Changes in angiotensin type 1 receptor binding and angiotensin-induced pressor responses in the rostral ventrolateral medulla of angiotensigen knockout mice

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MATERIALS AND METHODS

General Preparations

All experiments were performed in accordance with the National Health and Medical Research Council of Australia, Code of...
Practice for the Care and Use of Animals for Scientific Purposes, and were approved by the University of Melbourne Animal Ethics and Experimentation Committee. Experiments were performed on adult male mice of the C57BL6 strain, in which the coding region for the $\text{Agt}^{-/-}$ gene had been deleted by homologous recombination ($\text{Agt}^{+/+}$). These heterozygous mice, from a line ($\text{Agt}^{+/+}$) generated by H. S. Kim and colleagues (23), were bred at the Howard Florey Institute to derive homozygous $\text{Agt}^{+/+}$ (wild-type) and $\text{Agt}^{-/-}$ (knockout) mice. All mice were genotyped, as described previously, prior to experimentation (30). The $\text{Agt}^{-/-}$ mice used in this study showed general characteristics that are consistent with previous observations. As observed by others, only a small percentage of $\text{Agt}^{-/-}$ mice survive past weaning (23, 36). To overcome this, $\text{Agt}^{-/-}$ pups in this study were given subcutaneous injections of warm isotonic saline (0.15 mol/l NaCl) during the first week of life (30). Despite attempts to use littermate homozygous $\text{Agt}^{+/+}$ and $\text{Agt}^{-/-}$ mice, the reduced survival of the $\text{Agt}^{-/-}$ mice meant this was not always possible, and a small difference in the mean ages of the populations occurred ($\text{Agt}^{+/+} 7 \pm 1$ mo vs. $\text{Agt}^{-/-} 5 \pm 1$ mo). Mice were maintained under 12:12-h light-dark cycle with ad libitum access to standard chow and water.

**Localization of Receptor Binding Sites**

The distribution of angiotensin binding sites was mapped in the brain of littermate $\text{Agt}^{+/+} (n = 3)$ and $\text{Agt}^{-/-} (n = 3)$ mice using standard methods (27). Power calculations using standard deviations derived from previously published data from this laboratory (3, 21, 27), an expected change of 20% in mean levels, power of 0.8, and 0.05 were used to calculate sample size. Examining a minimum number of mice enabled all sections to be incubated at once, thus removing the need to normalize data and control for interassay variability. Briefly, the mice were deeply anesthetized, by inhalation of isoflurane in an enclosed container, and decapitated. The brain was removed, frozen in isopentane on dry ice and 20-μm coronal sections cut on a cryostat. Slide-mounted sections were incubated using standard solutions containing 125I-[Sar1, Ile8] ANG II as radioligand (Prosearch International Australia Pty Ltd, Vic, Australia). Either 1 μM angiotensin II (to determine non-specific binding; Aus-Pep Pty Ltd, Vic, Australia), 1 μM candesartan (to displace binding from AT1 receptors; gift from Astra Hässle, AB, Sweden) or 10 μM PD123319 (to displace binding from AT2 receptors; Sigma-Aldrich, St. Louis, MO) were added to the incubation media to define receptor ligands and ANG II injected unilaterally (50 pmol: 50 nl of 1 mM solution), in the RVLM.

**Measurement of BP and HR**

For physiological studies, mice were initially anesthetized by inhalation of isoflurane, followed by intraperitoneal injection of a mixture of urethane (0.75 mg/g ip) and chloralose (0.05 mg/g ip). A stable surgical plane of anesthesia was determined by the loss of the pedal withdrawal reflex to a strong noxious stimulus and loss of the corneal reflex. Supplemental doses of urethane (75 μg/g ip) were administered if necessary. Mice were tracheotomized and artificially ventilated (UGO Basile mouse ventilator 28025; Comerio, VA, Italy) with room air. The left carotid artery was cannulated for BP measurement with heat stretched polyethylene tubing (0.96 mm OD, 0.58 mm ID), as previously reported (26). This cannula was filled with sterile saline containing heparin (50 IU/ml) to prevent clotting. The catheter was connected to a BP transducer (Neurolog System Digitimer, Hertfordshire, England) and the signal amplified (Neurolog System Digitimer, Hertfordshire, England), digitized (2-kHz sampling rate), and stored on computer (MacLab, Takoma Park, MD) for later analysis. Mean arterial pressure (MAP) and heart rate (HR) were derived from this signal. Rectal temperature was maintained at 37°C with a heating pad (ATC1000 Animal Temperature Controller, World Precision Instruments, Sarasota, FL). In preliminary experiments, blood samples (0.1 ml) were taken from the carotid artery to measure arterial blood gases and pH. Ventilation parameters (stroke volume: 0.3–0.4 ml, and rate: 135–150 strokes/min) were then determined to maintain arterial PCO2 at ~4.5%. (Continued in the next section.)
ANG II RECEPTORS AND ANGIOTENSINOGEN-DEFICIENT MICE

Perihaperal ANG II and Phenylephrine Bolus

Agt+/+ and Agt−/− mice were prepared as described above for measurement of BP and HR. The left external jugular vein was cannulated with polyethylene tubing (0.61 mm OD, 0.28 mm ID) for the administration of ANG II and phenylephrine. The AP response to intravenous injection of a 0.1 ml bolus of ANG II (0.01, 0.05, or 0.1 μg/kg) was examined in Agt+/+ (n = 7) and Agt−/− (n = 8) mice. In between each ANG II dose, saline was used to wash out residual ANG II in the catheter, and mice were allowed to recover for at least 20 min. In a subset of these mice [Agt+/+ (n = 5) and Agt−/− (n = 4)], a 0.1 ml bolus of phenylephrine (4 μg/kg and 2 μg/kg) was also administered intravenously.

Statistical Analysis

Data are expressed as means ± SE. Student’s t-test was used to compare the receptor-binding densities in specific regions between Agt+/+ and Agt−/− mice using GraphPad Prism (GraphPad software, San Diego, CA). Baseline MAP and HR are calculated based on 5-min averages at the start of each recording. For analysis of the responses to RLVM microinjections, 30-s averages of MAP and HR signals were determined for the period 5 min prior to the microinjections and for at least 15 min afterwards. The peak response to each microinjection was the highest 30-s average value after microinjection and was compared with the baseline measure. Comparisons of the peak ANG II and glutamate responses were made between Agt+/+ and Agt−/− mice with a two-way repeated-measures ANOVA followed by the Newman-Keuls multiple-comparisons test [Sigma Stat ver. 3.5 (Jandel, San Jose, CA)]. For analysis of intravenous ANG II and phenylephrine responses, 10-s averages of MAP and HR were calculated for the 1-min period prior to injection (baseline) and for at least 5 min after injection. The peak response to each injection was determined by the highest 10-s point and was compared with the baseline measure.

Comparisons of the intravenous ANG II and phenylephrine AP changes were made between Agt+/+ and Agt−/− mice within each dose by two-way ANOVA followed by Holm-Sidak multiple-comparison test (Sigma Plot 11, Jandel, San Jose, CA). Different statistical programs and post hoc tests were used in data analysis to conduct the most stringent comparisons. Statistical significance was set at P < 0.05.

RESULTS

General Parameters

Wild-type (n = 14) mice were 7 ± 1 mo of age, and Agt+/+ (n = 14) mice were 5 ± 1 mo of age. The average weights of the Agt+/+ mice was 29 ± 1 g and Agt−/− mice was 24 ± 1 g, (P < 0.05). Under anesthesia, the baseline BP was significantly higher in Agt+/+ mice compared with Agt−/− mice (systolic BP: 81 ± 4 vs. 58 ± 3 mmHg, respectively and MAP: 64 ± 4 mmHg vs. 39 ± 1 mmHg, respectively, P < 0.0001) (Fig. 3A). HR was not different in Agt+/+ and Agt−/− [519 ± 35 bpm vs. 473 ± 23 bpm, respectively, (not significant, N.S.)].

Receptor Distribution

The distribution of binding sites for 125I-[Sar1, Ile8] ANG II was examined throughout the brain of Agt+/+ and Agt−/− mice. A similar overall distribution was observed for each genotype, which was comparable to the distribution in the mouse brain that has been published previously (17, 21). The sites of highest density binding were in the lamina terminalis, the PVN, the NTS, area postrema, and IO. Comparison of the density of AT1 and AT2 receptors in a selection of these regions indicated that, in general, there was little difference in density between the Agt+/+ and Agt−/− mice (Fig. 1). The exceptions to this were the NTS, where there was a small, but significant decrease in AT1 receptor binding density, with no alteration in the AT2 receptor density in the Agt−/− mice; the IO, with a small increase in AT2 receptor density in the Agt−/− mice; and the RVLM, with a small increase in AT1 receptor density in the Agt−/− mice.

Injection Studies in Anesthetized Mice

Glutamate response. Identification of the RVLM was based upon the AP response to microinjection of the excitatory amino acid L-glutamate and histological verification of the placement of fluorescent microspheres (Fig. 2B) within the anatomical boundaries of the nucleus. Mice were excluded from the study...
Fig. 2. A: schematic coronal sections of mouse medulla oblongata [modified from the atlas of Franklin and Paxinos (14)], showing the centre of microinjection sites of ANG II in all mice (gray-shaded ovals). These microinjections were considered to be within the anatomical boundary of the RVLM. The numbers represent the distance from bregma. B: photomicrograph of a coronal section of mouse medulla taken under fluorescence illumination, showing the distribution of rhodamine-labeled microspheres that were included in a microinjection of ANG II. C: photomicrograph of the same section as shown in B, taken under light illumination after thionin staining. The clump of parasympathetic preganglionic neurons located in the rostral portion of the compact formation of nucleus ambiguus is clearly shown. D: ratemeter recordings showing arterial pressure (AP), mean arterial pressure (MAP), and heart rate (HR) recordings from a Agt+/+ mouse. The time calibration bar is shown underneath the HR trace. Glutamate (100 pmol in 10 nl) was microinjected at the time indicated by the arrow. This example illustrates the pressor effect evoked by microinjection into the RVLM and the activation of respiratory activity that was observed in some animals. Amb, nucleus ambiguus; 7N, facial motor nucleus; RVLM, rostral ventrolateral medulla; IO, inferior olivary nucleus.

if these conditions were not met. Microinjection of glutamate into the RVLM (100 pmol in 10 nl) caused a rapid pressor response that was not different in Agt+/+ and Agt−/− mice as shown in Fig. 3B (peak response of +17 ± 3 mmHg vs. +13 ± 2 mmHg, respectively, N.S.). Even when expressed as a percentage change in BP, to account for the lower basal AP of the Agt−/− mice, there was no difference in the glutamate response between the genotypes (28 ± 4% in each case). In approximately half the mice, glutamate microinjection into the RVLM also induced changes in respiratory activity with increased breathing against the cycle of the respirator (Fig. 2D). Vehicle microinjections (saline, 10 nl and 50 nl) into the RVLM had no effect on BP, HR, or respiratory parameters (data not shown).

ANG II response in RVLM. Unilateral microinjections of ANG II (50 pmol in 50 nl) were made into the RVLM in Agt−/− and Agt+/+ mice (either left or right side of the brain). Comparable responses were observed between the left and right side injections; therefore all injections sites are mapped on the one side in Fig. 2A. Microinjection of ANG II into the RVLM of Agt+/+ and Agt−/− mice caused a prompt increase in AP with no change in HR (Fig. 3A). The ANG II-induced pressor response was significantly larger in the Agt+/+ mice (+23 ± 4 mmHg) compared with the Agt−/− mice (+10 ± 1 mmHg, P = 0.004) (Fig. 3B). The difference in response remained significant when expressed as a percentage of the baseline AP (44 ± 9% and 24 ± 2% in Agt+/+ and Agt−/− mice, respectively, Student’s t-test, P < 0.05). The time for the peak ANG II pressor response to occur was similar in Agt+/+ and Agt−/− mice (83 ± 19 s vs. 75 ± 20 s, respectively, N.S.). In four (3 Agt+/+ and 1 Agt−/−) mice, where injections were histologically verified to be dorsal and rostral to the anatomical boundary of the RVLM, ANG II had no effect on AP or HR. Unlike glutamate, microinjection of ANG II did not cause any significant changes in respiratory activity when injected into the RVLM. In two wild-type mice, the response to microinjection of ANG II was assessed after microinjection of the AT1 receptor antagonist, candesartan (50 nl of a 10 mM solution). In both cases, the pressor response to ANG II was abolished (data not shown).

Intravenous ANG II response. Intravenous injection of ANG II in anesthetized Agt+/+ and Agt−/− mice caused a brisk pressor response that was not different between the genotypes (Fig. 4A). Because of the lower basal BP of the Agt−/− mice, when these changes were expressed as a percentage of basal BP, there was a significant increase in the response of the Agt−/− mice to the higher doses of ANG II [0.1 μg/kg ANG II: +105 ± 5% vs. 143 ± 8% (P < 0.05); 0.05 μg/kg ANG II: +79 ± 11% vs. 117 ± 16% (P < 0.05); 0.01 μg/kg ANG II: 45 ± 10% vs. 61 ± 11% (N.S.); Agt+/+ vs. Agt−/−, respectively].

Intravenous phenylephrine response. Intravenous injection of two different doses of phenylephrine (2 μg/kg and 4 μg/kg) in anesthetized Agt+/+ and Agt−/− mice caused similar responses in both groups of mice (Fig. 4B). When expressed as a percentage of basal BP, there was no significant difference in the responses between the genotypes [4 μg/kg phenylephrine: 80 ± 18% vs. 81 ± 16%; 2 μg/kg phenylephrine: 31 ± 8% vs. 47 ± 9%; Agt+/+ vs. Agt−/−, respectively, (N.S.)].

DISCUSSION

Small, but statistically significant, changes in ANG II receptor density were observed in some brain nuclei in the Agt−/−.
mice in this study, but the overriding conclusion is that genetic deletion of AGT expression in mice does not alter the overall distribution of AT1 or AT2 receptor binding sites. Small alterations were observed in AT2 receptor density in the IO and in AT1 receptor density in the NTS and the RVLM. Despite the elevation in AT1 receptor density in the RVLM, the pressor response to microinjection of ANG II into the RVLM was decreased in the Agt−/− mice compared with the wild-type controls. This decrease in response to ANG II is not observed in the vasculature, where the increase in BP produced by intravenous ANG II is either not altered between strains or increased in the Agt−/− when expressed as a percent change from the decreased basal BP.

The changes in AT1 receptor density occur in two medullary regions that play a key role in central regulation of cardiovascular function. The NTS, which is the primary synapse for visceroafferent afferents, showed a small decrease in AT1 receptor density, whilst the RVLM, the site of sympathetic premotor neurons, showed a small increase in AT1 receptor density. It is not clear from the current studies whether these changes are due to the loss of AGT directly or to another factor such as the altered resting AP displayed by the Agt−/− mice. Genetically modified mice lacking AGT show decreased resting AP, abnormal kidney morphology, increased body fluid turnover, and reduced body weight (23, 28, 30, 36, 45, 46). Therefore, it is possible that any of the changes we observed between the Agt+/+ and Agt−/− mice in this study could be secondary responses to these dramatic alterations in basal physiology.

Genetic deletion of components of the RAS results in substantial decreases in AP. Using tail-cuff methods in conscious mice, researchers have reported the basal systolic BP to be ~120 mmHg (Agt+/+) vs. ~98 mmHg (Agt−/−) (23) and 100 mmHg (Agt+/+) vs. 67 mmHg (Agt−/−) (46). In this study, we used a combination of urethane and chloralose in an attempt to minimize the dampening effect of anesthesia. However, clearly this does result in further decreases in BP in these mice, with a suggestion that the effect of anesthesia is greater in RAS knockout mice (Agt+/+; systolic pressure 84 mmHg; MAP 67 mmHg vs. Agt−/−; systolic pressure 58 mmHg; MAP 39 mmHg). Interestingly these BP are very similar to those reported by Gembardt et al. (15) in anesthetized mice with all three ANG II receptors deleted [MAP 62 mmHg (wild-type) vs. 34 mmHg (all ANG II receptor knockout)]. Whilst the BP in the anesthetized Agt−/− mice in this study are relatively low, the mice maintained stable BP and blood gases throughout the experiment and appeared to be well perfused.

The expression level of the AT1 receptor is a major contributor to the biological efficacy of ANG II (12); hence, altered expression of the AT1 receptor is one potential control mechanism for physiological and pathological regulation of the cardiovascular system. Modulation of AT1 receptor expression is observed in a number of tissues, including the brain, in response to both physiological challenges and pathological
ANG II RECEPTORS AND ANGIOTENSINOGEN-DEFICIENT MICE

conditions (10, 43). In addition to altered gene expression (12, 13), posttranscriptional regulation of the AT₁ receptor occurs (25, 41). Whilst the factors involved in this regulation are numerous, one is homologous regulation by ANG II (25, 35). Therefore, we predicted that the expression of AT₁ receptors would be altered in Agrp⁻/⁻ mice. As reported previously, the Agrp⁻/⁻ mice from the colony used in this study have no detectable levels of circulating or tissue ANG I or II (1). However, the distribution of AT₁ receptor binding was not altered in the brain of the Agrp⁻/⁻ mouse compared with Agrp⁺/⁺ mice, and only small changes in density were observed. The relative densities as seen in our experiments are similar to those reported by others (17, 21). Our observations are in partial agreement with Tamura and colleagues (45), who demonstrated that AT₁ mRNA expression was not altered in the brain stem of Agrp⁻/⁻ mice. These data suggest that homologous regulation of the AT₁ receptor by ANG II might not be a significant factor in the adult brain.

One brain region that did show a small increase in AT₁ receptor density was the RVLM. The RVLM contains neurons whose activity is essential for the generation and regulation of sympathetic activity to the vasculature (11, 16). In all species studied, the RVLM has moderate to high concentrations of AT₁ receptors (3). Microinjection of ANG II into the RVLM induces a sympathetically mediated increase in BP (2, 5, 18, 33, 39, 42). Our study is the first to demonstrate that microinjection of ANG II into the RVLM of anesthetized mice also results in increased BP. Despite the slightly increased receptor density in the Agrp⁻/⁻ mouse, the AP response to microinjection of ANG II into the RVLM was significantly reduced compared with the Agrp⁺/⁺ mouse. The explanation for this decrease in the response to ANG II is not clear from this study.

To investigate whether altered responsiveness was a general phenomenon, we measured the pressor response to intravenous administration of ANG II in Agrp⁻/⁻ and Agrp⁺/⁺ mice. Our results show a similar increase in absolute BP between Agrp⁻/⁻ and Agrp⁺/⁺ mice. When this change is expressed as a percentage of basal BP, the response to the higher doses of ANG II is greater in the Agrp⁻/⁻ mice. The reason for this difference is not apparent from these studies but might indicate a ceiling to the pressor response elicited by ANG II. The pressor response to intravenous administration of phenylephrine was not different between the genotypes. Together, these results suggest that overall the vascular responsiveness between the mouse strains is not vastly altered.

Furthermore, the response to microinjection of glutamate into the RVLM is not different between the Agrp⁻/⁻ and Agrp⁺/⁺ mice, indicating that the neural pathway for sympathetic regulation of BP, at least from the level of the sympathetic premotor neurons, is not affected in these mice. This latter observation should be treated with caution as the primary purpose of the glutamate microinjections was to localize the RVLM. The dose of glutamate used was relatively low (100 pmol compared with 5–10 times higher doses used by many other investigators), and the injected volume was small. As the pressor response to intravenous phenylephrine was similar between both groups of mice, the observations do point toward a specific decrease in the responsiveness of RVLM neurons to ANG II rather than a decrease in efficacy of the sympathetic vasomotor pathway. One possibility might be that the intracellular second messenger pathways are altered, resulting in a decrease in effector response. Surprisingly, there do not appear to be any published reports on the ANG II responsiveness of other tissues from Agrp⁻/⁻ mice or on ANG II receptor signaling in these mice.

One explanation that should be considered for the lack of any obvious changes in the distribution or density of ANG II receptors in the brain of the Agrp⁻/⁻ mice is the suggestion of an alternative ligand for brain angiotensin receptors. Two studies have reported the presence of a non-angiotensin ligand derived from brain homogenates that can bind to angiotensin receptors (8, 37). To date, the identity of this ligand has not been reported, but it is possible that a non-angiotensin ligand could be released in the absence of AGT to act on and regulate brain angiotensin receptors.

Using a different approach, Schinke et al. (40) developed a transgenic rat line with decreased levels of brain AGT. This model uses the glial fibrillary acidic protein promoter to drive expression of antisense specific for AGT and decrease AGT transcription (40). The greater than 90% reduction in brain AGT production by glia caused a 50% reduction of hypothalamic ANG I levels but no significant effect on hypothalamic ANG II concentrations (19). These rats have reduced plasma vasopressin levels, decreased basal AP, altered baroreceptor reflex regulation of HR, and partial central diabetes insipidus.
Receptor binding studies, using methods similar to those described in this study, demonstrated that rats with decreased brain AGT exhibit an increase in AT₁ receptor binding in areas inside the blood-brain barrier, and a decrease in AT₁ receptor binding in the circumventricular organs which, being outside the blood-brain barrier, are exposed to systemic ANG II (32). Interestingly, when the drinking response to intracerebroventricular administration of ANG II was tested, the antisense rats displayed an increased sensitivity compared with controls (32). Similarly, an increased AP response to microinjection of ANG II into the RVLM was observed in the transgenic rats with a decreased brain AGT content compared with their Sprague-Dawley controls (6). These differences between the observations in the current study and those published on the antisense rat are perplexing, and a plausible explanation is not apparent. The major difference between the Agr⁻/⁻ mouse and the brain AGT antisense rat is that ANG II is completely absent in the mouse, both systemically and centrally, while the antisense rat has a brain-selective decrease in AGT production without reduced brain ANG II levels. Clearly, the results indicate that regulation of expression and responsiveness of the RAS in the brain has considerable complexity.

We conclude that complete absence of angiotensin peptides throughout development does not dramatically alter the overall distribution of ANG II receptor binding sites in the adult brain and causes only small changes in receptor density in restricted sites. The changes in AT₁ receptor density occurred in medullary nuclei involved in neural regulation of autonomic activity to the cardiovascular system. Thus, it is feasible that such changes were secondary to the altered arterial pressure observed in the Agr⁻/⁻ mouse. Interestingly, despite increased AT₁ receptor density in the RVLM, a critical sympathetic vasomotor control nucleus, the response to microinjection of ANG II was diminished, suggesting impaired coupling between the receptor and intracellular transduction pathways.

Perspectives and Significance

Altered expression of components of the RAS plays an important role in the homeostatic responses to various physiological and pathological perturbations and in regulating the activity of this system. This has been demonstrated both systemically and in the brain. In some peripheral tissues, altered levels of ANG II alter transcription and translation of the AT₁ receptor, and this could, therefore, be an important mechanism for modulation of ANG II activity. Our results in the adult mouse indicate that this may not occur in the brain, at least following chronic and complete absence of angiotensin peptides. Rather, and in distinct contrast to our hypothesis, the absence of AGT expression appears to result in downregulation of the responsiveness of the AT₁ receptor, indicating that presence of the ligand may be required for maintenance of coupling between the receptor and its effector pathways. Our study adds to evidence that regulation of the components of the RAS is tissue specific and points to the need for further information regarding regulation of RAS components in the brain.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


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