Brain-specific Restoration of Angiotensin II Corrects Renal Defects Seen in Angiotensinogen-deficient Mice*

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Mice deficient for angiotensinogen (AGT), or other components of the renin-angiotensin system, show a high rate of neonatal mortality correlated with severe renal abnormalities including hydronephrosis, hypertrophy of renal arteries, and an impaired ability to concentrate urine. Although transgenic replacement of systemic or adipose, but not renal, AGT in AGT-deficient mice has previously been reported to correct some of these renal abnormalities, the tissue target for this complementation has not been defined. In the current study, we have used a novel transgenic strategy to restore the peptide product of the renin-angiotensin system, angiotensin II, exclusively in the brain of AGT-deficient mice and demonstrate that brain-specific angiotensin II can correct the hydronephrosis and partially correct renal dysfunction seen in AGT-deficient mice. Taken together, these results suggest that the renin-angiotensin system affects renal development and function through systemically accessible targets in the brain.

The renin-angiotensin system (RAS) plays a critical role in the regulation of blood pressure and volume homeostasis in mammals. The main effector of the RAS is the octapeptide hormone angiotensin II (Ang II) produced by the successive enzymatic cleavage of the hepatic glycoprotein angiotensinogen (AGT) by kidney-derived renin and angiotensin-converting enzyme (ACE), a metalloprotease located at the surface of endothelial cells (1). Although Ang II acts through two receptors, type I and type II (AT1 and AT2, respectively), all of the known cardiovascular effects of Ang II are mediated by the AT1 receptor. In addition to this circulating RAS, the mRNA and protein for all of the RAS components have been identified in different tissues as brain, heart, adrenal gland, reproductive organs, and kidney (reviewed in Ref. 2), leading to the suggestion that tissue-specific (tRAS) might mediate cardiovascular and other functions within tissue sites.

Inactivation of the RAS during development either by pharmacological inhibition or gene ablation also causes severe anomalies in the neonatal kidney, leading to hydronephrosis and impaired urine concentration (3). In humans, inhibition of the RAS during pregnancy is associated by a high rate of spontaneous abortion and in some strains of mice with a high rate of mortality in the first 3–5 weeks of life (4–8). In an effort to understand the mechanisms that implicate the RAS in renal development, all of the genes coding for the components of the RAS from AGT to the angiotensin receptors have been disrupted in mice (9–15). Although these results confirm that Ang II acting through the AT1 receptor is required for normal renal development, renal defects in mouse pups are only observed when both of the two AT1 isoforms present in mice (AT1A and AT1B (16–18)) are inactivated, leading to the suggestion that each isoform can compensate in the absence of the other in the target tissue (19). Because only limited tissues, including the brain, adrenal gland, and testis, express both the AT1 receptor isoforms in mice, it is possible that the effects of the RAS on renal development are mediated through one of these non-renal tissues. Supporting this hypothesis, specific restoration of Ang II in the circulation by targeting adipocytes (20) or multi-tissue restoration of AGT by using the ubiquitously expressed metallothionein promoter (21) or the native angiotensinogen promoter (22) prevents kidney abnormalities seen in AGT−/− mice, whereas targeted restoration of AGT only in the kidney of these animals does not prevent the renal anomalies (8). In the current study, we have used a novel transgenic strategy to directly address the ability of brain Ang II to correct the renal anomalies in AGT−/− mice and have compared these results to those obtained when Ang II is specifically restored in the circulation of these mice.

MATERIALS AND METHODS

Generation of Transgenic and Angiotensinogen Knockout Mice—The expression vector used for releasing Ang II is shown in Fig. 1, and the details of its construction have been described previously (23). Briefly, the signal peptide of human prorenin was linked to a fragment of the heavy chain constant region of IgG2b mouse immunoglobulin lacking the BIP binding, hinge, and intermolecular disulfide bridge regions. This domain is followed by a portion of the human prorenin prosegment, which includes a consensus cleavage site (RVRTRK) for furin, a ubiquitinous protease, immediately linked to the human Ang II peptide sequence. This fusion protein coding sequence was placed downstream of the BIP binding, hinge, and intermolecular disulfide bridge regions. The transgene was excised from the plasmid vector and co-injected with a tyrosinase gene for pigmentation marking in the pronucleus of fertilized eggs from FVB/N mice as previously described (25). The resulting transgenic founder lines were called GFAP-AngII. Generation and characterization of transgenic mice with cardiac-specific expression of frog skin Ang II (fAngII 2C) has been described previously (26).

Knockout mice for angiotensinogen (AGT−/−), a gift from Drs. Kim and Smithies, University of North Carolina, Chapel Hill, NC were...
crossed from the original C57BL/6 strain onto the FVB/N background for 10 generations to generate AGT−/− (FVB) mice. GFAP-Ang II or fsAng II 2C transgenic mice were crossed into the AGT−/− (FVB) background. All mice were used at 12–16 weeks of age unless otherwise specified. The animal protection committee of the Clinical Research Institute of Montreal approved all animal protocols.

Transgene Expression Analysis in GFAP-Ang II Mice—Total RNA from brain or other tissues was prepared by the guanidinium-thiocyanate-phenol-chloroform method (25), and RNase protection assays were performed as described previously (27) with minor modifications. Labelled RNA probes corresponding to the transgene or to histone H4 (an internal control for RNA loading) were synthesized in presence of the [α-35S]CTP (Amersham Biosciences). Total RNA (10 μg) from tissue samples was hybridized with a mixture of the labeled probes (8 × 10^4 cpm/mg RNA) and was hybridized overnight at 55 °C. Non-hybridized probes were digested by incubation in RNase A solution (200 μg/ml of RNase A in 1× SSC), and the slides were extensively washed and dipped in photographic emulsion (Kodak NTB-2). Slides were exposed for 6 days and developed in Kodak D19 solution.

Characterization of GFAP-Ang II Mice—The levels of Ang II in brain, tissues, and plasma were determined by radioimmunoassay (RIA) (29). Ang II in tissue or plasma was obtained by acid/alcohol extraction (80% ethanol, 0.1 M HCl) and purified on Sep-Pak hydrophobic C18 cartridges (Waters, Milford, MA). The peptides were eluted with ethanol, and lyophilized peptides were quantified by RIA using a polyclonal rabbit antiserum which has 100% cross-reactivity with Ang II (angiotensin 1–8), Ang III (angiotensin 2–8), and Ang IV (angiotensin 3–8) but no reactivity with Ang I (angiotensin 1–10) (29). To determine the form of angiotensin peptides released by the fusion protein in brain, whole brain acid/alcohol extracts from three mice were pooled and fractionated by reverse-phase HPLC and elution fractions were lyophilized and subjected to RIA using an antibody specific for Ang II and its metabolites (26, 29).

Plasma renin activity was determined as described previously (26). Briefly, blood (5 drops) was obtained by orbital puncture of mice under light ether anesthesia in cold tubes containing 25 μl of EDTA (pH 8.0). Blood was immediately centrifuged, and the plasma was frozen in liquid nitrogen and stored at −80 °C until the assay was performed. Plasma renin activity was determined by an Ang I generation assay in presence of excess angiotensinogen substrate from sheep (26). The resulting Ang I was measured by RIA using antibody specific for Ang I.

Physiological Measurements—Systolic blood pressure (BP) was measured by tail-cuff plethysmography (BP-200 system, Visitech Systems, Apex, NC). Mice were trained for 7 days and BP was recorded for an additional 3 days. After the initial 10 days of BP recording, some mice were treated with an ACE inhibitor (Captopril, Sigma) at 100 mg/kg/day by intraperitoneal injection in 0.9% NaCl or an angiotensin AT1 receptor antagonist (Candesartan, a gift from Astra-Zeneca) 15 mg/kg/day by gavage for a further 5 or 3 days while measuring BP during the treatment.

For determination of water intake and urine output, animals were placed in metabolic cages with free access to water and food. After a 24-h adaptation period, water and urine output volume were measured for three consecutive days. Urine and plasma osmolality were determined by the freezing-point depression method (μOsometry). Precision was controlled by calibrating bottles were removed for 24 hr, and urine volume and osmolality were again measured to evaluate the capacity of the mice to concentrate urine when faced with dehydration.

Histology—Animals were killed by CO2 inhalation, and the kidneys were fixed in Bouin’s fixative and embedded in paraffin. Serial cuts (5 μm) were prepared and stained with either Sirius Red or hematoxylin/cosin. Morphological analysis by standard techniques.

Statistical Analysis—Results are expressed as mean ± S.E. One-way ANOVA with Dunnett’s post test or unpaired t test was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

Transgene Expression—A fusion protein capable of releasing Ang II into the secretory pathway of expressing cells was placed under the control of the human GFAP promoter (Fig. 1) to drive over-production of Ang II specifically in the brain of transgenic mice. Six founder lines were obtained, and the tissue distribution of transgene expression in the brain was determined by RNase protection and in situ hybridization. Three lines (GFAP58.8, GFAP58.5, and GFAP58.2) were found to express the transgene (Fig. 2A) at different levels in brain. Line GFAP58.8 (which had the highest expression level in the brain) and was used for most of the experiments in this study) was found to express the transgene exclusively in the brain (Fig. 2B) and in situ hybridization demonstrated that this expression was evenly distributed throughout the entire brain (Fig. 2C).

Notably, expression of the fusion protein had no effect on the pattern of expression of the endogenous GFAP gene (data not shown).

**Dosage and Characterization of Ang II in Brain of GFAP-Ang II Transgenic Mice—**Angiotensin peptides were extracted from various tissues and plasma from line GFAP58.8 by acid/ethanol extraction and quantitated by radioimmunoassay. The results show that brain Ang II was increased by 9–7-fold in the transgene-expressing lines as compared with non-transgenic littersmates (Fig. 3A), and Ang II production in the brain of each line was roughly proportional to the level of transgene expression (Fig. 2A). Measurement of the levels of Ang II in various tissues of line GFAP58.8 confirmed that Ang II production was only increased in the brain of this line as we could not detect any increase of Ang II content in non-brain tissues tested (Fig. 3B).

Importantly, we were also unable to detect any increase in circulating Ang II in the plasma of GFAP-Ang II mice as compared with control littersmates, suggesting that the Ang II being produced in the brain of the transgenic animals is not spilling over into their circulation (Fig. 3C). To confirm this finding, we measured plasma renin activity, a sensitive indicator of circulating Ang II levels, because renin secretion from the kidney is potently suppressed by increases in circulating Ang II. Notably, GFAP58.8 mice actually show a slight increase in plasma renin activity (GFAP58.8, 1721 ± 404; non-transgenic 1193, ± 185 ng Ang I/ml/h; p < 0.05), supporting the conclusion that these animals do not liberate brain-derived Ang II into the circulation. Because the brain is rich in aminopeptidases that can break down Ang II to less active metabolites (30, 31), we investigated the form of Ang II contained in the brain of line GFAP58.8 by RIA-coupled HPLC of whole brain acid/alcohol extracts. The results clearly demonstrate that the majority of the peptide extracted from the brain of GFAP58.8 mice migrates as bona fide Ang II (Fig. 4).

**Blood Pressure Measurement of GFAP-Ang II Mice**—To determine the effect of increases in brain Ang II on cardiovascular functions, systolic BP was measured by the non-invasive tail-cuff method (32). We found that the BP was significantly in-

[Fig. 1. Diagram of the vector used to release Ang II in the brains of transgenic mice. A 2.2-Kb fragment of the human GFAP (hGFAP) promoter was used to drive expression of a fusion protein comprised of a signal peptide sequence (Pre), a fragment of the heavy chain constant region of mouse immunoglobulin IgG2b (Ig), and a human prorenin prosegment as molecular spacer (Pro) containing the furin cleavage site (RVRTKR) followed by the sequence encoding Ang II peptide (Ang II). The coding sequence for Ang II is followed by an intron and polyadenylation signal from rabbit β-globin.](http://www.jbc.org/Downloaded from)
increased in the transgenic mice (GFAP58.8, 150 ± 12; control, 123 ± 6 mmHg; \( p < 0.001 \)). Despite the differences observed in the levels of Ang II in brain of the three transgenic lines we characterized, there was no significant difference in the degree of hypertension seen in mice from these lines (data not shown). Treatment of line GFAP58.8 mice with a specific angiotensin II AT1 receptor antagonist (Candesartan) at a dose of 15 mg/kg/day by gavage normalized BP after 3 days of treatment (Fig. 5), confirming that the hypertension seen in these mice was due to the action of Ang II on the AT1 receptor. In contrast, treatment of these mice with the angiotensin-converting enzyme inhibitor Captopril at a dose of 100 mg/kg/day intraperitoneally for 4 days did not reduce the BP of transgenic mice to a greater extent than seen in non-transgenic littermates (Fig. 5). This dose of Captopril was effective, however, in correcting RAS-dependent hypertension in mice, which over-express human renin in the liver under the control of transthyretin promoter (TTRhRen-A3 before treatment 169 ± 10 and after treatment 112 ± 9; \( p < 0.001 \) (29)). These results clearly demonstrate that the hypertension seen in line GFAP58.8 is due to the direct action of Ang II on the AT1 receptor and not due to activation of the endogenous mouse RAS, which would have responded to Captopril treatment.

**Fig. 2.** Transgene expression analysis in GFAP-Ang II mice. An RNase protection assay was performed on total RNA from brain (A) or non-brain tissues from line GFAP58.8 (B). TG, expected size of the protected transgene mRNA. Histone H4, expected size for the histone internal control mRNA; tRNA, negative control; C, mRNA from GH4 cells transfected with the vector in Fig. 1 (10 \( \mu \)g RNA by lane). In situ hybridization on brain sections from line GFAP58.8 (C) or non-transgenic control mice (D) with an antisense transgene probe (AS).

**Fig. 3.** Level of Ang II in tissues and plasma of GFAP-Ang II mice. Angiotensin peptides were extracted from brain of the three founder lines of mice (A), tissues (B), or plasma (C) from line GFAP58.8 mice (solid bars) or control (open bars) and subjected to RIA using an antibody specific for Ang II and its metabolites, \( n = 4 \). Values are expressed as means ± S.E.; ***, \( p < 0.001 \) compared with control.

**Fig. 4.** Characterization of angiotensin peptides present in brain of GFAP-Ang II mice. Tic marks indicate the migration of angiotensin peptide standards for Ang II (angiotensin 1–8), Ang III (angiotensin 2–8), and Ang IV (angiotensin 3–8). Peptides extracted from the brains of three transgenic (closed circles) or three control (open diamonds) mice were separated by HPLC, and fractions were subjected to RIA with an antibody specific for Ang II and its metabolites.

**Fig. 5.** Blood pressure of GFAP-Ang II mice. Systolic blood pressure of male GFAP58.8 (solid bars) and non-transgenic or control (open bars) mice at 10–12 weeks of age (BP) was recorded by the tail cuff method. Shown is the pressure difference after treatment with the ACE inhibitor Captopril or the angiotensin II AT1 receptor antagonist Candesartan. Values are expressed as means ± S.E., \( n = 6 \); ***, \( p < 0.001 \) compared with control relative.
magnification was 2×. Arrowheads noted thickening of arterial walls, increased perivascular, glomerular and interstitial fibrosis, and hypertrophy of the interlobular arteries (D, note thickening of arterial walls as denoted by open arrows) as compared with control FVB mice (A and B). Hydronephrosis was corrected in GFAP-Ang II/AGT−/−(FVB) (E) and 2C/AGT−/−(FVB) (G) mice while cortical cysts (E and G, arrowheads) and hypertrophy of interlobular arteries (F and H, note thickening of arterial walls, arrowheads) persist.

mortality rate by weaning (6–8). To test for the effect of complementation of Ang II on these phenotypes, we first bred the AGT−/− mice onto the FVB/N background (hereafter referred to as AGT−/−(FVB)). After 10 generations, AGT−/−(FVB) mice had all of the expected phenotypes but did not exhibit any increased mortality at weaning (data not shown). These mice were bred to GFAP58.8 transgenic mice to transgenic mice over-expressing frog skin Ang II in the heart (line 2C; Ref. 26). The 2C line has an approximate 1,000-fold increase in cardiac Ang II and spills this peptide into the circulation as evidenced by a suppression of plasma renin content (line 2C; Ref. 26). Blood pressure in line 2C mice is not increased at 5 weeks, but is slightly elevated by 10–12 weeks as compared with non-transgenic littermates (26). The GFAP58.8 and line 2C transgenic mice were bred into the AGT−/−(FVB) background, and the resulting mice were characterized. Selective restoration of Ang II either in brain or in plasma normalizes BP of the AGT−/−(FVB) mice (∗AGT−/−(FVB) 106 ± 7; GFAP-Ang II/AGT−/−(FVB) 110 ± 9; 2C/AGT−/−(FVB) 112 ± 12 and control 120 ± 5 mmHg; *, p < 0.01 versus control).

The kidneys of AGT−/−(FVB) mice present hydronephrosis, increased perivascular, glomerular and interstitial fibrosis, medullary cysts, and hypertrophy of interlobular arteries (Fig. 6, C and D). All (22/22) kidneys examined from GFAP-Ang II/AGT−/−(FVB) mice were free of hydronephrosis (Fig. 6E), whereas the vast majority (8/10) of kidneys from 2C/AGT−/−(FVB) were hydronephrosis-free (Fig. 6G). Interestingly, some glomerular atrophy and hypertrophy of interlobular arteries persists despite restoration of Ang II in brain or the circulation of AGT−/− animals (Fig. 6, F and H). Small cysts were also present in the renal cortex of animals restoring Ang II only in the circulation of the AGT-deficient animals (Fig. 6H).

Effect of Brain or Circulating Ang II on Kidney Function of AGT−/−(FVB) Mice—To test for the effect of brain or circulatory Ang II on kidney function in AGT−/−(FVB) mice, animals were placed individually in metabolic cages with free access to water and food, and urine output and water consumption were measured. As expected, AGT−/−(FVB) mice show an increased water intake and urine output (Fig. 7, A and B), but both were significantly attenuated in GFAP-Ang II/AGT−/−(FVB) and 2C/AGT−/−(FVB). Urine osmolality of AGT−/−(FVB) and GFAP-Ang II/AGT−/−(FVB) was significant lower than that of control mice (Fig. 7C). In contrast, 2C/AGT−/−(FVB) mice correct this decrease in urine osmolality (Fig. 7C), consistent with the known importance of circulating Ang II for kidney function (8). As previously reported, AGT−/− mice are unable to concentrate urine when deprived of water (Fig. 7C). After a 24-h period of water deprivation, GFAP-Ang II/AGT−/−(FVB) mice show the same capacity as control mice to concentrate urine (an approximate 2-fold increase) even though the starting osmolality is lower than that of control mice (Fig. 7C). The 2C/AGT−/−(FVB) as well AGT−/−(FVB) cannot concentrate their urine after being water-deprived as compared with control littermates (Fig. 7C). These results demonstrate that brain Ang II plays an important role in regulating fluid balance in conditions of dehydration.

DISCUSSION

We have used a novel technique to generate transgenic mice with a chronic over-production of Ang II peptide in the brain. This locally produced Ang II does not spill over into the circulation as evidenced by an absence of transgene mRNA or Ang II peptide increases in the non-brain tissues tested, a lack of detectable increases in circulating Ang II, and a lack of suppression of circulating renin, a very sensitive indicator of circulating Ang II. Our results demonstrate that restoration of brain Ang II in mice deficient for an endogenous renin-angiotensin system prevents the hydronephrosis seen in the non-complemented AGT−/− mice and restores their ability to concentrate urine when challenged by dehydration. In contrast, although restoration of circulating Ang II in the AGT−/− mice also corrects hydronephrosis, it is unable to correct their ability to concentrate urine when challenged with dehydration, suggesting differences in the site of action of Ang II in these two models. Although both complementation strategies partially corrected the increased drinking and urine output seen in AGT−/− mice, neither improved the observed hypertrophy of the interlobular renal arteries. Taken together, these results suggest that the panoply of renal phenotypes described in RAS-deficient mice may be due to the action of Ang II on multiple target tissues.

Newborn mice deficient for a RAS have histologically normal kidneys at birth and develop frank hydronephrosis in the first 3–5 weeks of life (8, 33), a period when the newborn kidney must adapt to a much higher flux in fluid than it was exposed to in utero. Although both transgenic models reduce drinking behavior and urine output in the AGT−/− background, these...
values do not return to levels seen in control mice, and it seems unlikely that this partial reduction in urine flow could account for the complete disappearance of the hydronephrosis. Taniguchi et al. (33) have suggested that the hydronephrosis seen in RAS-deficient C57bl/6 mouse pups is due to an incomplete development of a smooth muscle layer around the upper ureter, which results in a loss of ureteral peristalsis and hydronephrosis due to reflux pressure. Surprisingly, in the FVB background we see no deficiency in the formation of the smooth muscle cell layer around the upper ureter of the AGT−/− animals (data not shown) even though all of these animals develop hydronephrosis, raising the possibility that both brain and circulating Ang II act at the same sites in the brain to influence either postnatal renal development or function in newborn mice. Although the actual mechanisms need further investigation, both central and circulating Ang II could influence renal sympathetic nerve activity, which in turn could have a direct effect on kidney function by stimulating the peristalsis of the ureter and in this way prevent the urinary reflux (41). Interestingly, we found that the high rate of neonatal mortality that has been associated with renin-angiotensin system deficiency depends on genetic background use. By using AGT−/−(FVB) it seems that the kidney anomalies seen in mice with RAS disruption are an adaptation problem instead of a developmental problem, because obvious renal defects are not seen until 4 to 5 weeks of age.

In conclusion, we have demonstrated that chronic elevations of Ang II specifically restricted to the brain can prevent the hydronephrosis and urine concentration deficit seen in RAS-deficient mice. These findings may have important implications for understanding the fetotoxic effects of RAS inhibitors seen in humans (4, 5).

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