histamine were inhibited by pretreatment with thioperamide or diphenhydramine (Tanida et al. 2007b). This observation indicates a role of central histamine receptors in sympathetic and cardiovascular regulation. Thus, on the basis of our findings, we suggest that anserine might act in the brain, and cause the autonomic nerves

to modulate cardiovascular functions via the histaminergic neurons. Because the effect of anserine injection on histamine release in the hypothalamus was not examined, microdialysis study focusing on the hypothalamic release of histamine after anserine injection might be helpful to determine the precise role of histaminergic mechanism on cardiovascular responses to anserine.

Present study has several limitations that need to be addressed. First, anesthetized rats were used in the study; therefore, it is possible that the results would have been different if free-moving, conscious rats had been used. The effects of anserine on conscious rats were not determined in the study because it was difficult to measure RSNA in conscious rats. However, in our previous study of both unanesthetized and anesthetized rats, low-dose administration of L-carnosine suppressed hyperglycemia induced by 2-deoxy-D-glucose (2DG) in conscious rats, and enhanced the activity of celiac parasympathetic nerves in urethane-anesthetized rats (Yamano et al. 2001). In addition, our preliminary study confirmed that peripheral administration of a low dose of anserine inhibited 2DG-induced hyperglycemia in free-moving rats, and activated the gastric parasympathetic nerves in urethane-anesthetized rats (unpublished data). Thus, it is possible that there is little effect of anesthesia on the sympathetic and cardiovascular responses to anserine. Second, in the present study, the effects of anserine on neural activity of the parasympathetic nerve branches were not investigated. With respect to the role of parasympathetic nerves on blood glucose regulation, the parasympathetic nerves stimulate the glucose metabolism in the pancreas and liver via insulin

release and glycogen synthesis, respectively. In general, elevated parasympathetic nerve activity results in a reduction in blood glucose concentrations via increases in insulin release and glycogen synthesis. In support of this observation, the injection of a low dose of L-carnosine was shown to reduce adrenal sympathetic nerve activity and increase celiac parasympathetic nerve activity (Yamano et al. 2001) after the suppression of 2DG-induced hyperglycemia. Our preliminary observation that peripheral administration of a low dose of anserine inhibited 2DG-induced hyperglycemia, and increased parasympathetic nerve outflow to the stomach (unpublished data), suggests a possible role of anserine on parasympathetic control. Third, the effects of anserine on the autonomic center of the hypothalamus were not determined. However, the suprachiasmatic nucleus (SCN), one of the hypothalamic nuclei and a master circadian oscillator, plays an important role in the control of glucose metabolism, and BP via the regulation of the autonomic nervous system (Nagai et al. 1996, Tanida et al. 2005). Furthermore, the bilateral lesions of the SCN have been shown to completely eliminate the responses of both RSNA and BP to L-carnosine (Tanida et al. 2005), which suggests that the SCN might be one of the regions of L-carnosine activity. Therefore, the SCN might not only be involved in the above mentioned mechanisms, but may also be involved in the mechanisms responsible for the effects of anserine on RSNA and BP. Precise investigations such as the study of electric lesion or a microinjection study might be helpful to determine the action area in the hypothalamus during cardiovascular responses to anserine.

In conclusion, present findings suggest that anserine affects RSNA, BP, and HR in a

dose-dependent manner. The histaminergic nervous system in the brain might be involved in the mechanism responsible for these effects. However, further study is required to determine the precise pathway responsible for the effects of anserine on sympathetic and cardiovascular system functions.

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(Figure legend)

Fig.1. Effects of IV injection of anserine on the renal sympathetic nerve activity (RSNA), blood pressure (BP) and heart rate (HR).

Representative trace data from recordings of RSNA (A) and BP (B) before and after the IV injection of saline or anserine (1 μg and 1000 μg). Time-course data of RSNA (C), MAP (D) and HR (E) after IV injection of saline or anserine (1 μg and 1000 μg) are expressed as mean ± SEM. *Significant differences between saline group and anserine groups ($P < 0.05$).

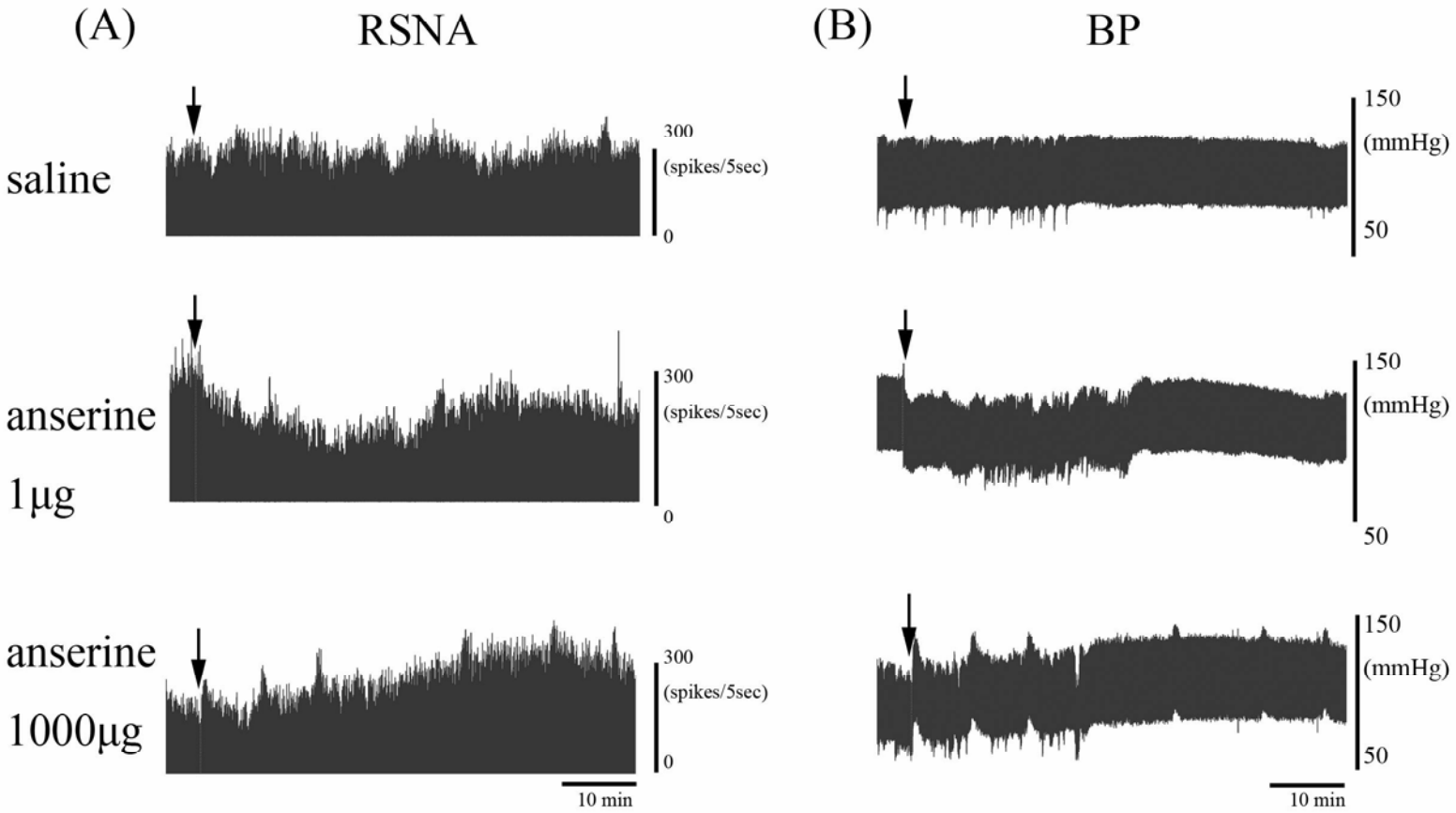
Fig.2. Dose-dependent effects of IV injection of anserine on the RSNA, MAP and HR.

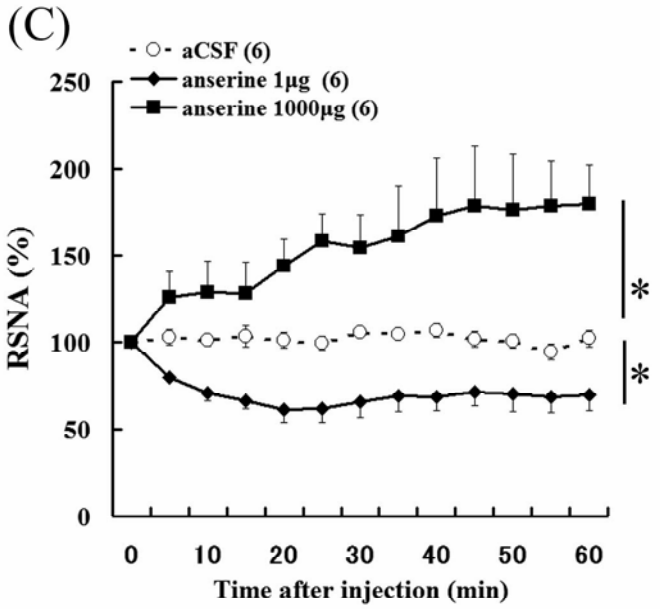
Bars in A, B and C show RSNA, MAP and HR values 60 min after injection of five doses (0.1, 1, 10, 100 and 1000 μg) of anserine and saline. Numbers of animals used in each group are 6 rats. *Significant differences between saline group and anserine groups ($P < 0.05$).

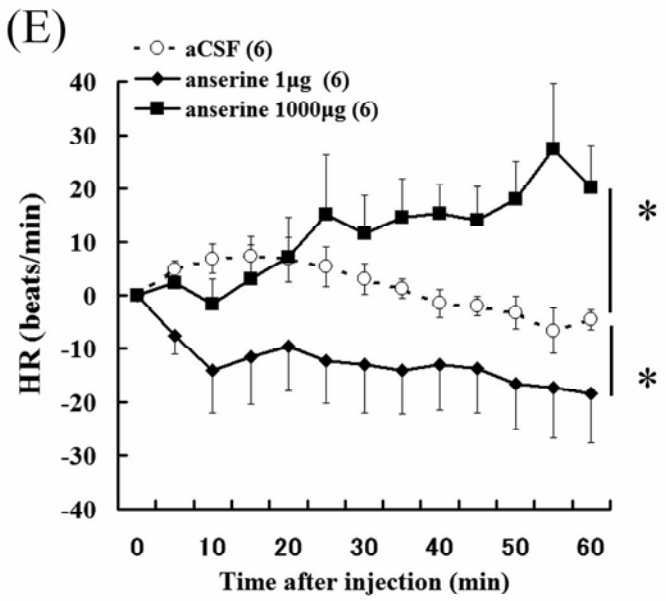
Fig.3. Effects of thioperamide and diphenhydramine on changes in RSNA, MAP and HR following IV injection of 1 μg and 1000 μg of anserine.

RSNA (A), MAP (B) and HR (C) after IV injection of saline (0.1 ml) or anserine (1 μg or 1000 μg) are expressed as mean ± SEM. LCV injection of aCSF, thioperamide (thiop) or diphenhydramine (diphen) were given 15 min before IV injection of either saline or anserine.

Significant differences between control group (aCSF ± saline) and anserine groups (aCSF ± anserine 1 μg or 1000 μg) were analyzed by ANOVA ($P < 0.05$).







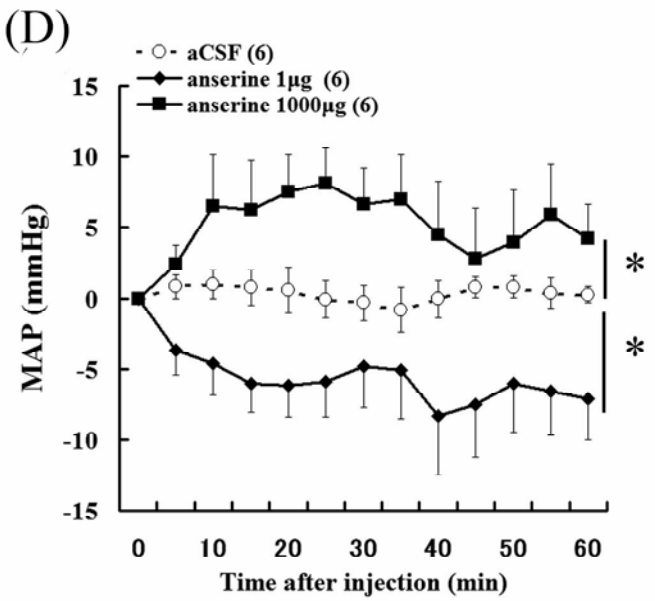


Fig. 1 Tanida et al.

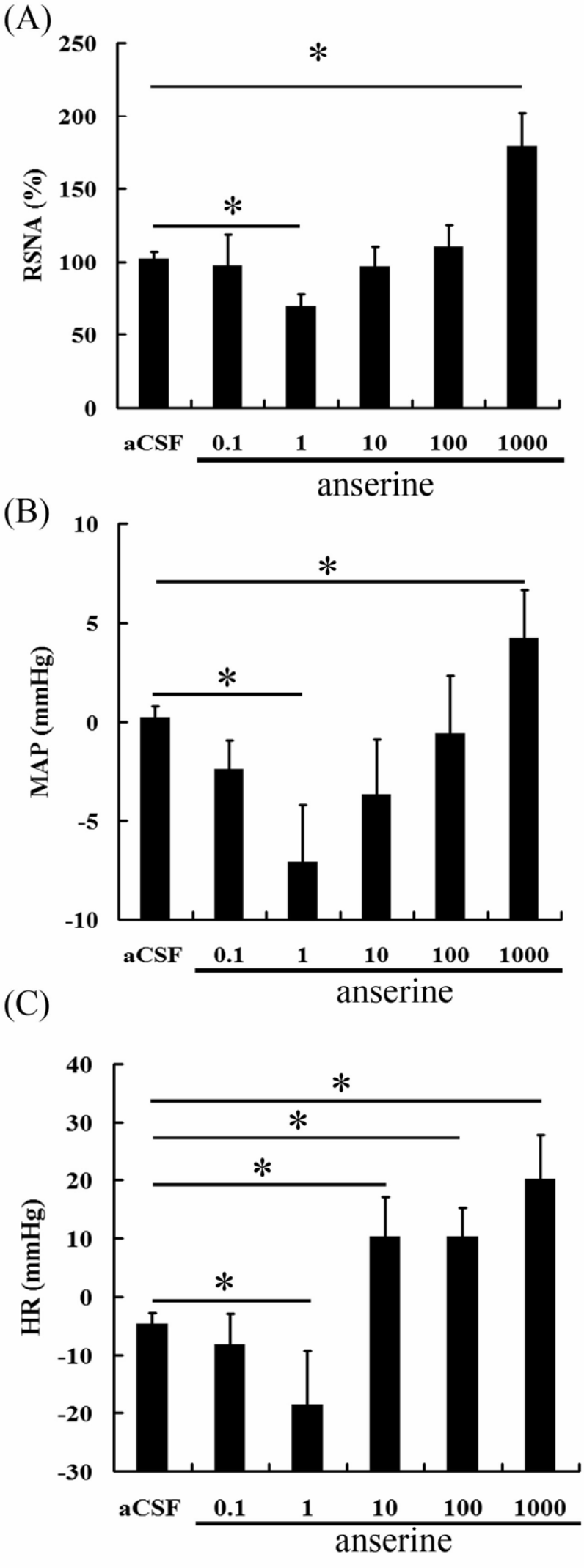


Fig. 2 Tanida et al.

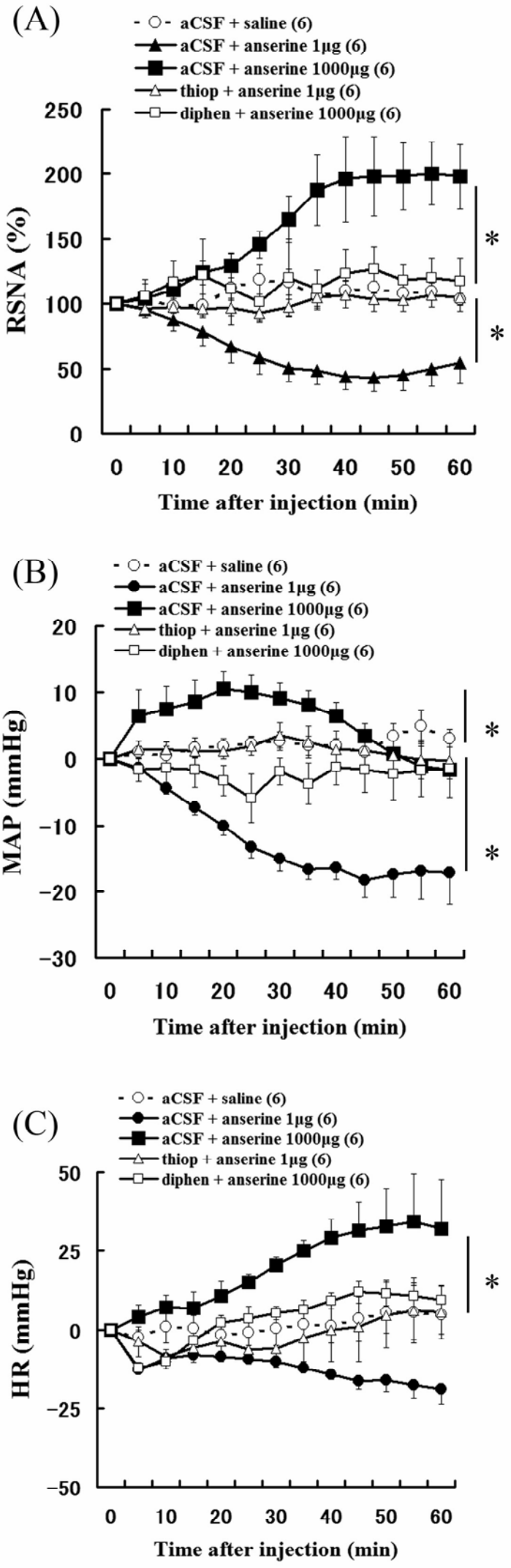


Fig. 3 Tanida et al.

Table 1, Basal levels of the RSNA, MAP and HR in the groups of rats.

	(n)	RSNA (spikes/sec)	MAP (mmHg)	HR (beats/min)
(Group of Fig.2)				
aCSF	6	119.8 ± 31.6	94.6 ± 7.2	362 ± 54
anserine 0.1μg	6	195.9 ± 22.2	94.6 ± 9.5	358 ± 37
anserine 1μg	6	178.8 ± 24.1	95.2 ± 9.0	389 ± 18
anserine 10μg	6	228.7 ± 75.8	100.9 ± 9.7	390 ± 18
anserine 100μg	6	200.4 ± 18.8	86.9 ± 10.7	369 ± 23
anserine 1000μg	6	259.6 ± 96.3	92.2 ± 5.6	353 ± 32
(Group of Fig.3)				
aCSF + saline	6	136.3 ± 26.5	80.7 ± 7.1	363 ± 26
aCSF + anserine 1μg	6	131.4 ± 7.2	88.5 ± 8.5	371 ± 13
aCSF + anserine 1000μg	6	168.6 ± 22.5	85.2 ± 6.5	332 ± 23
thiop + anserine 1μg	6	150.1 ± 22.0	86.7 ± 8.6	405 ± 13
diphen + anserine 1000μg	6	148.6 ± 21.4	88.9 ± 10.9	372 ± 13

RSNA, renal sympathetic nerve activity; MAP, mean arterial pressure; HR, heart rate

Data are presented means ± SE. (n) = number of rats.