Bidirectional Transport of Angiotensin II Binding Sites in the Vagus Nerve

DEBRA I. DIZ AND CARLOS M. FERRARIO

SUMMARY We previously showed that specific angiotensin II (Ang II) binding sites are present in the canine nodose ganglion and peripheral vagus nerve, and that unilateral removal of the nodose ganglion results in loss of binding in the ipsilateral nucleus tractus solitarii and the dorsal motor nucleus of the vagus. An association of Ang II binding sites with both afferent and efferent vagal fibers is consistent with actions of the peptide on cardiac vagal tone and the baroreceptor reflex. To investigate possible transport of Ang II binding sites, quantitative in vitro receptor autoradiography was used to visualize binding after double ligation of the peripheral process of the cervical vagus nerve. One ligature was tied 0.2 to 0.5 cm distal to the nodose ganglion; the second ligature was tied on the same nerve 1.0 to 1.5 cm from the nodose ganglion. Twenty-four hours later, high-affinity Ang II binding sites ($K_i = 0.46 \pm 0.08$ nM) accumulated at the first ligature (the side nearest the nodose ganglion), indicating anterograde transport. Since accumulations of similar affinity sites were seen distal to the second ligature, retrograde transport of binding sites also occurred. These data reveal the existence of a mechanism for the bidirectional axonal transport of Ang II binding sites in the cervical portion of the vagus nerve. (Hypertension 11 [Suppl I]: I-139–I-143, 1988)

KEY WORDS • axonal transport • cardiovascular reflexes • brainstem • nodose ganglion

EARLY observations that baroreceptor reflex resetting occurred during renal hypertension provided a link between angiotensin II (Ang II) and modulation of central nervous system control of mean arterial pressure. In recent years, it became clear that even short-term infusions of Ang II inhibit the baroreceptor reflex, since increases in pressure produced by the peptide are not accompanied by reflex decreases in heart rate. Previous studies suggested that the medulla oblongata is a primary site for the inhibitory action of Ang II on baroreceptor reflex control of heart rate. Specifically, Ang II binding sites were shown to exist in the area postrema, nucleus tractus solitarii (NTS), and dorsal motor nucleus of the vagus (DMNX). These findings are consistent with site-specific, dose-dependent actions of the peptide at these nuclei. However, our most recent investigations revealed that Ang II binding sites were also present extracranially in the canine nodose ganglion and peripheral process of the vagus nerve. These experiments also showed that unilateral removal of the nodose ganglion causes the disappearance of Ang II binding sites in the NTS and DMNX, while cervical vagotomy causes a selective loss in the DMNX. These data indicate that Ang II binding sites in the medulla oblongata are dependent upon an intact vagal system. In addition, the data established a relationship between Ang II receptors in the peripheral and central nervous systems.

Recently, there has been increasing evidence for transport of peptide and other transmitter receptors in the vagus nerve, at times associated with bound ligand. Since information on the translocation of putative receptors can provide important information on the possible cellular functions of these proteins, we assessed the translocation of Ang II binding sites in the cervical vagus nerve in order to gain new insights into the possible mechanisms by which Ang II influences the baroreceptor reflex.

Materials and Methods

Eight male mongrel dogs (8–15 kg) were premedicated with morphine sulfate (2 mg/kg intramuscularly) and anesthetized with 2% halothane. Either the right ($n = 4$) or left ($n = 4$) cervical vagus nerve was exposed at the level of the nodose ganglion and isolated from the sympathetic nerve bundle. Silk sutures (3-0) were placed around the isolated cervical vagus nerve; care was taken not to tie any vessels present in the vagal sheath. One ligature (LIG1; Figure 1A) was placed on
FIGURE 1. A. Longitudinal 20-μm section through the nodose ganglion (NG) and peripheral process of the cervical vagus nerve (X) stained with cresyl violet. Upper section is from a double-ligated (LIG1 and LIG2) nerve and the lower section is through the contralateral intact control nerve from the same dog. B. Autoradiographic image generated from the same section as in Panel A after incubation with 0.8 nM ¹²⁵I-Ang II. Arrows indicate the seven regions in the ligated nerve (upper section) and two regions in the contralateral control nerve that were evaluated for binding. Accumulations of specific Ang II binding sites were consistently seen in segment 1 immediately above (to the left of) LIG1 and in segment 3 immediately below (to the right of) LIG2. Uniform densities of binding sites were seen in the process of the control nerve. C. Nonspecific binding in a section adjacent to the one in Panel A incubated with 0.8 nM ¹²⁵I-Ang II in the presence of 1 μM unlabeled Ang II. (Bar = 2 mm; magnification is the same for all three panels.)

the vagus nerve 0.2 to 0.5 cm distal to the nodose ganglion. A second ligature (LIG2) was placed 1.0 to 1.5 cm distal to the nodose ganglion on the same nerve. For ease of reference, the portion of the ligated nerve above LIG1 (containing the nodose ganglion) was designated as Segment 1, the intermediate portion between the two ligatures as Segment 2, and the remaining portion of the peripheral vagal nerve process as Segment 3. Twenty to 24 hours later the dogs were deeply anesthetized with sodium pentobarbital (35 mg/kg intravenously), and their heads were perfused with cold phosphate-buffered saline containing 0.04% formalin. Both the intact control (contralateral) and the ligated vagus nerves (about 3 cm in length with the nodose ganglion attached) were removed from each dog and frozen on dry ice. Longitudinal sections (14–20 μm) were cut on a cryostat, mounted onto chrom-alum-coated slides, and stored at −70°C for less than 1 week.

Ang II binding was determined using an in vitro autoradiographic technique as described previously. Slide-mounted serial sections were preincubated for 30 minutes in 35 mM sodium phosphate buffer (25°C) containing 2.5 mM dithiothreitol, 2.5 mM EDTA, 75 mM NaCl, 5 mM MgCl₂, and 0.5% bovine serum albumen. Sections were then incubated for 45 minutes in the same buffer to which moniodinated ¹²⁵I-Ang II (New England Nuclear, Boston, MA, USA) was added in the range of 0.01 to 3.0 nM for quantitative (n = 4) and at 0.5 nM for qualitative analyses (n = 4). Non-specific binding was determined in adjacent sections in the presence of excess (1 μM) unlabeled Ang II (M. Khosla, Cleveland, OH, USA). Labeled sections were rinsed in fresh buffer at 4°C, dipped in cold water, dried under a cooled and desiccated stream of air, and apposed to SB-5 x-ray film (Kodak, Rochester, NY, USA) in cassettes for 2 to 6 days. Aliquots of the incubation mixture were analyzed by high performance liquid chromatography to demonstrate purity of the ligand initially (99%) and to assess degradation during incubations. Because the ¹²⁵I-Ang II remained intact during incubation (less than 3% peptide fragments or free iodine was observed), no correction factor was necessary to determine free ligand concentrations for data analysis.

Film images generated from the sections of nerves and ganglia of four dogs, after incubation with seven concentrations of ¹²⁵I-Ang II, were quantitated on a Zeiss IBAS II microdensitometer (Zeiss, San Antonio, TX, USA) according to the procedures developed by Israel, Plunkett, and Saavedra for Ang n, and used by us previously to provide quantitative analysis of binding sites in the canine medulla oblongata. Two determinations were made in each animal at each of the seven concentrations of ¹²⁵I-Ang II. Densities of binding sites in the nerve segments are expressed as femtomoles of Ang II per milligram protein as determined from ¹²⁵I brain paste standards. Saturation isotherm binding data were analyzed by Scatchard plots to determine the binding affinity (Kᵣ) and density (B₉₅₀), and by Hill plots to assess homogeneity of binding sites.
Statistical analysis of differences in \( K_t \) or \( B_{\text{max}} \) values was performed using analysis of variance (ANOVA) for repeated measures, followed by contrasts of individual means or paired \( t \) tests when the ANOVA yielded a significant \( F \) value. Significance levels \((p)\) were adjusted for multiple comparisons.

Results

Analysis of autoradiograms from the intact control nerves confirmed our previous findings of specific Ang II binding sites overlying the cell body region of the nodose ganglion and in the cervical portion of the peripheral vagus nerve process (see Figure 1B). Quantitative measurements revealed a single population (Hill coefficient [slope] = 0.96 ± 0.06) of high-affinity binding sites in the nodose ganglion (Table 1). Although binding sites in the nerve process had a similar affinity to those in the nodose ganglion, there appeared to be a slight heterogeneity of binding sites in this portion of the nerve since the Hill coefficient for binding was slightly less than 1 (0.87 ± 0.07). 13

The ligated nerves from all eight dogs were evaluated qualitatively for binding in seven areas (labeled in Figure 1B). Discrete accumulations of specific Ang II binding sites were observed with regularity in Segment 1 immediately above LIG1 and in Segment 3 immediately below LIG2. It was also possible to visualize a build-up of binding sites within the intermediate portion (Segment 2) of the nerve immediately adjacent to each ligature. Moderate to low specific binding was also observed in the middle portion of Segment 2, and in Segment 3 in the peripheral vagal nerve process.

Quantitative characterization of binding in four regions of the ligated nerves from four dogs revealed a single population (Hill coefficient approximately 1) of Ang II binding sites with similar affinities (see Table 1). The tendency was for a lower-affinity value in the peripheral process of the ligated nerve and a Hill coefficient slightly less than 1 (0.84 ± 0.03). The \( B_{\text{max}} \) was similar in the intact contralateral control and ligated nodose ganglion and vagus nerve process. Approximately 50 to 150% higher densities were consistently observed for the areas above LIG1 and below LIG2 compared with those observed in the peripheral process of the normal nerve. Nonspecific binding in the above regions ranged from 18% in the area above LIG1 to 39% in the ligated nerve process. In the midportion of Segment 2, nonspecific binding averaged 65%, and in two of the four animals in which binding was quantitated, no specific binding was present.

Discussion

The present findings confirm our previous demonstration of the existence of high-affinity Ang II binding sites in the nodose ganglion and peripheral and central processes of the vagus nerve. 6 The affinity of the binding site present in both the control and ligated nodose ganglion is similar to that reported by us in the canine medulla oblongata \((K_t \sim 0.6 \text{ nM})\). Recent studies indicated that these medullary Ang II binding sites required the integrity of the vagus nerves, and the data obtained in the present experiments demonstrate that the extracranial portion of the vagus nerve contains a population of Ang II binding sites with an affinity constant identical to that determined in the dorsal medulla. 6

More important, these data are the first demonstration of the bidirectional translocation of Ang II binding sites in the cervical vagus nerve. The accumulation of binding sites above LIG1 indicates transport in the anterograde direction (away from the ganglion or cell body), while the accumulation of binding sites below LIG2 indicates retrograde transport. Quantitative analysis of the binding sites revealed that the build-up of sites in both the anterograde and retrograde directions are of similar affinity. Because the peripheral process of the vagus nerve contains both motor and sensory fibers, further studies are necessary to determine whether transport occurs selectively in afferent or efferent fibers or in a combination of both. However, bidirectional transport is consistent with the concept of synthesis of the receptor in the cell body, transport to a site of action, and return to the neuronal cell body for reprocessing or metabolism. 7-10

The demonstration that Ang II binding sites undergo transport in the vagus nerve is consistent with the observations by others of translocation of peptide and transmitter receptors in the vagus, sciatic, splenic, and splanchnic nerves of several species. 7-10 Although our present observations indicate that Ang II binding sites are bidirectionally transported, only anterograde transport was reported for cholecystokinin receptors. 7 The bidirectional transport of cholinergic muscarinic and opiate receptors has also been reported; however, the muscarinic binding sites moving in the retrograde direction were shown to possess a lower affinity than sites moving anterogradely. 7-10 Our observations do suggest heterogeneity of binding sites in the peripheral process of both control and ligated nerves; therefore, we cannot rule out the possibility that studies of Ang II binding sites at different times may reveal multiple affinity states. It has been suggested that the directionally different affinity states represent stages in the life cycle of a receptor, such that a high-affinity site leaves

### Table 1. Affinity \( (K_t) \) and Maximum Binding Capacity \( (B_{\text{max}}) \) for \( ^{125}\text{I}-\text{Ang II} \) Binding in the Nodose Ganglion and Vagus Nerve

<table>
<thead>
<tr>
<th></th>
<th>( K_t ) (nM)</th>
<th>( B_{\text{max}} ) (fmoles/mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodose ganglion</td>
<td>0.65 ± 0.06</td>
<td>74 ± 22</td>
</tr>
<tr>
<td>Peripheral process</td>
<td>0.83 ± 0.26</td>
<td>46 ± 18</td>
</tr>
<tr>
<td>Ligated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodose ganglion</td>
<td>0.83 ± 0.26</td>
<td>76 ± 20</td>
</tr>
<tr>
<td>Above LIG1</td>
<td>0.46 ± 0.09</td>
<td>112 ± 14*</td>
</tr>
<tr>
<td>Below LIG2</td>
<td>0.53 ± 0.08</td>
<td>88 ± 16</td>
</tr>
<tr>
<td>Peripheral process</td>
<td>1.96 ± 0.03</td>
<td>76 ± 30</td>
</tr>
</tbody>
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Values are means ± SEM for three to four dogs per area. At least two determinations at each of four to six concentrations were made in each dog. Regression coefficients for Scatchard plots averaged 0.89 ± 0.05.

*Significant difference when compared with the corresponding value in the control nerve process \((p < 0.05)\).
the sensory ganglion or motor neuron for the site of action in the nerve process, while a low-affinity site reflects receptors destined for degradation or recycling. The physiological significance of the similar affinity states for bidirectionally transported Ang II binding sites found in our studies is not yet known. However, that a returning site maintains a high affinity is consistent with possible transport of bound ligand after uptake and internalization, as we suggested previously.

The relative lack of specific binding in the intermediate portion of Segment 2 between the two ligatures indicates that most of the Ang II binding sites appear to be undergoing transport and are not stationary in the nerve. Again, this finding differs from that of Zarbin et al., who found that most cholecystokinin, opiate, and muscarinic receptors were stationary in the vagus nerve. Since it is well known that cholinergic agents have direct actions on vagal axons, a distinction between Ang II and cholinergic receptors in this instance may reflect differences in function of the binding proteins. However, no studies to date have evaluated the direct effects of Ang II on vagal afferent or efferent axons.

A further implication of the present findings is that transport occurs in an isolated segment of the nerve, since specific binding sites tended to accumulate in Segment 2 both below LIG1 and above LIG2. Previous studies documented that only fast axonal transport occurs in an isolated nerve segment and double ligation of a nerve effectively isolates the segment of the nerve to the peripheral side of the ligatures. Thus, the present findings also are consistent with a fast axonal transport mechanism for the Ang II binding sites. Since other receptor proteins have been reported to move by fast transport, the data further support the concept that the Ang II binding sites are functional receptors.

The physiological significance of the axonal location of Ang II binding sites has not been studied yet. We previously reported that Ang II binding sites in the medulla exhibit characteristics of functional receptors, since site-specific and dose-dependent actions were demonstrated for the exogenous application of Ang II in the NTS and DMNX. The direct actions of Ang II in the NTS may be due to presynaptic actions of the peptide on vagal afferent fibers, since we have documented an association between Ang II receptors in the NTS and vagal sensory afferent fibers. In addition, the actions of the peptide in the DMNX are likely to be postsynaptic on vagal motor neurons, since vagotomy abolishes the effects of Ang II injection in this site and Ang II binding sites were shown to be associated with vagal motor neurons in the DMNX.

More recently, experiments by Campagnole-Santos et al. provided evidence for a role for endogenous Ang II in baroreceptor reflex control of heart rate since NTS injections of the Ang II antagonist [Sar1,Thr1]Ang II facilitated the baroreceptor reflex. These studies extended the observations of Casto and Phillips that NTS infusions of Ang II attenuate baroreceptor reflex control of heart rate. Because the effects of local application of either Ang II or its antagonist produce effects similar to those following peripheral infusion, the above data indicate that the NTS is a site of action for the effects of both endogenous and exogenously administered Ang II (of either central or peripheral origin) on the baroreceptor reflex control of heart rate. However, these studies do not rule out the additional possibility that sites along the vagus nerve or nodose ganglion also participate in the effects of Ang II on cardiovascular reflex function. In fact, while endogenous NTS Ang II may act at sensory afferent nerve terminals or motor neurons in the medulla, the present findings suggest that peripherally administered or endogenously circulating Ang II may modify the activity of sensory and motor systems at multiple sites. Finally, uptake and internalization of the peptide from the peripheral circulation by binding sites in the vagus may occur in both sensory and motor systems as well. This mechanism may function as part of an intraneuronal transport of peptides to the cell body or even the central process of the pseudounipolar sensory neurons.

Acknowledgment
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