Central Angiotensin Type 1 Receptor Blockade Decreases Cardiac But Not Renal Sympathetic Nerve Activity in Heart Failure

Rohit Ramchandra, Sally G. Hood, Anna M.D. Watson, Andrew M. Allen, Clive N. May

Abstract—In heart failure (HF), cardiac sympathetic nerve activity (SNA; CSNA) is increased, which has detrimental effects on the heart and promotes arrhythmias and sudden death. There is evidence that the central renin-angiotensin system plays an important role in stimulating renal SNA in HF. Because SNA to individual organs is differentially controlled, we have investigated whether central angiotensin receptor blockade decreases CSNA in HF. We simultaneously recorded CSNA and renal SNA in conscious normal sheep and in sheep with HF induced by rapid ventricular pacing (ejection fraction: <40%). The effect of blockade of central angiotensin type 1 receptors by intracerebroventricular infusion of losartan (1 mg/h for 5 hours) on resting levels and baroreflex control of CSNA and renal SNA were determined. In addition, the levels of angiotensin receptors in central autonomic nuclei were determined using autoradiography. Sheep in HF had a large increase in CSNA (43±2 to 88±3 bursts per 100 heart beats; P<0.05) and heart rate, with no effect on renal SNA. In HF, central infusion of losartan for 5 hours significantly reduced the baseline levels of CSNA (to 69±5 bursts per 100 heart beats) and heart rate. Losartan had no effect in normal animals. In HF, angiotensin receptor levels were increased in the paraventricular nucleus and supraoptic nucleus but reduced in the area postrema and nucleus tractus solitarius. In summary, infusion of losartan reduced the elevated levels of CNSA in an ovine model of HF, indicating that central angiotensin receptors play a critical role in stimulating the increased sympathetic activity to the heart. (Hypertension. 2012;59:634-641.) ● Online Data Supplement

Key Words: angiotensin ■ cardiac sympathetic nerve activity ■ losartan ■ heart failure

Heart failure (HF) is a common cause of hospital admission and death in adults >55 years of age, and with the aging of the population it is becoming a leading cause of death worldwide. Activation of the renin-angiotensin system (RAS) and sympathetic nervous system is a hallmark of HF,1,2 and inhibition of these systems is a major focus of therapy.3–5 The sustained and excessive level of sympathetic nerve activity (SNA) has adverse effects that contribute to the progression of HF, and this is particularly the case for the increase in cardiac SNA (CSNA). The high level of norepinephrine release at the heart causes downregulation of cardiac β-adrenoceptors,6 has toxic effects on the sympathetic nerve terminals,6 induces left ventricular fibrosis and hypertrophy,7 and promotes the development of arrhythmias and sudden death.8 Underscoring the detrimental effect of the increased sympathetic drive to the heart in HF is the effectiveness of treatment with β-blockers8,9 and the finding that cardiac norepinephrine spillover is the strongest prognostic marker in HF patients.8

The mechanisms causing the increase in CSNA in HF are not well defined. The majority of studies examining the increase in SNA in HF have focused on sympathetic activity to the kidney in animal experiments10–13 and to skeletal muscle in patients.14,15 There is strong evidence from these studies supporting a role for both peripheral and central mechanisms. Regarding central mechanisms, a focus has been the RAS, which acts at multiple brain nuclei to regulate sympathetic outflow.16,17 Blockade of central angiotensin type 1 receptors (AT1Rs) with losartan reduces the elevated renal SNA (RSNA) in rats with HF induced by myocardial infarction10,18 and restores arterial baroreflex sensitivity in rats and rabbits with HF.10,12

The factors that control CSNA are, however, different from those controlling SNA to other beds. For example, we have
demonstrated that, in the normal animal, the resting level of CSNA is set much lower than that of RSNA\textsuperscript{19}; that, in an ovine pacing-induced model of HF, there is a much larger increase in CSNA than RSNA\textsuperscript{19,20}; that, in this model, the inhibition of CSNA in response to volume expansion is abolished, whereas the inhibition of RSNA is only attenuated\textsuperscript{20,21}; and, finally, in normal sheep we have shown that ICV infusion of angiotensin II (Ang II) or hypertonic saline has an opposite effect on CSNA and RSNA.\textsuperscript{22,23} The finding that ICV Ang II increased CSNA, together with findings that central AT\textsubscript{1}R blockade reduced RSNA in rodent models of HF, suggested that increased activity of the central RAS could contribute to the cardiac sympathoexcitation in HF.

In the present studies we have, therefore, investigated whether central administration of the AT\textsubscript{1}R blocker losartan reduces the large increase in CSNA that occurs in conscious sheep with pacing-induced HF. In addition to investigating the effects of ICV infusion of losartan on CSNA and RSNA in normal and HF sheep, we have compared the levels of angiotensin receptors in central cardiovascular nuclei using autoradiography.

**Methods**

Experiments were performed on 9 normal and 7 HF adult Merino ewes (30–40 kg) acclimatized to laboratory conditions and housed with other sheep. All of the experiments were approved by the animal experimentation ethics committee of the Howard Florey Institute, following guidelines from the National Health and Medical Research Council of Australia.

**Surgery**

Before the studies, sheep underwent 3 aseptic surgical procedures, separated by recovery periods of ≥2 weeks (for full details please see the online-only Data Supplement). For all of the normal animals, anesthesia was induced with IV sodium thiopental (15 mg/kg) and maintained with 1.5% to 2.0% isoflurane/O\textsubscript{2}. For animals in HF, anesthesia was induced and maintained with isoflurane. In the first operation, a carotid arterial loop was constructed, and in HF animals a cardiac pacemaker lead was inserted into the right ventricle. For the second operation, guide tubes were inserted over the lateral cerebral ventricles. The final operation was to implant electrodes in cardiothoracic and renal sympathetic nerves.\textsuperscript{22,23} Experiments were started ≥3 days after implantation of electrodes. One day before experiments, a further cannula was inserted into the carotid artery for measurement of arterial pressure.

**Pacing-Induced HF**

Sheep with pacemaking leads underwent left ventricular echocardiograph measurement (Hewlett Packard Sonos 1000) prepacing. Sheep were then paced at 200 to 220 bpm, and echocardiographs were repeated weekly with the pacing off. When ejection fraction was <40%, electrode placement surgery was performed. The sheep used in this study were paced for 8±1 weeks before nerve-recording electrodes were implanted.

**Nerve Recording**

SNA was recorded differentially between pairs of electrodes.\textsuperscript{22,23} The signal was amplified (×100 000) and filtered (bandpass 400 to 1000 Hz). SNA and blood pressure were recorded on a computer using a CED micro 1401 interface and Spike 2 software (Cambridge Electronic Design). Details of burst identification are included in the online-only Data Supplement.

**Experimental Protocols**

After a 5-minute baseline recording, baroreflex responses were generated by measuring CSNA, RSNA, and HR responses to changes in arterial pressure induced by increasing doses of phenylephrine hydrochloride and sodium nitroprusside. After baseline recordings, the AT\textsubscript{1}R antagonist losartan (Merck) was infused ICV (1 mg/h in artificial cerebrospinal fluid at 1 mL/h for 5 hours), and toward the end of the infusion another baseline recording was made and the baroreflex responses were retested. Effectiveness of this dose of losartan has been demonstrated in previous studies in sheep.\textsuperscript{22} Baroreflex curves were drawn using a 4-parameter sigmoidal logistic equation.\textsuperscript{24} The following control experiments were also conducted: (1) ICV infusion of cerebrospinal fluid (1 mL/h for 5 hours) in normal and HF sheep (n=3 per group); (2) IV losartan (1 mg/h for 5 hours) in 3 HF sheep; and (3) ICV candesartan (0.25 mg/h for 5 hours) in 2 HF sheep.

**Autoradiography of Angiotensin Receptors**

To determine changes in the levels of Ang II receptors in HF, the distribution of angiotensin binding sites was mapped in the brains of normal (n=5) and HF (n=5) sheep. Detailed methods of the autoradiography procedure are available in the online-only Data Supplement. Briefly, 20-μm coronal slide-mounted sections were incubated using standard solutions containing \textsuperscript{125}I-[Sar\textsuperscript{1}, Ile\textsuperscript{8}] Ang II and radiogalid (Prosearch International Australia Ltd, Victoria, Australia) and different angiotensin receptor subtype antagonists. After washing and then drying, the sections were apposed to x-ray film (UM-MAC HC, Fujifilm, Tokyo, Japan), and all of the binding was normalized to total binding in the normal brains.

**Statistics**

Results are expressed as mean±SEM. For baseline data, an unpaired t test was used to compare normal versus HF animals. Differences within a group before and after losartan were analyzed using 2-way repeated-measures ANOVA. A significant result was considered to be P<0.05.

**Results**

**Resting Levels in Normal and HF Sheep**

Ejection fraction (85.2±1.5% to 35.1±3%; P<0.001) and fractional shortening (53.6±2.0% to 16.7±1.5%; P<0.001) decreased in HF sheep over 5 to 13 weeks of rapid ventricular pacing. In the HF group, HR was significantly increased (100±6 versus 74±6 bpm; P<0.05) compared with the normal group, and systolic, diastolic, and mean arterial pressures tended to be higher (3.3 to 8.8 mm Hg, respectively) and central venous pressure tended to be higher (3.3 to 8.8 mm Hg, respectively) and central venous pressure tended to be higher (3.3±1.4 versus 4.0±0.8 mm Hg), but the differences did not reach statistical significance. In normal sheep, the resting level of CSNA was significantly lower than that of RSNA (Figures 1 and 2). In HF, there was a dramatic increase in resting CSNA (from 43±2 to 88±3 bursts per 100 heart beats; P<0.05), whereas the resting levels of RSNA were unchanged (90±4 to 82±5 bursts per 100 heart beats). Similar changes were seen when total nerve activity, expressed as spikes per second, was analyzed (Figure 2).

**Effects of ICV Losartan in Normal and HF Sheep**

Infusion of losartan into the cerebral ventricles had no effect on the resting levels of blood pressure in either group, but in the HF group it significantly reduced the raised HR back to normal levels (Figure 2). In the HF group losartan caused a gradual decrease in resting levels of CSNA over the 5-hour
infusion period toward normal levels (62±10–27±7 spikes per second; \( P<0.05 \); Figures 1 and 2). This was because of a significant decrease in the average burst incidence (88±3–69±5 bursts per 100 heart beats; \( P<0.05 \)), as well as a decrease in the burst amplitude (40±8–29±5 spikes per burst; \( P<0.05 \)). Losartan infusion tended to decrease CSNA in the normal animals, but this was not significant. In contrast, ICV losartan had no effect on the resting levels of RSNA in either group (Figures 1 and 2).

Central infusion of candesartan, another AT1R antagonist, had similar effects to losartan, causing a large fall in CSNA with no effect on RSNA. IV infusion of losartan (1 mg/h), the same dose as that given centrally, had no effect in HF sheep, and ICV infusion of artificial cerebrospinal fluid had no effect on CSNA, RSNA, HR, or mean arterial pressure in either group (data not shown).

**Effects of Losartan on Baroreflex Responses in Normal and HF Sheep**

To compare the effects of losartan within each group, arterial baroreflex curves of SNA were constructed from data normalized to the resting activity immediately before starting losartan infusion. In HF animals, the decrease in the resting level of CSNA after 5 hours of losartan infusion was associated with a decrease in the range and upper plateau of the CSNA baroreflex curve (Figure 3 and Table). In contrast, there was no change in any of these parameters in the normal group. Losartan had no effect on RSNA baroreflex parameters in either group. In HF, there was a decrease in the range and maximum gain of the HR baroreflex compared with normal animals (Figure 3 and Table). After 5 hours of ICV losartan, the sensitivity of the HR baroreflex curve tended to be increased in the heart failure group (−3.26±0.36 to −5.22±0.77 bpm/mm Hg; \( P=0.1 \)).

**Central Angiotensin Receptor Distribution**

The distribution of binding sites for \(^{125}\text{I}-[\text{Sar}^1, \text{Ile}^8]\) Ang II was examined in the medulla and the hypothalamus of normal and HF sheep brains. The sites of highest density were in the paraventricular nucleus of the hypothalamus (PVN), the nucleus of the solitary tract (NTS), and the dorsal motor nucleus (Figure 4), as has been noted previously in sheep. The receptor type was predominantly AT1R in all of the regions examined, because coichubation with losartan decreased the binding intensity to background levels. Comparison of the density of AT1Rs in regions associated with

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**Figure 1.** Raw traces of arterial pressure (AP), cardiac sympathetic nerve activity (SNA), and renal SNA in a conscious sheep in heart failure (HF) before (A) and after (B) 5 hours of ICV infusion of losartan (1 mg/h).
cardiovascular control showed differential changes in angiotensin receptor binding in the HF group (Figure 4). Of note, there was an increase in the AT1R levels in the PVN and hypothalamic supraoptic nucleus of HF sheep and a decrease in AT1R levels in the area postrema and in the NTS at the level of the area postrema. The AT1R levels were also examined in the NTS and the dorsal motor nucleus at the level of the opening of the central canal and in the organum vasculosum of the lamina terminalis, and there was no significant change in AT1R levels in these areas.

Discussion

The main findings of this study were that, in an ovine model of HF, central infusion of losartan significantly decreased the elevated level of CSNA almost back to control levels. In contrast, RSNA was not increased, and treatment with losartan had no effect on RSNA. In HF, losartan infusion decreased the range and threshold of the CSNA arterial baroreflex but had no effect on the baroreflex control of RSNA. In the HF animals, we observed differential alterations in AT1R binding densities in specific brain regions, including an increase in AT1R density in the PVN and a decrease in the NTS. In normal animals, losartan had no significant effects on the resting levels or baroreflex control of CSNA, RSNA, or HR.

Effects of Losartan on Baseline Levels of CSNA and RSNA

Pacing-induced HF caused a large increase in baseline levels of CSNA but no change in the baseline levels of RSNA (Figure 2), confirming our previous findings.19,26 These findings are similar to those in patients with mild HR where cardiac, but not renal, norepinephrine spillover was increased,27 whereas in more severe HF there was a significant increase in renal norepinephrine spillover.28,29 The finding that central AT1R blockade in HF restored baseline levels of CSNA and heart rate toward normal levels indicates a critical role for central angiotensinergic mechanisms in setting the high level of CSNA in HF. A similarly important role for the central RAS in driving the increased RSNA has been observed in HF models in rats, induced by myocardial infarction, and in rabbits, induced by rapid pacing.10,11,30 The lack of a fall in RSNA with losartan in sheep with mild HF is probably because at this level of HF in sheep there was no stimulation of RSNA.

Effects of Losartan on Baroreflex Control of CSNA and RSNA

In this model of HF we found that the sensitivity and range of the HR arterial baroreflex were significantly reduced, which is in accord with findings in humans and other animal models of HF.31,32 Treatment with ICV losartan did not increase the gain of the HR arterial baroreflex, in contrast to rodent models of HF where inhibition of the central RAS improved HR baroreflex sensitivity.11,18 As we have reported previously,19,24 the arterial baroreflex control of CNSA was not altered in this model of HF when the relation between CSNA and diastolic pressure was calculated using CSNA levels normalized to the maximum level obtained during severe hypotension. This finding indicates that altered vagal tone and cardiac β-adrenoceptor downregulation are probably the major factors leading to the decreased gain of the arterial baroreflex control of HR in this model of HF.
In the present experiments, the CSNA data used for the arterial baroreflex curves were normalized to the values immediately before losartan infusion to allow for direct comparison of the reflex before and after treatment with losartan. Central infusion of losartan caused a reduction in the upper plateau and the range of the CSNA baroreflex curve in HF but not normal animals (Figure 3). This effect of losartan on CSNA baroreflex sensitivity in HF is in contrast to the findings in rodent models of HF where the decreased RSNA baroreflex sensitivity was restored by ICV infusion of losartan.12 The reason for the different responses of the cardiac and renal sympathetic nerves to ICV losartan in HF are unclear but may relate to the degree of HF or different roles of central angiotensin in the control of these 2 sympathetic outflows, as demonstrated by their opposite responses to central infusion of angiotensin, which increased CSNA and decreased RSNA.22,23

Changes in Central Angiotensin Receptor Levels During HF

The ability of centrally administered AT_{1}R blockers to reduce SNA in HF is likely to be because of their actions to inhibit the effects of both the increase in central angiotensin levels, indicated by the increased cerebrospinal fluid levels of angiotensin found in dogs with pacing-induced HF,33 and the increased density of AT_{1}R, observed in brain nuclei associated with central sympathetic control in rodent models of HF.34–36 For example, increased mRNA expression of AT_{1}R was found in the rostral ventral lateral medulla of rabbits with pacing-induced HF,30,34 and AT_{1}R density measured by autoradiography was increased in the subfornical organ, organum vasculosum laminae terminalis, PVN, and median preoptic nucleus in rats with HF produced by aortocaval shunt.36

Our results in sheep with HF indicate decreased AT_{1}R density in the area postrema and in the NTS at the level of the area postrema (Figure 4). The reasons for the discrepancies regarding changes in the AT_{1}R levels in the NTS in the sheep and rodent models of HF are not clear. An increase in systemic levels of Ang II, as has been observed during HF, can decrease heart rate baroreflex sensitivity through actions at the area postrema and the NTS.39 It is possible that the decrease in AT_{1}R in the area postrema and the NTS may be a secondary response to limit further decreases in

Figure 3. Baroreflex relations of percentage of cardiac sympathetic nerve activity (CSNA; A) and percentage of renal sympathetic nerve activity (RSNA; B; both normalized to prelosartan values in each group) to diastolic blood pressure (dBP) and of heart rate (HR) to systolic blood pressure (sBP; C). Data are from normal (left) and heart failure (HF) animals (right) before (solid line) and after (dashed line) ICV infusion of losartan (1 mg/h for 5 hours). Resting points before (○) and after (▼) losartan are indicated as mean±SEM. †Significant decrease in upper plateau with losartan. †Decreased range with losartan. *Decreased range with HF.
Baroreflex sensitivity. In addition, our study indicated increased AT1R density in the PVN and supraoptic nucleus during HF. It is well known that Ang II can stimulate vasopressin release through actions at the PVN, as well as the supraoptic nucleus.40–43 Whether the increase in AT1R in these areas plays an important role in mediating the high baroreflex sensitivity. In addition, our study indicated increased AT1R density in the PVN and supraoptic nucleus during HF. It is well known that Ang II can stimulate vasopressin release through actions at the PVN, as well as the supraoptic nucleus.40–43 Whether the increase in AT1R in these areas plays an important role in mediating the high levels of vasopressin observed during HF remains to be established. In addition to actions on vasopressin release, the increase in AT1R density in the PVN of HF animals may also play an important role in mediating the increased SNA in HF, but whether it specifically drives the increased CSNA requires further studies examining the responses to PVN microinjections.

We have shown previously that short-term systemic treatment with an angiotensin receptor blocker in the sheep pacing model of HF prevented the reflex increase in CSNA in response to the drug-induced fall in arterial pressure.45 This intravenous treatment did not, however, reduce the high level of CSNA, probably because, as we demonstrated, it only blocked AT1R in the periphery and not in the brain. The findings from this and other studies that increased sympathetic outflow to different organs in HF is blocked by central AT1R blockade suggest that the beneficial effects seen with long-term treatment with this class of drugs in HF patients may result in part from these drugs crossing the blood-brain barrier and having a central action. For example, daily treatment with candesartan decreased CSNA, evaluated by iodine-123 meta-iodobenzylguanidine scintigraphy.46 It is important to note, however, that drug-induced improvements in hemodynamics are also likely to have acted to reduce CSNA.

**Perspectives**

Patients with HF die for 2 main reasons, circulatory insufficiency or sudden death, and because the excessive level of CSNA in HF can induce fibrosis and hypertrophy and promote arrhythmias and sudden death, it is critical to find new mechanisms to decrease CSNA to reduce patient morbidity and mortality. The present findings indicate that central inhibition of AT1R substantially reduced the elevated CSNA in HF, indicating a primary role for central angiotensin receptors in driving the increased CSNA. These results suggest that oral treatment with high doses of AT1R blockers that have a greater tendency to cross the blood-brain barrier may have beneficial actions to reduce the elevated CSNA in HF and the associated detrimental effects.

### Table. Arterial Baroreflex Relations in Conscious Normal and Heart Failure Sheep Before and After 5-h ICV Infusion of Losartan (1 mg/h)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal</th>
<th>Losartan (5 h)</th>
<th>Heart Failure</th>
<th>Losartan (5 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dBP and % CSNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper plateau</td>
<td>363.1±46.8</td>
<td>335.7±31.5</td>
<td>167.7±17.2</td>
<td>112.9±10.7†</td>
</tr>
<tr>
<td>Range</td>
<td>372.2±46.2</td>
<td>330.2±29.5</td>
<td>167.6±18.1</td>
<td>105.6±9.3†</td>
</tr>
<tr>
<td>Gain</td>
<td>−6.01±1.37</td>
<td>−4.74±0.75</td>
<td>−4.98±1.12</td>
<td>−3.48±0.9</td>
</tr>
<tr>
<td>BP50</td>
<td>73.8±2.8</td>
<td>65.1±4.3</td>
<td>73.8±4.4</td>
<td>69.3±3.2</td>
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<tr>
<td>Threshold, mm Hg</td>
<td>84.6±3.5</td>
<td>79.1±4.7</td>
<td>88.5±5.9</td>
<td>79.5±4.5†</td>
</tr>
<tr>
<td>Saturation, mm Hg</td>
<td>49.2±7.7</td>
<td>51.2±4.9</td>
<td>59.1±5.0</td>
<td>59.0±3.7</td>
</tr>
<tr>
<td><strong>dBP and % RSNA</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper plateau</td>
<td>172.2±16.8</td>
<td>173.5±10.3</td>
<td>201.6±27.5</td>
<td>209.1±40.8</td>
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<tr>
<td>Range</td>
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<td>185.5±13.4</td>
<td>216.5±29.8</td>
<td>214.8±45.0</td>
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<td>Gain</td>
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<td>−8.88±2.67</td>
<td>−7.89±1.02</td>
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<td>BP50</td>
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<tr>
<td>Threshold, mm Hg</td>
<td>99.3±1.8</td>
<td>97.7±3.3</td>
<td>102.3±12.1</td>
<td>96.8±5.9</td>
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<tr>
<td>Saturation, mm Hg</td>
<td>59.7±6.8</td>
<td>58.3±5.0</td>
<td>50.0±5.4</td>
<td>48.9±7.2</td>
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<tr>
<td><strong>sBP and HR</strong></td>
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<tr>
<td>Upper plateau, bpm</td>
<td>167.4±10.7</td>
<td>166±15.3</td>
<td>140.0±3.7</td>
<td>147.9±14.5</td>
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<td>Range, bpm</td>
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<td>115±12.6</td>
<td>87.0±3.9*</td>
<td>92.1±12.9</td>
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<tr>
<td>Gain, bpm/mm Hg</td>
<td>−4.83±0.54</td>
<td>−5.14±0.86</td>
<td>−3.26±0.36*</td>
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<td>BP50, bpm</td>
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<td>97.0±5.1</td>
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<td>Threshold, mm Hg</td>
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<td>113.7±3.8</td>
<td>118.0±6.5</td>
<td>109.6±4.0</td>
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<td>Saturation, mm Hg</td>
<td>74.3±5.0</td>
<td>73.9±4.5</td>
<td>76.0±4.3</td>
<td>82.0±2.8</td>
</tr>
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</table>

BP50 indicates the pressure at 50% of the range of sympathetic nerve activity or heart rate; CSNA, cardiac sympathetic nerve activity; RSNA, renal sympathetic nerve activity; dBP, diastolic blood pressure; sBP, systolic blood pressure; HR, heart rate. The percentage of CSNA and RSNA values during infusion of losartan are normalized to the resting levels prelosartan. Values are mean±SEM.

*Data show the significant effect of HF.
†Data show the significant effect of losartan.
23. Watson AM, Mogulkoc R, McAllen RM, May CN. Stimulation of cardiac sympathetic nerve activity by central angiotensinergic mechanisms in...


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CENTRAL ANGIOTENSIN AT1 RECEPTOR BLOCKADE DECREASES CARDIAC BUT NOT RENAL SYMPATHETIC NERVE ACTIVITY IN HEART FAILURE

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Short title: Central Losartan decreases CSNA in HF.

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Methods – Expanded Methods

Experiments were performed on 10 normal and 10 HF adult Merino ewes (30-40kg) acclimatised to laboratory conditions and housed with other sheep. Sheep were fed 800g/day oaten chaff, and had access to water ad libitum. All experiments were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute, following guidelines from the National Health and Medical Research Council of Australia.

Surgery

Prior to the studies, sheep underwent three aseptic surgical procedures, separated by recovery periods of at least two weeks. For all normal animals, anesthesia was induced with intravenous (IV) sodium thiopental (15mg/kg) and following intubation anesthesia was maintained with 1.5-2.0% isoflurane/O2. Once the animals were in HF, anesthesia was induced and maintained with isoflurane. In the first operation a carotid artery loop was constructed, and in HF animals a cardiac pacemaker lead was inserted into the right ventricle via the right jugular vein (Excellence SS+, Viatron, Minneapolis, MN, USA). The lead was exteriorised at the neck and connected to an external pacemaker. For the second operation, animals were placed in a stereotaxic frame and a two stainless steel guide tubes were inserted over the lateral cerebral ventricles. The flow of CSF into and out of the guide tubes was used to indicate placement in the ventricle. The final operation was to implant electrodes in cardiothoracic and renal sympathetic nerves, as described previously 22, 23. For the cardiac sympathetic nerves, an incision was made above the fourth rib, the periosteum was opened, and the rib was removed. The thoracic cardiac nerves were identified, and the facia over the nerves was removed. The recording electrodes consisted of stainless steel entomological pins (0.05 mm diameter) etched to a fine point, and glued into the end of Teflon coated 25-strand silver-coated copper wire (model CZ1174SPC; Cooner, Chatsworth, CA). The exposed point of the electrode (1.5–2.0 mm in length) was inserted obliquely through the nerve sheath, ensuring that the tip was positioned in the center of the nerve. Up to five electrodes were pushed obliquely through the nerve sheath, ensuring that the tip was positioned in the center of the nerve. Electrodes were fixed in place with cyanoacrylate glue, the implantation site was covered with a layer of Kwik-Sil (WPI, Glen Waverly, Vic, Australia), and the wires were exteriorized next to the sutured wound. For the renal sympathetic nerves, the right or left renal artery was exposed via a paracostal retroperitoneal approach. With the aid of a dissection microscope, the renal nerve was identified running along or parallel to the renal artery and cleared of surrounding fat. Up to five electrodes were implanted along the exposed length of nerve and fixed in place with cyanoacrylate glue. The wires were looped and exteriorized through the sutured wound. A stainless-steel suture looped through the skin was used as a ground.

A day prior to electrode placement surgery, using aseptic techniques, two cannulae were inserted into a jugular vein for measurement of central venous pressure (CVP) and for intravenous infusions. Antibiotic (900 mg procaine penicillin, Troy Laboratories, NSW, Australia) was administered prophylactically on the day prior to each surgery, at the start of surgery and for 2 days post-surgery. Post-surgical analgesia was maintained with intramuscular injection of flunixin meglumine (1 mg/kg; Mavlab, Qld, Australia) at the start of surgery, and after 24hrs recovery.

Experiments were started at least 3 days after implantation of electrodes. One day prior to experiments a further cannula was inserted into the carotid artery for measurement of arterial pressure.

Pacing-induced heart failure

Sheep with pacemaking leads underwent left ventricular echocardiograph measurements (Hewlett Packard Sonos 1000) pre-pacing, and were then paced at 200-220 beats/min. Echocardiographs were repeated every 7 days with the pacing off to monitor progression of heart failure, and when ejection fraction was below 40%, electrode placement surgery was performed.
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 (x100,000) and filtered (bandpass 400 to 1000 Hz), displayed on an oscilloscope and passed through an audio amplifier and loud speaker. Sympathetic nerve activity and blood pressure were recorded on computer using a CED micro 1401 interface and Spike 2 software (Cambridge Electronic Design, UK). The smallest burst was identified in the entire recording from a spreadsheet of data and its correct position in the cardiac cycle and absence of artefacts was confirmed visually. The number of spikes over threshold between the corresponding diastolic pressures of this burst was noted, and this was defined as the minimum number of spikes for a burst. When the number of discriminated spikes over threshold in any heart beat was greater than the minimum number defined, this was determined to constitute a burst. For each sheep the accuracy of burst determination was checked by eye for the data collected over the entire file. The burst incidence was calculated as the number of bursts per 100 heart beats; the burst frequency was calculated as the number of bursts per minute; the burst amplitude was calculated as the average number of spikes per burst and the mean level of SNA was calculated as the number of discriminated spikes over threshold per second.

Experimental protocols

After a 5 minute baseline recording, baroreflex responses were generated by measuring CSNA, RSNA, and HR responses to changes in arterial pressure induced by increasing doses of phenylephrine hydrochloride (33, 67, 133, and 330 mg/min, Neosynephrine, Abbot Australasia, Kurnell, NSW Australia) and sodium nitroprusside (42, 83, 167 and 417 mg/min; David Bull Laboratories, Mulgrave, Vic, Australia). Following baseline recordings, the AT1R antagonist losartan (Merck, New Jersey, USA) was infused ICV (1 mg/hour in artificial cerebrospinal fluid (CSF) at 1 mL/h for 5 h), and towards the end of the infusion another baseline recording was made and the baroreflex responses were re-tested (conducted in 9 normal and 7 HF animals). Effectiveness of this dose of losartan has been demonstrated in previous studies in sheep showing that ICV infusion of this dose of losartan for 1 h blocked the pressor effect and inhibition of RSNA in response to ICV Ang II. Baroreflex curves were drawn using a four-parameter sigmoidal logistic equation as described previously. The following control experiments were also conducted: ICV infusion of CSF (1 mL/h for 5 h) in normal and HF sheep (n=3/group), IV losartan (1 mg/h for 5 h) in 3 HF sheep, and ICV candesartan (0.25 mg/h for 5 h) in 2 HF sheep.

Autoradiography of angiotensin receptors

To determine changes in the levels of the Ang II receptor in the heart failure animals, the distribution of angiotensin binding sites was mapped in the brains of normal (n=5) and heart failure (n=5) sheep. Briefly, the sheep were killed using a lethal dose of sodium pentobarbitone (100 mg/kg). The brains were removed, frozen on dry ice and 20 μm coronal sections cut on a cryostat. Slide-mounted sections were incubated using standard solutions containing 125I-[Sar1, Ile8] Ang II as radioligand (Prosearch International Australia Ltd, Vic, Australia). Either 1 μM Ang II (to determine non-specific binding; Auspep Pty Ltd, Vic, Australia), 1μM candesartan (to displace binding from AT1R) or 10 μM PD123319 (to displace binding from AT2R) were added to the incubation media to define receptor subtypes. After washing, then drying, the sections were apposed to X-ray film (UM-MAC HC, Fujifilm, Tokyo, Japan) for 14 days in X-ray cassettes that also contained standards of known radioactivity. Radioactivity binding densities were quantified using Scion Image 4.0.2 (Scion, Frederick, MD), and all binding was normalised to total binding in the normal brains. Alternate sections were stained with cresyl violet to enable identification of brain nuclei for quantification.

Statistics
Results are expressed as mean ± SEM. For baseline data an unpaired t-test was used to compare normal vs. HF animals. For differences within a group before and after losartan, 2 way repeated measures ANOVA was performed. A significant result was considered to be $p < 0.05$. 