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Regulation of blood pressure by the type 1A angiotensin II receptor gene

(gene targeting/G protein-coupled receptor/hypertension)

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ABSTRACT **The renin–angiotensin system plays a critical role in sodium and fluid homeostasis. Genetic or acquired alterations in the expression of components of this system are strongly implicated in the pathogenesis of hypertension. To specifically examine the physiological and genetic functions of the type 1A receptor for angiotensin II, we have disrupted the mouse gene encoding this receptor in embryonic stem cells by gene targeting. *Agtr1A*(−/−) mice were born in expected numbers, and the histomorphology of their kidneys, heart, and vasculature was normal. AT_1 receptor-specific angiotensin II binding was not detected in the kidneys of homozygous *Agtr1A*(−/−) mutant animals, and *Agtr1A*(+/−) heterozygotes exhibited a reduction in renal AT_1 receptor-specific binding to ≈50% of wild-type [*Agtr1A*(+/+)] levels. Pressor responses to infused angiotensin II were virtually absent in *Agtr1A*(−/−) mice and were qualitatively altered in *Agtr1A*(+/−) heterozygotes. Compared with wild-type controls, systolic blood pressure measured by tail cuff sphygmomanometer was reduced by 12 mmHg (1 mmHg = 133 Pa) in *Agtr1A*(+/−) mice and by 24 mmHg in *Agtr1A*(−/−) mice. Similar differences in blood pressure between the groups were seen when intraarterial pressures were measured by carotid cannulation. These studies demonstrate that type 1A angiotensin II receptor function is required for vascular and hemodynamic responses to angiotensin II and that altered expression of the *Agtr1A* gene has marked effects on blood pressures.**

The renin–angiotensin system (RAS) is one of the primary physiological regulators of sodium and fluid balance (1). The RAS regulates body-fluid homeostasis through several distinct mechanisms—including effects on hemodynamics and vascular tone, direct stimulation of sodium reabsorption by the kidney, and stimulation of aldosterone production by the adrenal glands (2). The propensity of the RAS to cause elevated blood pressure was first recognized in acquired disorders such as renovascular hypertension (3), and alterations in the activity of this system have been strongly implicated in the pathogenesis of essential hypertension. For example, variations in genes encoding renin (4, 5), angiotensinogen (6, 7), angiotensin-converting enzyme (8, 9), and angiotensin receptors (10–12) have been associated with hypertension in human populations and in animal models of sodium-sensitive hypertension.

The major biologically active product of the RAS is the multifunctional peptide angiotensin II (1). The physiological effects of angiotensin II are elicited through binding to specific cell-surface receptors (13). Angiotensin II receptors belong to the large family of rhodopsin-like G protein-associated receptors and have been divided into two pharmacologically distinct types designated type 1 (AT_1) and type 2 (AT_2) (13–17). AT_1 receptors are thought to mediate the known functions of angiotensin II (13–15); they are defined pharmacologically by their high-affinity binding to the nonpeptide antagonist losartan (Dup 573). AT_2 receptors exhibit high-affinity binding to the antagonists PD 123177 and CGP 42112, but the physiological function of and signaling mechanisms used by AT_2 receptors are not known (13, 16, 17).

Among the AT_1 receptors, two subtypes AT_{1A} and AT_{1B} have been identified in human, rat, and mouse (18–21). These receptors are products of separate genes, share substantial sequence homology, and have wide tissue distributions. The AT_{1A} receptor seems to predominate in most tissues except the adrenal gland and the anterior pituitary (18–26), and expression of AT_{1A} and AT_{1B} receptors may be differentially regulated in the heart and the adrenals (22, 25, 26). This differential tissue distribution and regulation of AT_1 receptor subtypes may serve to modulate the biological effects of angiotensin II. However, due to the lack of discriminatory pharmacological antagonists, the individual functions of the two AT_1 receptor subtypes (A and B) have not been defined.

Associations between alterations in AT_1 receptor-encoding genes and hypertension have been identified in previous studies. For example, Deng and associates (10, 11) found that the AT_{1B} receptor gene is in a region of rat chromosome 2 that has an effect on blood pressure in both Lyon and Dahl rats. The same group found no association between the AT_{1A} receptor locus and hypertension in Dahl rats (27). However, in a recent study in humans with essential hypertension, Bonnardeaux *et al.* (12) identified an association between several AT_{1A} receptor-encoding gene polymorphisms and hypertension. Thus, there are data from humans and rats suggesting that AT_1 receptor genes might play a role in the pathogenesis of hypertension. In the present study, we have used gene targeting to determine which distinct physiological functions are specific to the AT_{1A} receptor gene. We have also defined the potential for naturally occurring mutations of the AT_{1A} receptor gene to affect the regulation of blood pressures.

MATERIALS AND METHODS

Gene Targeting. The gene encoding the AT_{1A} receptor (*Agtr1A*) was isolated from an E14TG2a embryonal stem cell (28) genomic library, using a probe generated by PCR from primers synthesized on the basis of published sequences for the rat AT_{1A} receptor (15). A restriction map of the genomic clone is shown in Fig. 1*A*. To construct the targeting vector, shown in Fig. 1*B*, a 0.5-kb *Eco*RI fragment containing coding sequences from the *Agtr1A* gene was replaced by the neomycin

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Abbreviations: AT_{1A} receptor, type 1A angiotensin II receptor; AT_{1B} receptor, type 1B angiotensin II receptor; AT_2 receptor, type 2 angiotensin II receptor; RAS, renin–angiotensin system.

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resistance cassette (neo) from pMC1neopolA (29). The *Herpes simplex* thymidine kinase gene from pMC1TK was inserted downstream of the 3′ homology arm. The embryonic stem cell line BK4, a subclone isolated from E14TG2a, was electroporated with the linearized targeting vector followed by positive–negative selection (29) with G418 at 0.15 mg/ml and 2 μM ganciclovir. G418/ganciclovir-resistant colonies were expanded and analyzed by PCR (30). Targeted embryonal stem cell lines were identified by the presence of a 1.7-kb PCR product using the PCR primers indicated by the arrows in Fig. 1*C*.

Blastocyst Injection and Mouse Genotype Analysis. Targeted embryonal stem cell lines containing the disrupted *Agtr1A* gene were injected into blastocysts to generate male chimeras, which were then mated with inbred females of strain C57BL/6J. F_1 progeny heterozygous for the disrupted *Agtr1A* gene were interbred to generate F_2 animals. To determine animal genotypes, genomic DNA was purified from tail biopsies, digested with *Bam*HI, and analyzed by Southern blot analysis. Incorporation of the targeting vector into the locus introduces another *Bam*HI site, so that the targeted allele can be identified by the presence of a diagnostic 3.8-kb *Bam*HI fragment using the probe shown in Fig. 1*C*. A representative autoradiogram of a Southern blot analysis of tail DNA samples is shown in Fig. 1*D*.

[Sar^1,Ile^8]Angiotensin II Receptor Autoradiography. Kidneys were removed from anesthetized *Agtr1A*(+/+), (+/−), and (−/−) mice, placed in liquid plastic (OCT compound), and frozen in isobutane chilled by liquid nitrogen. Twenty-micrometer sections were thaw-mounted on glass slides, and receptor autoradiography was done as described (31) with some modifications. Tissue sections were incubated with 200 pM ^{125}I-labeled [Sar^1,Ile^8]angiotensin II in buffer alone or in the presence of 5 μM concentrations of [Sar^1,Ile^8]angiotensin II (DuPont/NEN), PD 123319 (provided by D. Taylor, Parke–Davis, Ann Arbor, MI) (AT_2 receptor antagonist), Dup 753 (provided by R. Smith, DuPont/Merck, Wilmington, DE) (AT_1 receptor antagonist), or a combination of Dup 753 and PD 123319. After multiple washes, the sections were dried and exposed to Hyperfilm βmax (Amersham) for 3 days. Prints were made from the autoradiograms using F5 Kodabromide paper (Eastman Kodak).

Angiotensin II Infusions. Mice were anesthetized with isoflurane. Flexible plastic catheters (0.015 i.d.; Norton, Akron, OH) were placed in the carotid artery and jugular vein. The catheters were tunneled under the skin and exteriorized posteriorly at the base of the neck. After allowing the animals at least 4 hr to recover from anesthesia, arterial blood pressure was measured under unrestrained conditions by an investigator (C.F.B.) who did not know the genotypes of the animals. Pulse wave forms were monitored and recorded at a rate of 200 samples per sec through the carotid catheter using Windaq data acquisition and playback software (Dataq Instruments, Akron, OH). After an adequate baseline recording was established, angiotensin II at 10 μg/kg was administered as an i.v. bolus, and mean arterial pressures were recorded continuously. Mean pressures were calculated at 20-sec intervals for 10 min after the administration of angiotensin II.

Blood Pressure Measurements. Systolic pressure was measured in conscious mice using a computerized noninvasive tail cuff system that determines tail blood flow using a photoelectric sensor. The validity of this system and its correlation with intraarterial pressure measurements have been demonstrated (32). Consistent with these previous studies, there was a significant linear correlation between blood pressure measured by tail cuff and by intraarterial catheter within our present experiments (correlation coefficient = 0.68; $P < 0.0001$). All blood pressure determinations were obtained by an investigator (C.F.B.) who was unaware of the genotypes of the animals being tested. Mice were trained to the tail cuff apparatus for at least 5 days. Blood pressures were then measured and recorded for 5 consecutive days, and a mean value was generated for each individual mouse. To obtain intraarterial pressures, carotid artery catheters were placed as described above. After the animals had recovered from anesthesia, they were maintained in a quiet environment, unrestrained and conscious, and blood pressure was recorded continuously over a period of 30 min. Based on the blood pressure recordings during this period, a mean value for systolic pressure was calculated by using the Windaq software package (Dataq Instruments).

Statistical Analysis. Data are presented as the means ± SEMs. Statistical significance was assessed by using analysis of variance and the unpaired *t* test.

RESULTS AND DISCUSSION

Gene Targeting. After introduction of the targeting plasmid (Fig. 1*B*) into embryonic stem cells by electroporation, colonies resistant to G418 and ganciclovir (29) were screened for homologous recombinants by PCR. Targeting was confirmed by Southern blot analysis using the probe shown in Fig. 1*C*. The targeting frequency was ≈1 per 100 doubly resistant colonies. Four male chimeras that were mated with C57BL/6 females transmitted the disrupted *Agtr1A* gene to their progeny producing (129 × C57BL/6)F_1 mice heterozygous (+/−) for the mutation. These F_1(+/−) heterozygotes were interbred to generate F_2 mice that were homozygous for the mutant AT_{1A} receptor gene.

Generation of Mice That Lack AT_{1A} Receptors. Previous studies have suggested that the RAS plays a role in fetal development and organogenesis. This hypothesis is based, in

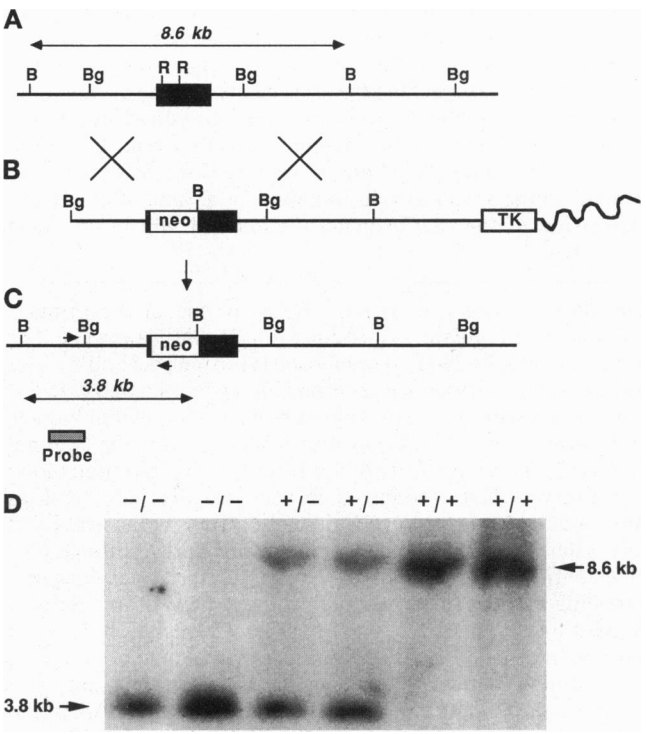

FIG. 1. Strategy for disrupting the *Agtr1A* gene. (*A*) Endogenous AT_{1A} receptor-encoding locus. The intronless coding region is indicated by the black box. (*B*) Targeting construct. neo, Neomycin resistance cassette; TK, thymidine kinase. (*C*) Structure of the disrupted gene. The PCR primers, the lengths of diagnostic restriction fragments, and the probe used for Southern analysis are shown. B, *Bam*HI; Bg, *Bgl* II; and R, *Eco*RI. (*D*) Southern blot of offspring of an *Agtr1A*(+/−) × *Agtr1A*(+/−) cross. An 8.6-kb *Bam*HI fragment indicates the wild-type allele, and a 3.8-kb fragment identifies the targeted locus.

part, on the observation that there is programmed expression of both AT_1 and AT_2 receptors within the developing fetus (33–36). To determine the relative survival of animals with the disrupted *Agtr1A* gene, we analyzed 20 $F_1 \times F_1$ matings yielding 152 weanlings. The proportion of animals of each genotype conformed to Mendelian expectations: 39 (+/+), 77 (+/−), and 36 (−/−); this is in marked contrast with our observation that most mice homozygous for a targeted disruption of the angiotensinogen locus (*Agt*) die before weaning (37). The kidneys of adult *Agtr1A*(−/−) mice were normal; this again contrasts with surviving *Agt*(−/−) adults that have distinctive renal histological abnormalities characterized by arteriolar wall thickening and focal areas of renal cortical atrophy (37). The survival and normal renal histomorphology of *Agtr1A*(−/−) animals suggest that the marked detrimental effects of angiotensinogen deficiency are not caused by the absence of signaling through the AT_{1A} receptor; they may result from the lack of AT_{1B} receptor- or AT_2 receptor-mediated responses or from the absence of other peptides that are generated from angiotensinogen (38).

Angiotensin II Binding in Mutant Mice. To define the contribution of AT_{1A} receptors to angiotensin II binding, we performed receptor autoradiography with ^{125}I-labeled [Sar^1,Ile^8]angiotensin II. Fig. 2 shows that both AT_1 receptor- and AT_2 receptor-specific binding could easily be detected in the kidneys of (+/+) animals: AT_1 receptor binding (Fig. 2*C*) is distributed in cortical areas and around glomeruli; AT_2 receptor binding (Fig. 2*D*) is also present in the cortex but extends into the medulla with a vascular and/or tubular distribution. In the kidneys of *Agtr1A*(−/−) mice, total angiotensin II binding (Fig. 2*F*) is significantly reduced compared with controls (Fig. 2*A*) and consists almost entirely of AT_2 receptor-specific binding (Fig. 2*I*). AT_1 receptor-specific binding (Fig. 2*H*) is virtually absent in *Agtr1A*(−/−) mice, confirming the efficacy of the gene disruption and suggesting that most AT_1 receptor-specific binding in the kidney is due to binding to AT_{1A} receptors. Heterozygous (+/−) mice have levels of AT_1 receptor-specific binding intermediate between the (−/−) and (+/+) animals (data not shown); thus, absence of a single *Agtr1A* allele results in a substantial reduction in the binding level that is not compensated through other regulatory mechanisms.

***In Vivo* Responses to Angiotensin II.** To determine the effects of altered AT_{1A} receptor expression on *in vivo* responses to angiotensin II, we infused angiotensin II peptide i.v. to conscious mice (Fig. 3). In (+/+) mice, an i.v. 10 μg/kg bolus of angiotensin II causes an initial increase in blood pressure that peaks at 20 sec followed by a delayed depressor response. Despite the reduced level of receptor expression in *Agtr1A*(+/−) mice, the magnitudes of the peak pressor and depressor effects of angiotensin II seen in these heterozygotes resembled those in *Agtr1A*(+/+) animals. However, the pattern of the response was qualitatively different: the positive pressor response was short-lived in the *Agtr1A*(+/−) mice compared with controls, and the depressor response was clearly evident within 400 sec in *Agtr1A*(+/−) mice compared with >500 sec in *Agtr1A*(+/+) animals. This result suggests that the character of the pressor–depressor responses mediated by angiotensin II can be altered when receptor density is reduced. Infusions of angiotensin II had virtually no effect on the blood pressures of *Agtr1A*(−/−) mice, demonstrating that this acute hemodynamic response to angiotensin II requires AT_{1A} receptors.

Blood Pressures in *Agtr1A* Mutant Mice. We determined the steady-state effects of changes in AT_{1A} receptor expression on blood pressures in F_2 animals between 8 and 12 weeks of age. The three genotypes differed significantly (Fig. 4): compared with *Agtr1A*(+/+) controls, systolic blood pressures measured

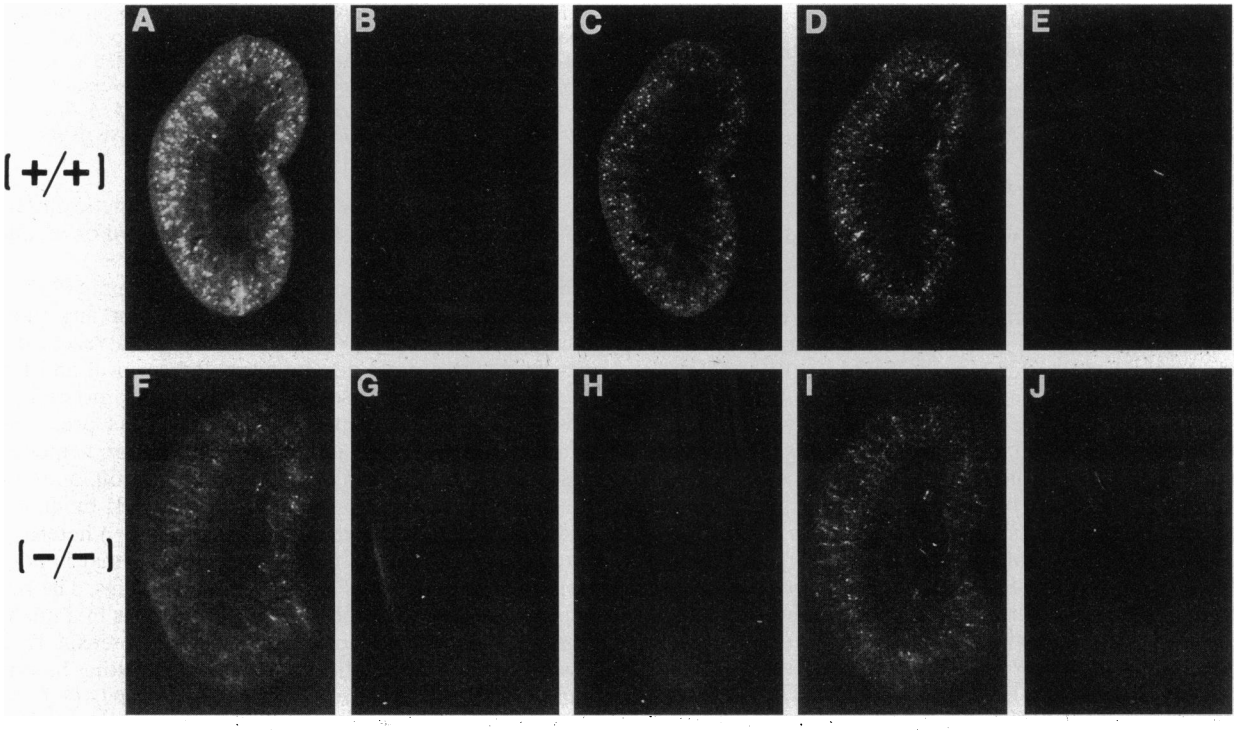

FIG. 2. [Sar^1,Ile^8]Angiotensin II receptor autoradiography in kidneys from *Agtr1A*(+/+) (*A–E*) and AT_{1A}(−/−) (*F–J*) mice. *A* and *F* show total ^{125}I-labeled [Sar^1,Ile^8]angiotensin II binding; it is substantially reduced in the *Agtr1A*(−/−) kidney (*F*). *B* and *G* show binding in the presence of excess concentrations of unlabeled [Sar^1,Ile^8]angiotensin II, which results in complete displacement of radioligand. *C* and *H* show binding of ^{125}I-labeled [Sar^1,Ile^8]angiotensin II in the presence of the AT_2 receptor antagonist PD 123319. Residual radioactivity represents AT_1 receptor-specific binding; it is virtually absent in the *Agtr1A*(−/−) mouse (*H*). *D* and *I* show ^{125}I-labeled [Sar^1,Ile^8]angiotensin II binding in the presence of the AT_1 receptor antagonist losartan (Dup 753). Residual radioactivity represents AT_2-specific binding; it is present in both the *Agtr1A*(+/+) (*D*) and *Agtr1A*(−/−) kidneys (*I*). *E* and *J* depict ^{125}I-labeled [Sar^1,Ile^8]angiotensin II binding in the presence of a combination of PD 123319 and losartan. The levels of residual radioactivity are not different from the levels of nonspecific binding seen in *B* and *G*.

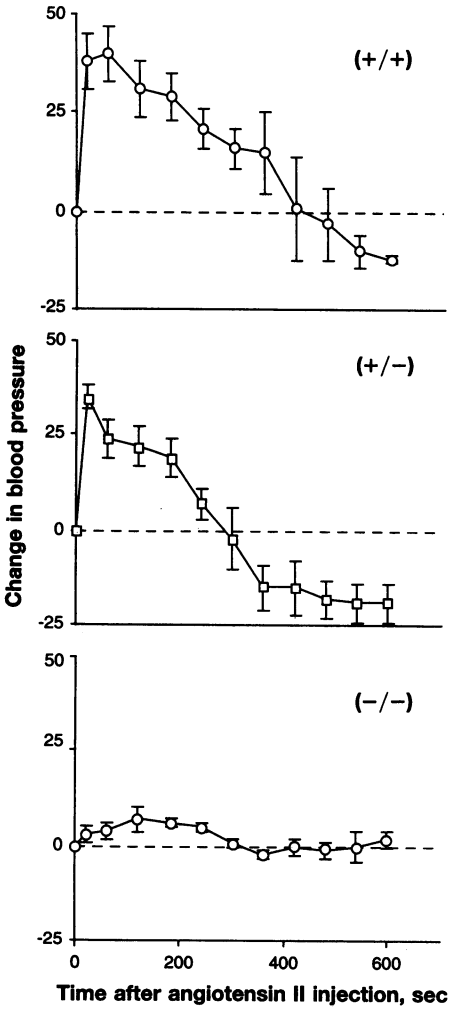

FIG. 3. Effects of an i.v. bolus of angiotensin II at 10 μg/kg on blood pressure in conscious mice. Changes in mean arterial pressure at 20-sec intervals after angiotensin II infusion are depicted for *Agtr1A*(+/+) (n = 7), (+/−) (n = 9), and (−/−) (n = 6) animals. Data are presented as the means ± SEMs.

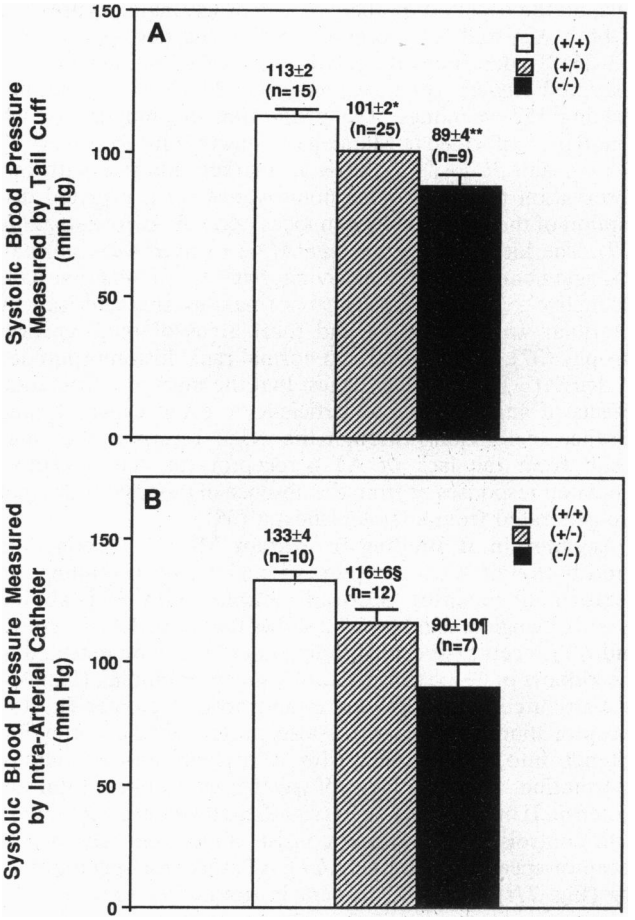

FIG. 4. Effect of the AT_{1A} receptor mutation on systolic blood pressure measured by tail cuff (A) and systolic blood pressure measured by intraarterial catheter (B). Data are presented as the means ± SEMs [*, P = 0.011 versus (+/+); **, $P < 0.0001$ versus (+/+), and P = 0.0013 versus (+/−); §, P = 0.026 versus (+/+); ¶, P = 0.0003 versus (+/+), and P = 0.02 versus (+/−) by analysis of variance and unpaired t test].

by tail cuff (Fig. 4*A*) were reduced by 12 mmHg in *Agtr1A*(+/−) mice [P = 0.011 versus (+/+)] and by 24 mmHg in *Agtr1A*(−/−) mice [$P < 0.0001$ versus (+/+) and P = 0.0013 versus (+/−)]. When intraarterial pressures were measured by carotid cannula, similar relative differences were observed: arterial pressures were reduced by 17 mmHg in (+/−) mice [P = 0.026 versus (+/+)] and by 43 mmHg in (−/−) mice compared with controls [P = 0.0003 versus (+/+) and P = 0.02 versus (+/−)]. Thus, there is a direct relationship between blood pressures and expression of the *Agtr1A* gene. Even a partial reduction in expression has a demonstrable effect, and this effect is seen in animals with all of their homeostatic mechanisms otherwise intact.

Exclusion of the Effects of Linked Genes. To be certain that the differences in blood pressures observed in *Agtr1A* mutant mice do not depend on genes linked to the *Agtr1A* locus that differ in mouse strains 129 and C57BL/6, we also examined blood pressures in F_1(+/+) and F_2(+/−) mice. These animals differ systematically in having either a wild-type or a mutant AT_{1A} gene derived from strain 129, but they do not differ systematically in any other genes, linked or unlinked to the *Agtr1A* locus. Blood pressures were measured by tail cuff sphygmomanometer. Systolic blood pressures in the F_2(+/−) mice (n = 7) were significantly lower than in the F_1(+/+) animals (n = 9) [103 ± 3 mmHg versus 111 ± 6 mmHg; P = 0.037]. Thus, the effects of the *Agtr1A* mutation on blood pressure are independent of any other genetic differences between strain 129 and C57BL/6 mice, linked or unlinked to the *Agtr1A* locus.

Conclusions. In these studies, we have examined the physiological functions of the AT_{1A} receptor for angiotensin II using gene targeting. We find that the AT_{1A} receptor is not necessary for normal development and survival and that the heart, kidney, and vascular system appear normal in the absence of AT_{1A} receptors. AT_{1A} receptors are, however, required for both the pressor and depressor hemodynamic responses to exogenous angiotensin II infusion, and virtually all of the AT_1 receptor-specific angiotensin II binding in the kidney is due to AT_{1A} receptors. *Agtr1A*(+/−) heterozygotes have reduced levels of receptor expression that is not compensated through other regulatory mechanisms. The reduced receptor density in *Agtr1A*(+/−) mice results in a qualitative alteration in the pressor response to angiotensin II and is associated with a significant reduction in resting blood pressure. Blood pressure is further reduced in *Agtr1A*(−/−) mice. These studies demonstrate the importance of the *Agtr1A* gene in regulating blood pressure in mice and suggest that variants of the human *AGTR1A* gene, which alter its level of expression, may likewise affect blood pressures.

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