Cardiovascular responses elicited by a new endogenous angiotensin in the nucleus tractus solitarius of the rat

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Chitravanshi VC, Sapru HN. Cardiovascular responses elicited by a new endogenous angiotensin in the nucleus tractus solitarius of the rat. Am J Physiol Heart Circ Physiol 300: H230–H240, 2011. First published November 12, 2010; doi:10.1152/ajpheart.00861.2010.—Cardiovascular effects of angiotensin-(1–12) [ANG-(1–12)] were studied in the medial nucleus of the tractus solitarius (mNTS) in anesthetized, artificially ventilated, adult male Wistar rats. Microinjections (100 nl) of ANG-(1–12) (0.06 mM) into the mNTS elicited maximum decreases in mean arterial pressure (MAP; 34 ± 5.8 mmHg) and heart rate (HR; 39 ± 3.7 beats/min). Bilateral vagotomy abolished ANG-(1–12)-induced bradycardia. Efferent greater splanchnic nerve activity was decreased by microinjections of ANG-(1–12) into the mNTS. Blockade of ANG type 1 receptors (AT1Rs; using ZD-7155 or L-158,809), but not ANG type 2 receptors (AT2Rs; using A-779 [ANG-(1–7) antagonist] did not attenuate ANG-(1–12)-induced responses. Pressure ejection of ANG-(1–12) (0.06 mM, 2 nl) caused excitation of barosensitive mNTS neurons, which was blocked by prior application of the AT1R antagonist. ANG-(1–12)-induced excitation of mNTS neurons was also blocked by prior sequential applications of captopril and chymostatin. These results indicate that 1) microinjections of ANG-(1–12) into the mNTS elicited depressor and bradycardic responses by exciting barosensitive mNTS neurons; 2) the decreases in MAP and HR were mediated via sympathetic and vagus nerves, respectively; 3) AT1Rs, but not AT2Rs, mediated these actions of ANG-(1–12); 4) the responses were mediated via the conversion of ANG-(1–12) to ANG II and both ACE and chymase were involved in this conversion; and 5) ANG-(1–7) was not one of the metabolites of ANG-(1–12) in the mNTS.

A NEW ENDOGENOUS ANGIOTENSIN (ANG), ANG-(1–12) has been identified recently (24, 44). Intravenous administration of this peptide has been reported to elicit pressor responses in the rat, and this effect was blocked by prior administration of an angiotensin-converting enzyme (ACE) inhibitor or ANG type 1 receptor (AT1R) antagonist (24). These data indicated that in the periphery, ANG-(1–12) may exert its actions via a rapid conversion to ANG II. Therefore, ANG-(1–12) was also named as "proangiotensin-12" (24). The presence of ANG-(1–12) has been reported in various peripheral tissues, including the heart and kidneys (17, 24). High concentrations of ANG-(1–12) have also been reported in the rat brain, and the presence of cells immunoreactive for ANG-(1–12) has been demonstrated in the nucleus tractus solitarius (NTS) of the rat (1, 24). It has recently been reported that bilateral microinjections of ANG-(1–12) into the NTS attenuate the bradycardic responses to intravenous injections of phenylephrine (PE) (1). In another study, chronic immunoneutralization of endogenous brain ANG-(1–12) by intracerebroventricular injections of anti-ANG-(1–12) IgG in the hypertensive phenotype of the (mRen2)27 rat elicited antihypertensive effects (16). The medial subnucleus of the NTS (mNTS) has been reported to be the first site where peripheral baroreceptor, chemoreceptor, and cardiopulmonary afferents make their primary synapse (10, 14, 33–35, 39). The present investigation was undertaken to test the hypothesis that ANG-(1–12) exerts an excitatory effect on barosensitive mNTS neurons, causing the activation of sympathetic and parasympathetic pathways, which results in depressor and bradycardic responses.

MATERIALS AND METHODS

General procedures. Experiments were done in adult male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 300–360 g (n = 121). All animals were housed under controlled conditions with a 12:12-h light-dark cycle. Food and water were available to the animals ad libitum. The experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (7th ed., 1996) and with the approval of the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

We have previously published the details of the procedures used in this study (18). Briefly, rats were anesthetized with an inhalation of isoflurane (2–3% in 100% oxygen), one of the veins was cannulated, and urethane (1.2–1.4 g/kg) was injected intravenously in eight to nine aliquots at 2-min intervals (total volume of the anesthetic solution was 0.4–0.45 ml injected over a period of ~16–18 min). Isoflurane inhalation was terminated as soon as urethane administration was completed. The absence of a blood pressure (BP) response and/or withdrawal of the limb in response to a pinch of a hind paw indicated that the rats were properly anesthetized. Using this procedure, administration of supplemental doses of urethane was not usually necessary. Rectal temperature was maintained at 37.0 ± 0.5°C. Femoral arterial BP and heart rate (HR) were recorded by standard techniques. All of the tracings were stored on a computer hard drive.

Vagotomy. Silk sutures were placed loosely around the vagus nerves bilaterally for the subsequent identification and sectioning of the nerves.

Microinjections. All microinjections into the mNTS were unilateral unless otherwise indicated. Rats were placed in a prone position in a stereotaxic instrument with a bite bar 18 mm below the interaural line. The dorsal medulla was exposed, and microinjections were made using multibarreled glass micropipettes (tip size: 20 – 40 μm). Each barrel was connected to a channel on a picospritzer. Three barrels contained L-glutamate (1-Glu), artificial cerebrospinal fluid (aCSF), and ANG-(1–12). The remaining barrels contained either an ANG
receptor antagonist or an ACE or chymase inhibitor. The coordinates for the mNTS were 0.5–0.6 mm rostral and 0.5–0.8 mm lateral to the calamus scriptorius and 0.5–0.7 mm deep from the dorsal medullary surface. The mNTS sites eliciting depressor and bradycardic responses were identified by microinjections of L-Glu (5 mM) (8, 39). The volume of all microinjections into the mNTS was 100 nl; the selection of this volume was based on our previous study (6). The volumes were pressure ejected and visually confirmed by the displacement of fluid meniscus in the barrel containing the solution using a modified binocular horizontal microscope with a graduated reticle in one eye piece. The duration of the microinjection was 5–10 s. Microinjections of aCSF (100 nl, pH 7.4) were used as controls. The interval between microinjections of L-Glu and ANG-(1–12) was at least 5 min. The interval between injections of ANG-(1–12) was 60 min to avoid the possibility of desensitization. The interval between the microinjection of an AT1R antagonist and ANG-(1–12) was between 2 and 5 min.

Greater splanchnic nerve recording. The greater splanchnic nerve (GSN) was sectioned at its junction with the celiac ganglion, a few millimeters of the central end of the nerve were desheathed, and whole nerve activity was amplified (×20,000–30,000), filtered (100–5,000 Hz), digitized, and stored on a computer hard drive. The digitized signals were full wave rectified and integrated over 1-s intervals using CED Spike 2 software (Cambridge Electronic Design, Cambridge, UK). The baroreflex sensitivity of GSN activity (GSNA) was indicated by its inhibition in response to pressor responses elicited by intravenous bolus injections of PE (10 μg/kg). At the end of the experiment, the nerve was sectioned centrally, and the remaining activity was considered to be the noise level, which was subtracted from the whole GSNA.

Extracellular neuronal recording. The mNTS area where baroreceptors are known to terminate (i.e., 0.5–0.6 mm rostral and 0.5–0.8 mm lateral to the calamus scriptorius and 0.5–0.7 mm deep from the dorsal medullary surface) was explored for single unit recording (7, 39). First, a site in the mNTS, where microinjections of L-Glu (5 mM) elicited depressor and bradycardic responses, was identified. The coordinates were noted, the microinjection pipette was withdrawn, and multibarreled glass micropipettes were then used for neuronal recording at the same site. The central barrel used for recording, and whole nerve activity was amplified (×20,000–30,000), filtered (100–5,000 Hz), digitized, and stored on a computer hard drive. The digitized signals were full wave rectified and integrated over 1-s intervals using CED Spike 2 software (Cambridge Electronic Design, Cambridge, UK). The baroreflex sensitivity of GSN activity (GSNA) was indicated by its inhibition in response to pressor responses elicited by intravenous bolus injections of PE (10 μg/kg). At the end of the experiment, the nerve was sectioned centrally, and the remaining activity was considered to be the noise level, which was subtracted from the whole GSNA.

Histology. Typical sites of microinjections in the mNTS were marked by microinjections of diluted India ink. Animals were perfused and fixed with 4% paraformaldehyde, and serial sections of the medulla were cut (40 μm), mounted on slides, dehydrated, cleared, and stained with cresyl violet. The microinjection sites were identified, photographed, and compared with a standard atlas (28).

Drugs and chemicals. The following drugs and chemicals were used: ANG-(1–7), ANG-(1–12), d-Ala7-ANG-(1–7) [A-779; ANG-(1–7) antagonist] (5, 32), captopril (ACE inhibitor) (23), chymostatin (chymase inhibitor) (15), isoflurane, L-158,809 (AT1R antagonist) (4), ZD-7155 (AT1R antagonist) (27), and PD-123319 (AT2R antagonist) (3). All of the solutions for the microinjections were freshly prepared in aCSF. Where applicable, the concentration of drugs refers to their salts. The sources of drugs were as follows: ANG-(1–12) and A-779 (American Peptide, Sunnyvale, CA); ANG-(1–7), captopril, and chymostatin (Sigma-Aldrich Chemicals, St. Louis, MO); L-158,809 (Merck, Whitehouse Station, NJ); PD-123319 and ZD-7155 (Tocris-Cookson, Ellisville, MO); and isoflurane (Baxter Pharmaceutical Products, Deerfield, IL).

Statistical analyses. Means and SEs were calculated for maximum changes in mean arterial pressure (MAP), HR, GSNA, and neuronal responses. In different groups of rats, comparisons of the decreases in MAP and HR in the concentration-response experiments, vagotomy, microinjections of L-Glu, and ANG-(1–12) and its blockade by AT1R and AT2R antagonists into the mNTS were made using one-way ANOVA followed by Tukey-Kramer’s multiple-comparison tests. For the analysis of GSNA, the control value represented the average amplitude of the nerve activity during the 35-s period before the microinjections of ANG-(1–12) or L-Glu into the mNTS. The maximum inhibition in GSNA amplitude elicited by microinjections of L-Glu or ANG-(1–12) into the mNTS was expressed as the percent decrease from the basal value of the GSNA amplitude. Mean values of the integrated nerve signals were compared using Student’s paired t-test. For the analysis of neuronal activity, the basal firing rate represented the average of firing during 1– to 2-s periods before neuronal activation. The maximum increase in firing rate in response to the intravenous bolus injections of PE or pressure ejection of ANG-(1–12) or L-Glu onto the mNTS neuron was averaged for 1–2 s and compared using ANOVA followed by Tukey-Kramer’s multiple-comparison tests. The blockade of excitation of neuronal activity by AT1R antagonist, captopril, and chymostatin was also compared using ANOVA followed by Tukey-Kramer’s multiple-comparison tests. In all cases, differences were considered significant at P < 0.05.

RESULTS

Average baseline values for MAP and HR in urethane-anesthetized rats were 102 ± 5.8 mmHg and 407.8 ± 12 beats/min, respectively (n = 121).

Concentration response of microinjections of ANG-(1–12) into the mNTS. In this and other series of experiments, the mNTS was always identified by microinjections of L-Glu (5 mM), which elicited decreases in MAP (32.3 ± 2.6 mmHg) and HR (50.5 ± 9.8 beats/min) (n = 15). The interval between the microinjections of L-Glu and other agents was at least 5 min. The decreases in MAP and HR elicited by microinjections of different concentrations of ANG-(1–12) are shown in Table 1. The depressor and bradycardic responses to 0.06 and 0.12 mM concentrations of ANG-(1–12) were significantly greater than those of 0.015, 0.03, and 0.5 mM concentrations (P < 0.05). The depressor and bradycardic responses elicited by 0.06 and 0.12 mM concentrations of ANG-(1–12) were not significantly different (P > 0.05). Therefore, the 0.06 mM concentration of ANG-(1–12) was selected for other experiments. The onset and

| Table 1. Concentration response of microinjections of ANG-(1–12) into the mNTS |
|-----------------|-----------------|-----------------|
| Concentration of ANG-(1–12) | Decrease in MAP, mmHg | Decrease in HR, beats/min |
| 0.015 mM | 9.6 ± 2.9 | 6.5 ± 2.9 |
| 0.03 mM | 14 ± 1.9 | 17 ± 2.6 |
| 0.06 mM | 34 ± 5.8* | 39 ± 3.7* |
| 0.12 mM | 21.4 ± 2.1* | 31 ± 8.1* |
| 0.25 mM | 20 ± 1.6 | 20 ± 3.2 |
| 0.50 mM | 15 ± 4.2 | 17 ± 3.7 |

Values are means ± SE; n = 15. mNTS, medial nucleus tractus solitarius; MAP, mean arterial pressure; HR, heart rate. *Significantly greater than the responses to other concentrations (P < 0.05).
duration of cardiovascular responses to microinjections of ANG-(1–12) (0.06 mM) were 15.9 ± 3 s and 5–7 min, respectively. The peak effect was observed at 30–90 s. In this and other experiments, microinjections of aCSF (100 nl) into the mNTS elicited no cardiovascular responses.

The decreases in MAP in response to three consecutive microinjections of ANG-(1–12) (0.06 mM) at 60-min intervals were 31 ± 7.3, 27 ± 4.7, and 29.2 ± 4.3 mmHg, respectively, and the decreases in HR were 29.6 ± 6.8, 27.6 ± 5.5, and 28.6 ± 4.9 beats/min, respectively (n = 5). Repeated-measures ANOVA showed that these values were not statistically different (P > 0.05). Thus, no desensitization was observed with repeated microinjections of ANG-(1–12) at 60-min intervals. Therefore, the interval between the microinjections of ANG-(1–12) was at least 60 min in all experiments to avoid the possibility of desensitization.

The dose of ANG-(1–12) that elicited maximal depressor and bradycardic responses when microinjected into the mNTS (0.06 mM, 100 nl) elicited no detectable cardiovascular responses when injected intravenously.

**Comparison of responses to microinjections of ANG-(1–12) and ANG II into the mNTS.** The decreases in MAP elicited by microinjection of ANG-(1–12) (0.06 mM) into the mNTS (23 ± 4.6 mmHg) were significantly smaller (P < 0.01) compared with the decreases in MAP (31 ± 4 mmHg) elicited by microinjections the same concentration of ANG II at the same site (n = 4). In the same group of rats, the decreases in HR elicited by microinjection of the same concentration of ANG-(1–12) into the mNTS (31.8 ± 1.1 beats/min) were significantly lesser (P < 0.01) compared with the decreases in HR (44 ± 2.5 beats/min) elicited by microinjections of ANG II (0.06 mM) at the same site. The onset of cardiovascular responses (15.5 ± 2 s) elicited by ANG-(1–12) (0.06 mM) was significantly longer (P < 0.05) compared with the onset (8.1 ± 0.9 s) of cardiovascular responses elicited by microinjections of the same concentration of ANG II.

**Site specificity of the responses to microinjections of ANG-(1–12).** Microinjections of ANG-(1–12) (0.06 mM) into the mNTS elicited usual depressor and bradycardic responses (n = 4). Microinjection of the same concentrations of ANG-(1–12) into an adjacent site in the cuneate nucleus (0.5 mm rostral and 1.5 mm lateral to the calamus scriptorius and 0.5 mm ventral to the dorsal surface of the medulla) did not elicit any cardiovascular responses.

**Effect of vagotomy on the responses induced by microinjections of ANG-(1–12) into the mNTS.** ANG-(1–12)-induced bradycardia was abolished by bilateral vagotomy; the decreases in HR before and after vagotomy were 33.6 ± 7.3 and 2 ± 0.9 beats/min, respectively (n = 5, P < 0.05). Bilateral vagotomy also abolished bradycardia elicited by microinjections of l-Glu into the mNTS; l-Glu-induced decreases in HR before and after bilateral vagotomy were 40 ± 7.4 and 4 ± 0.4 beats/min, respectively (P < 0.01). Bilateral vagotomy did not affect the decreases in MAP induced by microinjections of either ANG-(1–12) (0.06 mM) or l-Glu (5 mM) into the mNTS. ANG-(1–12)-induced decreases in MAP were 35 ± 6.8 and 35.8 ± 7.7 mmHg before and after bilateral vagotomy (P > 0.05), and l-Glu-induced decreases in MAP were 33 ± 6.2 and 31 ± 4.4 mmHg (P > 0.05) before and after bilateral vagotomy, respectively. Baseline MAP was not altered by bilateral vagotomy; MAP before and after the bilateral vagotomy was 96.6 ± 8.4 and 94.2 ± 6.8 mmHg, respectively (P > 0.05). Baseline HR was increased by bilateral vagotomy; HR before and after bilateral vagotomy was 401.8 ± 8.9 and 430.8 ± 8.3 beats/min, respectively (P < 0.05).

**Effect of AT1R antagonists on the responses induced by microinjections of ANG-(1–12) into the mNTS.** A tracing from a typical experiment showing the effect of an AT1R antagonist on ANG-(1–12)-induced responses is shown in Fig. 1. The mNTS site was identified by a microinjection of l-Glu (Fig. 1A). ANG-(1–12) (0.06 mM) was microinjected into the same site; decreases in pulsatile arterial pressure, MAP, and HR were elicited (Fig. 1B). After an interval of 60 min, an AT1R antagonist (ZD-7155, 2 mM) was microinjected at the same site; no cardiovascular responses were elicited (Fig. 1C). Two minutes later, ANG-(1–12) (0.06 mM) was again microinjected at the same site. The responses to ANG-(1–12) were blocked (Fig. 1D). The responses to l-Glu, tested 2 min later, were not blocked (Fig. 1E). aCSF (100 nl) was microinjected into the mNTS before microinjection of each drug (not shown). The blocking effects of lower (1 mM) and higher (3 mM) concentrations of ZD-7155 were also tested. Group data (n = 15) for the effects of ZD-7155 are shown in Table 2. The blocking effect of a 2 mM concentration of ZD-7155 on ANG-(1–12)-

![Fig. 1. Effect of angiotensin (ANG) type 1 receptor (AT1R) blockade on ANG-(1–12)-induced responses. Top trace, pulsatile arterial pressure (PAP; in mmHg); middle trace, mean arterial pressure (MAP; in mmHg); bottom trace, heart rate [HR; in beats/min (bpm)]. In this and other figures, microinjections refer to medial nucleus tractus solitarius (mNTS) sites. A: microinjection of l-glutamate (l-Glu; 5 mM) elicited a decrease in PAP, MAP, and HR. B: microinjection of ANG-(1–12) also elicited a decrease in PAP, MAP, and HR. C: 60 min later, microinjection of ZD-7155 (2 mM) elicited no significant responses. D: 2 min after the microinjection of ZD-7155, microinjection of ANG-(1–12) (0.06 mM) failed to elicit the usual depressor and bradycardic responses. E: 5 min after the microinjection of ANG-(1–12), l-Glu (5 mM) was microinjected; l-Glu-induced depressor and bradycardic responses persisted. In A–E, artificial cerebrospinal fluid (aCSF; 100 nl) was microinjected 5 min after the responses of the injected substance subsided; aCSF elicited no responses (not shown). The interval between the two injections of ANG-(1–12) (B and D) was at least 60 min to avoid desensitization.](http://ajpheart.physiology.org/Downloadedfrom/)
Effect of AT$_1$R on cardiovascular responses induced by microinjections of ANG-(1–12) into the mNTS. Microinjections of a selective AT$_1$R antagonist (A-779) into the mNTS did not alter the cardiovascular responses elicited by microinjections of ANG-(1–12) (0.06 mM) at the same site ($n = 5$). The decreases in MAP and HR induced by microinjections of ANG-(1–12) before and after the microinjections of A-779 (1 mM) are shown in Table 2. The dose of A-779 was selected based on its blocking effect of ANG-(1–7) ($n = 5$). The depressor responses elicited by microinjections of ANG-(1–7) (0.2 mM) into the mNTS before and after the microinjection of A-779 (1 mM) were 13.2 ± 1.2 and 2.7 ± 0.4 mmHg, respectively ($P < 0.05$). The bradycardic responses elicited by microinjections of ANG-(1–7) (0.2 mM) into the mNTS before and after the microinjection of A-779 (1 mM) were 11.2 ± 1.2 and 3.4 ± 1.2 beats/min, respectively ($P < 0.05$). Thus, the selected dose of A-779 blocked the cardiovascular responses elicited by microinjections of ANG-(1–7) into the mNTS. Similar results using A-779 have been reported by others (5).

Effect of captopril on the responses induced by microinjections of ANG-(1–12) into the mNTS. The decreases in MAP induced by microinjections of ANG-(1–12) (0.06 mM) into the mNTS before and after microinjections of captopril (50 mM) were 23 ± 2.2 and 11.4 ± 2.3 mmHg, respectively ($n = 5$, $P < 0.01$). The decreases in HR induced by microinjections of ANG-(1–12) before and after the same dose of captopril were 20 ± 4.1 and 8.4 ± 2.1 beats/min ($P < 0.01$), respectively (Fig. 2A). The decreases in MAP induced by microinjections of ANG-(1–12) (0.06 mM) into the mNTS before and after a higher dose of captopril were 37.4 ± 4.4 and 11.8 ± 2.8 mmHg, respectively ($P < 0.01$). The decreases in HR induced by microinjections of ANG-(1–12) before and after the higher dose of captopril were 39 ± 11 and 6.6 ± 2.3 beats/min, respectively ($n = 5$, $P < 0.01$; Fig. 2A). There were no significant differences ($P > 0.05$) between the blocking effects of the two concentrations of captopril. Therefore, the 50 mM concentration of captopril was selected for other experiments. ANG-(1–12)-induced decreases in MAP and HR did not completely recover to the initial values within 60 min of the microinjection of captopril (50 mM) into the mNTS. Microinjection of l-Glu into the mNTS before and after the microinjection of captopril into the same site elicited similar depressor and bradycardic responses. The decreases in MAP elicited by microinjection of l-Glu (5 mM) before and after captopril (50 mM) were 31.8 ± 5.7 and 34.8 ± 4.8 mmHg, respectively ($P < 0.05$), and the decreases in HR before and after the microinjection of captopril (50 mM) were 30.5 ± 1.6 and 31 ± 1.7 beats/min, respectively ($P > 0.05$). Unilateral microinjections of captopril alone (100 mM) into the mNTS did not elicit any response.

Effect of chymostatin on the responses induced by microinjections of ANG-(1–12) into the mNTS. The decreases in MAP induced by microinjections of ANG-(1–12) (0.06 mM) into the mNTS before and after microinjections of chymostatin (0.5 mM) were 30 ± 3.5 and 7.6 ± 0.2 mmHg, respectively ($P < 0.01$). The decreases in HR induced by microinjections of ANG-(1–12) before and after the same dose of chymostatin were 27.2 ± 4.6 and 5.6 ± 0.8 beats/min, respectively ($n = 5$, $P < 0.01$; Fig. 2B). The decreases in MAP induced by microinjections of ANG-(1–12) (0.06 mM) into the mNTS before and after a higher dose of chymostatin (1 mM) were

Table 2. Effect of AT$_1$R antagonists (ZD-7155 and L-158,809), AT$_2$R antagonist (PD-123319), and ANG-(1–7) antagonist (A-779) on ANG-(1–12)-induced responses

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>ANG-(1–12)-Induced Decrease in MAP, mmHg</th>
<th>ANG-(1–12)-Induced Decrease in HR, beats/min</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before After</td>
<td>Before After</td>
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<tr>
<td>ZD-7155</td>
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<td></td>
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<tr>
<td>1 mM</td>
<td>32.6 ± 2.8 22 ± 2.6*</td>
<td>30.4 ± 2.6 20 ± 1.6*</td>
</tr>
<tr>
<td>2 mM</td>
<td>32.8 ± 4.5 3.8 ± 1.7‡</td>
<td>29.4 ± 3.2 6 ± 1.1‡</td>
</tr>
<tr>
<td>3 mM</td>
<td>29.8 ± 2.1 1.8 ± 0.8‡</td>
<td>32.2 ± 2 9.8 ± 0.3‡</td>
</tr>
<tr>
<td>L-158,809 (10 mM)</td>
<td>38.2 ± 3.7 13 ± 1.2‡</td>
<td>37.6 ± 8.2 10.6 ± 1.2‡</td>
</tr>
<tr>
<td>PD-123319</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mM</td>
<td>31.2 ± 3.3 32.4 ± 3</td>
<td>27.6 ± 2.3 30 ± 5.2</td>
</tr>
<tr>
<td>5 mM</td>
<td>29.8 ± 3.5 25.4 ± 2.6</td>
<td>25.2 ± 2.4 21 ± 1.6</td>
</tr>
<tr>
<td>A-779 (1 mM)</td>
<td>24.2 ± 2 23 ± 2.6</td>
<td>23.7 ± 1.8 25.5 ± 3.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 15$ for ZD-7155 (1, 2, and 3 mM), 5 for L-158,809 (10 mM), 10 for PD-123319 (3 and 5 mM), and 5 for A-779. AT$_1$R, ANG type 1 receptor; AT$_2$R, ANG type 2 receptor. Values were significantly smaller compared with ANG-(1–12)-induced responses before microinjection of the antagonist; *$P < 0.05$, ‡$P < 0.01$, and †$P < 0.001$.

induced cardiovascular response was significantly greater than that of 1 mM concentration of this antagonist ($P < 0.01$). However, there were no significant differences ($P > 0.05$) between the blocking effect of 2 and 3 mM concentrations of this antagonist. Therefore, the 2 mM concentration of ZD-7155 was selected for blocking the effects of ANG-(1–12) in other experiments. In the same group of rats, l-Glu (5 mM) was microinjected into the same mNTS site 5 min after the microinjection of ZD-7155 (2 mM). The decreases in MAP (34.6 ± 13.1 mmHg) and HR (41.8 ± 6.7 beats/min) elicited by microinjection of l-Glu into the mNTS were not significantly changed after the microinjection of ZD-7155 (2 mM, $P > 0.05$).

The effects of another AT$_1$R antagonist (l-158,809) on the cardiovascular responses to microinjections of ANG-(1–12) (0.06 mM) into the mNTS were also studied ($n = 5$). ANG-(1–12)-induced depressor and bradycardic responses were blocked by prior microinjections of L-158,809 (10 mM; Table 2). This dose of L-158,809 has been reported to block AT$_1$R’s in other brain areas (e.g., the rostral ventrolateral medullary pressor area) (38). In the same group of rats, l-Glu (5 mM) was microinjected into the same mNTS site 5 min after the microinjection of L-158,809 (10 mM). The decreases in MAP (32.8 ± 5.6 mmHg) and HR (34.8 ± 4.9 beats/min) elicited by microinjection of l-Glu into the mNTS were not significantly changed after the microinjection of L-158,809 ($P > 0.05$).

The ANG-(1–12)-induced decreases in MAP and HR did not recover to the initial values within 60 min after the microinjection of either ZD-7155 (2 mM) or L-158,809 (10 mM) into the mNTS.

Effect of AT$_3$R antagonists on the responses induced by microinjections of ANG-(1–12) into the mNTS. Microinjections of a selective AT$_3$R antagonist (PD-123319) into the mNTS did not alter the cardiovascular responses elicited by microinjections of ANG-(1–12) (0.06 mM) at the same site ($n = 10$). The decreases in MAP and HR induced by microinjections of ANG-(1–12) before and after the microinjections of PD-123319 (3 and 5 mM) are shown in Table 2.
statin (1 mM) were 40.2 ± 2.2 and 20.6 ± 2.8 mmHg, respectively \((P < 0.05)\), and the decreases in HR before and after the microinjection of chymostatin (1 mM) were 68.8 ± 6.9 and 40.6 ± 4.3 beats/min, respectively \((P < 0.05)\). Because the higher concentration of chymostatin (1 mM) elicited nonspecific effects on \(\alpha\)-Glu-induced responses, the smaller concentration (0.5 mM) of chymostatin was selected for other experiments.

Effect of sequential microinjections of captopril and chymostatin on ANG-(1–12)-induced responses. The ANG-(1–12)-induced decreases in MAP before and after the sequential microinjections of captopril (50 mM) and chymostatin (0.5 mM) into the mNTS were 39.5 ± 3.7 and 4.4 ± 0.9 mmHg, respectively \((P < 0.001)\). Similarly, ANG-(1–12)-induced decreases in HR before and after the sequential microinjections of these blockers were 34.5 ± 1 and 3.8 ± 1 beats/min, respectively \((n = 5, P < 0.001)\). Thus, sequential microinjections of captopril and chymostatin elicited greater attenuation of ANG-(1–12)-induced responses compared with the attenuation elicited by either captopril or chymostatin alone (Fig. 2C).

Effect of microinjections of ANG-(1–12) into the mNTS on sympathetic nerve activity. Microinjections of ANG-(1–12) into the mNTS decreased the efferent sympathetic activity of the GSN. A typical recording from one experiment is shown in Fig. 3. A bolus injection of PE (10 \(\mu\)g/kg iv) increased MAP, which, in turn, elicited reflex bradycardia and inhibition of efferent GSN activity, which lasted for 9.9 ± 2 s (Fig. 3A). Ten minutes later, when the effects of PE subsided, microinjection of \(\alpha\)-Glu (5 mM) into the mNTS elicited an inhibition of GSN activity, which lasted for 6.6 ± 0.4 s (Fig. 3B). Within 5 min, microinjection of aCSF (100 nl) into the same mNTS site did not alter GSN activity (not shown). Two minutes later, ANG-(1–12) (0.06 mM) was microinjected at the same site; a decrease in efferent nerve discharge was elicited (Fig. 3C). Group data \((n = 7)\) for GSN recording experiments are shown in Fig. 4. All changes in GSN refer to comparison with basal nerve activity. An intravenous bolus injection of PE (10 \(\mu\)g/kg) decreased GSN activity significantly \((52 ± 3.2\%, P < 0.01;\) Fig. 4A). Microinjections of \(\alpha\)-Glu (5 mM; Fig. 4B) or ANG-(1–12) (0.06 mM; Fig. 4C) into the mNTS elicited significant \((P < 0.01)\) decreases in GSN activity \((48.2 ± 4.9\%\) and 15.6 ± 3\%, respectively\). After 60 min, ZD-7155 (2 mM) was microinjected at the same site, and no significant changes in GSN activity were observed (Fig. 4D). Two minutes after the microinjection of ZD-7155 into the mNTS, microinjection of ANG-(1–12) (0.06 mM) at the same site elicited a decrease in GSN activity \((2.2 ± 0.6\%;\) Fig. 4E), which was significantly attenuated \((P < 0.05)\) compared with the decrease in GSN before the microinjection of ZD-7155 (Fig. 4F). Five minutes later, \(\alpha\)-Glu (5 mM) microinjected into mNTS elicited significant decreases \((45.8 ± 5.6\%, P < 0.01)\) in GSN activity (Fig. 4F). The decreases in GSN activity elicited by \(\alpha\)-Glu (5 mM) before \((48.2 ± 4.9\%)\) and after \((45.8 ± 5.6\%)\) the microinjection of ZD-7155 (2 mM) were not significantly different \((P > 0.05)\).

Effect of pressure ejection of ANG-(1–12) on mNTS neurons. Electrical activity was extracellularly recorded from a total of 39 mNTS neurons in 12 rats. The neurons were spontaneously active at basal MAP. The firing rate of these neurons \((7.3 ± 0.8\) spikes/s\) was comparable with that reported by others (31). Consistent with a previous report (31), most mNTS barosensitive neurons did not exhibit pulse synchronicity, which was
confirmed by us using CED Spike 2 software (Cambridge Electronic Design). The increases in neuronal firing mentioned below refer to maximum changes in firing compared with firing just before the intravenous injection of PE or pressure ejection of drugs directly onto the neurons. The volume of aCSF or drugs directly ejected onto the neurons was always 2 nl. Involvement of the recorded neurons in baroreflex was tested by their excitation in response to intravenous bolus injections of PE (2–4 μg/kg). Sixteen of these neurons did not respond to the intravenous injection of PE; the firing rate before and after the PE was 7.7 ± 0.9 and 7.6 ± 1.1 spikes/s, respectively (P > 0.05), and these neurons were excluded from further experiments. The effect of ANG-(1–12) was studied in the remaining 23 barosensitive neurons. A typical recording of the changes in the firing rate of a mNTS neuron is shown in Fig. 5. The neuronal firing and MAP increased in response to an intravenous bolus injection of PE (Fig. 5A). Subsequent pressure ejection of L-Glu (5 mM) elicited a transient burst of firing immediately (Fig. 5B). Pressure ejection of aCSF onto the neuron elicited no changes in its firing (Fig. 5C). Subsequent pressure ejection of ANG-(1–12) (0.06 mM) onto the neuron elicited an increase in the firing rate; the onset and duration of the ANG-(1–12) effect was 15 and 5.5 s, respectively (Fig. 5D). Subsequent ejection of captopril (50 mM) and chymostatin (0.5 mM), sequentially within 4 s, elicited no significant change in the neuronal firing (Fig. 5E). Within 30 s, ANG-(1–12) (0.06 mM) was pressure ejected again; the increase in neuronal firing was no longer observed (Fig. 5F). In these experiments, the volume of drug or aCSF applications to the neurons was too small (2 nl) to elicit any changes in systemic MAP and HR. As shown in Fig. 5, B–F, the drugs were pressure ejected 5 s after the neuronal firing returned to the basal level. Group data for 23 barosensitive mNTS neurons are shown in Table 3; of note is the observation that the increase in mNTS neuronal firing induced by pressure ejection of ANG-(1–12) (0.06 mM) was significantly attenuated after sequential pressure ejection of captopril and chymostatin. The effect of the AT1R antagonist was studied on five barosensitive NTS neurons in another group of rats (n = 4). The increase in firing induced by pressure ejection of ANG-(1–12) on barosensitive NTS neurons was blocked by prior pressure ejection of ZD-7155 on the same neuron. These results are shown in Table 4.

Histology. The mNTS sites where microinjections of L-Glu and ANG-(1–12) elicited depressor and bradycardic responses were marked in 30 rats with microinjections of India ink (100

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**Fig. 3.** Effect of ANG-(1–12) on sympathetic nerve activity. Top trace, MAP (in mmHg); middle trace, integrated greater splanchnic nerve activity (GSNA, in μV/s); bottom trace, whole GSNA (in μV). A: inhibition of GSNA by a pressor response induced by phenylephrine (PE; 10 μg/kg iv) indicated that GSNA was barosensitive. B: microinjection of L-Glu (5 mM) into the mNTS elicited a decrease in MAP, HR (not shown), and GSNA. C: microinjection of ANG-(1–12) (0.06 mM) into the mNTS decreased MAP, HR (not shown), and GSNA.
Fig. 5. Typical tracings of a neuronal recording showing the effect of ANG-(1–12) on mNTS neurons. A: intravenous bolus injection of PE (2–4 μg/kg) elicited an increase MAP and neuronal firing. B: pressure ejection of L-Glu (5 mM) onto the neuron elicited a short burst of neuronal activity. C: pressure ejection of aCSF elicited no change in the neuronal firing rate. D: pressure ejection of ANG-(1–12) (0.06 mM) onto the neuron increased its firing. * The onset of the ANG-(1–12)-induced increase in neuronal activity was 15 s (only part of the segment showing basal neuronal firing is shown here). E: captopril (50 mM) and chymostatin (0.5 mM) were pressure ejected sequentially (within 4 s) onto the neuron; no change in neuronal firing was observed. F: subsequent pressure ejection of ANG-(1–12) (0.06 mM) within 30 s failed to elicit an increase in neuronal firing. Volume of pressure ejection was always 2 nl.

Table 3. Effect of angiotensin-converting enzyme and chymase inhibitors on mNTS neuronal activity

<table>
<thead>
<tr>
<th>Drug</th>
<th>Route</th>
<th>Before</th>
<th>After</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PE (2–4 μg/kg)</td>
<td>Intravenous</td>
<td>7.3 ± 0.8</td>
<td>18.1 ± 1.2</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>2. L-Glu (5 mM)</td>
<td>Pressure ejection</td>
<td>7.6 ± 0.9</td>
<td>20.8 ± 1.6</td>
<td>13.2 ± 1.4</td>
</tr>
<tr>
<td>3. aCSF</td>
<td></td>
<td>7.1 ± 0.9</td>
<td>7.3 ± 0.8</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>4. ANG-(1–12) (0.06 mM)</td>
<td></td>
<td>7.3 ± 0.8</td>
<td>16.7 ± 1.7</td>
<td>9.5 ± 1.1</td>
</tr>
<tr>
<td>5. Captopril (50 mM) + chymostatin (0.5 mM)</td>
<td>Pressure ejection</td>
<td>7.9 ± 0.8</td>
<td>7.3 ± 0.8</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>6. ANG-(1–12) (0.06 mM)</td>
<td></td>
<td>7.5 ± 0.8</td>
<td>8 ± 0.9</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 23 barosensitive neurons in 12 rats. Drugs were injected or pressure ejected in the same sequence as indicated (1–6). The volume of pressure ejection of each drug was 2 nl. Intravenous phenylephrine (PE) and pressure ejections of L-glutamate (L-Glu) and ANG-(1–12) significantly increased neuronal firing (P < 0.001 each). The increase in firing induced by pressure ejection of ANG-(1–12) was significantly attenuated after pressure ejection of captopril and chymostatin compared with the increase in firing before the application of these inhibitors (*P < 0.001). †The onset time of these effects was 15.9 ± 1.7 s.

DISCUSSION

The main results of this study were as follows: 1) unilateral microinjections of a new endogenous ANG, ANG-(1–12), into the mNTS elicited depressor and bradycardic responses, 2) these effects of ANG-(1–12) were mediated via AT1Rs, but not AT2Rs, in the mNTS, 3) inhibition of both ACE and chymase was necessary to completely block ANG-(1–12)-induced responses, 4) the ANG-(1–12)-induced decrease in MAP was mediated via a decrease in sympathetic nerve activity and the decrease in HR was mediated via vagus nerves, 5) direct application of ANG-(1–12) resulted in the excitation of barosensitive mNTS neurons, and 6) ANG-(1–12)-induced decreases in MAP and HR were not mediated by ANG-(1–7) in the mNTS.

The possibility that ANG-(1–12)-induced cardiovascular effects were due to the leakage of the peptide from the microinjection site in the mNTS into the peripheral circulation was excluded as follows. The dose of ANG-(1–12) that elicited maximum depressor and bradycardic effects when microinjected into the mNTS was 0.06 mM/100 nl (i.e., 6 pmol). Intravenous injections of this dose of ANG-(1–12) (6 pmol/300–360-g rats, i.e., 16.6–20 pmol/kg) did not elicit detectable cardiovascular responses in our experiments. However, intravenous injections of higher doses of ANG-(1–12) (e.g., 50 and 300 pmol/kg) elicited 6.4 ± 0.8 and 28.6 ± 3.7 mmHg increases in MAP, respectively. The increases in HR in response to the same doses of ANG-(1–12) were 5 ± 1.4 and 9.6 ± 3.0 beats/min, respectively. Our MAP responses to intravenous injections of ANG-(1–2) were comparable with those reported by Nagata et al. (24). These authors (24) did not determine HR responses to intravenous injections of ANG-(1–12).

The site specificity of ANG-(1–12)-induced cardiovascular responses was established by the lack of responses to ANG-(1–12) microinjections into the areas located adjacent to the mNTS, such as the cuneate nucleus. Local distortion of brain tissue was not responsible for the ANG-(1–12)-induced cardiovascular responses because microinjections of aCSF into the mNTS did not elicit any responses.
Based on current knowledge regarding medullospinal cardiovascular regulatory areas (10, 14, 33–35, 39), the mechanism of decreases in MAP elicited by microinjections of ANG-(1–12) into the mNTS can be explained as follows. Microinjections of ANG-(1–12) into the mNTS excited the mNTS neurons involved in cardiovascular regulation, causing an excitation of GABAergic neurons located in the caudal ventrolateral medullary depressor area, which, in turn, inhibited the presympathetic neurons located in the rostral ventrolateral medulla, causing a decrease in sympathetic nerve activity and systemic BP. Consistent with this explanation, microinjections of ANG-(1–12) into the mNTS elicited a decrease in efferent GSNA.

The proposed mechanism of ANG-(1–12)-induced cardiovascular actions was supported by our results of single unit recording in the mNTS. The presence of neurons with different patterns of firing has been reported in the mNTS (31, 36). Detailed studies on different types of mNTS neurons and determination of the type of baroreceptor input (monosynaptic or polysynaptic) they received was beyond the scope of this study. We tested the effects of ANG-(1–12) on only spontaneously firing mNTS neurons. The sensitivity of recorded mNTS neurons to afferent inputs from pressure sensitive cardiopulmonary and arterial baroreceptors was ascertained by their excitation in response to pressor responses elicited by intravenous bolus injections of PE. We recognize that barosensitivity of recorded mNTS neurons does not prove that the responsive neurons regulate the activity of cardiovagal motoneurons or the vasomotor sympathetic tone. We recorded from 23 barosensitive neurons in the mNTS at the site where baroreceptor afferents are known to terminate (e.g., 0.5–0.6 rostral and 0.5–0.8 mm lateral from the calamus scriptorius and 0.5–0.7 mm deep from the dorsal surface of the medulla) (7, 8). None of the recorded neurons showed clear pulse modulation, as determined by Spike 2 software (Cambridge Electronics Design). This observation is consistent with published literature in which very few barosensitive mNTS neurons exhibited a pulse synchronous pattern of firing (31, 36). Lack of pulse synchronous activity of barosensitive mNTS neurons is unexpected in view of the fact that most of the baroreceptor afferents show pulse rhythmicity. The fact that many NTS neurons were not pulse rhythmic has been interpreted by others to indicate that the processing of baroreceptor input in the NTS is complex and that baroreceptor afferent information is not always faithfully transmitted during each heart beat (31). In neuronal recording experiments, we preferred ejection of small volumes (2 nI) of drugs on the neurons, instead of microiontophoretic application, because known concentrations of drugs could be ejected by the small volume ejection method. The mNTS contains relatively small-size (12–15 μm) neurons (7). Therefore, the volume of pressure ejection was kept small (2 nI) so that the diffusion sphere of the...
injectate was limited. The application of ANG-(1–12) (0.06 mM, 2 nl) excited all barosensitive mNTS neurons. The application of aCSF (2 nl) did not alter firing of these neurons, thus excluding the possibility that an artifact may have caused this effect. Excitation of neurons by the direct application of L-Glu indicated that the recording was made from a neuronal cell body and not a passing fiber (46). The firing rate of the recorded barosensitive mNTS neurons was consistent with most published data (26, 31). Pressure ejection of ANG-(1–12) (0.06 mM) onto mNTS neurons elicited an ∼9–12 spikes/s increase in their firing. The onset of ANG-(1–12) (0.06 mM) effect on the mNTS neurons was ∼16 s. Prior pressure ejections of captopril and chymostatin blocked the actions of ANG-(1–12) on the mNTS neurons, suggesting the conversion of this peptide to ANG II. There are no reports in the literature on the effects of ANG-(1–12) on the extracellular activity of neurons for comparison with our results. However, even smaller concentrations of ANG II (e.g., 0.1 μM) have been reported to cause a similar excitation (an increase of ∼18 spikes/s) of hypothalamic neurons in Kyoto-Wistar rats (19).

The bradycardia elicited by microinjection of ANG-(1–12) into the mNTS was mediated via the activation of vagal innervation to the heart because bilateral vagotomy abolished these responses. Microinjections of ANG-(1–12) into the mNTS showed a nonlinear bell-shaped concentration response. A similar type of concentration response has been reported for microinjections of other peptides into the mNTS (25). This type of concentration response has been explained by homotropic allostery in which the agonist at higher concentrations binds to a modulator site, which is different from the primary binding site, and thereby affects the function of the receptor, resulting in attenuated responses (2). Another possibility is that at higher concentrations, ANG-(1–12) may activate inhibitory pathways located in the mNTS, causing an increase in sympathetic activity and BP. Thus, activation of inhibitory pathways in the mNTS by ANG-(1–12) at higher concentrations is likely to reduce the depressor and bradycardic effects of the peptide in the mNTS. In this context, it should be noted that the presence of inhibitory GABAergic neurons has been reported in the mNTS (13).

The depressor and bradycardic responses induced by unilateral microinjections of ANG-(1–12) into the mNTS were significantly attenuated by prior microinjections of ZD-7155 or L-158,809 (highly selective and potent antagonists for AT1Rs) at the same site. On the other hand, microinjections of PD-123319 (AT2R antagonist) did not significantly alter the ANG-(1–12)-induced responses. These results indicated that the effects of ANG-(1–12) in the mNTS were mediated via the activation of AT1Rs but not AT2Rs. This observation is in agreement with the report of Arnold et al. (1), who used losartan as an AT1R antagonist. These authors (1) did not test the effects of an AT2R antagonist on ANG-(1–12)-induced responses. Unilateral microinjections of ZD-7155 (2 mM) or L-158,809 (10 mM) alone into the mNTS did not significantly alter baseline MAP or HR. There are no reports in the literature for comparison with our results regarding the effects of microinjections of ZD-7155 or L-158,809 alone into the mNTS. In our study, the concentrations of ZD-7155 (2 mM) and L-158,809 (10 mM) did not exert any deleterious effects at the site of injection in the mNTS because they did not alter responses to another unrelated agonist, L-Glu, microinjected at the same site. In our study, the role of AT1Rs in the tonic control of cardiovascular function under normal physiological situations cannot be excluded based on the lack of cardiovascular responses to unilateral microinjections of AT1R antagonists alone into the mNTS. Bilateral microinjections of another AT1R antagonist (candesartan) into the mNTS have been reported to decrease resting MAP, suggesting that AT1Rs may play a role in the tonic control of BP and that they may be activated in pathological conditions such as high BP (1).

Attenuation of ANG-(1–12)-induced responses in the mNTS by prior microinjections of an ACE inhibitor (captopril) suggested that the responses elicited by ANG-(1–12) were partially mediated by its conversion to ANG II by ACE. In our experiments, the cardiovascular effects elicited by microinjections of ANG-(1–12) into the mNTS were generally similar to those reported for microinjections of ANG II (9, 40), supporting the contention that the cardiovascular effects of ANG-(1–12) in the mNTS were mediated via its conversion to ANG II. In our study, the onset of cardiovascular responses elicited by microinjections of ANG-(1–12) (0.06 mM) into the mNTS was ∼15 s. On the other hand, the onset of cardiovascular responses elicited by the same concentrations of ANG II was shorter (∼8 s). A similar onset time (∼10 s) has been reported by Tan et al. (40) for MAP responses elicited by microinjections of ANG II into the mNTS. The longer time interval needed for the onset of actions of ANG-(1–12) compared with that of ANG II supported our contention that ANG-(1–12) is converted to ANG II. Our results are in agreement with a report (24) in which the intravenous administration of ANG-(1–12) elicited immediate cardiovascular responses, indicating that the conversion of ANG-(1–12) to ANG II was rapid.

Microinjections of a chymase inhibitor (chymostatin) also significantly reduced the ANG-(1–12)-induced responses, suggesting that, in the mNTS, chymase also played a part in the conversion of ANG-(1–12) to ANG II. Higher doses of either captopril or chymostatin failed to completely block the effects of ANG-(1–12). The actions of ANG-(1–12) in the mNTS were almost abolished after sequential microinjections of captopril and chymostatin into the mNTS before the microinjections of ANG-(1–12). Using the isolated perfused rat heart, Prosser et al. (29) reported that ANG-(1–12) may be the circulating substrate for cardiac chymase-mediated ANG II production. Chymase has been identified as a major ANG II-forming enzyme in the heart (21). However, ours is the first report suggesting that chymase may be partially involved in the conversion of ANG-(1–12) to ANG II in the brain. The source of chymase in the brain may be mast cells (21, 37). The presence of mast cells has been reported in the brain (21, 26, 30, 37). Rat chymase has also been reported to be involved in the degradation of ANG II (20). However, this does not appear to the case in our study because inhibition of chymase by chymostatin would result in an exaggeration of ANG-(1–12) responses if chymase was involved in the degradation of ANG II. In our study, ANG-(1–12)-induced responses were attenuated by chymostatin.

ANG-(1–12) may also be metabolized to ANG-(1–7) (42–44). Microinjections of ANG-(1–7) into the mNTS have been reported to elicit depressor and bradycardic responses (5). However, prior microinjections of A-779, a potent ANG-(1–7) antagonist, into the mNTS, in doses that blocked the depressor effects of ANG-(1–7), did not alter the cardiovascular responses elicited by ANG-(1–12) into the mNTS.
and bradycardic effects of ANG-(1–7) in the mNTS, did not alter the cardiovascular responses to ANG-(1–12), suggesting that the generation of ANG-(1–7) was not involved in the cardiovascular actions of ANG-(1–12) in the mNTS.

A report (1) showing the impairment of baroreflex effects by microinjections of ANG-(1–12) in the NTS appeared in the literature during the writing of our article. Our results are in agreement with the observations of these authors in following respects: 1) ANG-(1–12) microinjections into the mNTS elicited depressor responses, 2) this effect was mediated via AT1Rs, and 3) this effect was partly mediated via the conversion of ANG-(1–12) to ANG II by ACE. Our study extends the observations of Arnold et al. (1) as follows: 1) microinjections of ANG-(1–12) into the mNTS elicited decreases in HR in our study, whereas Arnold et al. (1) failed to observe any changes in basal HR; 2) chymase, in addition to ACE, was involved in the conversion of ANG-(1–12) into ANG II in the mNTS; 3) we showed that the depressor responses of ANG-(1–12) were mediated via a decrease in sympathetic nerve activity and the bradycardic responses were mediated via vagal nerves; 4) the role of ANG-(1–7) in mediating the responses to ANG-(1–12) was excluded in our study by using a specific ANG-(1–7) antagonist (A-779); and 5) we showed that the direct application of ANG-(1–12) to mNTS neurons involved in baroreflex resulted in the excitation of these neurons. The onset of decrease in MAP and HR induced by microinjections of ANG-(1–12) into the mNTS in Fig. 1 of Arnold et al. (1) appeared to be longer (~1.5 min). These authors did not identify the cardiovascular site in the mNTS by prior microinjections of i-Glu (1). It is therefore possible that the microinjection of ANG-(1–12) was placed at a site slightly distant from where the cardiovascular neurons are located and, thus, a longer time was required for the diffusion of the drug to the site from where cardiovascular responses can be elicited.

In terms of the significance of newly identified ANG-(1–12), it may be pointed out that the discovery of ANG-(1–12) has challenged the idea that renin-dependent hydrolysis of angiotensinogen is the rate-limiting step for the formation of ANG peptides (11). It has been proposed that ANG-(1–12) may provide an alternate substrate for the formation of ANG peptides via a renin-independent pathway (43). Consistent with this notion is the report in which plasma and cardiac tissue expression of ANG-(1–12), ANG I, and ANG II was measured after 48 h of bilateral nephrectomy in Wistar-Kyoto rats. In these rats, the loss of kidney-derived renin caused a reduction of plasma ANG I and II to barely detectable levels, whereas there was only a small reduction in plasma and a 91% increase in the cardiac levels of ANG-(1–12), leading to the conclusion that ANG-(1–12) may serve as a precursor for the formation of ANG peptides in the absence of circulating renin (11, 12, 43). The importance of ANG-(1–12) is further highlighted by the observations that in spontaneously hypertensive and experimental anephric rats, there is an increase in ANG-(1–12) levels in cardiac tissue (12, 17). These reports suggest that there may be an increase in the formation of ANG-(1–12) in pathological states. This notion is strengthened by the observations that endogenous neutralization of ANG-(1–12) by the administration of a selective antibody via CSF decreased BP in transgenic hypertensive rats (16).

In summary, microinjections of ANG-(1–12) into the mNTS elicited depressor and bradycardic responses, which were mediated via sympathetic and vagus nerves, respectively. Both ACE and chymase inhibitors attenuated ANG-(1–12)-induced cardiovascular responses, suggesting that ANG-(1–12) was converted to ANG II in the mNTS. The direct application of ANG-(1–12) activated mNTS neurons involved in cardiovascular regulation. These observations suggest that this new ANG may play a role in the central regulation of cardiovascular function.

**Perspectives**

ANGs have been reported to play an important role in the long-term regulation of sympathetic activity and BP (22). In the NTS, AT1Rs are known to play a critical role in the modulation of cardiac baroreflex by circulating ANG II (41). In diseased states, such as chronic heart failure, enhanced cardiac sympathetic input contributes to the excitatory effect of chemoreflex, which is mediated via AT1Rs in the NTS (45). Thus, ANGs and AT1Rs in the mNTS play an important role in regulating sympathetic activity and BP in health and disease. ANG-(1–12) is the latest addition to the ANG family of peptides. Very little information is available about the role of this peptide in central cardiovascular regulation in health and disease. Our experiments are likely to provide the groundwork for future detailed studies on the central cardiovascular actions of this new peptide in normal and pathophysiological situations. As more data become available regarding the central and peripheral actions of this peptide, unique features of ANG-(1–12) actions, apart from those elicited by ANG II, may be revealed.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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