Glial-specific ablation of angiotensinogen lowers arterial pressure in renin and angiotensinogen transgenic mice

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Sherrrod, Mikhiela, Deborah R. Davis, Xizhou Zhou, Martin D. Cassell, and Curt D. Sigmund. Glial-specific ablation of angiotensinogen lowers arterial pressure in renin and angiotensinogen transgenic mice. Am J Physiol Regul Integr Comp Physiol 289: R1763–R1769, 2005. —Angiotensinogen (AGT) is mainly expressed in glial cells in close proximity to renin-expressing neurons in the brain. We previously reported that glial-specific overexpression of ANG II results in mild hypertension. Here, we tested the hypothesis that glial-derived AGT plays an important role in blood pressure regulation in hypertensive mice carrying human renin (hREN) and human AGT transgenes under the control of their own endogenous promoters. To perform a glial-specific deletion of AGT, we used an AGT transgene containing loxP sites (hAGT<sub>flox</sub>), so the gene can be permanently ablated in the presence of cre-recombinase expression, driven by the glial fibrillary acidic protein (GFAP) promoter. Triple transgenic mice (RAC) containing: 1) systemically expressed hREN transgene, 2) systemically expressed hAGT<sub>flox</sub> transgene, and 3) GFAP-cre-recombinase were generated and compared with double transgenic mice (RA) lacking cre-recombinase. Liver and kidney hAGT mRNA levels were unaltered in RAC and RA mice, as was the level of hAGT in the systemic circulation, consistent with the absence of cre-recombinase expression in those tissues. Whereas hAGT mRNA was present in the brain of RA mice (lacking cre-recombinase), it was absent from the brain of RAC mice expressing cre-recombinase, confirming brain-specific elimination of AGT. Immunohistochemistry revealed a loss of AGT immunostaining glial cells throughout the brain in RAC mice. Arterial pressure measured by radiotelemetry was significantly lower in RAC than RA mice and unchanged from nontransgenic control mice. These data suggest that there is a major contribution of glial-AGT to the hypertensive state in mice carrying systemically expressed hREN and hAGT genes and confirm the importance of a glial source of ANG II substrate in the brain.

renin-angiotensin system; glia; brain; hypertension

DIRECT INJECTION of ANG II into the brain can cause a rise in systemic blood pressure because of its ability to increase sympathetic outflow and vasopressin release (43). Brain ANG II can also cause long-term increases in arterial pressure through its dipsonic effects. The importance of the endogenous local production and action of ANG II is supported by experiments directly injecting inhibitors, antagonists, and antisense oligonucleotides directed against the renin-angiotensin system (RAS) into the brain (11, 27). Moreover, increased activity of the brain RAS has been implicated as a mechanism causing and maintaining hypertension in both genetic and experimental models (11, 13, 28, 32). Despite overwhelming evidence supporting a cardiovascular role for brain ANG II, the precise mechanisms for its local synthesis in the brain remain unclear. Uncertainties that need to be resolved include identifying the precise cellular and regional production of local ANG II and its relationship to ANG II receptor-containing cells in cardiovascular control regions and the relative importance of circulating and locally produced ANG II in circumventricular organs vs. that locally produced inside the blood-brain barrier (BBB).

In order for ANG II to be formed locally in the brain, there needs to be a source of substrate. We and others have demonstrated that angiotensinogen (AGT) is not only widely expressed in astrocytes but is also regionally expressed in neurons (33, 42, 44). Astrocytes secrete AGT into the extracellular fluid, and AGT can be detected in cerebrospinal fluid (33). We recently reported the close localization of renin- and AGT-containing cells in the brain (14). Therefore, once secreted, AGT can be processed by renin and angiotensin-converting enzyme to form ANG II. An intracellular pathway for the synthesis of ANG II has also been postulated on the basis of the presence of a novel form of unsecreted renin in the brain and the presence of cells coexpressing both renin and AGT (14, 17, 29, 37). To directly assess the contribution of local brain AGT production on arterial pressure, we previously generated mice that expressed both hAGT or human renin (hREN) under the control of the glial-specific glial fibrillary acidic protein (GFAP) promoter (23, 24). Double-transgenic mice expressing both proteins in glial cells exhibited a modest ANG II type 1 receptor-dependent increase in blood pressure, possibly due to an increase in sympathetic outflow.

These data support the hypothesis that glial-derived AGT is an important determinant for the synthesis and action of ANG II within the brain. Herein, we sought to test this hypothesis by examining the effects on blood pressure of a glial-specific loss of AGT. Specifically, we asked whether glial AGT is required for the elevated blood pressure observed in mice expressing both the hREN and hAGT genes. We previously reported that double transgenic mice systemically expressing hAGT and hREN exhibit hypertension, which can be lowered by intracerebroventricular injection of losartan (6, 26). We also reported the development of a novel hAGT transgene variant, in which exon II of the gene is flanked by LoxP sites (38, 39). This novel

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Fig. 1. Generation of triple transgenic mice. A: breeding scheme used to delete hAGT in glial cells. hAGT<sup>flox</sup> mice were first crossed with glial fibrillary acidic protein (GFAP)-cre mice to generate hAGT<sup>flox/GFAP-CRE</sup> (AC) double transgenic mice, and human renin (hREN) mice were crossed with GFAP-CRE mice to generate hREN/GFAP-CRE (RC) mice. We then intercrossed the RC and AC to generate triple transgenic mice (RAC) and the double transgenic (RA) controls. B: tail DNA was isolated and in separate experiments analyzed for the presence of hAGT<sup>flox</sup> (A), hREN (R), and GFAP-Cre (C). C: schematic showing the rationale for the model. RA mice exhibit expression of hAGT in liver (blue), kidney (green), and brain (red), whereas RAC mice should only express hAGT in liver and kidney but not brain (red to beige denoting reduction or ablation of brain hAGT).

transgene (hAGT<sup>flox</sup>) expresses a fully functional hAGT protein under baseline conditions, but in the presence of cre-recombinase, it causes a physical disruption of the gene-eliminating production of a functional protein (38). We previously demonstrated that double transgenic mice expressing hREN and hAGT<sup>flox</sup> are hypertensive under baseline conditions but become transiently normotensive after injection of an adeno virus, expressing cre-recombinase, which deletes the gene from the liver (39). Here, we assessed the effects on arterial pressure of a chronic loss of glial hAGT by creating triple transgenic mice expressing hREN, hAGT<sup>flox</sup> and GFAP-cre. Expressing cre-recombinase under the GFAP promoter restricts its expression to glial cells (46).

MATERIALS AND METHODS

Experimental mice. The hAGT<sup>flox</sup>, hREN, and GFAP-Cre mice were previously described (36, 38, 46). Of the two lines of hAGT<sup>flox</sup> mice previously described, we chose to use the line with the lower level of expression to facilitate its chronic elimination by cre-recombinase. Triple transgenic mice were generated by first breeding hAGT<sup>flox</sup> with GFAP-Cre, breeding hREN with GFAP-Cre mice followed by intercrossing the compound heterozygotes. Additional cross-breeding of compound heterozygotes from the second generation provided enough mice for these studies. Genotypes were determined by PCR of DNA isolated from tail biopsies using primers specific for each transgene, hAGT<sup>flox</sup>, and hREN, as previously described (36, 38), and 5'-ATCACTCCTTTCATCGACCG-3' and 5'-AAGTTTTTTTGTGGGCAGC-3' for GFAP-cre. All mice were fed standard mouse chow (LM-485; Teklad Premier Laboratory Diets, Madison, WI) and water ad libitum. Care of all mice used experimentally met or exceeded the standards established by the National Institutes of Health in their Guidelines for the Care and Use of Experimental Animals. All procedures received approval by the University of Iowa’s Animal Care and Use Committee.

Gene and protein expression. Mice were killed, and tissues were removed, snap frozen in liquid nitrogen, and stored at −80°C until RNA was prepared. RNA was isolated using TRI-Reagent, as described by the manufacturer (Molecular Research Center). For RPA analysis, the RPA III kit (Ambion) was used according to the manufacturer’s protocol. The hAGT probe directed complementary to exon II resulted in a protected fragment of 350 nucleotides, and the 28S probe resulted in a fragment of 115 nucleotides. For RPA analysis on renin mRNA, the protected products were 326, 301, and 250 nucleotides for mouse renin, hREN, and β-actin, respectively. Quantification was performed using a Storm phosphorimager. Levels of hREN and mREN mRNA were normalized to β-actin to control for loading. Each sample was assayed in duplicate. Plasma was obtained from killed mice, mixed with 2 µl of 0.5 M EDTA and centrifuged at 12,000 rpm for 10 min at 4°C. The plasma was removed and stored at −80°C. Five micrograms of diluted (∼100) plasma were used for Western blot analysis, as previously described (38).

Immunohistochemistry. Whole brains were isolated from two triple transgenic mice (RAC) and double transgenic mice (RA) mice each. Mice were killed and perfused transcardially with 20 ml PBS followed by 50 ml 4% paraformaldehyde in PBS. The brain was removed, postfixed overnight at 4°C in paraformaldehyde, and placed in 30% sucrose solution at 4°C. The brain was cut coronally (30- to 50-µm slices) in PBS using a vibratome. Free-floating brain slices were permeabilized with 0.1% Triton X-100 in PBS before using the Tyramide Signal Amplification (TSA) Biotin System protocol (Perkin Elmer) for immunostaining. The sections were blocked for 30 min in TNB blocking buffer (0.1 M Tris·HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent supplied by kit) and then incubated overnight at 4°C in 1 ml TNB containing 1:1,000

Fig. 2. Expression of hAGT<sup>flox</sup> mRNA in RA and RAC mice. Total RNA was extracted from brain, liver, and kidneys, and expression of hAGT was scored by RNase protection assay (RPA). The position of the hAGT and 28S internal control bands are indicated.
dilution of rabbit-anti-AGT polyclonal antibody and 1:100 dilution of mouse-anti-GFAP antibody. Sections were washed as above and incubated in 500 μl TNB containing 1:100 dilution of anti-mouse rhodamine conjugated secondary antibody (Jackson Immunological Laboratories) and anti-rabbit biotinylated secondary antibody (Jackson Immunological Laboratories) for 1 h at room temperature. The samples were then incubated with 1:100 dilution of HRP-conjugated streptavidin (supplied in kit) in TNB for 30 min before incubation with 300 μl biotinyl tyramide amplification reagent (supplied by manufacturer) for an additional 30 min, all at room temperature. The sections were washed and incubated with 1:100 dilution of FITC-conjugated streptavidin antibodies in TNB for 30 min at room temperature. All washes were performed in 0.1% Triton X-100/PBS except the last wash, which contained only PBS. Individual sections were rolled onto glass slides, dried, and then covered with glycerol in PBS and coverslips. Photos were taken using a fluorescent microscope at ×20 magnification. We previously validated and documented the specificity of the hAGT antisera used herein using both preabsorption and nontransgenic mice (23–25, 34). Sections lacking primary antisera were used as negative controls.

Blood pressure analysis. Age- and sex-matched littermates were anesthetized and the pressure-sensing tip of the radiotelemetry catheter (Data Sciences International) was placed in the left common carotid artery, as previously described (16). Mice were returned to their home cages, which were placed atop telemetry receivers, and allowed to recover for 5–7 days after which radiotelemeters were magnetically activated. Radiotelemetry data for heart rate and mean arterial pressure (MAP) were collected continuously (sampling every 5 min for 10-s intervals 24 h a day for 7 consecutive days) and were stored using the Dataquest ART data acquisition system (Data Sciences International). Data were compiled and grouped using Excel and Telemetry Analyzer (JCL Consultants).

Statistical analysis. Data were expressed as means ± SE. Group comparisons were performed by ANOVA using Bonferroni test for pairwise comparisons. A value of \( P < 0.05 \) was considered statistically significant.

Fig. 3. Identification of circulating hAGT protein. A plasma sample from each mouse was diluted, and 5 μg of protein was loaded onto an SDS-PAGE gel and subjected to Western blot analysis. The blot was incubated with hAGT polyclonal antibodies and visualized using enhanced chemiluminescence. Size markers in kilodaltons are indicated. A: plasma samples from 2 RAC and RC mice are shown. B: plasma samples from 3 RAC and 1 RA mouse are shown.

Fig. 4. Cell-specific ablation of hAGT. The cellular localization of hAGT (green) and GFAP (red) was visualized by immunohistochemistry on 30-μm brain slices from RAC and RA mice. All photographs were obtained at ×20 magnification using a fluorescent microscope. Shown are brain regions inside the blood-brain barrier: the paraventricular nucleus (PVN) and bed nucleus of stria terminalis (BNST) located in the thalamic/hypothalamic region, and the amygdala. White arrows point to regions of obvious overlap between GFAP and hAGT.
RESULTS

The purpose of this study is to determine the contribution of glial AGT to blood pressure control by examining the consequences of glial-specific ablation of AGT using a model that exhibits hypertension. To accomplish this, we chose to use a model previously developed by us in which expression of hREN and hAGT is driven by their endogenous promoters (36, 45). The hAGT gene contains loxP sites surrounding exon II, the exon encoding the translation start site, secretory peptide, ANG I, and ANG II peptides, as well as ~80% of the coding potential of the gene. Thus, as previously reported by us, cre-loxP-mediated deletion of exon II functionally cripples the gene (38). We performed the glial-specific deletion of hAGT by creating triple transgenic mice expressing hREN (R), hAGTlox (A), and cre (C) recombinase driven by the GFAP promoter. Although only two generations of breeding are required (in theory) to generate triple transgenic RAC mice (Fig. 1A), several additional generations were required to obtain a sufficient number of mice. Transgene-specific primers were used to genotype each mouse derived from these crosses (Fig. 1B). As illustrated schematically in Fig. 1C, RAC mice should retain hAGT expression in liver (blue) and kidney (green), while in the brain, we anticipate it will be substantially reduced or ablated (denoted as a change from red in RA to beige in RAC). We used double transgenic mice expressing hREN and hAGTlox, but lacking GFAP-cre (R+/A+/C-) as a control, as they should retain full expression of glial hAGT. It is important to note that there was no genetic manipulation of the endogenous mouse RAS.

We performed an RNase protection assay to verify that the deletion of hAGT was confined to the brain in RAC mice. Normal levels of hAGT mRNA were evident in kidney and liver of RA and RAC mice (Fig. 2). Whereas normal levels of hAGT mRNA were evident in the brain of RA mice (because they lack GFAP-cre), there was essentially no detectable exon II-containing hAGT mRNA in the brain of RAC mice, confirming the efficiency of the brain-specific ablation. The liver is the primary source of circulating AGT. We therefore measured circulating hAGT by Western blot analysis to verify that glial hAGT deficiency did not affect the levels of hAGT in plasma (Fig. 3). First, we validated the specificity of the antisera by comparing plasma from RAC mice with plasma from mice lacking hAGT, but retaining hREN and GFAP-cre (RC, Fig. 3A). The multiple forms of hAGT centering on 55 kDa reflect multiple glycosylated forms of the protein (5, 38). We previously reported that HepG2 cells also express multiple forms of hAGT protein, which become reduced to a single immunoreactive product after N-glycosidase treatment (34). Next, we demonstrated that equivalent levels of hAGT protein were detected in plasma of RA and RAC mice, confirming that removal of hAGT from the brain did not affect the ability of the liver to produce and secrete hAGT (Fig. 3B).

We next performed immunohistochemistry to examine the cell type(s) in which the deletion occurred (Fig. 4). In RA mice, a classic pattern of double staining for hAGT and GFAP, a glial-specific marker, was observed in all regions of the brain examined. Although a similar pattern and magnitude of GFAP marker staining were observed in paraventricular nucleus (PVN), amygdala, and bed nucleus of the stria terminalis of RAC mice, there was essentially no staining of hAGT in glia in these regions of the brain. In other regions of the brain, such as the rostral ventrolateral medulla (RVLM), where we previously reported both glial and neuronal expression of hAGT (14, 44), there was a loss of glial-hAGT, but a retention of neuronal-like hAGT staining (Fig. 5). Note that glial staining detected by GFAP avoids regions where AGT staining remains.

We next measured blood pressure using radiotelemetry to assess the importance of glial AGT in the model. We compared daytime and nighttime MAP in RAC mice (n = 13), compared with both RA mice (n = 7) and negative control (n = 12) littermates (Fig. 6). Initial analysis of variance indicated a highly significant difference in both daytime and nighttime recordings among the three groups (P < 0.001). Importantly, the presence of the GFAP-cre transgene had no significant effect on arterial pressure in control mice lacking the hAGTlox transgene (R-/A-/C-. 95 ± 10 mmHg vs. R-/A-/C+ 104 ± 4 mmHg, P = 0.33). Our analysis revealed that there was a significant decrease in both daytime (−26.3 mmHg) and nighttime (−29.0 mmHg) arterial pressure in RAC compared with RA mice. Interestingly, there was no difference in arterial pressure comparing RAC (101.6 ± 3.8 daytime, 108.0 ± 4.1 nighttime) to negative control mice (101.5 ± 4.2 daytime, 107.3 ± 4.1 nighttime), suggesting a reduction in arterial pressure back to baseline. These data suggest that glial cells are...
DISCUSSION

It is widely believed that in the brain, ANG II can control blood pressure acutely and chronically by regulating sympathetic outflow, secretion of arginine-vasopressin, drinking, salt appetite, and the baroreceptor reflex. What is much less well defined is the precise mechanism regulating the in situ production of ANG II in the brain. Whereas the circumventricular organs, regions of the brain outside the BBB, have access to AGT-generating cells and tissues.

In contrast to glial AGT, neuronal expression of AGT is much more regionally restricted to the nucleus of the solitary tract (NTS), PVN, parabrachial nucleus, RVLM, mesencephalic trigeminal nucleus, and the subfornical organ (SFO) (14, 44).

To determine the contribution of glial-derived AGT in hypertension, we generated a mouse model that systemically expressed the hAGT and hREN transgenes but exhibited gliospecific expression of cre-recombinase. In this model, expression of the hAGT and hREN genes are driven by their own endogenous promoters and therefore are expressed in numerous tissues and cells throughout the organism (36, 38, 45). The hREN transgene we bred with hAGTflox in this study is the same that we previously bred with a hAGT transgene lacking loxP sites (21, 39). Both hREN/hAGT and hREN/hAGTflox models are chronically hypertensive; the only difference between the two models is the presence of intronic loxP sites surrounding hAGT exon II. Importantly, both hAGTflox and hAGT transgenes exhibit the same tissue-specific expression profile, although expression of hAGTflox in the line of mice used in these studies was lower than hAGT (38). By using the hREN/hAGTflox model and exploiting an additional genetic element, that is, mice expressing GFAP-cre, we were able to retain overexpression of hREN and hAGT in all tissues, except glial cells in the brain, where hAGT was ablated. This “overexpression-selective elimination” provides an experimental scheme to dissect the individual contributions of different ANG II-generating cells and tissues.

Our data suggest that glial AGT plays an important role in maintaining hypertension, at least in the hREN/hAGT model. On the basis of the modest increase in blood pressure caused by gliospecific overexpression of ANG II (24), we were initially surprised that blood pressure in RAC mice decreased to baseline. Nevertheless, this result is in agreement with our previous study of hREN/hAGT mice, which showed a reduction in arterial pressure to baseline in response to ICV losartan (6). Therefore, like other experimental and genetic models of hypertension, the hREN/hAGT and hREN/hAGTflox models exhibit a very prominent neurogenic component to blood pressure regulation. Recent studies by Davison and colleagues (47, 48) implicate superoxide production in the brain in re-
sponse to ANG II as an important mechanism causing elevated blood pressure. It will therefore be of interest in future studies to establish whether there is a link between ANG II derived from glial AGT and oxidative stress-induced changes in arterial pressure.

Other evidence supports the importance of glial AGT as a source for ANG II substrate in the brain. For example, Schinke et al. (31) reported a significant decrease in arterial pressure and a diabetes insipidus-like syndrome in transgenic rats expressing an AGT antisense construct that caused a 90% reduction in brain AGT. Consistent with our findings, they demonstrated that double transgenic rats expressing both the mouse Ren-2 gene [TGR(mREN2)27] and the GFAP-driven AGT antisense exhibited much lower arterial pressure (∼20 mmHg) than TGR(mREN2)27 rats alone. Rats lacking glial AGT exhibit a decrease in AT1 receptor binding in circumventricular organs, an increase in AT1 receptors in areas inside the BBB, and an increased dispogenic response to ICV ANG II (22). Other studies report an increase in AT1 receptors in the SFO and PVN (12). Although increased baroreflex sensitivity was reported in some studies (2, 4), another study reported no change in baroreceptor reflex in rats lacking glial AGT (7). An alteration in circadian variation in blood pressure was also reported in these rats (1). The data obtained to date therefore support the conclusion that glial cells are an essential source of ANG II substrate in the brain in the regulation of cardiovascular function.

This data clearly leave in question the relative contribution of ANG II production from neuronal sources of hAGT. As indicated above, expression of neuronal AGT is much more regionally restricted than glial AGT. In the RAC model, cre-recombinase was driven by a glial-specific promoter, and therefore, neuronal expression of hAGT was retained. We recently reported differential modulation of the baroreflex in mice expressing ANG II in glial cells vs. neurons, suggesting that glial- and neuronal-derived AGT and ANG II may indeed serve different functions (30). The neuronal source of AGT is particularly interesting when one considers studies by us and others suggesting the presence of an intracellular form of active renin in the brain (17, 37); our data suggest that renin is primarily neuronal (14, 15), and unpublished results from our laboratory suggest that intracellular active renin in the brain plays a functional role in the regulation of arterial pressure (Lavoie JL and Sigmund CD, unpublished data). Indeed, the presence of intracellular renin in AGT-containing neurons may provide a molecular explanation for the long-known presence of ANG II in neurons and nerve fibers, which define angiotensinergic neural projections in the brain and use ANG II as a neurotransmitter (9). ANG II-immunoreactive nerve fibers have been identified in the SFO, medial preoptic nucleus, supraoptic nucleus, PVN, and posterior pituitary (10, 18, 19); and perhaps, the best characterized angiotensinergic pathways regulating cardiovascular function are those that originate as soma in the SFO and project through ANG II-immunoreactive axons, where they synapse in the hypothalamus. However, other projections from the PVN to the NTS and RVLM (and from NTS to RVLM) have also been reported to be involved in the regulation of blood pressure and to mediate the blood pressure changes during stress responses (3, 8, 20, 35, 41). These projections, although perhaps not directly angiotensinergic themselves, are clearly stimulated by ANG II, possibly as a consequence of local production of ANG II in these nuclei. What remains unclear is whether the source of ANG II substrate in these sites is derived from glial cells or neurons. Consequently, the overall blood pressure effect of brain-derived ANG II may result from a combination of neuronal signaling via intracellular angiotensinergic pathways (using ANG II as a classical neurotransmitter, i.e., SFO-PVN) and from locally produced extracellular ANG II-derived from either glial or neuronal sources of AGT (i.e., as in the NTS or RVLM). If this is indeed the case, we would predict that elimination of either arm of the pathway would have marked effects on arterial pressure, as observed in this study.

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