Modulation of Reflex Function by Endogenous Angiotensins in Older Transgenic Rats With Low Glial Angiotensinogen

Amy C. Arnold, Atsushi Sakima, Detlev Ganten, Carlos M. Ferrario, Debra I. Diz

Abstract—Age-related impairments in baroreflex sensitivity in Sprague-Dawley rats are associated with low solitary tract nucleus content of angiotensin-(1-7). However, transgenic rats with low-brain angiotensinogen resulting from glial overexpression of an antisense oligonucleotide to angiotensinogen (ASrAOGEN) are spared age-related declines in cardiovascular function characteristic of Sprague-Dawley rats. We examine whether cardiovascular and reflex actions of angiotensin-(1-7) persist in the solitary tract nucleus of older (16 to 22 months) ASrAOGEN rats. Baroreflex sensitivity for control of heart rate and chemosensitive vagal afferent activation in response to phenylbiguanide were measured before and after bilateral microinjection of the angiotensin II type 1 receptor antagonist candesartan and angiotensin-(1-7) receptor antagonist (D-Ala7)-angiotensin-(1-7) in urethane/chloralose-anesthetized rats. In older anesthetized ASrAOGEN rats, candesartan had no effect, whereas (D-Ala7)-angiotensin-(1-7) significantly reduced baroreflex sensitivity (1.80±0.43 versus 0.50±0.17 ms/mm Hg). Phenylbiguanide responses were attenuated by injection of candesartan (−79±6 versus −55±12 mm Hg and −277±12 versus −156±27 bpm; P<0.05). In addition, resting blood pressure was reduced by injection of candesartan or (D-Ala7)-angiotensin-(1-7) in urethane/chloralose-anesthetized rats. In older anesthetized ASrAOGEN rats, the antisense oligonucleotide results in decreased drinking responses to renin and lower hypothalamic tissue levels of Ang I with a trend for lower levels of Ang II compared with Sprague-Dawley (SD) rats.7 Consistent with expression of Aogen mRNA in neurons of the paraventricular nucleus (PVN), neuronal Ang II and Ang-(1-7) are preserved in PVN of these animals compared with SD rats.8 Functionally, conscious adult ASrAOGEN rats (3 to 5 months) have enhanced baroreceptor9 and chemoreceptor10 reflexes and lower resting mean AP (MAP) compared with age-matched SD rats.4,11 Solitary tract nucleus (NTS) blockade of Ang II type 1 (AT1) receptors has no effect on BRS, whereas blockade of Ang-(1-7) receptors impairs BRS in younger ASrAOGEN rats, suggesting a glial source of Aogen for Ang II and a nonglial source for Ang-(1-7) for modulating BRS for control of HR.12 A paradoxical enhanced response to cardiac vagal afferent chemosensitive fiber acti-

Key Words: transgenic rats ■ brain renin-angiotensin system ■ solitary tract nucleus ■ aging

Angiotensinogen (Aogen), the precursor of both angiotensin (Ang) II and Ang-(1-7), is expressed primarily in glial cells but is also present in neuronal cells in key cardiovascular nuclei. Double transgenic mice overexpressing both human Aogen and renin in either glial or neuronal cells suggests that glia-derived Ang peptides are responsible for impairments in the baroreflex sensitivity (BRS) for control of heart rate (HR), whereas neuronal overexpression of Ang peptides resets the baroreceptor set point without altering BRS. Both glial and neuronal overexpression contribute to increases in resting arterial pressure (AP).2

Transgenic rats with low-brain Aogen (ASrAOGEN) offer a targeted disruption of the endogenous renin-Ang system (RAS). The antisense oligonucleotide targeted to glia by a glial fibrillary acidic protein promoter results in a 90% reduction in brain Aogen. This promoter drives expression specifically to astrocytes in both cell culture and in vivo experiments.6 In ASrAOGEN rats, the antisense oligonucleotide results in decreased drinking responses to renin and lower hypotalamic tissue levels of Ang I with a trend for lower levels of Ang II compared with Sprague-Dawley (SD) rats. Consistent with expression of Aogen mRNA in neurons of the paraventricular nucleus (PVN), neuronal Ang II and Ang-(1-7) are preserved in PVN of these animals compared with SD rats. Functionally, conscious adult ASrAOGEN rats (3 to 5 months) have enhanced baroreceptor reflexes and chemoreceptor reflexes and lower resting mean AP (MAP) compared with age-matched SD rats. Solitary tract nucleus (NTS) blockade of Ang II type 1 (AT1) receptors has no effect on BRS, whereas blockade of Ang-(1-7) receptors impairs BRS in younger ASrAOGEN rats, suggesting a glial source of Aogen for Ang II and a nonglial source for Ang-(1-7) for modulating BRS for control of HR. A paradoxical enhanced response to cardiac vagal afferent chemosensitive fiber acti-

Received November 25, 2007; first decision December 19, 2007; revision accepted February 14, 2008.

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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.107.106005

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viation (CVA) and elevated resting MAP occurs under anesthesia compared with SD rats.\textsuperscript{10} The elevated MAP in anesthetized ASrAOGEN rats was reversed by blockade of either AT\textsubscript{1} or Ang-(1-7) receptors.\textsuperscript{10}\n
In contrast to older SD rats (16 months), ASrAOGEN rats do not show typical age-related deficits, such as increases in systolic blood pressure or indices of metabolic dysfunction.\textsuperscript{11} Although baroreflex function is reduced in conscious older ASrAOGEN rats compared with younger rats, the lower BRS of older ASrAOGEN rats is similar to levels seen in younger SD rats.\textsuperscript{12} A recent study in 16- to 18-month-old SD rats revealed that the age-related BRS impairment may result from low production of Ang-(1-7) in the NTS.\textsuperscript{13} The contribution of Ang II and Ang-(1-7) at the level of the NTS to cardiovascular and reflex function in older ASrAOGEN has not been studied. Thus, we clarified the unique central integration of Ang II and Ang-(1-7) in the NTS of 16- to 22-month-old anesthetized ASrAOGEN rats. Identification of factors that regulate brain stem areas controlling autonomic outflow is crucial in understanding the decline in baroreflex and cardiovascular functions that occurs with aging. The transgenic rat model used in this study is a tool to evaluate the contribution of different sources of Ang peptides in modulation of cardiovascular function.

**Methods**

For a detailed Methods section, please see http://hyper.ahajournals.org.

**Animals**

Experiments were performed in 16- to 22-month-old male transgenic TGR(ASrAogen)680 (ASrAOGEN) rats. The institutional animal care and use committee approved all of the procedures.

**Surgical Procedures**

As reported previously,\textsuperscript{10,13} rats were anesthetized with urethane chloralose (750 and 35 mg/kg) via intraperitoneal injections, instrumented with femoral artery and vein catheters and placed in a stereotaxic frame with the head tilted downward (45° angle) for surgical exposure of the dorsal medulla oblongata.

**AP and HR Measurements**

Pulsatile AP and MAP were monitored, recorded, and digitized using a Data Acquisition System (Acknowledgment software 3.8.1, BIOPAC System Inc), and HR was determined from the AP wave as reported previously.\textsuperscript{10,13}

**Reflex Testing**

Baseline responses to CVA and BRS were established by bolus intravenous administration of phenylbiguanide (10 μg/kg in 0.9% NaCl) or phenylephrine (2, 5, and 10 μg/kg in 0.9% NaCl), as reported previously.\textsuperscript{10,13} Peak MAP and HR responses to administration of each receptor antagonist were measured. BRS was determined by the slope of the relationship between changes in MAP and the pulse interval. CVA and BRS tests were repeated within 10 minutes of NTS microinjections so that each animal served as its own control.

**Microinjections**

The AT\textsubscript{1} receptor antagonist candesartan (CAN; CV-11974; 24 pmol/120 nL), Ang-(1-7) receptor antagonist (D-Ala\textsubscript{7})-Ang-(1-7) (D-Ala; 144 fmol/120 nL), or artificial cerebrospinal fluid (aCSF; pH 7.4; 120 nL) was bilaterally microinjected via pressure into the NTS (0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius [caudal tip of the area postrema], and 0.4 mm below the dorsal surface) using a glass micropipette connected to a syringe.\textsuperscript{10,13}

**Histology**

The brain was removed and frozen on dry ice at the end of each experiment to assure that the sites of microinjections were within the medial NTS at a rostro-caudal level −13.3 to −14.0 mm caudal to bregma.

**Analysis of Data**

Values are presented as means±SEM. Comparisons of changes in BRS, CVA, and resting MAP and HR over time were made by repeated-measures ANOVA with posthoc Student-Newman-Keuls multiple comparisons. Comparisons of overall absolute changes in MAP and HR in response to receptor antagonists or aCSF were analyzed by 1-way ANOVA with posthoc Dunnett multiple comparisons. Individual changes of MAP and HR in response to treatments were compared with baseline using a 1-sample t test. The criterion for statistical significance was \(P<0.05\). Tests were performed using Prism 4.0 and Instat 3 (GraphPad Software).

**Results**

**Effect of NTS Injection of CAN, D-Ala, or aCSF on Resting MAP and HR**

Maximal transient changes in MAP and HR were measured after NTS injection of receptor antagonists (Figure 1). CAN alone, D-Ala in the presence of CAN, D-Ala alone, and CAN in the presence of D-Ala significantly reduced MAP compared with baseline values. The effect of CAN alone was significantly greater than D-Ala alone. The combination of CAN and D-Ala was not different from treatment with either antagonist alone or each other. HR was reduced significantly from baseline values with CAN alone, D-Ala in the presence of CAN, and D-Ala alone. There were no differences in HR values among the groups. Injection of aCSF (n=3) had no

\[\text{Figure 1. Maximal transient changes in MAP and HR in response to NTS injection of CAN or D-Ala.}\]

A, All of the treatments, alone or in combination, significantly reduced MAP compared with baseline values. The reduction in pressure on CAN injection was significantly larger than when D-Ala was administered alone (#\(P<0.05\)). B, Injection of CAN alone, D-Ala in the presence of CAN, and D-Ala alone significantly reduced HR compared with baseline values. n=5 for each group; **\(P<0.05\), ***\(P<0.01\), and ****\(P<0.001\).
After and was not different from BRS after D-Ala alone. NTS combination treatment with D-Ala and CAN 60 minutes later reversed (Figure 2B), D-Ala alone decreased BRS by 69%. BRS administration of CAN resulted in a 72% decrease in BRS. In contrast, blockade of Ang-(1-7) receptors after previous administration of AT1 or Ang-(1-7) receptor antagonists revealed that administration of D-Ala alone (n=5) decreased BRS by 69%. CAN administered 60 minutes later in the presence of D-Ala (n=5) did not have any further effect on BRS compared with D-Ala alone. *P<0.05 vs after CAN, &P<0.01 versus baseline.

MAP and HR Immediately Before Reflex Tests
Values of MAP and HR immediately before microinjections (Table S1) remained stable throughout the study when CAN was administered first followed 60 minutes later by D-Ala or in aCSF experiments. In experiments where D-Ala was given first, followed 60 minutes later by CAN, the values of MAP and HR were significantly lower immediately before injection of CAN.

BRS After NTS Microinjection of CAN, D-Ala, or aCSF
Baseline values of BRS did not differ among the groups (Figure 2A). AT1 receptor blockade by CAN did not alter BRS (Figure 2A). In contrast, blockade of Ang-(1-7) receptors after previous administration of CAN resulted in a 72% decrease in BRS compared with baseline. When the order of receptor antagonists was reversed (Figure 2B), D-Ala alone decreased BRS by 69%. BRS was significantly lower compared with baseline values after combination treatment with D-Ala and CAN 60 minutes later and was not different from BRS after D-Ala alone. NTS injection of aCSF had no significant effect on BRS (Figure S1).

Responses to CVA After NTS Microinjection of CAN, D-Ala, or aCSF
CAN significantly attenuated both MAP and HR responses to CVA (Figure 3A). Responses to CVA were still attenuated when restet after D-Ala administration 60 minutes later in the presence of CAN. When the order of receptor antagonists was reversed (Figure 3B), D-Ala had no significant effect on MAP responses to CVA but significantly attenuated HR responses. CAN, administered 60 minutes later in the presence of D-Ala, attenuated both MAP and HR responses to CVA to a similar extent as when CAN was administered alone. Injections of aCSF had no significant effect on responses to CVA (Figure S2).

Discussion
NTS administration of AT1 or Ang-(1-7) receptor antagonists indicates repeatedly that endogenous Ang II attenuates and

Ang-(1-7) facilitates BRS for control of HR in response to increases in MAP evoked by the α-adrenergic receptor agonist phenylephrine. In older ASrAOGEN rats, there is no effect of AT1 receptor blockade on BRS for control of HR, whereas Ang-(1-7) receptor blockade impairs BRS to a greater extent than reported previously for younger ASrAOGEN or SD rats. The present results, combined with our previous observations in younger animals, show that the source of endogenous Ang II modulating BRS depends on expression of glial Aogen. Regulation of the BRS by glia-derived components of the RAS is consistent with studies in transgenic mice showing that glial overexpression of the RAS decreases, whereas neuronal overexpression does not change BRS. Moreover, blockade with either receptor antagonist lowered resting MAP in younger and older ASrAOGEN rats, indicating that nonglial sources of Aogen are responsible for support of resting MAP under anesthesia in ASrAOGEN rats, which is in contrast to anesthetized SD rats. Although tissue peptide levels from glial and nonglial sources have not been measured to date, studies reveal lower hypothalamic Ang I with a similar trend for Ang II in ASrAOGEN rats. Moreover, studies using the glial fibbrillary acidic protein promoter to express Aogen or renin indicate via immunostaining that the expression of these components is targeted to glia. Furthermore, preliminary studies in our laboratory indicate that neuronal Ang-(1-7) is preserved in neurons of the PVN of young and old ASrAOGEN animals. In both conscious and anesthetized SD rats, BRS for control of HR is highest in younger animals and declines with
aging.\textsuperscript{12,13} The age-related decline in BRS is partly a result of low endogenous Ang-(1-7) in the NTS.\textsuperscript{13} In conscious older ASrAOGEN rats, BRS is lower than in younger ASrAOGEN animals, but the level achieved is similar to that seen in younger SD rats,\textsuperscript{12} suggesting overall maintenance of reflex function in ASrAOGEN rats relative to normotensive rats during aging. BRS, as measured in older anesthetized ASrAOGEN rats in the present study, is comparable to that in younger anesthetized ASrAOGEN rats.\textsuperscript{10} Ang-(1-7) receptor blockade reduced BRS to a greater extent in older than in younger ASrAOGEN or SD rats\textsuperscript{10,11} and achieved levels seen in older SD rats,\textsuperscript{13} suggesting that maintenance of Ang-(1-7) actions within the NTS may serve to mitigate greater age-related declines in BRS in ASrAOGEN rats. Previous studies show that BRS for control of HR in response to decreases in MAP is not affected by Ang II injection or AT\textsubscript{1} receptor blockade in the NTS of anesthetized SD,\textsuperscript{16} Wistar-Kyoto, or spontaneously hypertensive rats,\textsuperscript{17} thus, reflex responses to nitroprusside were not measured in the present study.

We also evaluated responses to activation of cardiac vagal afferent chemosensitive fibers because Ang II receptors are present on these fibers as well.\textsuperscript{18} CVA was induced by intravenous administration of phenylbiguanide, a serotonin 5-hydroxytryptamine-3 receptor agonist, near the heart. To minimize changes resulting from direct activation at other potential sites of action, such as NTS neurons, only immediate responses to activation were determined. Younger and older ASrAOGEN rats have an enhanced depressor response to CVA under anesthesia. Direct activation of NTS neurons could result in a pressor response, which was not observed during the time course studied.\textsuperscript{19} In younger rats, the responses to CVA were not modulated by AT\textsubscript{1} or Ang-(1-7) receptor blockade in the NTS.\textsuperscript{10} In contrast, MAP responses to CVA were attenuated by AT\textsubscript{1} receptor blockade, and HR responses were attenuated by both AT\textsubscript{1} and Ang-(1-7) receptor blockade in older ASrAOGEN rats. Differential effects of Ang II on BRS and responses to CVA are possible, because baroreceptor and chemoreceptor modalities within vagal afferents to the NTS do not converge.\textsuperscript{20,21}

Furthermore, urethane-chloralose anesthesia induces 5-fold increases in plasma renin activity and increases in circulating Ang II.\textsuperscript{22} Circulating Ang II can facilitate cardiopulmonary reflexes, effects that are blocked by AT\textsubscript{1} receptor blockade.\textsuperscript{23} ASrAOGEN rats have elevated AT\textsubscript{1} receptor binding compared with SD rats in the subfornical organ, PVN,\textsuperscript{24} and NTS\textsuperscript{25} at the ages used in this study. Therefore, elevated AT\textsubscript{1} receptors in locations accessed by increased circulating Ang II could modulate CVA in the older rats. Ang-(1-7) alone does not modulate MAP responses but may potentiate the bradycardic responses to CVA in older ASrAOGEN rats. Previous studies using antisense oligonucleotides within the NTS determined that Ang II–induced HR effects require receptors on cell bodies, whereas blood pressure effects could be partially mediated by receptors on nerve terminals.\textsuperscript{26} Ang II and Ang-(1-7) differentially alter neurotransmitter release in the medulla, and these effects further depend on the level of activation of the cells and fibers involved.\textsuperscript{27} Thus, the site and mechanism of action of these peptides within the NTS may differ, allowing independent regulation of blood pressure and HR.

The reduction in MAP and HR seen in ASrAOGEN rats in response to AT\textsubscript{1} or Ang-(1-7) receptor blockade in the NTS is in contrast to other rat strains.\textsuperscript{14–16} These changes are not because of a volume stimulatory effect, because control aCSF injections produced no significant changes in MAP or HR. Although conscious resting MAP is lower in ASrAOGEN rats compared with anesthetized SD rats,\textsuperscript{4,11} it is elevated and to a similar extent in younger and older anesthetized ASrAOGEN rats.\textsuperscript{10} Reductions in cerebral levels of Ang II could lead to the upregulation of AT\textsubscript{1} receptors seen in ASrAOGEN rats during aging.\textsuperscript{24,25} This may contribute in part to enhanced resting MAP under anesthesia and to greater reductions in pressure on NTS injection of CAN in older versus younger ASrAOGEN rats. Others have shown reduced AT\textsubscript{1} receptor binding in the NTS of young (3 to 4 months) ASrAOGEN rats compared with age-matched SD rats,\textsuperscript{26} which may also, in part, explain the lack of modulation by Ang II on responses to CVA in younger rats and the lesser reduction in pressure with CAN injection compared with older ASrAOGEN rats.

Blockade of Ang-(1-7) receptors significantly reduced MAP to a similar magnitude as younger ASrAOGEN rats. We previously reported relative mas mRNA in the dorsal medulla for younger and older SD rats\textsuperscript{13} and younger ASrAOGEN rats\textsuperscript{10} from a data set that also included older ASrAOGEN rats. The relative value for mas mRNA in younger ASrAOGEN rats was significantly higher compared with younger SD rats as control (1.5±0.1 versus 1.2±0.1; P<0.05),\textsuperscript{10} with no significant difference in mRNA values between older SD rats (1.2±0.1)\textsuperscript{13} and older ASrAOGEN rats (1.1±0.1; P>0.05; unpublished data). Although mas receptor mRNA was higher in the dorsal medulla of younger ASrAOGEN rats, blockade of Ang-(1-7) receptors resulted in a smaller decrease in BRS in younger versus older ASrAOGEN rats. Also, there does not appear to be an age-related difference in modulation of resting MAP by Ang-(1-7). Thus, differences in mas receptor mRNA do not appear to account for the functional differences observed among strains or ages in our studies.

Previous studies showed that blockade of either AT\textsubscript{1} or mas receptors lowered MAP to a similar level in younger ASrAOGEN rats.\textsuperscript{10} To determine whether interactions between AT\textsubscript{1} and mas receptors were occurring, we administered the receptor antagonists alone and in combination in the same animals and looked for additive responses. Importantly, changes in CVA, MAP, or HR do not appear to be additive, because each antagonist, alone or in combination, changed responses by approximately the same amount, suggesting a common mechanism. The contribution of Ang II and Ang-(1-7) to increases in MAP in older ASrAOGEN rats in a nonadditive manner is consistent with several recent studies. Activation of pathways from PVN to NTS and rostral ventrolateral medulla to increase MAP involves AT\textsubscript{1} and Ang-(1-7) receptors at each site,\textsuperscript{10,29} with no evidence of additive effects.

Different mechanisms appear to be involved in modulation of resting MAP and BRS. Although AT\textsubscript{1} receptors are elevated in the SFO, PVN, and NTS of older ASrAOGEN rats, there was no

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effect of AT₁ receptor blockade on BRS. In contrast, AT₁ receptor blockade significantly reduced resting MAP and HR and the responses to CVA in these animals. Ang-(1-7) continues to modulate BRS during aging in ASrAOGEN rats and plays a role in modulating resting MAP and HR in the older animals, similar to younger ASrAOGEN rats. The source of the Aogen for both Ang II and Ang-(1-7) actions on CVA and resting MAP may be the PVN, because neuronal expression of the 2 peptides is preserved in ASrAOGEN rats. We propose that, under anesthesia, elevated levels of circulating Ang II activate descending angiotensinergic pathways, involving both Ang II and Ang-(1-7), from the PVN to the NTS and rostral ventrolateral medulla to increase sympathetic outflow, possibly contributing to the anesthesia-induced pressor responses seen in these animals.

**Perspectives**
Cardiovascular baroreflex function declines with age in healthy human populations. Age-related impairments in BRS originate from central neuronal dysfunction and peripheral vascular changes. Although plasma renin and circulating Ang peptides decrease with age, RAS blockade is efficacious in preventing age-related deficits and improving lifespan of normotensive rats, suggesting that, in various tissues, blockade of RAS may contribute to beneficial effects. Administration of either AT₁ receptor blockers or Ang-converting enzyme inhibitors improves BRS, suggesting that an increase in Ang II or its actions might be responsible for impaired BRS. These treatments, however, also shift the endogenous balance of Ang peptides toward Ang-(1-7). The present study indicates that neuronal preservation of Ang-(1-7) may be a mechanism to prevent the age-related decline in baroreflex function.

**Acknowledgments**
We thank Ellen Tommasi for technical assistance and are grateful to Takeda Chemical Industries for their generous donation of candesartan (CV-11974).

**Sources of Funding**
We gratefully acknowledge support from the National Heart, Lung, and Blood Institute grant HL-51952, Unifi (Greensboro, NC), and the Farley-Hudson Foundation (Jacksonville, NC).

**Disclosures**
None.

**References**


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Hypertension. 2008;51:1326-1331; originally published online March 17, 2008; doi: 10.1161/HYPERTENSIONAHA.107.106005
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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