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Abstract—The subfornical organ (SFO) of the brain has long been considered a critical integrating center for the cardiovascular actions of the renin-angiotensin system (RAS). Early reports of angiotensin II (Ang II) immunoreactivity in the SFO and its neural projections to downstream cardiovascular nuclei raised the possibility that Ang II is produced locally and functions as a putative neurotransmitter in these circuits. However, evidence of functionally significant de novo synthesis of Ang II in the SFO has been lacking. Here, implementing spatiotemporally restricted gene ablation by way of the Cre recombinase/loxP system, we provide the first direct evidence that the local RAS in the SFO has a critical role in blood pressure regulation. Using a transgenic mouse harboring an angiotensinogen (AGT) gene modified for Cre-mediated deletion (hAGTlox), in combination with gene transfer of an adeno virus encoding Cre targeted to the SFO, we show that deletion of the Ang II substrate in this brain region nearly abolishes the pressor and bradycardic effects of renin infused in the CNS. Immunohistochemical analyses verified intense and restricted expression of Cre in the SFO, which paralleled the decrease in AGT expression selectively in this site. Further physiological studies confirmed the integrity of central angiotensinergic and nonangiotensinergic cardiovascular response systems in the Cre-treated mice. In addition to establishing that AGT expression in the SFO and its local conversion to Ang II has a profound effect on blood pressure, this study provides proof-of-principle of the utility of this approach for dissecting the brain RAS and other complex systems in CNS cardiovascular circuits. (Circ Res. 2006;99:1125-1131.)

Key Words: renin-angiotensin system ■ hypertension ■ Cre recombinase ■ transgenic mice ■ adenovirus

A bundant evidence supports an important role for the brain renin-angiotensin system (RAS) in cardiovascular and volume regulation.1 However, it still remains unclear how angiotensin II (Ang II) is formed within the central nervous system (CNS), where it is located, and how regional Ang II production correlates with particular physiological responses. Furthermore, the relative role of circulating and brain-derived Ang II remains controversial. This is mainly because of the difficulties in experimentally dissecting this complex multigene system expressed in numerous sites and cell types both in the brain and in the periphery.2

The subfornical organ (SFO) has long been implicated as a pivotal cardiovascular regulatory center of the brain, and is particularly complex with regard to the RAS in these effects. Rich in Ang II (AT1) receptors,3 it is clearly an important target for Ang II. Microinjection of this peptide directly into SFO activates its neurons4 and elicits a robust pressor response.5 Furthermore, by virtue of the fact that it lies outside the blood-brain-barrier, the SFO has access to circulating Ang II. In fact, a number of classic6,7 and more recent studies8,9 demonstrate a significant role for the SFO in systemic Ang II-elicted hypertension.

In addition to being a target for Ang II, the SFO also has the potential to produce Ang II. It is one of the few discrete regions of the brain where angiotensinogen (AGT), the substrate of the RAS, is localized to neurons.10,11 Furthermore, the finding of “Ang II-like” immunoreactive neurons in the SFO and in neuronal projections from the SFO to the hypothalamic paraventricular nucleus (PVN)12 raised the intriguing possibility that Ang II may be locally produced and function as a cardiovascular regulatory neurotransmitter in this and related circuitry. However, 20 years later, this question still remains unanswered.

A number of experimental tools have recently been developed that provide new opportunities to determine the functional significance of the RAS in particular brain regions. First, we and others have shown that specific CNS cardiovascular nuclei, including the SFO, can be efficiently and selectively targeted for gene modification using recombinant viral vectors.13–16 This is critical be-
cause specific promoters have not been identified that could be used to drive transgenes selectively to these regions. Second, we have recently demonstrated that conditional gene deletion can be achieved in the SFO and other cardiovascular regions of the CNS using the Cre recombinase/loxP system in combination with viral gene transfer of Cre.14 Third, Sigmund and colleagues have generated a number of transgenic mouse lines expressing human RAS genes. These mice provide valuable tools for easily detecting and tracking the RAS in the brain because of the strict species specificity of the reaction between renin and AGT in humans and mice.17,18 Interestingly, we have shown in previous studies that mice expressing both the human renin (hREN) and human AGT (hAGT) transgenes exhibit brain RAS-dependent hypertension.19 However, the particular brain regions involved remain unknown.

Bringing these 3 experimental strategies together, here we tested the hypothesis that the SFO plays a critical role in central angiotensinergic control of blood pressure. Using a transgenic mouse harboring an AGT gene modified for conditional Cre-mediated deletion,20,21 in combination with gene transfer of an adenovirus encoding Cre targeted to the SFO, we show that selective genetic ablation of AGT in this brain region abolishes the pressor effects of renin infused in the CNS. In addition to establishing that local production of Ang II in the SFO causes a robust vasopressor effect, this study provides proof-of-principle of the utility of this approach for dissecting the brain RAS and other complex systems in CNS cardiovascular circuits.

Materials and Methods

Transgenic mice

Experiments were performed in adult (8 to 12 weeks of age, 26 to 30g) transgenic mice harboring a loxP-flanked (exon II) human AGT transgene (hAGTlox). This model has been generated and characterized by us previously,20 and the line has been maintained by successive generations of backcross breeding to C57BL/6 (Jackson Laboratory, Bar Harbor, Maine). All mice were fed standard mouse chow (LM-485; Teklad Premier Laboratory Diets) and water ad libitum. The Animal Care and Use Committee at the University of Iowa approved all protocols used in these studies.

Adenoviral Vectors

Replication-deficient adenoviruses encoding Cre recombinase (Ad-Cre) or a control empty vector (Ad-Con) were generated by the University of Iowa Gene Transfer Vector Core and characterized by us previously.13,20 Briefly, the Cre expression cassette was cloned into E1A and E1B regions of the adenovirus shuttle plasmid pAdCMV5. The recombinant plasmid was cotransfected with Ad5/ sub360 into HEK293 cells. Cell lysates were evaluated for Cre recombinase activity and recombinant Ad-Cre was plaque purified and viral titer was determined as described.22 The Ad-Con vector was prepared identically except that no complimentary DNA was cloned into the E1A and E1B regions. Titer-matched stocks of Ad-Cre and Ad-Con (5 × 10⁹ particles, in PBS) were used for CNS injections. We have shown previously that at this concentration, adenoviruses do not cause significant inflammatory responses in mouse brain.13-15,23

Surgical Procedures and Experimental Protocol For Physiological Studies

Transgenic hAGTlox mice were anesthetized with Nembutal (50 mg/Kg ip), blood samples were drawn by orbital eye bleed, and then radiotelemetry (DSI, St. Paul, Minn) were surgically implanted as described.24 At the time of telemetry surgery, mice also underwent gene transfer of Ad-Cre (n = 9) or Ad-Con (n = 6) to the SFO using procedures described by us previously.13-15 We have established that this results in robust transgene expression by 3 days that remains stable for at least 4 weeks.13-14 Following gene transfer, mice were also fitted with intracerebroventricular (ICV) cannulae (lateral ventricle) as described.59 After 1 week recovery, blood samples were drawn as above. The next day, radiotelemeters were activated and mean arterial pressure (MAP) and heart rate (HR) were recorded continuously using the Datquest ART data acquisition system (DSI) as described.25 On the first day of experimentation, both groups of mice received ICV infusions of hREN (200 ng, 500 nL over 1 minute; kind gift of Drs Walter Fischli and Klaus Lindpaintner, F. Hoffman-LaRoche, Basel, Switzerland). We have established previously that this dose of hREN in the brain produces robust pressor and bradycardic responses in hAGT transgenic mice.26 The next day, all mice underwent ICV injections of Ang II (200 ng, 200 nL) and the cholinergic agonist carbachol (50 ng, 200 nL) (the order was randomized, 1 each in the morning and afternoon) to confirm the integrity of angiotensinergic and nonangiotensinergic response systems, respectively. These doses have also been established previously.59 Finally, on the last day of the protocol, mice were pretreated with ICV losartan (20 μg, 200 nL) 20 minutes before ICV injection of hREN to confirm the role of AT1 receptors in the cardiovascular responses.

Immunofluorescence Studies to Localize and Quantify Cre and hAGT Levels

The day after conclusion of the physiologic experiments, mice were anesthetized as above, blood samples collected by orbital eye bleed, and then perfused transcardially with 4% paraformaldehyde. Coronal brain sections (30 μm) were cut and processed for immunofluorescent detection of hAGT expression using a rabbit polyclonal antibody (generous gift from Dr Duane Tewksbury, Marshfield Medical Research Foundation, 1:10,000 dilution). hAGT immunoreactivity was detected using the tyramide signal amplification technique and a fluorescein-avidin marker as described.13,27 After the amplification procedure, sections were then double labeled using an anti-mouse Cre-specific antibody (1:100 dilution, Covance, Berkeley, Calif) with 24 hour incubation at 4°C in the dark. Sections were washed and then incubated with a rhodamine-conjugated goat anti-mouse antibody (1:200, 1 hour, Molecular Probes) to detect Cre expression. Mounted sections were imaged using a Zeiss LSM 510 confocal microscope equipped with a krypton/argon laser. Identical laser settings and other parameters were used for acquisition of hAGT (green) and Cre (red) images, which were then converted to 8-bit gray scale and quantified with Image J (NIH) software as described.9 Analysis of hAGT and Cre staining was performed in 3 to 4 serial sections through the SFO for each mouse.

Plasma Angiotensinogen Assay

Plasma hAGT levels were measured by Western blot analysis as described.20 Equal volumes of plasma samples were subjected to electrophoresis on 7.5% SDS-polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose and incubated for 2 hours with the hAGT antibody (1: 1000 dilution, Covance, Berkeley, Calif) with 24 hour incubation at 4°C in the dark. Sections were washed and then incubated with a rhodamine-conjugated goat anti-mouse antibody (1:200, 1 hour, Molecular Probes) to detect Cre expression. Mounted sections were imaged using a Zeiss LSM 510 confocal microscope equipped with a krypton/argon laser. Identical laser settings and other parameters were used for acquisition of hAGT (green) and Cre (red) images, which were then converted to 8-bit gray scale and quantified with Image J (NIH) software as described.9 Analysis of hAGT and Cre staining was performed in 3 to 4 serial sections through the SFO for each mouse.

Statistics

All data are expressed as mean ± SEM, and were analyzed by Student’s t test (quantification of immunofluorescence), or ANOVA
Results

Effects of Selective Ablation of AGT in the SFO of hAGT<sup>fl</sup> Transgenics on Central hREN-Induced Cardiovascular Responses

We have shown previously that ICV infusion of the AT<sub>1</sub>-antagonist losartan ameliorates hypertension in double transgenic mice coexpressing hREN and hAGT. Although this suggests that overproduction of brain Ang II plays a role in the hypertension of this model, the fact that these mice exhibit increased Ang II levels both in the brain and in the systemic circulation (the transgenes are expressed in widespread tissues and cell types) leaves open the possibility that bloodstream Ang II acting at the SFO or other circumventricular organs plays an important role. Here we used a more selective genetic approach in which the hAGT gene could be site-specifically disrupted with Ad-Cre, and the functional human RAS was reconstituted by CNS-targeted infusion of hREN. Therefore, the transgene functions normally in the absence of Cre, and exhibits the same tissue- and cell-specific pattern of expression as nonfloxed hAGT transgenes. We hypothesized that ablation of AGT selectively in the SFO, thereby removing the substrate necessary for Ang II production in this site, would prevent the blood pressure effects of central renin infusion.

Consistent with previous findings, single transgenic hAGT<sup>fl</sup> mice are normotensive, and baseline MAP and HR were not altered by viral gene transfer of Cre to the SFO (MAP: Ad-Cre 110±6 versus Ad-Con 112±4 mm Hg; HR: Ad-Cre 647±42 versus Ad-Con 637±57 bpm, P<0.05, 7 days post-gene transfer, n=9 for Ad-Cre, n=6 for Ad-Con). As shown in representative recordings in Figure 1A (left panel), ICV hREN elicited a significant pressor and bradycardic effect in control hAGT<sup>fl</sup> mice. This response profile, with a latency of 8.0±1.1 minute, peak effects within 20.6±2.1 minute, and duration of 60.9±3.0 minutes is similar to that reported previously in hAGT transgenic mice. In contrast, these hREN-elicited cardiovascular effects were nearly abolished in hAGT<sup>fl</sup> mice that had undergone gene transfer of Ad-Cre to the SFO (Figure 1A, right panel). The peak effects of hREN on MAP and HR in hAGT<sup>fl</sup> mice are summarized in Figure 1B, and clearly demonstrate that Cre-mediated deletion of hAGT in the SFO resulted in a significant attenuation of the pressor (ΔMAP: Ad-Con 22±3 versus Ad-Cre 5±3 mm Hg, P<0.05) and bradycardic responses (ΔHR: Ad-Con −64±22 versus Ad-Cre −2±23 bpm, P<0.05). The role of Ang II-mediated activation of AT<sub>1</sub> receptors in the hREN-induced responses was confirmed by experiments infusing losartan ICV. Pretreatment with this AT<sub>1</sub> blocker prevented the blood pressure and HR effects of hREN in control-treated hAGT<sup>fl</sup> mice (Δ MAP: −6±2 mm Hg; Δ HR −21±25 bpm, P<0.05 versus Ad-Con data presented in Figure 1B). Losartan had no effect on the hREN-elicited responses in Ad-Cre-treated mice, likely because of the lack of Ang II production in the SFO as a result of ablation of hAGT (data not shown).

Finally, to confirm that Ad-Cre did not have a nonspecific effect on angiotensinergic or nonangiotensinergic response systems in the CNS, we examined the pressor effects of direct ICV injection of Ang II and the muscarinic agonist carbachol. At the doses used, these drugs produce pressor and bradycardic responses similar in magnitude to each other, and to the peak changes in MAP and HR elicited by hREN. In both groups of mice, Ang II caused equivalent increases in blood pressure (ΔMAP: Ad-Con 20±5 versus Ad-Cre 25±6 mm Hg, P>0.05) and decreases in HR (ΔHR: Ad-Con −56±16 versus Ad-Cre −63±24 bpm, P>0.05). Similarly, the pressor and bradycardic effects of carbachol were unchanged by Ad-Cre (Δ MAP: Ad-Con 21±3 versus Ad-Cre 19±6 mm Hg; Δ HR: Ad-Con −49±12 versus Ad-Cre −62±21 bpm P>0.05). This suggests that the systems necessary for CNS-driven cardiovascular responses remained intact in Ad-Cre-transduced hAGT<sup>fl</sup> mice.

Taken together, these data provide compelling evidence of the importance of the local RAS in the SFO for central angiotensinergic cardiovascular responses.

Localization and Quantification of hAGT and Cre Levels in Brains of hAGT<sup>fl</sup> Mice

To verify accurate stereotaxic targeting of Cre recombinase to the SFO, and to quantify expression of both Cre and hAGT,
immunohistochemical analyses of sections from throughout the brains of Ad-Con and Ad-Cre-treated hAGTflox mice were performed. First, Cre recombinase immunoreactivity was restricted to the SFO and was observed at very high levels in this region of Ad-Cre-injected mice (Figure 2A, lower right). As expected, no Cre was detected in any brain region of Ad-Con-treated mice. A typical example of Cre staining in SFO of control animals is shown in Figure 2A (upper right).

On the other hand, in control vector animals, hAGT was expressed at high levels in many regions of the brain and with a similar distribution profile as described previously for hAGT transgenics. For example, as shown in the representative image in Figure 2A (upper left), hAGT is expressed at intense levels in the SFO of Ad-Con mice. In contrast, hAGT levels were markedly reduced selectively in the SFO of Ad-Cre–treated mice (Figure 2A, lower left).

Quantification of fluorescence intensity for hAGT and Cre in multiple serial sections through the SFO were performed, and the summary data are presented in Figure 2B. These data confirm that Ad-Cre–treated hAGTflox mice show markedly reduced hAGT levels in SFO, which parallels the intense Cre staining observed in this region.

To further verify that the Cre-mediated reductions of hAGT levels were restricted to SFO, we examined brain regions of Ad-Cre–treated mice for hAGT immunoreactivity. There was no effect on hAGT expression outside the SFO, and this is shown in the typical examples of robust hAGT staining in cortex, mesensephalic trigeminal nucleus (MeV) and area postrema (AP) (Figure 3). Importantly, hAGT levels also were not affected in paraventricular nucleus (PVN), a region that receives projections from the SFO (Figure 3, lower left panel). The lack of Cre immunoreactivity in these regions is also shown (Figure 3, right panels).

Together, these data demonstrate that the Ad-Cre treatment in these experiments resulted in robust and selective decreases in hAGT in the SFO.

CNS Gene Transfer of Cre Has No Effect on Plasma hAGT Levels

To verify that Ad-Cre gene transfer to SFO does not affect circulating hAGT levels, we measured hAGT levels in the plasma of hAGTflox mice just before (day 0) and 7 days after Ad-Cre or Ad-Con treatment. Representative Western blot data presented in Figure 4A show that Ad-Cre had no effect on plasma hAGT levels. The densitometric data summarized as a ratio of hAGT values on day 7 to day 0 were similar between Ad-Cre- and Ad-Con-treated mice (Figure 4B). The specificity of the hAGT antibody was previously verified in hAGTflox mice after intravenous Ad-Cre, resulting in ablation of hepatic hAGT and elimination of circulating hAGT.

These data demonstrate that Ad-Cre-mediated deletion of hAGT in SFO of hAGTflox mice does not alter circulating hAGT levels in these mice.

Discussion

The brain exerts powerful influence on the control of blood pressure and fluid balance, and there is now compelling...
It is noteworthy that the pressor response was not completely abolished in the Ad-Cre-treated mice. With ICV administration of hREN, it is certainly possible that enzymatic conversion of hAGT to Ang II in brain regions other than the SFO accessed by hREN could explain this small residual response. However, it is also interesting that evidence that a number of cardiovascular diseases involve pathogenic dysfunction of CNS mechanisms. Angiotensinergic signaling in the CNS has emerged as a primary culprit in driving the neuro-cardiovascular dysfunction in diseases such as hypertension, however still relatively little is known about the pathways and mechanisms involved. Considering the complexity of the multigene RAS, the likely involvement of numerous brain circuits, not to mention different cell types, it is perhaps not surprising that the precise mechanisms involved in brain Ang II regulation of cardiovascular function still remain elusive.

The Cre/loxP system has proven to be a very powerful tool for dissecting gene function in the mammalian brain. However, use of this strategy for analysis of CNS cardiovascular circuits has been limited by the lack of promoters that can target Cre to these sites selectively. Recently we established proof-of-principle for achieving Cre-mediated conditional gene deletion in cardiovascular nuclei through viral gene transfer. Using a mouse model in which Cre activates reporter gene expression, these studies showed that recombination of loxP-flanked genes, ie, gene ablation, could be achieved in discrete cardiovascular nuclei of the brain using targeted delivery of recombinant viruses encoding Cre. Here, the goal was to implement this strategy in combination with a unique transgenic strain, hAGTlox, to determine the functional significance of the RAS in one of these brain regions, the SFO. This site has long been thought to be a pivotal integrating center for RAS functions in normal animals. Because these transgenics exhibit faithful AGT expression patterns in the brain by virtue of the fact that the transgene is under the control of its endogenous promoter, it is likely that this part of the system is a good model for the brain RAS under normal conditions. However, we acknowledge that reconstitution of the brain RAS by intraventricular injection of exogenous human renin may not reflect the normal situation. Experiments targeting the endogenous AGT gene for Cre-mediated deletion, along with generation of new transgenics harboring renin under the control of more specific CNS promoters are ongoing in our laboratories. These mice should provide additional insight into function of the brain RAS.

It will also be important in future studies to confirm that these findings in the hAGTlox mice reflect how the brain RAS functions in normal animals. Because these transgenics exhibit faithful AGT expression patterns in the brain by virtue of the fact that the transgene is under the control of its endogenous promoter, it is likely that this part of the system is a good model for the brain RAS under normal conditions. However, we acknowledge that reconstitution of the brain RAS by intraventricular injection of exogenous human renin may not reflect the normal situation. Experiments targeting the endogenous AGT gene for Cre-mediated deletion, along with generation of new transgenics harboring renin under the control of more specific CNS promoters are ongoing in our laboratories. These mice should provide additional insight into function of the brain RAS.
the extent of inhibition of the pressor response parallels the percent decrease in hAGT levels in the SFO of these mice, ie, ~75%. It is possible that there may be incomplete ablation of Ang II production in the SFO because of either incomplete gene transfer or Cre-mediated effects in some hAGT-synthesizing cells in this region. Given the high levels of hAGT immunoreactivity in the SFO, it is perhaps not surprising that ablation is not 100%.

We have shown previously that adeno vectors efficiently transduce both neurons and glial cells in the SFO (and other cardiovascular regions). As such, because hAGT is expressed in both cell types in the SFO of these mice, the results of the current study do not allow us to determine conclusively whether Ad-Cre-mediated ablation of AGT in neurons, glia, or both cell types was responsible for blocking the central renin-induced pressor response. With the previous identification of Ang II immunoreactivity in the SFO and its projections, it is certainly tempting to speculate that it was the loss of neuronal AGT that was critical in the physiological responses of the hAGT mice herein. We are testing this hypothesis directly in ongoing studies using feline immunodeficiency virus (FIV) to deliver Cre recombinase. We have shown that FIV has high tropism for neurons in this brain region, and that FIV-Cre induces neuron-specific gene deletion in SFO and other central cardiovascular nuclei.

In summary, these results demonstrate that local synthesis of AGT in the SFO is critical in central angiotensinergic cardiovascular responses, and provides support for the idea posited twenty years ago that Ang II functions as a putative neurotransmitter in this and related circuits. This study is also important because it provides us with a useful paradigm for dissecting the role of the brain RAS in specific cell types, in other CNS regions and in the context of additional physiological and pathophysiological states.

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Disclosures
None.

References
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