Stimulation of cardiac sympathetic nerve activity by central angiotensinergic mechanisms in conscious sheep

Anna M. D. Watson, Rasim Mogulkoc, Robin M. McAllen, and Clive N. May. Stimulation of cardiac sympathetic nerve activity by central angiotensinergic mechanisms in conscious sheep. Am J Physiol Regul Integr Comp Physiol 286: R1051–R1056, 2004.—Central actions of angiotensin play an important role in cardiovascular control and have been implicated in the pathogenesis of hypertension and heart failure. One feature of centrally or peripherally administered angiotensin is that the bradycardia in response to an acute pressor effect is blunted. It is unknown whether after central angiotensin this is due partly to increased cardiac sympathetic nerve activity (CSNA). We recorded CSNA and arterial pressure in conscious sheep, at least 3 days after electrode implantation. The effects of intracerebroventricular infusions of ANG II (3 nmol/h for 30 min) and artificial cerebrospinal fluid (CSF) (1 ml/h) were determined. The response to intracerebroventricular hypertonic saline (0.6 M NaCl in CSF at 1 ml/h) was examined as there is evidence that hypertonic saline acts via angiotensinergic pathways. Intracerebroventricular angiotensin increased CSNA by 23 ± 7% (P < 0.001) and mean arterial pressure (MAP) by 7.6 ± 1.2 mmHg (P < 0.001) but did not significantly change heart rate (n = 5). During intracerebroventricular ANG II the reflex relation between CSNA and diastolic blood pressure was significantly shifted to the right (P < 0.01). Intracerebroventricular hypertonic saline increased CSNA (+9.4 ± 6.6%, P < 0.05) and MAP but did not alter heart rate. The responses to angiotensin and hypertonic saline were prevented by intracerebroventricular losartan (1 mg/h). In conclusion, in conscious sheep angiotensin acts within the brain to increase CSNA, despite increased MAP. The increase in CSNA may account partly for the lack of bradycardia in response to the increased arterial pressure. The responses to angiotensin and hypertonic saline were losartan sensitive, indicating they were mediated by angiotensin AT-1 receptors.

angiotensin II; baroreflex; hypertonic saline; intracerebroventricular; losartan

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contact. Sheep were fed a diet of oaten chaff (800 g/day), and water was offered ad libitum. Experiments were conducted with the sheep standing, and access to water was removed during the experimental period. All experiments were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute.

Surgical Procedures

Anesthesia was induced with intravenous thiopental sodium (15 mg/kg) and after intubation was maintained with 1.5–2.0% isoflurane/O2. Animals were placed in a stereotaxic apparatus, and stainless steel guide tubes were implanted over the lateral cerebral ventricles.

After at least 2 wk recovery and on the day before implantation of recording electrodes, a sterile Tygon cannula (ID 1.0 mm, OD 1.5 mm) was inserted under aseptic conditions 15–20 cm into a carotid artery, toward the heart, for the measurement of arterial pressure. Under local anesthesia, a sterile polyethylene cannula (ID 0.58 mm, OD 0.97 mm) was inserted 15 cm into the jugular vein for intravenous infusion. The cannula for measurement of arterial pressure was connected to a pressure transducer (TDXIII, Cobe) tied to the wool on the sheep’s back. The arterial pressure was corrected to compensate for the height of the transducer above the level of the heart. Heart rate (HR) was recorded with a cardiotachometer triggered by the arterial pressure waveform.

Under general anesthesia, electrodes were implanted in the left cardiothoracic nerves (10). Briefly, an incision was made above the fourth rib, the periestem was opened, and the rib was removed. The thorax was held open with a rib retractor, and the lungs were held back with wet packs. With the use of a binocular microscope the thoracic cardiac nerves were identified and the fascia over the nerves was removed. Electrodes consisted of stainless steel entomological pins (0.05 mm diameter) etched to a fine point, glued into the end of Teflon-coated 25-strand silver-coated copper wire (CZ1174SPC, Cooner Wire, Chatsworth, CA) (17, 18). The exposed tip of the electrode (1.5–2.0 mm in length) was pushed obliquely through the nerve sheath, ensuring that the tip was positioned in the center of the nerve. Up to five electrodes were implanted into the nerve and fixed in place with cyanoacrylate glue. The implantation site was covered with a layer of Kwik-Sil (WPI, Glen Waverly, Victoria, Australia), and the wires were exteriorized through the sutured wound. A stainless steel suture looped through the skin was used as an earth.

Experiments were conducted on standing, conscious sheep and to minimize any effect of surgical stress were started on the third day after implantation of the electrodes.

Nerve Recording

Sympathetic nerve activity was recorded differentially between pairs of electrodes, and the pair with the best signal-to-noise ratio was selected. The signal was amplified (×100,000) and filtered (bandpass 400–1,000 Hz), displayed on an oscilloscope, and passed through an audio amplifier and loud speaker. Spikes above the noise level were detected with a discriminator (17). Sympathetic nerve activity, spike counts, and blood pressure were recorded on computer using a CED micro 1401 interface and Spike 2 software (Cambridge Electronic Design). Spike counts (in 10-s bins) were also plotted on a chart recorder (Gould RS3400), together with MAP and HR.

Experimental Protocols

Ganglion blockade. Ganglion blockade with hexamethonium (125 mg/h for 2 h) was used to confirm that nerve activity was recorded from postganglionic sympathetic nerves. We have demonstrated previously that this dose of hexamethonium inhibits RSNA (17).

Intracerebroventricular infusions. Before infusion into the lateral cerebral ventricles (intracerebroventricular), the cap was removed from one of the guide tubes and a sterile probe (20-gauge needle with luer lock fitting) of appropriate length was inserted through the guide tube into a lateral cerebral ventricle. A sterile polyethylene cannula filled with artificial cerebrospinal fluid (CSF) and joined to a syringe pump (Braun) was connected to the probe. The probe was considered patent if CSF flowed freely into and back from the ventricle under hydrostatic pressure, as the tubing was raised or lowered in relation to the ventricle. All solutions for intracerebroventricular infusion were filtered through a sterile 0.22-μm filter before administration and were given at 1 ml/h. Infusions of the test solutions were started after a 20-min stable control period. Not all experiments were performed in all sheep, because the order in which the treatments were given was randomized and the recording signal had deteriorated in some cases before all the experiments were completed.

Effect of intracerebroventricular infusion of losartan on the responses to intracerebroventricular ANG II. ANG II (Human, Auspep; 3 nmol/ml), dissolved in CSF, was infused intracerebroventricularly for 30 min at 1 ml/h in six conscious sheep. CSNA and MAP were measured during the control period, during the infusion, and for up to 2 h afterward. Two hours after the end of the intracerebroventricular infusion of ANG II (3 nmol/h) or when all parameters had returned to control for at least 30 min, an intracerebroventricular infusion of losartan (Dupont-Merck; 1 mg/ml in CSF) was commenced. After 1 h of intracerebroventricular infusion of losartan, ANG II (3 nmol/h) was infused intracerebroventricularly together with losartan for 30 min.

Recordings were continued for 60 min after the end of the infusion. Control experiments in which artificial CSF was infused at 1 ml/h for 30 min were also performed.

Effect of intracerebroventricular losartan on the responses to intracerebroventricular hypertonic saline. CSNA and MAP were measured during the 10-min control period, during intracerebroventricular infusion of CSF containing 0.6 M NaCl at 1 ml/h for 20 min, and during the 20-min postinfusion period in seven conscious sheep. Intracerebroventricular infusion of losartan (Dupont-Merck; 1 mg/ml in CSF, infused at 1 ml/h) was commenced a minimum of 40 min after the end of the intracerebroventricular hypertonic saline, when all variables had returned to control. The dose of losartan used has been shown to inhibit the effect of intracerebroventricular ANG II (10 nmol/h) on RSNA, MAP, and plasma renin activity in conscious sheep (17). After infusion of losartan for 1 h, 0.6 M NaCl in CSF containing losartan (1 mg/ml) was infused at 1 ml/h for 20 min. Recordings were continued for 30 min after the end of the infusion.

As an osmotic control for the infusion of CSF containing 0.6 M NaCl, the effects of intracerebroventricular infusion of hyposmotic CSF (0.9 M sorbitol in CSF at 1 ml/h) were examined, using the same protocol as that used for intracerebroventricular hypertonic saline. Control experiments in which CSF was infused at 1 ml/h for 30 min were also completed.

Baroreflex relations. The baroreflex relations between CSNA and diastolic pressure, during intracerebroventricular infusion of ANG II and during intravenous infusion of phenylephrine, were constructed as described previously for RSNA (17, 18). The intercept of the linear regression line with the diastolic blood pressure axis was used to assess shifts in the baroreflex relations, and differences in the slopes were used to assess differences in baroreflex sensitivity. The control baroreflex relation was determined by increasing arterial pressure from baseline levels by intravenous infusion of incremental doses of phenylephrine hydrochloride (Winthrop) at 2, 4, and 8 mg/h (at 30, 60, and 120 ml/h in normal saline) for 1–2 min at each dose. By this means, the control baroreflex relation was constructed over the range of arterial pressure that encompassed the entire range seen in response to intracerebroventricular infusion of angiotensin. Intracerebroventricular infusions of ANG II were started 15–20 min after the end of the phenylephrine infusion, when all variables had returned to control levels. Baroreflex relations during intracerebroventricular ANG II were obtained from spontaneous fluctuations in arterial pressure and CSNA from 25 to 30 min of the intracerebroventricular infusion (12).
and were compared with those obtained on the same day during intravenous infusion of phenylephrine. To avoid any influence from the prolonged actions of intracerebroventricular ANG II, the control relation was constructed before infusion of ANG II. Analogous procedures were used to compare the baroreflex relation of CSNA to diastolic blood pressure during intravenous infusion of phenylephrine with the relations during intracerebroventricular infusion of hypertonic saline and intracerebroventricular infusion of CSF.

Baroreceptor relations were constructed from ~5 min of data, analyzed on a beat-to-beat basis using custom-written routines in the Spike 2 program, giving 250–400 data points to generate each baroreflex relation. For each heartbeat the program determined diastolic pressure and, as a measure of CSNA, the number of discriminated spikes between the following diastolic pressures. The background noise was taken as the spikes per second during the highest dose of phenylephrine when CSNA was abolished, and this was subtracted from the data collected on that day. The number of discriminated spikes between successive diastolic pressures was divided by the heart period. These data were then sorted by diastolic pressure and meaned in groups of 10. To allow data to be grouped, spike counts were normalized by calculating the percent change in CSNA from the mean activity recorded during a 1-min control period, immediately before infusion of phenylephrine. The same control value was used to calculate the percentage change in CSNA during the subsequent intracerebroventricular II infusion. CSNA was plotted against diastolic pressure to obtain the baroreceptor reflex relations.

Statistics

CSNA is expressed as the percent change from the mean of the readings taken during the control period. Unless otherwise specified, a two-way ANOVA, repeated measures on one variable (time) and subsequent Bonferroni t-tests were used to compare the baseline value to the values obtained during each of the treatments or to compare various treatment values (SigmaStat, SPSS). The relations between CSNA and diastolic pressure were calculated on the linear portion of the curve using linear regression analysis. The slopes and intercepts with the diastolic blood pressure axis of the baroreceptor relations during intravenous infusion of phenylephrine were compared with those during intracerebroventricular infusion of angiotensin, hypertonic saline, and CSF using a paired, two-tailed Students t-test. Results are presented as means ± SE. P < 0.05 was considered significant.

RESULTS

CSNA was pulse related and was blocked by ganglion blockade with hexamethonium, indicating that the recordings were of postganglionic, efferent sympathetic nerve activity (data not shown).

Effect of Intracerebroventricular Losartan on the Responses to Intracerebroventricular ANG II

In six conscious sheep, CSNA increased progressively during the 30-min intracerebroventricular infusion of ANG II (3 nmol/h), reaching a maximum of 23 ± 7% above control (P < 0.001) (Figs. 1 and 2). Over the infusion period ANG II increased MAP from 89 ± 1.8 to 97 ± 2.1 mmHg (P < 0.001) but did not change HR. These responses to ANG II were prolonged, taking ~45 min to return to control. Intracerebroventricular infusion of losartan for 1 h before administration of ANG II had no effect on CSNA or MAP. The increases in CSNA and MAP in response to ANG II were blocked by intracerebroventricular infusion of losartan. Intracerebroventricular infusion of CSF for 30 min had no effect on CSNA, MAP, or HR (n = 6).

Effect of Intracerebroventricular Losartan on the Responses to Intracerebroventricular Hypertonic Saline

In seven conscious sheep, intracerebroventricular infusion of hypertonic saline caused a gradual increase in CSNA (P < 0.05), reaching a maximum increase of 9.4 ± 6.6% at the end of the 20-min infusion period (Fig. 3). This was accompanied by an increase in MAP from 87 ± 2 to 92 ± 2 mmHg (P < 0.01), but there was no change in HR. Both CSNA and MAP returned to control levels over the next 20 min.

Intracerebroventricular infusion of losartan for 1 h had no significant effects on CSNA or MAP. At the end of the infusion CSNA was 97 ± 5% of control and MAP was 87 ± 3 compared with 88 ± 3 mmHg before infusion. Losartan inhibited the increase in CSNA and MAP in response to intracerebroventricular hypertonic saline (Fig. 3). Central administration of sorbitol (1.2 M in CSF) for 20 min, an osmotic...
control for hypertonic saline, had no significant effects on CSNA or MAP (n = 5).

**Baroreflex Relations**

The slopes and intercepts of the baroreflex relations between CSNA and diastolic blood pressure were the same regardless of whether they were obtained from spontaneous fluctuations during intracerebroventricular infusion of CSF or during intravenous infusion of phenylephrine (n = 6) (Fig. 4A). During intracerebroventricular infusion of ANG II, there was a rightward shift of the intercept of the relation between CSNA and diastolic pressure when compared with the control relation constructed during intravenous infusion of phenylephrine on the same day (98.3 ± 2.3 vs. 90.0 ± 1.6 mmHg, respectively, P < 0.01, n = 9), without any change in the slope (−10.4 ± 1.7 vs. −11.3 ± 1.3% of control/mmHg, respectively) (Fig. 4B). With intracerebroventricular hypertonic saline, there was a nonsignificant trend toward a greater intercept (91.7 ± 3.2 vs. 90.4 ± 2.9 mmHg) with no change in the slope of the relation (−11.9 ± 1.6 vs. −11.1 ± 1.4% change/mmHg) (n = 8) (Fig. 4C).

**DISCUSSION**

This is one of the very few studies to examine the control of CSNA in conscious animals and the first to directly investigate the control of CSNA by central angiotensinergic mechanisms. The main findings of this study are that intracerebroventricular infusion of ANG II or hypertonic saline increased CSNA. This occurred despite the increase in arterial pressure that normally causes baroreceptor-induced inhibition of CSNA. These responses, an increase in CSNA and MAP, were inhibited by losartan, indicating that they were mediated by angiotensin AT-1 receptors.

There is extensive evidence that sympathetic activity to individual organs is controlled differentially. In a number of elegant studies in conscious cats, Ninomiya and Matsukawa (24, 25) simultaneously recorded CSNA and RSNA and dem-

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*Fig. 2. Changes in CSNA (A), heart rate (HR; B), and mean AP (MAP; C) in 6 conscious sheep during intracerebroventricular infusion of ANG II alone and then together with intracerebroventricular infusion of losartan (1 mg/h) after pretreatment with losartan for 1 h. Results are means ± SE. Comparisons are between treatments: *P < 0.05, †P < 0.01, ‡P < 0.001.*

*Fig. 3. Changes in CSNA (A), HR (B), and MAP (C) in 7 conscious sheep during intracerebroventricular infusion of hypertonic saline [0.6 M NaCl in artificial cerebrospinal fluid (CSF) at 1 ml/h] alone and then together with intracerebroventricular infusion of losartan (1 mg/h) after pretreatment with losartan for 1 h. Results are means ± SE. Comparisons are between treatments, *P < 0.05.*
onstrated a lack of concordance between bursts in the two nerves, different respiratory modulation of CSNA and RSNA in a given respiratory cycle, and a greater initial response of RSNA than CSNA with exercise. In anesthetized animals, activation of left atrial receptors caused an increase in CSNA, a decrease in RSNA, and no effect on lumbar and splenic nerve activities (11). The present findings that intracerebroventricular infusion of ANG II and hypertonic saline increased CSNA, in contrast to our previous findings that these stimuli caused profound and sustained renal sympathoinhibition (17, 18), are further examples of the differential control of CSNA and RSNA. In addition, the rightward shift of the baroreflex relation between CSNA and diastolic pressure during central infusion of ANG II is in striking contrast to the leftward shift in the relation between RSNA and diastolic pressure caused by this stimulus (17, 18).

The intracerebroventricular ANG II-induced increases in CSNA and MAP were prevented by losartan, indicating that these responses depended on stimulation of ANG II AT-1 receptors. Losartan also inhibited the increase in CSNA and MAP were prevented by losartan, indicating that this stimulus (17, 18), are further examples of the differential control of CSNA and RSNA. In addition, the rightward shift of the baroreflex relation between CSNA and diastolic pressure during central infusion of ANG II is in striking contrast to the leftward shift in the relation between RSNA and diastolic pressure caused by this stimulus (17, 18).

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The present finding that central administration of ANG II increased MAP but did not decrease HR is in agreement with our previous findings (17) and those of others in conscious rats (4) and conscious rabbits (29). The observation that intracerebroventricular ANG II increased CSNA indicates that the absence of a baroreceptor-induced bradycardia is not only due to inhibition of efferent vagal discharge by ANG II but is also due to its action to increase CSNA. The fact that during intracerebroventricular ANG II there was no increase in HR, despite the increase in CSNA, suggests that vagal tone increased, although there is evidence that the extent of the increase in vagal tone to the heart is attenuated by angiotensin (4). These effects of ANG II on the baroreflex control of HR have important physiological actions as they result in greater increases in arterial pressure for any degree of peripheral vasconstriction.

The site of action of ANG II to cause this increase in CSNA cannot be determined from these studies of intracerebroventricular infusions, but findings from other studies indicate that the lamina terminalis is a probable site (3, 19). The laminal terminalis, which is situated in the anterior wall of the third ventricle, consists of the subfornical organ, median preoptic nucleus, and organum vasculosum of the lamina terminalis. It is important to note that the responses to ANG II in the present study result from its actions behind the blood-brain barrier, suggesting that the median preoptic nucleus is a likely site of action as the other two nuclei within the lamina terminalis are circumventricular organs. Evidence supporting a role for the lamina terminalis in the central responses to ANG II is the finding that it contains a high density of ANG II receptors in the sheep (20) and that the neural activation marker c-fos is expressed in the lamina terminalis of rats after central administration of ANG II (21). Furthermore, lesion of the lamina terminalis blocked the increase in MAP and inhibition of RSNA in response to intracerebroventricular ANG II and hypertonic saline (19).

**Perspectives**

It has been suggested that central angiotensinergic mechanisms may play a critical role in the increase in sympathetic activity that occurs in heart failure. In conscious rats, intracerebroventricular administration of losartan returned basal RSNA and the reflex control of RSNA toward normal (5). To date the effect of central AT-1 receptor blockade on CSNA has not been studied in heart failure, but our finding that intracerebroventricular ANG II increases CSNA indicates that it is possible that overactivity of the central ANG II system may contribute to the large increase in CSNA reported in heart failure. There is evidence from studies of norepinephrine spillover in patients with heart failure that sympathetic drive to the heart increases before that to other organs (31) and that in established heart failure the increase in cardiac norepinephrine spillover is greater than that to other organs (6). Our findings

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**Fig. 4.** Comparisons of the relationships between CSNA and diastolic blood pressure (dBP) during intravenous (iv) phenylephrine (PE) and the last 5 min of intracerebroventricular infusion of artificial CSF (n = 6; A), ANG II (3 nmol/h, n = 9; B), and 0.6 M NaCl in artificial CSF (1 ml/h, n = 8; C). Data obtained during intracerebroventricular infusions were compared with those obtained during intravenous infusion of PE on the same day. P value refers to the difference in intercept with the dBP axis (B).

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**A**

- **IV PE**
- **ICV CSF (1 ml/h)**

**B**

- **IV PE**
- **ICV ANG II (3 nmol/h)**

**C**

- **IV PE**
- **ICV NaCl (0.6 M at 1 ml/h)**
that ANG II increases CSNA, but decreases RSNA, indicate that overactivity of this system could be one of the causes of a preferential increase in sympathetic activity to the heart compared with other organs.

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