Sustained Activation of the Central Baroreceptor Pathway in Angiotensin Hypertension

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Abstract—Recent studies indicate that renal sympathetic nerve activity is chronically suppressed in angiotensin (Ang II) hypertension and that baroreflexes play a critical role in mediating this response. To support these findings, we determined whether the hypertension associated with chronic infusion of Ang II at 4.8 pmol/kg per minute (5ng/kg per minute) produces sustained activation of medullary neurons that participate in the central baroreceptor reflex pathway. We used Fos-like (Fos-Li) protein immunohistochemical methods to determine activation of neurons in the nucleus tractus solitarius (NTS), caudal ventrolateral medulla (CVLM), and rostral ventrolateral medulla (RVLM). Results were compared in three groups of chronically instrumented dogs subjected to infusion of: 1) saline (control); 2) Ang II-2 hours (acute); and 3) Ang II-5 days (chronic). Mean arterial pressure increased 22±3 and 35±3 mm Hg during acute and chronic Ang II infusion, respectively. There was little Fos-Li immunoreactivity in medullary neurons in control dogs. In contrast, during acute Ang II infusion there was a 2- to 3-fold increase in Fos-Li staining in the NTS and CVLM, but no increase in staining in RVLM neurons. As baroreceptor suppression of sympathoexcitatory cells in the RVLM is mediated by activation of neurons in the NTS and CVLM, these results were expected. More importantly, this same pattern of central neuronal activation was observed during chronic Ang II hypertension. Therefore, these results support recent findings indicating that baroreflex suppression of renal sympathetic nerve activity is a long-term compensatory response in Ang II hypertension. (Hypertension. 2002;39[part 2]:550-556.)

Key Words: angiotensin • baroreflex • central nervous system • brain

There has been a long-standing interest in the mechanisms that contribute to the hypertension induced by pathophysiological levels of angiotensin (Ang II) in the circulation. Although circulating Ang II has sustained actions on the kidneys, vasculature, and adrenal glands that promote chronic hypertension, acute studies have also demonstrated that circulating Ang II can act in the central nervous system to increase sympathetic activity and arterial pressure.1 However, the relevance of these acute studies to the pathophysiological role of Ang II in hypertension has not been settled. In large part, this is because of technical limitations that prevent determination of both the long-term changes in sympathetic activity and the sustained influence of the sympathetic nervous system on renal excretory function.

It is well established that the kidneys play a critical role in the long-term regulation of arterial pressure.2 The few studies that have determined the temporal changes in renal sympathetic nerve activity and the resultant neurally-induced renal excretory responses during chronic Ang II infusion strongly indicate interactions between the renin-angiotensin and sympathetic nervous systems in the genesis of the hypertension.3-7 In direct opposition to the notion that the sympathetic nervous system contributes to Ang II hypertension, these studies indicate that suppression of renal sympathetic nerve activity and attendant increases in sodium excretion are sustained responses in Ang II hypertension.3-7 These findings therefore suggest that the sympathetic nervous system actually attenuates rather than contributes to the severity of Ang II hypertension. Furthermore, based on a recent study in chronically instrumented dogs, it seems that chronic renal sympathoinhibition in Ang II hypertension is mediated by baroreflexes.8 As it is well established that baroreceptors undergo rapid adaptation and resetting,8 this recent study is rather surprising. In fact, the implication of this study is that the baroreflex does not completely reset in chronic hypertension and, therefore, may play a role in the chronic regulation of arterial pressure. The primary objective of the present study was to use complementary methodology in chronically instrumented dogs to support the novel observation that there is sustained activation of the baroreflex in Ang II hypertension.

Over the last decade, the method of immunohistochemical labeling of Fos, the protein product of the immediate early gene c-fos, has been used widely to identify central neurons involved in the regulation of arterial pressure.9 Studies employing Fos immunohistochemistry have clearly identified...
both medullary neurons of the baroreflex and other central neurons that are activated in response to acute increases in arterial pressure induced by pressor agents including Ang II.9–12 However, relatively few studies have used this methodology to determine sites of neuronal activation in chronic hypertension. More specifically, Fos immunohistochemistry has not been used previously to identify excitatory and inhibitory neurons activated in chronic Ang II hypertension. Thus, in accordance with the results mentioned above indicating that the baroreflex accounts for chronic renal sympathoinhibition in Ang II hypertension, we hypothesized that this model of hypertension should be associated with sustained activation of neurons that are part of the central baroreflex pathway. To test this hypothesis, we determined the expression of Fos and Fos related antigens (Fos-Li) in the brain stem of dogs subjected to chronic Ang II infusion. In addition to emphasizing the activity of neurons comprising the baroreflex, we also examined Fos-Li immunoreactivity in central regions that have been previously reported to either mediate the acute neuroendocrine effects or the long-term hypertensive actions of circulating Ang II.13–16

Methods

Animal Preparation

Six female and 12 male dogs weighing 17 to 23 kg were used in this study. All procedures were in accordance with National Institutes of Health Guidelines and approved by the Institutional Animal Care and Use Committee. In all dogs, catheters made of Tygon microbore tubing were implanted in the lower abdominal aorta and inferior vena cava and exteriorized between the scapulae.9–12 The dogs were maintained in metabolic cages as described previously.5–7

Experimental Protocol

During a 2-week training and equilibration period, and throughout the study, the dogs were given free access to water and maintained on a fixed daily diet of two 15.5-oz cans of prescription heart diet (H/D; Hill’s Pet Products) supplemented with 5 mL of vitamin syrup. Two cans of H/D provide ~5 mmol of sodium and ~60 mmol of potassium. They were also continuously infused intravenously with isotonic saline at a rate of 350 mL/d. Thus, sodium intake was provided by both the food and the 24-hour saline infusion. This rate of Ang II infusion increases plasma levels of the peptide to ~5 times control.17

On the morning of the terminal day of the experimental period (Day 5), the dogs were placed in a recumbent position on the cage floor. While in a resting state, pentobarbital sodium (20 to 25 mg/kg) was quickly injected into a venous catheter to anesthetize the dogs. In Groups 1 and 3, pentobarbital sodium was injected after 1 hour in the recumbent position. In Group 2, a 1-hour control period and 2 hours of Ang II infusion preceded pentobarbital sodium administration. Subsequently, heparin (6000 U) and sodium nitrite (60 mg) were injected intravenously. Within a few minutes, the dogs were deeply anesthetized with additional pentobarbital sodium before opening the chest and cross-clamping the descending aorta. They were then perfused transcardially with 2 L of cold 0.1 mol/L phosphate-buffered saline (PBS) followed by 10 L of cold 4% paraformaldehyde in PBS. The brains were then removed and postfixed for several hours in 4% paraformaldehyde in PBS. Subsequently, they were cryoprotected in 30% sucrose in PBS solution for several days. Then, they were frozen and 6 series of 40-μm coronal sections were cut through the medulla and hypothalamus on a sliding microtome, and collected in PBS. The sections were stored in cryoprotectant at −20°C until group sections were stained for Fos-Li activity. During staining for Fos-Li activity, an equal number of dogs from each of the 3 groups were analyzed together. This eliminated the possibility that any group differences could be caused by different batches of immunological reagents.

Fos Immunocytochemistry

Free-floating sections from each brain were processed for Fos immunocytochemistry using an avidin-biotin peroxidase procedure as previously described.12 In the present study, a c-Fos polyclonal antibody (K-25, sc-253; Santa Cruz Biotechnology, Inc) was used which reacts with other Fos-related antigens including Fos B, Fra-1, and Fra-2. This antibody has been used previously to examine long-term changes in Fos-Li staining in hypertensive animals.18 The anti-Fos antibody was diluted 1:400 in PBS, and the sections were incubated in the primary antibody at 4°C for 72 hours. On completion of all reactions, the sections were placed on gelatin-coated slides, dried, and cover slipped.

Microscopic Analysis and Quantification

The sections were examined using light microscopy to identify Fos-Li stained cells in central neurons subserving the baroreflex, and in forebrain and hindbrain regions we believed might be activated during chronic Ang II hypertension. Specifically, the regions examined were the nucleus tractus solitarius (NTS), caudal ventrolateral medulla (CVLM), rostral ventrolateral medulla (RVLM), area postrema (AP), paraventricular nucleus (PVN), and supraoptic nucleus (SON). Digital images from all regions of interest were recorded using an Olympus IX50 microscope (Olympus American, Inc) and a Dage camera (ccd-72; Dage MTI) connected to a Pentium computer containing a frame grabber board (LG-3-PCI; Scion Corp). Fos-Li neurons were mapped and quantified using Scion Image software (version 3b).

Anatomical structures were identified using the atlas of Lim et al.19 Additionally, for the NTS, we emphasized regions that have been shown in the dog to contain cells sensitive to baroreceptor stimulation.20 The CVLM was defined as the region of the ventral brain stem between the nucleus reticularis ventralis, the nucleus reticularis, and the nucleus ambiguus. The RVLM was defined as a region ventromedial to the nucleus n. facialis, as described by Dormer et al.21–23 Images for each region were captured from at least two different sections from each brain with the exception of the NTS and ventrolateral medulla where 5 to 6 sections were used. Counts were taken unilaterally from each region. The numbers of Fos-Li positive cells, as indicated by black nuclear staining, were counted by observers who were blind to the experimental conditions of the dogs. In each dog, the number of Fos-Li cells per section for each brain region was calculated for statistical analysis.

Measurement of Urinary Electrolyte Concentration

Urine concentrations of sodium and potassium were determined by flame photometry (IL 943; Instrumentation Laboratories).

Measurement of Hemodynamics

Throughout the study, arterial pressure and heart rate were monitored continuously from an arterial catheter as previously described.5–7
Results

Hemodynamics and Urinary Electrolyte Excretion

The Table shows the daily values for hemodynamics and urinary electrolyte excretion. With the exception of urinary electrolyte excretion during the experimental period, the values presented in the Table are for the last day of the control and experimental periods. Because of incomplete urine collection on the morning of the terminal experiment, the values for urinary electrolyte excretion are for the preceding day (Day 4 of the experimental period). With the exception of the increased MAP during chronic Ang II infusion, there were no significant differences in MAP, heart rate, urinary sodium excretion, or urinary potassium excretion either between or within groups. In Groups 1 and 2, urinary electrolyte excretion equaled intake and hemodynamic values were stable throughout the control and experimental periods. This indicates that steady state conditions were achieved at the time of the terminal experiment. Further, based on our earlier studies, we also expected virtually stable hemodynamics and body fluid volumes by Day 5 of Ang II infusion in Group 3 dogs. As in our previous studies, marked sodium retention occurred on Days 1 to 2 of Ang II infusion before sodium excretion returned to control levels at an elevation in MAP of 35±3 mm Hg on Days 4 and 5. After Day 5 of Ang II infusion, there is little further change in either sodium balance or MAP.

Figure 1 illustrates 30-minute hemodynamic values during the morning sessions when the dogs were resting quietly. The last control and experimental days are shown for each of the three groups of dogs. The last set of values for each group represents the average MAP and heart rate for the 30-minute period immediately preceding anesthesia and subsequent brain perfusion. By comparing Figure 1 with the Table, one can see that the values for MAP and heart rate during these morning sessions were very similar to the values calculated from the 24-hour hemodynamic recordings. Additionally, in Figure 1 the last set of values for the acute Ang II group shows that MAP was elevated 22±3 mm Hg (control MAP just before Ang II infusion=101±3 mm Hg) after 90 to 120 minutes of Ang II infusion. In comparison, during the morning sessions MAP was 37±3 mm Hg higher than control when Ang II was infused chronically. The difference in the MAP response to acute and chronic Ang II infusion was statistically significant.

Fos-Li Immunoreactivity in the Medulla

As illustrated by the photomicrographs of the NTS in Figure 2, cells that exhibited Fos-Li immunoreactivity had darkly stained nuclei. In the NTS, as in other regions of the medulla, there were few Fos-Li positive cells in control dogs (Figure 2A). In contrast, dogs subjected to either acute or chronic Ang II infusion exhibited distinctly more Fos-Li positive cells.
(Figure 2B and 2C) than controls. These cells were located in regions of the NTS that have been previously reported to contain baroreceptor sensitive neurons.20

Figure 3 summarizes the number of Fos-Li neurons/section in various regions in the medulla, in control dogs and in dogs infused either acutely or chronically with Ang II. Values are mean±SEM (n=6 for each group). *P<0.05 versus control period. AP indicates area postrema; NTS, nucleus tractus solitarius; CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla.

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pathway. Acute infusion of Ang II at a rate that increased MAP 22±3 mm Hg resulted in a 2- to 3-fold increase in the number of Fos-Li cells in the NTS and CVLM, but no significant change in the number of Fos-Li cells in the RVLM. Most importantly, this same pattern of Fos-Li expression was sustained during the hypertension produced by chronic Ang II infusion. In addition, during both acute and chronic Ang II infusion there was also a significant increase in the number of Fos-Li cells in the AP. In fact, there were no significant differences in Fos-Li staining in any of the above medullary neurons during acute and chronic Ang II infusion.

**Fos-Li Immunoreactivity in the Hypothalamus**

As illustrated in Figure 4, Ang II infusion also increased Fos-Li staining in hypothalamic nuclei. During acute infusion of Ang II there was a significant increase in the number of Fos-Li cells in the SON, as well as in the parvocellular, but not in the magnocellular neurons of the PVN. Further, there was even greater Fos-Li staining in the PVN during chronic infusion than acute Ang II infusion. Only during chronic Ang II infusion did the increase in the number of Fos-Li cells in magnocellular neurons of the PVN reach statistical significance. However, in parvocellular neurons there was a further
Discussion

Although a number of studies indicate that endogenous Ang II stimulates the sympathetic nervous system in hypovolemic and/or hypotensive states, it is controversial whether the sympathetic nervous system contributes to the hypertension induced by circulating Ang II.\textsuperscript{1,15,23} On one hand, it may be argued that arterial baroreflexes and/or cardiac reflexes chronically oppose the sympathoexcitatory actions of Ang II. On the other hand, this contention is inconsistent with the notion that baroreceptors undergo rapid adaptation and resetting and, therefore, would be unable to participate in long-term regulation of sodium excretion and arterial pressure.\textsuperscript{2,8,23} It should be emphasized, however, that because of inherent difficulties in achieving chronic nerve recordings which faithfully provide accurate quantitative time-dependent changes in sympathetic activity, it is unknown to what extent baroreflex control of sympathetic activity may adapt in chronic hypertension. A recent study in chronically instrumented dogs provides strong support for the hypothesis that baroreflexes do not totally reset in chronic Ang II hypertension and account for chronic renal sympathoinhibition.\textsuperscript{6} In this regard, the results of the present study are both consistent with and complimentary to this recent investigation in that they demonstrate that there is sustained activation of neurons in the central baroreflex pathway during chronic Ang II hypertension.

Because numerous acute studies have shown that circulating Ang II stimulates sympathetic activity by both central and peripheral actions,\textsuperscript{1} there has been considerable interest in the possibility that the sympathetic nervous system may play a role in mediating Ang II hypertension. However, there is little evidence from chronic studies to support this possibility.\textsuperscript{1,15,23} In fact, recent findings in chronically instrumented animals suggest that the sympathetic nervous system may actually attenuate the hypertension induced by high circulating Ang II.\textsuperscript{1,3-7,21} These studies clearly indicate that renal sympathetic nerve activity is chronically suppressed in Ang II hypertension and that this response is critically dependent on the integrity of the baroreflex.

To support the contention that the baroreflex may account for sustained renal sympathoinhibition in Ang II hypertension, we used Fos methodology to determine whether there is sustained activation of central neurons involved in the baroreflex.\textsuperscript{7} This methodology is based on the principle that stimulation of neurons results in the expression of immediate early genes, including \(c\text{-}fos\), which in turn leads to the production of transcriptional regulatory proteins, including Fos. Fos and Fos related proteins can be measured by immunohistochemistry and, therefore, quantification of Fos-Li staining can serve as a convenient marker for neuronal activation. One advantage of this methodology over the use of electrophysiological techniques is that it can be used to map a large number of activated neurons rather than the activity in individual neurons. Another important advantage is that Fos methodology can be used in conscious animals and, therefore, circumvent the confounding influence of anesthesia and surgical stress on neuronal activation. To reduce the possible effects of excitement on Fos-Li expression in the present study, experiments were conducted under resting conditions and only after the dogs were thoroughly conditioned to their home environment. Further, to minimize the potential influence of variations in body fluid volumes and arterial pressure on neuronal activation, independent of the effects of Ang II infusion, experiments were initiated only after steady-state conditions were achieved, as reflected by sodium balance and hemodynamic values that were stable from day to day. The low levels of Fos-Li immunoreactivity in the control group indicates that these experimental concerns were adequately addressed. Finally, because Fos expression in response to a number of stimuli seems to be rather transient, we were uncertain whether measurement of Fos alone would be an appropriate marker for neuronal activation in the present chronic study. In contrast to Fos, the expression of Fos-related proteins persists during chronic neuronal activation.\textsuperscript{18,24} Therefore, we used an antibody that reacts not only with Fos, but with Fos family proteins. This antibody has been used previously to identify central neurons activated in hypertension.\textsuperscript{18}

Our study is the first to determine the activity of neurons in the central baroreceptor pathway during chronic Ang II hypertension. It is well established that spinally-projecting neurons in the RVLM provide tonic excitatory drive to sympathetic preganglionic neurons that control sympathetic output to the heart and blood vessels.\textsuperscript{14} It is also recognized that activation of neurons in the NTS, the site of termination of baroreceptor inputs, and the subsequent activation of inhibitory neurons in the CVLM, plays an important role in mediating baroreflex inhibition of sympathoexcitatory cells in the RVLM. In the present study, the increase in Fos-Li staining in cells in the NTS and CVLM during acute infusion of Ang II is entirely consistent with the above concept. Further, as Fos-Li expression is a marker of neuronal activation rather than inhibition, the absence of a change in the number of Fos-Li cells in the RVLM is also an expected finding. The above pattern of Fos staining has been reported previously in both rats and rabbits during acute elevations in arterial pressure induced by either Ang II or phenylephrine infusion.\textsuperscript{10-12} These studies have also shown that sinoaortie baroreceptor denervation abolishes these responses, confirming the critical role of baroreceptor input in mediating these central changes in neuronal activity when arterial pressure is acutely increased. However, the single most important and novel finding in the present study is that this same activation pattern of central baroreceptor neurons persists during chronic Ang II hypertension. This suggests that there is sustained activation of the baroreflex during chronic Ang II hypertension and provides complementary data to support the contention that baroreflex-mediated renal sympathoinhibition is a long-term compensatory response in this and in other forms of hypertension.\textsuperscript{23}

Acute studies have demonstrated that circulating Ang II also acts directly on the central nervous system to elicit a
number of neuroendocrine responses, in addition to indirectly activating neurons of the central baroreflex pathway by increasing blood pressure.1,14–16 These neuroendocrine responses include activation of the sympathetic nervous system and stimulation of vasopressin secretion. However, the central sites of action and the physiological significance of these direct neural effects of Ang II are unclear. The neuroendocrine responses to circulating Ang II are achieved by activation of neurons in circumventricular organs, including the area postrema, and involve hypothalamic regions that regulate autonomic outflow and neuroendocrine function. Unfortunately, it was not technically possible for us to quantify Fos-Li labeling in all of the central regions mediating these effects. We did, however, determine Fos-Li immunoreactivity in the PVN and SON of the hypothalamus. These nuclei are in areas of the forebrain that play an important role in mediating the neuroendocrine effects of circulating Ang II.1,14–16

In the present study there was a significant increase in Fos-Li staining in both the PVN and SON during acute infusion of Ang II infusion. Similar findings have been reported in both intact and baroreceptor denervated rabbits.11 Moreover, the current study also showed that Fos-Li staining in the parvocellular neurons of the PVN increased even further during chronic Ang II infusion. While the explanation for this response is unclear, this is a particularly intriguing finding because cells of the PVN provide substantial input into cell groups regulating sympathetic activity.14 Activation of the PVN has been reported to both increase and decrease sympathetic activity and arterial pressure. On one hand, the especially high degree of Fos staining during chronic Ang II hypertension may reflect a sustained sympathoexcitatory effect of Ang II, as there is some evidence that the integrity of the PVN is essential for the production of some forms of neurogenic hypertension.14 On the other hand, other studies indicate that reflex activation of the PVN inhibits renal sympathetic nerve activity and plays a role in the defense of body fluid volumes during acute volume expansion.24,25 Thus, activation of this reflex pathway, if sustained, could contribute to the suppression of renal sympathetic nerve activity associated with chronic Ang II hypertension.

The increments in Fos-Li staining in the magnocellular neurons of the PVN and SON during acute Ang II infusion were quantitatively similar to those reported in both intact and baroreceptor denervated rabbits subjected to a 1 hour infusion of Ang II.11 However, the magnitude of the increase in Fos staining in these cells during Ang II infusion is vastly smaller than that which occurs in response to a moderate degree of hypotension,10 a known stimulus for vasopressin and oxytocin secretion. Furthermore, in the dog, infusions rates of Ang II (including the rate employed in the present study) producing physiological increments in circulating levels of Ang II do not increase plasma vasopressin concentration either acutely or chronically.17,24 Therefore, if the acute and chronic increase in Fos-Li staining in the magnocellular cells of the SON and PVN reflects activation of vasopressinergic neurons, it would seem that the degree of activation was insufficient to increase plasma levels of the hormone. An alternate and interesting possibility is that the increase in Fos-Li activity in these neurons reflected acute and sustained activation of oxytocinergic cells. As continuous infusions of oxytocin that produce physiological levels of the hormone increase sodium excretion,27 a persistent increase in oxytocin secretion could provide a compensatory mechanism, other than suppression of renal sympathetic nerve activity, to attenuate Ang II hypertension. However, to our knowledge, it is not known whether oxytocin levels are elevated in chronic Ang II hypertension.

Cells in the AP, like the parvocellular neurons in the PVN, are a source of afferent input into neural centers regulating sympathetic activity.1,14–16 Further, the AP seems to be an important central site at which circulating Ang II acts to increase sympathetic activity.1,14–17 A finding consistent with increased Fos-Li expression in the AP during acute Ang II infusion in the present and in an earlier study.11 A novel finding in the present study is that there is a sustained increase in Fos-Li staining in the AP during chronic Ang II hypertension, a response which could reflect a persistent central sympathoexcitatory influence of Ang II. However, it should be recognized that even if Ang II were to have a sustained effect on the AP to stimulate sympathetic activity, this action in itself would be insufficient to promote hypertension. This is because the baroreflex suppresses renal sympathetic nerve activity in Ang II hypertension, a response expected to attenuate rather than contribute to hypertension.2,23 A central action of Ang II at the AP, however, could account for the sustained increase in renal sympathetic nerve activity associated with chronic Ang II hypertension. Therefore, sympathoexcitatory actions of Ang II mediated via activation of neurons in the AP, PVN, or other central sites could contribute to hypertension if baroreflex suppression of renal sympathetic nerve activity were to wane under more chronic conditions, due either to chronic baroreflex resetting or to progressive baroreflex dysfunction.

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