Impaired Heart Rate Baroreflex in Older Rats
Role of Endogenous Angiotensin-(1–7) at the Nucleus Tractus Solitarii

Atsushi Sakima, David B. Averill, Patricia E. Gallagher, Sherry O. Kasper, Ellen N. Tommasi, Carlos M. Ferrario, Debra I. Diz

Abstract—Age-related baroreflex reductions in function may originate from central neural dysregulation as well as vascular structural/functional changes. We determined the role of 2 angiotensin (Ang) peptides at the nucleus tractus solitarii in age-related baroreflex impairment. Baroreflex sensitivity control of heart rate in response to increases in blood pressure was tested in younger (3 to 5 months) and older (16 to 20 months) anesthetized male Sprague-Dawley rats before and after bilateral solitary tract injections of the Ang II type 1 (AT$_1$) receptor antagonist candesartan (24 pmol) or the Ang-(1–7) antagonist (d-Ala$^3$)-Ang-(1–7) (144 fmol or 24 pmol). Basal reflex sensitivity of older rats was significantly lower than younger rats. In younger rats, the reflex was facilitated by bilateral candesartan injections and attenuated by bilateral (d-Ala$^3$)-Ang-(1–7) injections. In older rats, the reflex was facilitated by AT$_1$ blockade; however, (d-Ala$^3$)-Ang-(1–7) injected into the solitary tract nucleus had no effect. Neprilysin mRNA in the medulla was lower in older rats compared with younger rats, whereas angiotensin-converting enzyme (ACE), ACE2, and mas receptor mRNA levels of older rats did not differ from values of younger rats. Thus, opposing actions of endogenous Ang II and Ang-(1–7) in the solitary tract nucleus contribute to baroreflex function in response to increases in mean arterial pressure of younger rats. The attenuated counterbalancing effect of Ang-(1–7) on baroreflex function is lost in older rats, which may be attributable to diminished production of the peptide from neprilysin. (Hypertension. 2005;46:333-340.)

Key Words: baroreceptors ■ angiotensin ■ aging

Emerging evidence suggests that changes in the central nervous system contribute to the autonomic reflex impairment associated with aging$^{1,2}$ characterized by enhanced sympathetic outflow.$^3$ Diminished baroreflex control of heart rate (HR) and splanchnic sympathetic nerve activity occurs in older female Sprague-Dawley rats.$^4$ Aging also attenuates neurotransmitter actions in the nucleus tractus solitarii (solitary tract nucleus; NTS), where baroreceptor and chemoreceptor afferents terminate.$^5$–$^8$ Cardiovascular responses to serotonin,$^9$ clonidine,$^7$ or the $\gamma$-aminobutyric acid type A agonist muscimol$^9$ microinjected into the NTS of older rats were significantly attenuated compared with younger rats. NTS lesions by 6-hydroxydopamine reduced reflex bradycardia in 3- but not 14-month-old Sprague-Dawley rats, suggesting that catecholaminergic pathways within the NTS that regulate reflex bradycardia become impaired with aging.$^5$

Aging may also alter formation of components of the brain renin-angiotensin system (RAS). mRNA levels of angiotensin II type 1 (AT$_1$) receptor subtypes in rat brain gradually decreased during aging (3 to 20 months).$^9$ Angiotensinogen mRNA levels in medulla oblongata in old rats were slightly but significantly decreased compared with adult rats.$^{10}$ Angiotensin II (Ang II) participates in cardiovascular reflex control in the NTS.$^{11–16}$ Microinjection of Ang II in the NTS attenuated baroreceptor reflex sensitivity,$^{14,15}$ whereas microinjection of nonselective Ang II antagonist [Sar$^1$Thr$^8$]-Ang II or selective AT$_1$ antagonist candesartan (CV-11974) enhanced baroreceptor reflex sensitivity.$^{14,16}$ Ang-(1–7) also mediates actions of the brain RAS.$^{17}$ Ang-(1–7) opposes the actions of Ang II in baroreflex control of HR.$^{11,12,18–21}$ Baroreflex function was improved after injection of Ang-(1–7) and impaired by the Ang-(1–7) antagonist (d-Ala$^3$)-Ang-(1–7) injections into NTS.$^{18–21}$ Formation of Ang-(1–7) from Ang I may be catalyzed by the endopeptidase neprilysin,$^{22–24}$ although other enzymes may contribute as well. Because neprilysin activity is downregulated during the aging process,$^{25–27}$ formation of Ang-(1–7) may decrease with aging. We hypothesized that with aging, baroreflex impairment or alterations in reflex responses to activation of cardiopulmonary chemosensitive afferent fibers would result, in part, from reduced formation of Ang-(1–7) by neprilysin because transgenic rats with upregulation or downregulation of the brain RAS exhibit alterations in these responses.$^{13}$ We determined whether blockade of either AT$_1$ or Ang-(1–7) receptors altered responses to activation of either reflex and measured mRNA levels for enzymes potentially participating

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The Hypertension and Vascular Disease Center, Department of General Surgery and Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC.
Correspondence to Debra I. Diz, PhD, Professor, Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1032. E-mail ddiz@wfubmc.edu
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in formation (neprilysin and angiotensin-converting enzyme 2 [ACE2]28,29) or metabolism (ACE) of Ang-(1–7) as well as the Ang-(1–7) receptor mas30 in medulla oblongata.

Methods

Animals
Experiments were performed in 3- to 5-month-old and 16- to 20-month-old male Sprague-Dawley rats (Hannover Sprague-Dawley, from the Hypertension and Vascular Disease Center Colony, Wake Forest University School of Medicine, Winston-Salem, NC, or Harlan Sprague-Dawley, Indianapolis, Ind). Rats were housed in group cages in temperature- and humidity-controlled rooms (12-hour light/dark cycle) with free access to standard rat chow and water. All procedures were approved by the institutional animal care and use committee.

Surgical Procedures
Anesthesia was induced with a combination of urethane and chloralose (750 mg/35 mg per kg IP) and supplemental doses were given intravenously. Polyethylene catheters (PE-50 tubing; Clay Adams) were inserted into a femoral artery and vein. The venous catheter was positioned near the right atrium. Rats were placed in a stereotaxic frame (David Kopf Instruments) for surgical exposure of the dorsal medulla oblongata.13,14 Animals breathed a mixture of 70% room air and 30% oxygen. Body temperature was maintained at 37.0 ± 1.0°C by a heating pad (model 39 DP; Braintree Scientific Inc.). Corneal reflexes, respiratory rhythm, and stability of mean arterial pressure (MAP) and HR were used to monitor depth of anesthesia throughout the experiment and supplemental doses of anesthetic were administered as required.

Microinjection and Reflex Study
Pulsatile arterial pressure was monitored via strain gauge transducer connected to a femoral arterial catheter and data acquisition system (Acknowledge software; V 3.7.2; BIOPAC System Inc.). HR was determined from the arterial pressure wave. Multibarreled glass microinjection needles (Kimpl Klemp Inc.) with a tip diameter of 30 to 50 μm were coated with silicone (Silmacote; Sigma Chemical Co.) and placed into medial NTS (0.4 mm rostral and 0.4 mm lateral to calamus scriptorius, and 0.4 mm below dorsal surface).13-15 The AT1, antagonist candesartan (CV-11974; 24 pmol in 120 nL) and the Ang-(1–7) antagonist (D-Ala7)-Ang-(1–7) (144 fmol in 120 nL) dissolved in artificial cerebrospinal fluid (aCSF; pH 7.4, 120 nL) as vehicle control was microinjected bilaterally into NTS. The concentration of candesartan was found previously to effectively increase baroreflex sensitivity (BRS) of spontaneously hypertensive rats (SHR) or Wistar-Kyoto rats (WKY).16 Injections were given over a 50-s interval via hand-held syringe as described previously.15 One group of younger or older animals received injections of candesartan into the NTS first, followed by a second NTS injection ~60 minutes later of (D-Ala7)-Ang-(1–7). Another group at each age received (D-Ala7)-Ang-(1–7) into the NTS and then a second injection of (D-Ala7)-Ang-(1–7) ~60 minutes later, followed within 5 minutes by candesartan. In a separate group of older rats (n=4), 24 pmol (D-Ala7)-Ang-(1–7) in 120 nL was injected into the NTS.

Baseline values of MAP and HR were obtained for ≥30 minutes before administration of test agents. Phenylbiguanide and phenylephrine were administered through a venous catheter before microinjections to establish baseline responses of the cardiopulmonary vagal afferent activation (CVA) and the BRS, respectively. The sequence began with phenylbiguanide (10 μg/kg; diluted in 0.9% NaCl) followed by graded doses of phenylephrine (2, 5, and 10 μg/kg; diluted in 0.9% NaCl). A period of 10 to 20 minutes was allowed between injections of phenylbiguanide and phenylephrine, and 5 minutes was allowed between various doses of phenylephrine so that all reflex tests were completed within 30 minutes. The volume of intravenous infusion of each drug was 0.05 mL. MAP and HR were allowed to return to baseline values before the next dose was administered. The baseline before each drug dose was used to calculate the peak MAP responses (ΔMAP; mm Hg) and the associated reflex changes in HR (ΔHR; bpm). The series of injections for reflex testing using phenylbiguanide and phenylephrine was repeated starting within 10 minutes of injection of the first antagonist into the NTS and again within 10 minutes of NTS injection of the second antagonist ~60 minutes later.

Changes in HR were converted to change in pulse interval (PI; ms) by the formula 60 000/HR. The slope of line fit through the increase in MAP, and corresponding change in PI (ΔPI) in response to graded doses of phenylephrine was used as an index of BRS for control of HR.14,15 Data were also analyzed for direct changes in HR versus changes in MAP yielding comparable results; the BRS using PI was used for all data presentation for consistency with previous studies.14,15 At the end of each experiment, the brain was removed and frozen, and correct location of injection sites within the medullary NTS at rostrocaudal level −13.3 to −14.0 mm caudal to bregma20 was assessed histologically.13

Quantification of ACE, ACE2, Neprilysin and mas Receptor mRNAs
Total RNA was isolated from the medulla of each rat with Trizol reagent (GIBCO/BRL) as described by the manufacturer. The RNA concentration was determined by UV spectroscopy at 260 nm and an estimate of purity assessed by optical density ratio at 260/280 nm. RNA integrity was examined by ethidium bromide staining intensity of 28S and 18S ribosomal RNA after agarose gel electrophoresis and then incubated with RNase (Promega) to eliminate residual DNA that might amplify during polymerase chain reaction (PCR). Approximately 1 μg of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase in a mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in reverse transcriptase buffer; heating at 95°C terminated the reaction. PCR assays were performed as described previously33 using gene-specific primers (Table 1). Amplification conditions were as follows: denaturation for 1 minute at 94°C; annealing for 1 minute at 60°C, except the neprilysin primers, which had an annealing temperature of 56°C; and elongation for 1 minute at 72°C, with a final elongation step for 7 minutes at 72°C. Primers for control elongation factor 1a (EF1α) sequence (347 bp) were added to all reactions at the cycle number indicated in Table 1, except in the neprilysin reactions in which a primer pair for a shorter 260-bp EF1α segment was added. Amplification products were separated on 6% polyacrylamide gels, visualized using a PhosphorImager, and quantified.

### Table 1. Primer Pairs for ACE, ACE2, Neprilysin, and mas Receptor mRNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pair</th>
<th>Fragment Size (bp)</th>
<th>Total Cycles*</th>
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<tbody>
<tr>
<td>mas receptor</td>
<td>5′-CTCTCGGTCCTCGGTCGAGC-3′-forward</td>
<td>474</td>
<td>32 (10)</td>
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<tr>
<td></td>
<td>5′-AGGGAGTGACACGCGAGAGTA-3′-reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neprilysin</td>
<td>5′-CTATCCGATGACATCCTC-3′-forward</td>
<td>318</td>
<td>29 (7)</td>
</tr>
<tr>
<td></td>
<td>5′-TGTGATTTCATGCTGCGATGACC-3′-reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>5′-TGATGGTGCAGAATTCCG-3′-forward</td>
<td>421</td>
<td>26 (5)</td>
</tr>
<tr>
<td></td>
<td>5′-GGTGCAAGCTCTGGTGATA-3′-reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE2</td>
<td>5′-GTCGACAAAGGTCGACAAATG-3′-forward</td>
<td>410</td>
<td>30 (10)</td>
</tr>
<tr>
<td></td>
<td>5′-GTGGTCATGACGAGGACAG-3′-reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF1α</td>
<td>5′-GGAATGGTGCAGACACATGG-3′-forward</td>
<td>347</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5′-GTTGGAA6CTCATTGTTGC-3′-reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF1α</td>
<td>5′-GGAATGGTGCAGACACATGG-3′-forward</td>
<td>260</td>
<td>—</td>
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</table>

*Total cycles signifies the No. of amplification cycles performed for the specific target gene. The primer pairs for the EF1α control were added after the No. of cycles indicated in the parenthesis.
by computerized densitometry. The mRNA concentration was expressed as the ratio of target to control \( \text{EF1_{H9251}}/\text{H9251} \) to account for variations in the RT-PCR assay.

**Data Analysis**

Values are presented as mean±SEM. To assess baseline (before microinjection) differences between the 2 ages, all baseline values were pooled. Comparison of baseline values of MAP, HR, BRS, and CVA between younger and older rats was made by the Student’s unpaired \( t \) test, and changes in these variables in the separate group of older rats receiving 24 pmol \((\text{D-Ala}^7)-\text{Ang-(1–7)}\) injection were made by the Student’s paired \( t \) test. We also analyzed the baseline values of BRS between younger and older rats by linear regression. Comparisons of changes in MAP and HR and BRS and CVA over time and in response to different drug treatments in a given animal were made by ANOVA for repeated measures with post hoc Scheffe’s multiple comparisons. ANOVA with post hoc Scheffe’s multiple comparisons was used to compare group baselines (before microinjections) for MAP and HR. In separate groups of rats, comparisons of ACE, ACE2, neprilysin, and \( \text{mas} \) receptor mRNA levels between younger and older rats were made by Student’s unpaired \( t \) test. The criterion for statistical significance was \( P<0.05 \) and tests were performed using Prism (GraphPad Software) and StatView-15.0 (SAS Institute Inc.).

**Results**

**Baseline MAP, HR, Adrenergic Cardiovascular Responsiveness and Reflex Function**

Baseline MAP of anesthetized animals was not different between younger and older rats. In contrast, baseline HR of older rats was significantly lower than younger rats. As shown in Figure 1A, increases in MAP in response to graded doses of phenylephrine were significantly attenuated in older rats compared with younger rats \( (*P<0.0001) \). The increases in PI in response to phenylephrine-induced increased MAP were less in older rats compared with younger rats \( (P<0.02) \). C. The relationship between increases in blood pressure and reflex bradycardia was determined in each rat and the slope used as an index of BRS. The baseline BRS of older rats was significantly attenuated compared with younger rats \( (P<0.01) \). D. The relationship between increases in blood pressure and reflex bradycardia analyzed by linear regression for older and younger rats is shown (95% confidence intervals of slope are from 0.43 to 0.83 in the older rats and from 0.86 to 1.15 in the younger rats). \( *P<0.0001 \) when compared with younger rats; \( **P<0.02 \) when compared with younger rats; \( #P<0.01 \) when compared with younger rats.

\( \text{Figure 1. Baseline } \alpha_1\text{-adrenergic cardiovascular responsiveness and HR BRS in younger and older rats. A, The increases in MAP in response to graded doses of phenylephrine were significantly attenuated in older rats compared with younger rats (\(*P<0.0001\)). B, The increases in PI in response to phenylephrine-induced increased MAP were less in older rats compared with younger rats (\(P<0.02\)). C, The relationship between increases in blood pressure and reflex bradycardia was determined in each rat and the slope used as an index of BRS. The baseline BRS of older rats was significantly attenuated compared with younger rats (\(P<0.01\)). D, The relationship between increases in blood pressure and reflex bradycardia analyzed by linear regression for older and younger rats is shown (95% confidence intervals of slope are from 0.43 to 0.83 in the older rats and from 0.86 to 1.15 in the younger rats). \(*P<0.0001\) when compared with younger rats; \(**P<0.02\) when compared with younger rats; \(#P<0.01\) when compared with younger rats.} \)
TABLE 2. Baseline MAP, HR, BRS, and CVA in Younger and Older Rats

<table>
<thead>
<tr>
<th>Variables</th>
<th>Younger (n=24)</th>
<th>Older (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>95±2</td>
<td>90±3</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>318±5</td>
<td>294±7*</td>
</tr>
<tr>
<td>BRS, ms/mm Hg</td>
<td>1.11±0.06</td>
<td>0.68±0.04†</td>
</tr>
<tr>
<td>CVA-ΔMAP, mm Hg</td>
<td>−59±3</td>
<td>−59±3</td>
</tr>
<tr>
<td>CVA-ΔHR, bpm</td>
<td>−225±15</td>
<td>−222±11</td>
</tr>
</tbody>
</table>

Values are means±SEM. n indicates No. of rats.

*P<0.01 when compared with younger rats; †P<0.0001 when compared with younger rats.

Differences in these values. Bilateral injection of candesartan, d-Ala2-Ang-(1–7), or aCSF into NTS had no effect on MAP or HR or pressor responses to phenylephrine injection during reflex testing (data not shown).

As shown in Figure 2A, bilateral injection of candesartan into NTS significantly improved BRS, as calculated using changes in PI in response to increases in MAP in younger (P<0.001) and older rats (P<0.01). In younger rats, augmentation of BRS induced by blockade of AT1 receptors in NTS was reversed by d-Ala2-Ang-(1–7) injection [P<0.001, candesartan versus (d-Ala2)-Ang-(1–7) in the presence of candesartan], whereas this was not observed in older rats. Bilateral injection of (d-Ala2)-Ang-(1–7) into NTS significantly attenuated BRS in younger (P<0.001) but not older rats (Figure 2B). In younger rats, attenuation of BRS induced by Ang-(1–7) blockade into NTS was reversed by candesartan (P<0.001; (d-Ala2)-Ang-(1–7) versus candesartan in the presence of (d-Ala2)-Ang-(1–7)). Moreover, the BRS was facilitated compared with baseline (control) values when candesartan was given in the presence of (d-Ala2)-Ang-(1–7). These data suggest that the contribution of endogenous Ang II to attenuate BRS is greater in the absence of endogenous Ang-(1–7) in younger animals.

In older rats, (d-Ala2)-Ang-(1–7) injection did not attenuate BRS; further, pretreatment with (d-Ala2)-Ang-(1–7) did not affect subsequent BRS enhancement induced by candesartan (P<0.002; (d-Ala2)-Ang-(1–7) versus (d-Ala2)-Ang-(1–7) followed by candesartan). Moreover, the magnitude of the increase in BRS after AT1 blockade was comparable whether in the absence or presence of (d-Ala2)-Ang-(1–7). In a separate group of older rats (n=4), a higher dose (24 pmol) of (d-Ala2)-Ang-(1–7) injected into NTS did not affect resting values of MAP (from 103±4 to 100±2 mm Hg), HR (from 291±3 to 300±14 bpm), BRS (from 0.66±0.19 to 0.66±0.16 s/mm Hg), or CVA (ΔMAP from −61±5 to −59±9 mm Hg; ΔHR from −203±29 to −207±47 bpm).

Control aCSF injection did not alter BRS in either age group of rats (Figure 2C). The reflex reductions in MAP and HR evoked by CVA with phenylbiguanide are shown in Table 4. Neither injection of candesartan, (d-Ala2)-Ang-(1–7) nor aCSF into NTS altered responses to CVA in younger or older rats.

Changes in ACE, ACE2, Neprilysin, and mas Receptor mRNA in Rat Medulla

ACE, ACE2, neprilysin, and the Ang-(1–7) receptor mas mRNA levels in rat medulla oblongata were determined by RT-PCR, as shown in Figure 3. Densitometric analysis demonstrated that the ratio of neprilysin mRNA to EF1α

TABLE 3. Prevailing Values of MAP and HR Before Reflex Testing in Younger and Older Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Younger</th>
<th>Old er</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAP (mm Hg)</td>
<td>HR (bpm)</td>
</tr>
<tr>
<td>Candesartan/d-Ala2-Ang-(1–7)</td>
<td>Baseline</td>
<td>8 95±3</td>
</tr>
<tr>
<td></td>
<td>Candesartan</td>
<td>8 97±2</td>
</tr>
<tr>
<td>d-Ala2-Ang-(1–7)</td>
<td>Baseline</td>
<td>8 96±3</td>
</tr>
<tr>
<td>d-Ala2-Ang-(1–7)/candesartan</td>
<td>Baseline</td>
<td>8 92±3</td>
</tr>
<tr>
<td></td>
<td>d-Ala2-Ang-(1–7)</td>
<td>8 92±3</td>
</tr>
<tr>
<td></td>
<td>Candesartan</td>
<td>8 94±3</td>
</tr>
<tr>
<td>aCSF/aCSF</td>
<td>Baseline</td>
<td>8 97±3</td>
</tr>
<tr>
<td></td>
<td>aCSF1</td>
<td>8 92±3</td>
</tr>
<tr>
<td></td>
<td>aCSF2</td>
<td>8 94±3</td>
</tr>
</tbody>
</table>

Values are means±SEM. n indicates No. of rats.

Values were determined in the minute preceding each series of reflex testing.

For candesartan/d-Ala2-Ang-(1–7), rats first received candesartan injections into the NTS before reflex testing followed by d-Ala2-Ang-(1–7) before the second set of reflex tests; d-Ala2-Ang-(1–7)/candesartan, rats received d-Ala2-Ang-(1–7) first followed by candesartan injection into the NTS; aCSF/aCSF, rats received control aCSF injection into the NTS to serve as a time control.
mRNA was significantly reduced in older compared with younger Hannover Sprague-Dawley rats. ACE, ACE2, and mas receptor mRNA levels of older rats did not differ from values of younger rats.

**Discussion**

We found the following: (1) AT1 blockade improved BRS, as assessed by changes in PI in response to increases in MAP in young and older rats, whereas Ang-(1–7) blockade attenuated BRS only in younger rats. In older rats, Ang-(1–7) blockade at the NTS was without effect on BRS. (2) Neprilysin mRNA in medulla was significantly lower in older rats compared with younger rats. Thus, an attenuated counterbalancing effect of endogenous Ang-(1–7) on baroreflex function in older rats may, in part, reflect decreased production of the peptide by neprilysin. Although our studies were performed in Sprague-Dawley rats at a relatively early time point in aging, the loss of endogenous effects of Ang-(1–7) on the reflex in the NTS occurred at a time when increases in systolic blood pressure, insulin resistance, and memory deficits have been reported in conscious Sprague-Dawley rats.34–36

**TABLE 4. Effects of Candesartan or D-Ala7-Ang-(1–7) Injection on the Response to CVA in Younger and Older Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Younger</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔMAP (mm Hg)</td>
<td>ΔHR (bpm)</td>
</tr>
<tr>
<td>Candesartan/D-Ala7-Ang-(1–7) Baseline</td>
<td>8</td>
<td>55±6</td>
</tr>
<tr>
<td>Candesartan</td>
<td>8</td>
<td>61±4</td>
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<tr>
<td>D-Ala7-Ang-(1–7) Baseline</td>
<td>8</td>
<td>59±3</td>
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<tr>
<td>D-Ala7-Ang-(1–7)/candesartan Baseline</td>
<td>8</td>
<td>62±5</td>
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<td>D-Ala7-Ang-(1–7)</td>
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<td>Candesartan</td>
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<td>58±4</td>
</tr>
<tr>
<td>aCSF2</td>
<td>8</td>
<td>60±4</td>
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</tbody>
</table>

Values are means±SEM. n indicates No. of rats. For candesartan/D-Ala7-Ang-(1–7), rats received candesartan followed by D-Ala7-Ang-(1–7) injection into the NTS; D-Ala7-Ang-(1–7)/candesartan, rats received D-Ala7-Ang-(1–7) followed by candesartan injection into the NTS; aCSF/aCSF, rats received control aCSF injection into the NTS.
The existence of receptors for Ang II\textsuperscript{17} and Ang-(1–7)\textsuperscript{12,17} in NTS has been documented.\textsuperscript{17} Microinjection of CV-11974, the AT\textsubscript{1} selective antagonist used in this study, into NTS of SHR and WKY improved baroreflex function.\textsuperscript{16} HR BRS was improved after injection of Ang-(1–7) and impaired by an Ang-(1–7) antagonist (D-Ala\textsubscript{7})-Ang-(1–7) injection into NTS in normotensive WKY as well as in SHR or renal hypertension.\textsuperscript{18–21} The current study of younger rats confirms these previous experiments. The magnitude of the BRS increase with candesartan or decrease with (D-Ala\textsubscript{7})-Ang-(1–7) was similar in older rats whether the antagonist was given as the first injection or after previous blockade of the other receptor. In contrast, in younger animals, facilitation of BRS after AT\textsubscript{1} blockade appears greater in the absence of Ang-(1–7). Thus, the design of our studies shows that the 2 peptides work independently of each other, which is not surprising because Ang II but not Ang-(1–7) increases release of substance P and norepinephrine from brain medullary slices.\textsuperscript{37}

Ang-(1–7) levels in plasma increase in patients with hypertension, SHR, [mRen-2]\textsuperscript{27} rats, and rat models of myocardial infarction during treatment with ACE inhibitors or AT\textsubscript{1} antagonists.\textsuperscript{38–43} Blockade of endogenous Ang-(1–7) reverses \approx 30\% of antihypertensive effects of these treatments\textsuperscript{39,39,41} and the improvement in BRS of HR in SHR or renovascular hypertensive rats after treatment with an ACE inhibitor.\textsuperscript{18,44} Furthermore, in salt-depleted losartan-treated SHR, the depressor effect of losartan was reversed in part by (\textalpha-Ala\textsubscript{3})-Ang-(1–7) via non AT\textsubscript{1}/AT\textsubscript{2} receptor mechanisms.\textsuperscript{41} Our observation in younger rats shows that endogenous Ang II and Ang-(1–7) at the NTS independently and oppositely modulate BRS.

AT\textsubscript{1} blockade at the NTS improved BRS in Sprague-Dawley rats to a similar extent at older and younger ages. Kalinyak et al showed that AT\textsubscript{1} receptor mRNA in rat brain gradually decreased during aging.\textsuperscript{9} There were no significant differences in AT\textsubscript{1} receptor density in medulla oblongata of 15-, 48-, and 68-week-old male Sprague-Dawley rats, despite a decline in AT\textsubscript{1a} mRNA.\textsuperscript{45} Interestingly, AT\textsubscript{1b} mRNA increased in these animals over time, perhaps contributing to lack of change in overall AT\textsubscript{1} receptor density.\textsuperscript{45} Small but significant decreases in angiotensinogen mRNA occurred in medulla oblongata of older compared with younger adult rats.\textsuperscript{19} The lack of change in AT\textsubscript{1} receptor density defined pharmacologically in medullary tissue would be consistent with maintenance of tonic inhibition of BRS by Ang II in younger and older Sprague-Dawley rats.

Ang-(1–7) formation from Ang I is by a variety of endopeptidases, including neprilysin, and metabolism is primarily by ACE.\textsuperscript{46} Lower expression of neprilysin mRNA in medulla of older compared with younger rats suggests that attenuated counterbalancing effect of endogenous Ang-(1–7) on baroreflex function may partly reflect decreased production of the peptide. A novel ACE-related carboxypeptidase, ACE2, was identified recently.\textsuperscript{28,29} ACE2 converts Ang I to Ang-(1–9) and Ang II to Ang-(1–7). The Ang II-to-Ang-(1–7) conversion exhibits the highest kinetics of 100 other potential substrates. Finally, Santos et al determined recently that Ang-(1–7) is an endogenous ligand for the G-protein–coupled receptor mas.\textsuperscript{30} That the present data show no alterations of ACE, ACE2, or mas receptor mRNA in rat medulla oblongata at 2 ages suggests that age-related baroreflex impairment and altered responses to blockade of endogenous Ang-(1–7) are not a result of altered peptide metabolism through ACE, production from Ang II via ACE2, or reduced actions at mas receptors. We cannot rule out that ACE2 and mas receptor mRNA in other brain sites (eg, hypothalamus) with projections to medulla may be altered during the aging process or that more localized changes in mRNA such as in the dorsomedial medulla oblongata may occur. Demonstration of reduced enzyme activity as well as reduced protein levels is also necessary to support the change in mRNA seen for neprilysin. Nonetheless, the decrease in neprilysin mRNA in brain medulla in older animals is consistent with previous findings of reduced neprilysin activ-

![Figure 3. ACE, ACE2, neprilysin, and mas receptor mRNA in medulla oblongata of younger and older Rats. ACE (A), ACE2 (B), neprilysin (C), and mas receptor (D) mRNA in rat medulla oblongata was quantified by RT-PCR. Densitometric analysis of mRNA normalized against EF\textalpha{1} mRNA in each experiment (younger n=9; older n=7).](http://hyper.ahajournals.org/)

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**Figure 3.** ACE, ACE2, neprilysin, and mas receptor mRNA in medulla oblongata of younger and older Rats. ACE (A), ACE2 (B), neprilysin (C), and mas receptor (D) mRNA in rat medulla oblongata was quantified by RT-PCR. Densitometric analysis of mRNA normalized against EF\textalpha{1} mRNA in each experiment (younger n=9; older n=7).
ity in forebrain areas, kidney, and plasma in older rats and mice.25–27

Peripheral functional or structural changes as well as central neural impairment may contribute to diminished BRS in older rats.1–2 The attenuated α1-adrenergic vascular responsiveness and lower resting HR in older rats may partly account for baroreflex impairment during aging. In older rats, an attenuated pressor response to phenylephrine may elicit inadequate carotid sinus stretch, which, in turn, may reduce baroreflex afferent input to NTS. In rats, a decrease64 and no change65,66 in HR have been found with aging. These discrepancies may be attributed to differences in rat strain, age, or anesthetics used, but dysfunction of atrial cardiac pacemaker cells2,48 or cardiac β-adrenoceptor density and responsiveness2 may decrease resting HR and contribute to attenuated BRS in older rats. However, because we tested the reflex before and during local blockade of Ang II and Ang-(1-7) within NTS, the findings implicate alterations at this brain site involving Ang-(1-7) in age-related differences as well. In addition, use of ~150-fold higher doses of the Ang-(1-7) antagonist in older animals did not reveal a contribution of Ang-(1-7) to the reflex. Although higher doses of the antagonist may ultimately reveal some contribution of the peptide in older rats, major differences in sensitivity to blockade of Ang-(1-7) exist between younger and older animals.

Cardiopulmonary baroreflex function is preserved,49 diminished,50,51 or augmented52 during aging. Responses to CVA were preserved in older rats in this study, and cande-sartan did not alter responses at either age. Ang II acts at AT1 receptors in the periphery to enhance53 and centrally to attenuate16 activation of carotid chemoreceptors, whereas captopril treatment facilitates responses to CVA.54 Genetically engineered animals with long-term elevations in endogenous Ang II in medulla oblongata are associated with reduced responses to CVA in [mRen-2]27 rats,12 whereas animals with reductions of brain angiotensinogen have enhanced responses.12 Thus, differences exist in central versus peripheral actions, and biphasic actions may occur with low and high doses of Ang II.55,56 (d-Ala5)-Ang-(1-7) injected in NTS did not affect responses to CVA in either age group. Differential actions of Ang II and Ang-(1-7) on barosensory versus chemosensation may also relate to lack of convergence of sensory inputs within NTS.57

In summary, comparison of cardiovascular and reflex effects induced by AT1 versus Ang-(1-7) blockade in NTS of younger and older Sprague-Dawley rats demonstrated that opposing actions of endogenous Ang II at AT1 receptors and Ang-(1-7) at its unique receptor within the NTS contributed to baroreflex function of younger rats. In older rats, improvement of BRS by AT1 blockade in NTS was not reversed by d-Ala5-Ang-(1-7) injection, and d-Ala5-Ang-(1-7) injected into NTS did not further attenuate BRS of older rats. Neprilysin mRNA in medulla oblongata was significantly lower in older than younger rats. Therefore, an attenuated counterbalancing effect of endogenous Ang-(1-7) at NTS on baroreflex function in older rats may reflect decreased production of the peptide by neprilysin and contribute to diminished BRS in older rats.

**Perspectives**

Blockade of RAS may provide a favorable outcome for older individuals. Indeed, clinical studies indicate beneficial effects of RAS blockade in elderly hypertensive patients.58,59 The present results support this concept. Blood pressure-lowering effects of ACE inhibitors and AT1 antagonists are attributable not only to direct inhibition of Ang II synthesis and direct AT1 receptor blockade but also to accumulation of Ang-(1-7).38–41 Chronic RAS blockade may reverse the attenuated production of brain Ang-(1-7) with aging and elicit beneficial effect for older individuals. Recently, Tagawa and Dampney demonstrated that AT1 receptors mediate activation of rostral ventrolateral medulla presynaptic neurons evoked by disinhibition of neurons in the hypothalamus.60 Because sympathetic nerve activity increases with aging, activation of this pathway may contribute to the increased sympathetic outflow with aging.

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Impaired Heart Rate Baroreflex in Older Rats: Role of Endogenous Angiotensin-(1–7) at the Nucleus Tractus Solitarii
Atsushi Sakima, David B. Averill, Patricia E. Gallagher, Sherry O. Kasper, Ellen N. Tommasi, Carlos M. Ferrario and Debra I. Diz

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