Differential modulation of baroreflex control of heart rate by neuron- vs. glia-derived angiotensin II

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Sakai, Koji, Mark W. Chapleau, Satoshi Morimoto, Martin D. Cassell, and Curt D. Sigmund. Differential modulation of baroreflex control of heart rate by neuron- vs. glia-derived angiotensin II. Physiol Genomics 20: 66–72, 2004. First published October 5, 2004; doi: 10.1152/physiolgenomics.00168.2004.—We developed transgenic mice with targeted expression of human renin (hREN) and human angiotensinogen (hAGT) to either neurons (N-AII mice) or glia (G-AII mice) to test the hypothesis that neuronal and glial ANG II may have differential function. Since baseline blood pressure (BP) did not differ between the models (109 ± 3 vs. 114 ± 4 mmHg), we stressed the BP regulatory pathway by measuring the heart rate (HR) (baroreflex) response to phenylephrine- and nitroprusside-induced changes in arterial BP. The midpoint of the baroreflex curve (BP50) was reset to a significantly higher BP in N-AII mice (131 ± 5 mmHg) compared with littermate controls (115 ± 3 mmHg). Baroreflex gain (slope of BP-HR relation) was similar in N-AII and control mice (12 ± 1 vs. 14 ± 2 beats·min⁻¹·mmHg⁻¹). In contrast, G-AII mice exhibited less of an increase in BP50 (125 ± 5 mmHg) but a larger decrease in baroreflex gain (8 ± 1 beats·min⁻¹·mmHg⁻¹) compared with both control and N-AII mice. Differences in BP50 and gain between N-AII, G-AII, and control mice persisted after parasympathetic blockade with atropine but were eliminated after sympathetic blockade with propranolol, indicating the effects of ANG II were selective for cardiosympathetic arm of the reflex. ANG II-like immunoreactivity was observed more prominently around the paraventricular nucleus and nucleus tractus solitarii in G-AII mice but more prominently in the ventrolateral medulla in N-AII mice. We conclude that ANG II differentially modulates baroreflex control of HR in mice producing ANG II in neurons vs. glia, and its differential function may reflect regional differences in the production of ANG II in cardiovascular control nuclei of the brain.

renin-angiotensin system; brain; transgenic animal; sympathetic nervous system

THE ARTERIAL BAROREFLEX is a major regulator of arterial pressure (AP) and cardiovascular function and acts to buffer changes in AP in part by changing heart rate (HR). The baroreflex has been studied extensively in animal models and humans. Clinical studies have demonstrated that hypertension is accompanied by a modulation of baroreflex function (3, 4, 11, 23, 32); and it is known that this modulation occurs by one of two mechanisms. In the first, the baroreflex curve is shifted or reset to a higher pressure, whereas in the second there is a decreased sensitivity of the reflex as measured by a decreased slope (or attenuated gain) of the baroreflex curve. These mechanisms have been documented in many experimental animal models of hypertension (10, 17, 25, 26).

There is considerable evidence that circulating ANG II plays a critical role in the baroreflex control of HR by modulating either the set point or sensitivity of the reflex (7, 24, 25, 34). For example, chronic intracerebroventricular (ICV) infusion of ANG II decreased baroreflex gain and blockade of ANG II AT1 receptors with losartan increased baroreflex gain in conscious rabbits (9). In the brain, ANG II receptors and ANG II-immunoreactive nerve fibers, terminals, and cell bodies are localized in several discrete regions. Evidence also supports the functional and anatomical diversity of ANG II in the rostral ventrolateral medulla (RVLM), the caudal ventrolateral medulla (CVLM), the nucleus tractus solitarii (NTS), and the paraventricular nucleus (PVN), all regions controlling cardiovascular function (5, 8). Current evidence suggests that the mechanism by which ANG II modulates the baroreflex likely depends on the site of ANG II action. In support of this are studies in rats showing that acute microinjection of AT1 receptor blocker losartan into the NTS increased the gain of the baroreflex without resetting the baroreflex curve (24), whereas microinjection of ANG II into the RVLM elicited a shift in the baroreflex curve to a higher pressure without altering the gain (31). At the present time, the relative importance of these two mechanisms in blood pressure (BP) control remains controversial.

Angiotensinogen (AGT), the precursor of ANG II, is widely expressed in glial cells throughout the brain but is also expressed in neurons in several important cardiovascular control regions. Consistent with this are reports that ANG II exists in nerve terminals and glial cell populations (8, 21). We previously demonstrated that ANG II produced by either neurons or glial cells is functionally active (28). This was accomplished by generating transgenic mice expressing both human AGT (hAGT) and/or human renin (hREN) driven by either the synapsin-I (SYN) promoter, a neuronal promoter, or the glial fibrillary acidic protein (GFAP) promoter, a glial promoter (27–29). Baseline AP was modestly increased in both models but did not differ from each other. In this report, we investigated the baroreflex, one of the mechanisms regulating BP, to test the hypothesis that neuron- and glia-derived ANG II may provide contrasting functions in BP control. We report that the neuron-derived ANG II (N-AII mice) and glia-derived ANG II (G-AII mice) have different physiological effects on the baroreflex control of HR.

METHODS

Generation of double transgenic mice. We previously generated double transgenic mice encoding both the hREN and hAGT genes under the control of either neuron-specific promoter synapsin-I or...
glia-specific promoter GFAP by breeding heterozygous SYN-hREN transgenic mice with heterozygous SYN-hAGT transgenic mice or heterozygous GFAP-hREN transgenic mice (22) with heterozygous GFAP-hAGT transgenic mice (27–29). Approximately 25% of the offspring from these breedings were double transgenic, and there was no apparent increased mortality in either of the GR+/GA+ mice (glia-derived angiotensin II mice, herein termed G-AII mice) and SR+/SA+ mice (neuron-derived angiotensin II, mice herein termed N-AII mice). Double transgenic mice were identified by PCR of genomic DNA purified from tail biopsy samples using hREN- and hAGT-specific primers as previously described (28). All mice were fed standard mouse diet (LM-485; Teklad Premier Laboratory Diets) and water ad libitum. We used age-matched N-AII, G-AII, and control mice, 16–20 wk old, in all studies. Care of the mice used in the experiments met the standards set forth by the National Institutes of Health in their “Guide for the Care and Use of Laboratory Animals.” All procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

Surgical procedures. Mice were anesthetized with ketamine/xylazine (90 mg/kg and 10 mg/kg) mixture intraperitoneally and surgically instrumented with sterile heparinized saline-filled (100 U/ml) intraarterial catheters (heat-elongated polyethylene tubing, PE-50; Becton-Dickinson) for direct measurement of mean arterial pressure (MAP) and venous catheters (MicroRenathane tube MRE-033; Braintree Laboratories, Braintree, MA) for administration of drugs by the right femoral approach. Both catheters were tunnelled subcutaneously and sutured in place between the scapulae. Overnight recovery was allowed before any experimental manipulation. On the day following this operation, the mice were allowed to rest about 1 h before we began any measurements. MAP and HR were recorded with computer-based BP recording unit (PowerLab/4SP data acquisition system; ADInstruments, Colorado Springs, CO) for 1 h. In some mice exhibiting a narrow pulse pressure, small sterile copper wires attached directly to the right and left thoracic muscles were used for HR determination via electrocardiogram monitoring system (BioAmp; ADInstruments). This was performed in a small number of mice (less than 1 per group in untreated mice, 1–2 per group in mice treated with atropine or propranolol).

Analysis of baseline MAP and HR. After the connection to the recording equipment, the mice were allowed a 30- to 60-min accommodation period before the data acquisition. Mice were allowed to move freely. BP and HR were recorded continuously for 60 min, and the data from the entire measurement session were averaged.

Analysis of baroreflex control of HR. Baroreflex control of HR was determined by producing acute changes in MAP with manual intravenous infusion (30 µl over about 2 min) of phenylephrine (0.05–20 mg/kg body wt) (Fig. 1A) and nitroprusside (SNP, 1–10 mg/kg body wt) (Fig. 1B). Each drug was administered in a random order. MAP was altered at a rate of 1–2 mmHg/s from baseline to peak change. At least 15 min was allowed for AP and HR to return to baseline before administering the next drug. Administration of the same volume of saline did not alter baseline AP and HR in normal mice (data not shown). HR and MAP data were acquired by the multi-channel recorder system (PowerLab, ADInstruments). Readings were averaged every 500 ms using a macro enabling us to analyze the changes in BP evoked by phenylephrine and SNP using a computer-based application (Chart, ADInstruments). A sigmoid logistic function was applied to the data using a nonlinear regression program (SigmaPlot2000; SPSS, Chicago, IL) (Fig. 1C).

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HR = \frac{P1}{1 + e^{(P2\text{MAP} - P3)}} + P4
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where \( P1 = (\text{maximum } HR - \text{minimum } HR) \) (range), \( P2 = \text{slope coefficient} \), \( P3 = \text{MAP at 50% of the } HR \text{ range (BP50)} \), and \( P4 = \text{minimum } HR \). The maximum slope (maximum gain) was calculated as \((P1 \times P2)/P4\). The threshold pressure (Pth, lowest pressure that produces a significant decline in HR) and saturation pressure (pressure necessary to achieve maximal inhibition of HR) were calculated from the third derivative of the equation (16). The data were analyzed using a computer-based program (SigmaPlot2000, SPSS).

In separate groups of mice, either atropine methyl nitrate (Sigma Aldrich, St. Louis, MO) or propranolol (Sigma Aldrich) was used to study the cardiopreventive effect of angiotensin II. After 30-min recovery, MAP and HR were recorded for 15 min, and either atropine (1 mg/kg body wt) or propranolol (1 mg/kg body wt) was administered intravenously over 1 min. Thirty minutes after the injection, MAP and HR were again recorded for 15 min. Subsequently, intravenous infusion of phenylephrine or SNP was performed to determine the baroreflex control of HR as mentioned above under the effect of either atropine or propranolol.

Immunohistochemistry for the angiotensin peptide. Angiotensin peptide in the mouse brain was visualized with ABC immunohistochemistry combined with tyramide signal amplification and a fluorescein isothiocyanate (FITC)-conjugated fluoroprobe. Mice were deeply anesthetized with pentobarbital and then perfused transcardially with 20 ml PBS followed by 50 ml of a 1% glutaraldehyde-4% formaldehyde mixture. Brains were removed, postfixed overnight (4°C) in 4% formaldehyde, and placed in 30% sucrose solution. Coronal sections were cut serially with a Vibratome. After incubation in SuperBlock (Pierce, Rockford, IL) for 30 min and permeabilization with 0.3% Triton X-100 in PBS, the sections were incubated in goat IgG polyclonal antibody to angiotensin I/II (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 to 2 wk, as described for other neuropeptides (14, 15). The sections were then incubated sequentially in biotinylated anti-goat IgG (1:1,000; Vector Laboratories, Burlingame, CA) at room temperature for 2 h, HRP-conjugated streptavidin (1:100, PerkinElmer, Boston, MA) at room temperature for 30 min,
RESULTS

G-AII mice express the hREN and hAGT genes under the control of the glial-specific GFAP promoter, whereas N-II mice express both transgenes under the control of the neuron-specific synapsin-I promoter. The tissue specificity and cell specificity of expression in those models was previously reported (28, 29). To obtain concurrent measurements, we first compared resting BP in G-AII, N-AII, and control mice. Baseline MAP in G-AII mice (114 ± 4 mmHg, n = 10, P < 0.05 vs. control) was not significantly different from MAP in N-AII mice (109 ± 3 mmHg, n = 10, P > 0.05 vs. control) but was significantly increased compared with control mice (103 ± 3 mmHg, n = 10). Although N-AII mice exhibited less of an increase in baseline MAP, there was no significant difference in resting MAP comparing G-AII mice to N-AII. Baseline HR did not differ between the three groups of mice.

Since we did not observe any significant difference in baseline AP, we next performed experiments to determine whether the mechanisms regulating AP in the G-II and N-II models were similar. This was accomplished by measuring the change in HR in response to infusion of phenylephrine and nitroprusside. Figure 2 shows the baroreflex results obtained with N-II mice and G-AII mice. N-II mice exhibited a rightward shift in the baroreflex, resetting it to a higher BP. BP50 (131 ± 5 vs. 115 ± 3 mmHg, n = 10, P < 0.05) and threshold pressure (Pth) (120 ± 4 vs. 106 ± 3 mmHg, n = 10, P < 0.05) were significantly higher in N-AII mice compared with control mice. There was no change, however, in the gain of the baroreflex (12 ± 1 vs. 14 ± 2 beats-min⁻¹-mmHg⁻¹, n = 10, P > 0.05) between control mice and N-AII mice. Baroreflex range was not significantly different in N-AII vs. control mice, nor was the maximum HR and minimum HR (data not shown).

On the contrary, G-AII mice exhibited a smaller increase in BP50 (9.8 ± 1.6 mmHg, n = 10, P > 0.05) than did N-AII mice (16.1 ± 2.6 mmHg, n = 10, P < 0.05), suggesting a smaller resetting of the baroreflex to a higher AP (Fig. 2B). A smaller increase in Pth was also observed in G-AII (3.7 ± 0.7 mmHg) than N-AII (3.6 ± 2.6 mmHg) mice (Fig. 2C). The gain of the baroreflex decreased significantly compared with both control and N-AII mice (8 ± 1 vs. 14 ± 2 vs. 12 ± 1 beats-min⁻¹-mmHg⁻¹, respectively, n = 10, P < 0.05). As with N-AII mice, the baroreflex range, maximum HR, and minimum HR were not significantly different in G-AII compared with control mice.

We next used atropine and propranolol to dissect the relative roles of sympathetic and parasympathetic nervous system on the baroreflex in the N-AII and G-AII mouse models. Neither atropine nor propranolol affected the baseline BP in any of the groups significantly (data not shown). Blockade of parasympathetic control of HR with atropine did not prevent baroreflex resetting in N-AII mice or decreased baroreflex gain in G-AII mice. BP50 was still higher in N-AII mice (136 ± 5 mmHg, n = 5) compared with control mice (110 ± 5 mmHg, n = 11) and G-II mice (105.4 ± 6.1 mmHg, n = 8) after administration of atropine. (Fig. 3, P < 0.01) Likewise, Pth also remained higher in N-AII mice (129 ± 6 mmHg) than control mice (102 ± 5 mmHg) after atropine (Fig. 3, P < 0.01). In the G-AII model, baroreflex gain (3.6 ± 0.6 beats-min⁻¹-mmHg⁻¹, n = 8) was still decreased significantly compared with control mice (8.5 ± 1.9 beats-min⁻¹-mmHg⁻¹, n = 11) and N-AII mice (8.5 ± 1.9 beats-min⁻¹-mmHg⁻¹, n = 6) after atropine (Fig. 3, P < 0.01). In contrast, blockade of sympathetic control with propranolol eliminated both the baroreflex resetting in N-AII mice and
the decreased baroreflex gain in G-AII mice. After administration of propranolol, BP50 and Pth were no longer significantly different in N-AII (BP50, 126 ± 7 mmHg; Pth, 114 ± 10 mmHg, n = 7), control mice (BP50, 127 ± 4 mmHg; Pth, 116 ± 4 mmHg, n = X), or G-AII mice (BP50, 117.9 ± 3.9 mmHg; Pth, 107.9 ± 5.9 mmHg, n = 8) (Fig. 4). Similarly, there was no significant difference in baroreflex gain in G-AII mice (6.2 ± 1.0 beats·min⁻¹·mmHg⁻¹, n = 8), N-AII mice (7.4 ± 1.8 beats·min⁻¹·mmHg⁻¹, n = 11), or control mice (5.7 ± 0.7 beats·min⁻¹·mmHg⁻¹, n = 11) after administration of propranolol (Fig. 4).

To assess the potential mechanism by which glial-specific and neuron-specific production of ANG II may differentially modulate the baroreflex, we examined the localization of ANG II-like immunoreactivity in the brain of these mice. We reasoned that it was more likely that the differences in baroreflex control in the models were caused by regional differences in ANG II production in the model than a bona fide differential effect of ANG II produced in glia vs. neurons. Brain ANG II-like immunoreactivity was detected mainly in nerve terminals in the NTS, hypothalamus, subfornical organ, and cerebellar cortex as reported previously in control mice (data not shown) (18, 30, 31). ANG II-like immunoreactivity was observed mainly around NeuN-positive neurons in N-AII mice (Fig. 5, A–C) and in GFAP-positive glia in the G-AII mice (Fig. 5, D–F). ANG II-like immunoreactivity was observed as a punctate pattern surrounding both neuronal somata and dendrites. There were dramatic regional difference in ANG II staining between G-AII and N-AII (Fig. 6). Using identical staining methods, increased staining was seen in the VLM in N-AII mice, whereas there was increased staining in NTS and PVN in G-AII mice.

**DISCUSSION**

We previously generated double transgenic mice carrying both the hREN and hAGT genes (25). These mice exhibited a marked elevation in BP and a resetting of the baroreflex control of HR to a higher pressure without significantly changing the gain of the reflex (25). ICV administration of losartan in these mice lowered BP acutely, demonstrating the functional importance of brain ANG II even when systemically overexpressed (6). To examine the mechanisms regulating production of ANG II in the brain, we generated double transgenic mice carrying both the hREN and hAGT genes under the control of the neuron-specific synapsin-I promoter (N-AII mice) or the glia-specific GFAP promoter (G-AII mice). We previously demonstrated that both hREN and hAGT mRNA are selectively overexpressed in the brain of these mice and specifically expressed in either glial cells or neurons. In neither model was measurable hREN or hAGT protein detected in the systemic circulation (27–29). We reported that both models exhibited a modest increase in baseline MAP (about 10 mmHg), along with an increase in salt appetite and water consumption (28). The elevated BP in G-AII mice could be corrected by ICV injection of losartan but not by intravenous injection of the same dose of losartan (28). Those data coupled with our immunohistochemistry analysis of ANG II shown herein demonstrate that ANG II derived from local synthesis of renin and AGT in the brain influences systemic cardiovascular regulation through an AT₁-dependent mechanism.
Given that both models cause a similar increase in AP and appear, at least under baseline conditions to be phenotypically equivalent, we sought to determine whether AP is similarly regulated in the models. The results of the present study demonstrate for the first time that neuron-derived ANG II and glia-derived ANG II may have disparate effects on AP in part by differentially modulating the baroreflex. Neuronal production of ANG II caused a selective resetting of the baroreflex to a higher pressure without changing the sensitivity of the reflex, whereas ANG II derived from glia decreases baroreflex sensitivity. In our previous studies, both the N-AII and G-AII mice exhibited a significant and similar increase in baseline AP (28). However, in this study only the G-AII mice exhibited a significant increase in AP compared with control mice. Nevertheless, there was no significant difference between AP in G-AII and N-AII mice in this study.

Baroreflex control of HR is regulated by both sympathetic and vagal mechanisms. Consequently, we considered whether the modulation of the cardiac baroreflex by ANG II in this study could result from the changes in cardiosympathetic or cardiovagal baroreflex function. We compared the effects of cholinergic blockade with β-adrenergic blockade thus examining the cardiosympathetic and cardiovagal components of the baroreflex in isolation. Atropine is known to reduce baroreflex gain and range, mainly by blocking muscarinic cholinergic receptors and increasing the minimum HR. In contrast, propranolol is known to reduce baroreflex gain and range, mainly by blocking β1-adrenergic receptors and decreasing the maximum HR. N-AII mice still exhibited baroreflex resetting under parasympathetic blockade, whereas it was eliminated after sympathetic blockade. Similarly, G-AII mice still exhibited decreased baroreflex sensitivity under parasympathetic blockade, but normal sensitivity under sympathetic blockade. Our results demonstrate that ANG II in the brains of N-AII and G-AII mice modulates the cardio sympathetic arm of the reflex, suggesting that differences in the relative contribution of sympathetic and parasympathetic mechanisms cannot explain the difference observed in our mice.

Many investigators have observed that ANG II decreases baroreflex sensitivity while blockade of the renin-angiotensin system increases it (7, 9, 12, 19, 34). Others have observed resetting with no change in sensitivity (9, 13, 24, 30). These two mechanisms have different physiological and pathophysiological implications since baroreflex resetting preserves short-term buffering of acute BP changes, whereas reduced baroreflex sensitivity impairs short-term buffering of acute BP changes. Studies of baroreceptor afferents have demonstrated dissociation between resetting and changes in gain. For example, acute increases in BP lead to resetting of the BP-afferent baroreceptor activity curve (1). In contrast, aging and atherosclerosis can decrease baroreceptor gain in the absence of any baroreceptor resetting (2, 20, 22). Decreased baroreceptor gain translates to an impaired ability to buffer fluctuations in BP and is thought to be important pathologically. This raises the question of whether neuron-derived ANG II has greater physiological significance while glia-derived ANG II has a greater influence during pathological states. Certainly, it is accepted that neuron-derived ANG II plays an important role within the brain and is normally associated with nerve terminals (8). We also must recognize some of the limitations of our experimental models. Whereas our models allow us to assess the conse-

Fig. 5. Cell-type-specific localization of ANG II-like immunoreactivity in the brain. Representative photomicrographs of double labeling for ANG I/II peptide and neuronal nuclei (NeuN), or ANG I/II peptide and glial fibrillary acidic protein (GFAP) in brain. ANG II-like immunoreactivity (A) was observed in NeuN-positive cells (B) in the brain of N-AII mice. ANG II-like immunoreactivity (D) was observed in GFAP-positive cells (E) in the brain of G-AII mice. Merged images demonstrate colocalization of ANG II and neuron in N-AII mice (C) and ANG II and glia in G-AII mice (F).

Fig. 6. ANG II-like immunoreactivity in brain. ANG II-like immunoreactivity in the paraventricular nucleus (PVN), nucleus tractus solitarii (NTS), and rostral ventrolateral medulla (VLM) of either control, N-AII, or G-AII mice. ANG II-like immunoreactivity in PVN and NTS was more prominent in G-AII mice compared with N-AII and control mice. In contrast, ANG II-like immunoreactivity in VLM was more prominent in N-AII mice compared with G-AII and control mice. Sections were immunostained and photographed under identical conditions.
quences of ANG II overproduction in the brain, the promoters employed do not provide sufficient discrimination to target only those populations of neurons and glial cells which normally express renin and AGT. Indeed, recent data using reporter transgenes suggests AGT is expressed in glial cells and neurons whereas renin is mainly neuronal (18). Moreover, renin expression in neurons is not widespread but rather regional. There are no promoters that allow us to target expression of a transgene only to specific regions of the brain (e.g., neurons in the RVLM).

Given these limitations, what have we learned? We know there is considerable evidence supporting regional specificity in the action of ANG II. That is, ANG II differentially modulates baroreflex control of HR depending on its specific site of action (8, 24, 31, 33). For example, it was reported that ANG II acts in the NTS to reduce the gain of the baroreceptor reflex (24, 33) but acts in the CVLM and RVLM to shift the baroreflex control of HR to a higher pressure (31, 33). In this sense, our results could be explained by the different local distribution of ANG II within the brain in the G-AII and N-AII mice. Indeed, we found increased staining of ANG II-like peptide in the NTS and PVN in G-AII mice, regions where ANG II reduces baroreflex gain. In contrast, we found increased staining of ANG II-like peptide in the VLM in N-AII mice, consistent with its role in shifting the baroreflex relationship to a higher pressure. Although not explicitly quantitative, we presume that this difference in staining reflects regional differences in ANG II synthesis. Based on this, we hypothesize that the different modes of baroreflex regulation by brain ANG II, observed in our models more likely reflects relative differences in regional expression of ANG II from neurons and glial. Indeed, the differences in regional production of ANG II in our models are in general agreement with studies where ANG II, ACE inhibitors, and AT1 receptor blockers were microinjected directly into specific brain nuclei.

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