AT₁ Antisense Distinguishes Receptors Mediating Angiotensin II Actions in Solitary Tract Nucleus

Debra I. Diz, Brian Westwood, David B. Averill

Abstract—Angiotensin (Ang) II receptors in the solitary tract nucleus (nTS) are located on vagal sensory-afferent fiber terminals as well as on neuronal cell bodies. Results from in vitro slice preparations indicate that ≈50% of the neuronal excitatory actions of Ang II result from actions at presynaptic receptors. The differential contribution of actions on fiber terminals versus neuronal cell soma to the cardiovascular effects of Ang II in the nTS is not known. We used antisense oligonucleotides to the angiotensin type 1 (AT₁) receptor, which should reduce receptors on neurons within the injection site but not those on fiber terminals projecting to the nTS. Ang II injections (250 fmol/30 nL) into the nTS reduced blood pressure by 14±1 mm Hg and heart rate by 13±1 bpm (n=8) in male Sprague-Dawley rats anesthetized with chloralose/urethane. Although there was still a significant fall in pressure that was induced by Ang II at 90 and 150 minutes after AT₁ antisense (164 pmol/120 nL) was injected into the nTS, the response was blunted 50% (P<0.01). Heart rate responses were completely blocked at the 150-minute time point. Scrambled sequence oligonucleotides did not alter Ang II responses at any time. There was a 40% reduction in ¹²⁵I[Sar¹ Thr⁸]-Ang II binding when antisense-injected and noninjected sides of the nTS were compared with receptor autoradiography. This finding is consistent with the continued presence of AT₁ receptors on afferent fibers. This unique strategy illustrates that both presynaptic fiber terminals and nTS neurons are involved in the blood pressure lowering actions of Ang II, whereas heart rate responses are largely due to actions directly on nTS neurons and activation of vagal efferent pathways. (Hypertension. 2001;37:1292-1297.)

Key Words: solitary tract nucleus □ medulla oblongata □ circulation □ angiotensin II □ microinjections □ oligodeoxynucleotides, antisense □ receptors, angiotensin

Angiotensin (Ang) II receptors are present on vagal sensory-afferent fiber terminals in the nucleus of the solitary tract (nTS), consistent with the known localization of receptors on neuronal cell bodies in the nodose ganglion.¹ ² In addition, neurons in the medial nTS and in the dorsal motor nucleus of the vagus (dmnX) also express Ang II receptors.¹ ⁶ While it is not known whether the hemodynamic actions of Ang II are mediated by actions at fiber terminals or neuronal cells within the nTS, electrophysiological evidence supports actions at both sites. Ang II elicits excitation of nodose ganglion neurons⁵ ⁶ as well as neurons of the medial nTS and the dmnX.⁹ Other studies lend support to the idea that ≈50% of the responses to Ang II in the nTS may be a result of actions at presynaptic receptors.¹⁰ ¹¹ In fact, unilateral nodose ganglionectomy results in a 36% to 48% reduction in the density of Ang II receptors in the nTS of rats.¹² Antisense oligodeoxynucleotides have been used to examine the role of Ang II in neural control of the cardiovascular system. Recently, we showed that local paraventricular nucleus administration of angiotensin type 1 (AT₁) receptor antisense oligonucleotides could reverse the excess hypertension caused by drinking hypertonic sodium chloride for 4 days in (mRen2)27 transgenic rats.¹³ A unique feature of using antisense techniques to block receptors is that distinct receptor populations (those on cell bodies versus those on nerve terminals) can be targeted. The antisense oligomer is taken up locally to interfere with synthesis of receptors in cell bodies at the injection site. However, receptors synthesized at remote locations and transported to the site via cells with fiber terminals projecting to the area are spared. Thus, unlike receptor antagonists that block all receptors in the area of the injection, antisense treatment affords a window of selectivity. We took advantage of this ability of AT₁ antisense oligonucleotides to decipher whether exogenous Ang II produces its depressor and bradycardic effects in the nTS through receptors made in local nTS neurons or those synthesized elsewhere (ie, vagal afferent neurons of the nodose ganglion).

Methods

Animals Experiments were performed in male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Ind [n=6 antisense and n=3 scrambled] and Hannover Sprague-Dawley rats from the Hypertension and Vascular Disease Center Colony, Wake Forest University School of Medicine, Winston-Salem, NC. Animals were provided by Harlan Sprague-Dawley and the Hypertension and Vascular Disease Center. Animals were housed under conditions of constant temperature and 12-hour light-dark cycle. Food and water were available ad libitum. Animals were fasted for 18-24 hours before experiments. Experiments were performed in male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Ind [n=6 antisense and n=3 scrambled] and Hannover Sprague-Dawley rats from the Hypertension and Vascular Disease Center Colony, Wake Forest University School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157-1032. E-mail ddiz@wfubmc.edu

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School of Medicine, Winston-Salem, NC [n=2 antisense and n=2 scrambled]; 9 to 12 weeks of age). Rats were housed in group cages with free access to standard rat chow and water in a temperature- and humidity-controlled room (12:12 hour light:dark cycle). Anesthesia was induced by urethane/chloralose (750 mg/35 mg per kg, intraperitoneal administration) with supplemental doses given intravenously. Catheters were inserted into a femoral artery and vein. Rats were placed in a stereotaxic frame with the head flexed downward at 45° for exposure of the dorsal medulla via atlanto-occipital membrane incision. The pipette was placed into the nTS (0.4-mm rostral and 0.4-mm lateral to calamus scriptorius, and 0.4- to 0.6-mm below the dorsal surface). Arterial pressure was monitored by a strain gauge transducer (Model DTX, Spectramed, Inc) connected to the femoral arterial catheter. Heart rate was determined from the arterial pressure wave. Pressure and heart rate were digitized and recorded with a computerized acquisition system as published previously.\(^{14,15}\) Depth of anesthesia throughout the experiment was monitored by corneal reflexes, respiratory rhythm, arterial pressure, and heart rate stability.

### AT\(_{1}\) Antisense and Scrambled Sequence Oligodeoxynucleotides

In this study, we used the same sequence oligomers as in a previous report.\(^{13}\) The sequence of the 15-mer antisense to the AT\(_{1}\) receptor was 5’-AGAGTTAAGGGCCAT-3’. The scrambled sequence used as a control was 5’-CCCTTTGAAGGTTCC-3’. The DNA Synthesis Core Laboratory of the Comprehensive Cancer Center of the Wake Forest University School of Medicine synthesized both oligomers.

### Protocol

Microinjections of 30 to 60 nL were made with either 3- or 4-barreled glass micropipettes (50- to 100-μm outer diameter) over a 40- to 60-second interval, as described previously.\(^{14-17}\) Ang II (250 fmol, Bachem) was injected into the nTS in a volume of 30 nL to locate depressor sites. A second Ang II injection was given several minutes before a 120-nL injection of AT\(_{1}\) receptor antisense (n=8) or scrambled sequence (n=5) oligodeoxynucleotides in artificial cerebrospinal fluid. The oligomers were given at a dose of 164 pmol/120 nL based on our previous studies.\(^{13}\) Arterial blood pressure and heart rate responses to Ang II injections were repeated ~45, 90, and 150 minutes after injection of the oligomer.

### Ang II Receptor Autoradiography

The medulla oblongata was removed at the end of the experiment from rats that received scrambled sequence or AT\(_{1}\) receptor antisense oligonucleotide injections. The tissue was frozen and sectioned. Consecutive adjacent 14-μm sections were preincubated in a sodium phosphate buffer that contained 2.5 mmol/L EGTA, 5 mmol/L MgCl\(_2\), and 0.5% bovine serum albumin. The sections were then incubated with 0.5 mmol/L \(^{125}\)I-Sarcosine, \(^{123}\)Threonine, \(^{125}\)Ang II (Sarthran) in the same buffer. The presence of 5 pmol/L losartan, 5 pmol/L PD123319, or the combination of the 2 competitors was used to determine the proportion of AT\(_{1}\) or AT\(_{2}\) receptors and nonspecific binding, respectively.\(^{16,17,18}\) Slides were rinsed and dried according to published protocols, and then placed against x-ray film (Kodak Biomax MR Scientific Imaging film, Kodak) in cassettes for 2 days. After exposure and development, film images were analyzed by computerized densitometry. Images were measured on both the right and left sides of the nTS along the rostro-caudal extent of the injection site as determined from the injection tract and from the tissue damage that identified the injection area in each rat. Values from all sections within the area of the injection site were averaged to obtain 1 value for each rat for each side of the brain (injected versus noninjected). Data are expressed as the ratio of the injected to noninjected side as well as in absolute values for binding for each side of the brain. To verify the location of the injections, the injection tract was visually identified on cresyl violet-stained brain stem sections for each animal. All injections were localized within the intermediate portion of the medial nTS within the rostro-caudal level –13.3 to –14 according to the atlas of Paxinos and Watson.\(^{19}\)

### Analysis of Data

Baseline values for blood pressure and heart rate were obtained in the minute preceding each injection. The maximum change in pressure and heart rate was recorded after each injection. Significant treatment and interaction terms were identified by 2-way ANOVA. Therefore, further analyses were made with 1-way ANOVA for repeated measures (before versus after treatment) with post hoc Dunnett’s multiple comparisons to identify the source of the differences in each treatment group. For between-group differences, unpaired t tests were used to compare the scrambled-treated versus AT\(_{1}\) antisense-treated animals at each time point and for evaluation of the binding data in antisense- versus scrambled-treated animals. Paired comparisons were made between the injected and noninjected sides of the brain for the binding data in each treatment group. Changes in blood pressure or heart rate after scrambled or AT\(_{1}\) antisense treatments were also compared with a constant (0) to determine whether significant changes in these variables occurred. The criterion for statistical significance was \(P<0.05\), and all tests were performed using InStat or Prism (GraphPad Software). Numerical values are given as mean±SEM.

### Results

#### Baseline Hemodynamics

Mean arterial pressure and heart rate at various time points during the experiment are shown in the Table. Baseline values did not differ between AT\(_{1}\) antisense and scrambled-sequence treatment groups before Ang II injections at any time point. There was no long-term change in blood pressure or heart rate as a result of AT\(_{1}\) antisense or scrambled-sequence injections relative to the initial values (the before value).

<table>
<thead>
<tr>
<th>Time</th>
<th>AT(_{1}) Antisense Oligo</th>
<th>Scrambled Oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial baseline</td>
<td>80±2</td>
<td>81±3</td>
</tr>
<tr>
<td>45 minutes after oligo</td>
<td>79±2</td>
<td>82±4</td>
</tr>
<tr>
<td>90 minutes after oligo</td>
<td>81±2</td>
<td>83±3</td>
</tr>
<tr>
<td>150 minutes after oligo</td>
<td>81±3</td>
<td>81±7</td>
</tr>
</tbody>
</table>

Values are mean±SE; n=8 rats for the AT\(_{1}\) receptor antisense group and n=5 for the scrambled-sequence group. Baseline refers to the values for mean arterial pressure (MAP) and heart rate (HR) obtained in the minute preceding the Ang II microinjection given before treatment with either the scrambled or AT\(_{1}\) antisense oligonucleotide (Oligo). Other values were taken throughout the experiment at the times indicated prior to Ang II microinjections.

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**Table:** Mean Arterial Pressure and Heart Rate Before Each nTS Injection

<table>
<thead>
<tr>
<th>Time</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial baseline</td>
<td>80±2</td>
<td>318±11</td>
<td>81±3</td>
<td>310±25</td>
</tr>
<tr>
<td>45 minutes after oligo</td>
<td>79±2</td>
<td>326±14</td>
<td>82±4</td>
<td>313±17</td>
</tr>
<tr>
<td>90 minutes after oligo</td>
<td>81±2</td>
<td>329±10</td>
<td>83±3</td>
<td>325±17</td>
</tr>
<tr>
<td>150 minutes after oligo</td>
<td>81±3</td>
<td>332±8</td>
<td>81±7</td>
<td>316±29</td>
</tr>
</tbody>
</table>
Effects of AT1 Antisense on Cardiovascular Responses to Ang II Injected into the nTS

Ang II (250 fmol/30 nL) injected into the nTS produced a significant decrease in blood pressure of 14±6 mm Hg and heart rate of 15±3 bpm in 8 rats before injection of AT1 receptor antisense into the nTS. The fall in blood pressure was reduced by ~50% at 90 and 150 minutes after 120 nL of the AT1 receptor antisense oligonucleotide (164 pmol/120 nL; n=8) or scrambled-sequence oligonucleotide (164 pmol/120 nL; n=5). The Ang II-induced reduction in pressure and heart rate was significantly reduced at the 150-minute time point compared with the response before antisense treatment. At the 90- and 150-minute time points after antisense administration, the change in heart rate was not significantly different from 0. The mean arterial pressure and heart rate responses to Ang II were not altered after the nTS injection of the scrambled sequence oligonucleotide. *P<0.05 compared with Before value.

Effects of AT1 Receptor Antisense on Ang II Receptor Binding in the Dorsal Medulla

As illustrated in Figure 3, there was a unilateral reduction in 125I-Sartrhan binding in the dorsal medulla of animals given the AT1 antisense injections into the nTS. The density of 125I-Sartrhan binding was 218±649 fmol/mg on the antisense-injected side and 389±84 fmol/mg on the noninjected side of medulla (P<0.03). The loss in binding did not occur in the nTS of animals injected with scrambled-sequence oligonucleotides compared with the noninjected side of the brain (402±122 fmol/mg protein versus 430±144 fmol/mg protein, respectively). The ratio of binding on the injected to noninjected side of the medulla averaged 0.61±0.04 (n=8) in AT1 antisense-treated rats. In comparison, the ratio of binding in the injected to noninjected side of the animals receiving the scrambled sequence oligonucleotide...
The nTS occurred unilaterally in the AT1 receptor antisense-shown (dotted line indicates midline). A reduction in binding in oligonucleotides are given on the right side of the images (Oligo). Injections of either antisense or scrambled-sequence and 1 rat treated with scrambled-sequence oligonucleotides were compared. There was no effect of the scrambled-sequence oligonucleotide on binding (ie, right and left sides are of comparable density at all 3 levels shown). Sections are 84-μm apart and show binding over 252-μm rostro-caudally in each rat. Scale bar=1 mm.

Figure 3. Effect of unilateral AT1 receptor antisense injections into the nTS on Ang II receptors. Digitized images of film autoradiographs are shown to illustrate 0.5 nmol/L 125I-Sarthran labeling of Ang II receptors in the dorsal medulla of the rat. Sections are from 1 rat treated unilaterally with the AT1 receptor antisense and 1 rat treated with scrambled-sequence oligonucleotides (Oligo). Injections of either antisense or scrambled-sequence oligonucleotides are given on the right side of the images shown (dotted line indicates midline). A reduction in binding in the nTS occurred unilaterally in the AT1 receptor antisense-injected animals. This is seen most prominently in the middle section from the antisense-treated rat when right and left sides are compared. There was no effect of the scrambled-sequence oligonucleotide on binding (ie, right and left sides are of comparable density at all 3 levels shown). Sections are 84-μm apart and show binding over 252-μm rostro-caudally in each rat. Scale bar=1 mm.

was 0.98±0.07 fmol/mg protein (n=4). Thus, there was a significant (P<0.05, AT1 antisense versus scrambled oligonucleotide) reduction of 38% in angiotensin binding at 3 hours after the AT1 antisense injections. The rostro-caudal extent of the injection site in which the receptors were measured averaged 248±26 μm. In the presence of losartan, there was no residual specific binding in the nTS of antisense- or scrambled-treated animals. This confirms that AT1 receptors predominate in the intermediate region of the nTS of rats, which is consistent with previous reports.20

Discussion
Ang II lowers blood pressure and heart rate after injection of low (fmol) doses in the nTS, as previously reported by us and others in several strains of rats.14-17,21 This response can be blocked completely by the nTS injection of an AT1 receptor antagonist.22 We found that the bradycardia in response to Ang II was totally abolished at 150 minutes after injection of AT1 receptor antisense into the nTS. At this time, the depressor response induced by Ang II was reduced by only 50%. The reduction in cardiovascular responses was associated with a 40% decrease in the density of Ang II receptors in the nTS.

The pattern of distribution of Ang II receptors in the dorsal medulla is known to overlap that of the vagal sensory and motor systems.1,3,5 Receptors have been identified on local neurons of the nodose ganglion, nTS, and dmnX as well as fiber terminals that project to these sites by anatomical,2 molecular,23,24 or electrophysiological9,25,26 techniques. Knowledge of the role of each of these populations of receptors in the fall in blood pressure and heart rate in response to Ang II was limited previously. This was because techniques using receptor antagonists did not allow the selective interruption of each component, whereas selective denervations did not maintain the necessary efferent pathways as functional. The present study takes advantage of the unique features of antisense technology, which would include sparing receptors on neurons with fiber terminals projecting to the area of the injection. We reveal that the actions of Ang II on heart rate may be mediated entirely by angiotensin receptors made in nTS cells within the injection site. The fall in pressure, however, results from actions at receptors on both local nTS cells and terminal processes projecting to the area.

The mechanism by which antisense oligodeoxynucleotides interfere with synthesis of the protein products of a certain gene allows for their use in the central nervous system to dissect out actions at local elements versus pathway components with neuronal cell bodies in remote locations.27,28 We previously used a similar approach to illustrate the role of AT1 receptors in the paraventricular nucleus in the blood pressure response of (mRen2)27 transgenic rats to sodium loading.13 In that study and in earlier work by Phillips et al,29 the maximal effects of the AT1 receptor antisense were observed within 2 to 3 hours. A similar time course of action occurred in the present study, because the effects of the antisense took at least 90 minutes to develop, and the responses remained suppressed at 150 minutes postinjection. AT1 receptors exhibit rapid turnover apparently explained by the internalization and loss of receptors after exposure to Ang II, even before any change in mRNA.30 Thus, our study and at least 2 other reports using AT1 receptor antisense indicate that after interruption of synthesis, there is also a relatively rapid loss of surface AT1 receptors developing over a 3-hour time period. We cannot completely rule out, however, that additional actions might develop over a longer time period that leads to complete inhibition of the blood pressure as well as heart rate responses in our study.

To assess the reduction of Ang II receptors after treatment with the AT1 antisense injections, we used the in vitro receptor autoradiographic technique to quantify 125I-Sarthran-labeled receptors in the nTS at the end of the experiments. There was a reduction in receptors of 40% in the nTS of the antisense-treated rats with no reduction seen in the nTS of the animals treated with scrambled-sequence oligonucleotides. This indicates that the reduction in binding is not simply a result of damage in the area of the injections, given that the ratio of the injected and noninjected sides of scrambled-treated rats was 0.98. The magnitude of reduction of Ang II receptors in the nTS after unilateral nodose gangliectomy in the rat, which would remove afferent fibers, is 40% to
50%. This implies that at least 50% of the receptors in this brain area are located on nTS neurons. Because we observed a reduction of \(\approx40\%\) after antisense injections, the data indicate that the majority of the binding remaining is not due to incomplete effects of the antisense but rather reflects the remaining receptors located on afferent fibers. The reduction in binding appeared limited to the vicinity of the injection sites, which averaged \(\approx250\ \mu\text{mol/L}\) in the rostro-caudal direction. This area of distribution for the 120-nL injection volume of antisense is less than what we estimated after \(\text{I-Sarthran}\) injections into the nTS in a volume of 100 nL in a previous study. While the area of reduction in receptors appeared to be confined to the nTS, it is possible that the actions of Ang II before or after the antisense injections were a result of actions at both nTS and dmnX sites. The remaining fall in blood pressure may then result from actions on receptors outside the area of the loss of binding. This possibility is not likely, because the volume of the Ang II injection was only 30 nL; previous studies indicate that this volume does not reach the dmnX from injection sites in the nTS. Finally, we saw no residual non-AT\(_1\) angiotensin receptor binding in the nTS in the present study. Thus, there is no strong evidence for AT\(_2\) receptors in the medial nTS of the rat that would account for the residual actions of Ang II.

The differential effects of the AT\(_1\) receptor antisense treatment on blood pressure and heart rate are similar to what we observed in early studies investigating the peripheral mechanisms responsible for the Ang II–induced effects. Vagotomy or treatment with methylatropine blocked the bradycardia by 80% but only reduced the hypotension by \(\approx50\%\). Thus, Ang II acts directly on nTS neurons to stimulate efferent pathways responsible for parasympathetic control of heart rate. In contrast, at least 50% of the Ang II–mediated effect on the blood pressure results from inhibition of sympathetic nervous system activity. The data in the present study indicate that receptors on presynaptic fiber sites not affected by the AT\(_1\) receptor antisense might account for these actions. There are direct connections of vagal sensory afferent fibers with cells in the A2-catecholamine cell group in the ventral nTS. The A2 cells project to ventral medullary sites involved in control of sympathetic outflow. Thus, the remaining actions of Ang II appear to be mediated by receptors on presynaptic afferent-fiber terminals in the nTS most likely of vagal origin.

The data obtained in the current experiments are consistent with previous electrophysiology studies noting both a presynaptic and postsynaptic contribution to the neuronal excitatory actions of Ang II. The percentage of Ang II excitatory responses linked to presynaptic effects of the peptide in the nTS is 50%. Moreover, with the use of high-resolution autoradiography, we were able to see Ang II receptors overlying nTS neurons. Combined with the findings after AT\(_1\) receptor antisense treatment, it appears that at least 50% of the actions of Ang II on blood pressure and most of the heart rate effects involve actions directly on neurons within the nTS. However, the remaining fall in blood pressure obtained after AT\(_1\) receptor antisense treatment suggests additional actions on fiber terminals projecting to the region. Unilateral denervation of the sino-aortic nerves (which contribute \(\approx2\%\) of the primary sensory afferent input to the nTS) is accompanied by an 11% to 15% reduction in Ang II receptors in the nTS at an intermediate rostro-caudal level.

On the basis of the available electrophysiological and anatomical evidence, presynaptic vagal sensory afferent fibers other than those carrying baroreceptor reflex input would be likely candidates to mediate the residual blood pressure component of the response. However, the only way to test this will be to use the antisense approach to inhibit AT\(_1\) receptors in the nodose ganglion.

The physiological significance of the acute depressor and bradycardic actions of Ang II are not known. We previously showed that the entire blood pressure and heart rate response elicited by Ang II in the nTS is blocked by either of 2 different substance P antagonists. Substance P is contained in both vagal sensory afferent fiber terminals projecting to the nTS and in nTS interneurons projecting to the dmnX. Thus, substance P–containing cells within the nTS appear to be responsible for the direct actions of Ang II. In addition, the nerve fibers within the nTS that result in the Ang II–induced actions must also contain substance P. A link has been made recently between substance P–containing pathways and the effects of Ang II on the chemoreceptor reflex.

Ang II is present in nerve terminals projecting to the nTS from forebrain sites and may access the nTS by blood-borne routes via the area postrema or cerebrospinal fluid routes (see reviews). Regardless of the source of the angiotensin acting in the nTS, it is well accepted that endogenous Ang II plays a tonic role in modulation of the baroreceptor reflex control of heart rate and the response to activation of cardio-pulmonary vagal chemoreceptors at the level of the nTS. The inhibitory role of the peptide on these predominantly vagally mediated responses is present in normotensive conscious or anesthetized animals and is even more pronounced in hypertensive rats. To conclude, the present studies show the utility of using AT\(_1\) antisense to discern the presynaptic versus postsynaptic sites for the acute hemodynamic actions of Ang II in the nTS. Future work using antisense technology can be used to establish the involvement of vagal sensory-afferent fibers or local nTS cell soma in the baroreceptor or chemoreceptor reflex actions of Ang II.

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References


