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Genetics

Reduced growth, abnormal kidney structure, and type 2 (AT_2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT_{1A} and AT_{1B} receptors for angiotensin II

MICHAEL I. OLIVERIO‡, HYUNG-SUK KIM*, MASAKI ITO†, THU LE‡, LAURENT AUDOLY‡, CHRISTOPHER F. BEST‡, SYLVIA HILLER*, KIMBERLY KLUCKMAN*, NOBUYO MAEDA*, OLIVER SMITHIES*, AND THOMAS M. COFFMAN‡§

‡Duke University and Durham Veterans Affairs Medical Centers, Durham, NC 27705; *Department of Pathology, University of North Carolina, Chapel Hill, NC 27599-7524; and †School of Pharmaceutical Sciences, University of Shizuoka, Yada Shizuoka-Shi, Japan

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ABSTRACT **The classically recognized functions of the renin–angiotensin system are mediated by type 1 (AT_1) angiotensin receptors. Whereas man possesses a single AT_1 receptor, there are two AT_1 receptor isoforms in rodents (AT_{1A} and AT_{1B}) that are products of separate genes (*Agtr1a* and *Agtr1b*). We have generated mice lacking AT_{1B} (*Agtr1b* −/−) and both AT_{1A} and AT_{1B} receptors (*Agtr1a* −/−*Agtr1b* −/−). *Agtr1b* −/− mice are healthy, without an abnormal phenotype. In contrast, *Agtr1a* −/−*Agtr1b* −/− mice have diminished growth, vascular thickening within the kidney, and atrophy of the inner renal medulla. This phenotype is virtually identical to that seen in angiotensinogen-deficient (*Agt*−/−) and angiotensin-converting enzyme-deficient (*Ace* −/−) mice that are unable to synthesize angiotensin II. *Agtr1a* −/−*Agtr1b* −/− mice have no systemic pressor response to infusions of angiotensin II, but they respond normally to another vasoconstrictor, epinephrine. Blood pressure is reduced substantially in the *Agtr1a* −/− *Agtr1b* −/− mice and following administration of an angiotensin converting enzyme inhibitor, their blood pressure increases paradoxically. We suggest that this is a result of interruption of AT_2-receptor signaling. In summary, our studies suggest that both AT_1 receptors promote somatic growth and maintenance of normal kidney structure. The absence of either of the AT_1 receptor isoforms alone can be compensated in varying degrees by the other isoform. These studies reaffirm and extend the importance of AT_1 receptors to mediate physiological functions of the renin–angiotensin system.**

The renin–angiotensin system (RAS) regulates blood pressure and body fluid balance and plays a role in growth and development (1–3). The biological functions of the RAS and its major effector peptide, angiotensin II, are mediated by specific receptors. Two angiotensin receptor subtypes, type 1 angiotensin receptor (AT_1) and AT_2, can be distinguished pharmacologically. The classically recognized actions of the RAS are mediated by AT_1 receptors (1, 2). Whereas man possesses a single AT_1 receptor, rodents possess two AT_1 receptor isoforms, designated AT_{1A} and AT_{1B}. These receptors are the products of distinct but highly homologous genes (*Agtr1a* and *Agtr1b*) located on separate chromosomes (3, 4). Expression of the AT_{1A} receptor subtype predominates in nearly all tissues except the anterior pituitary gland and the adrenal cortex, where AT_{1B} receptors are more highly expressed (5–9). Because pharmacological AT_1 receptor antagonists block both AT_{1A} and AT_{1B} receptors, it has been difficult to separate their distinct functions (2).

Experiments using gene targeting have provided insight into the roles of RAS genes in regulating blood pressure, body fluid homeostasis, and development. For example, mice that are unable to generate angiotensin II because of targeted mutations in the angiotensinogen (*Agt* −/−) or angiotensin-converting enzyme (*Ace* −/−) genes have virtually identical phenotypes characterized by reduced survival, low blood pressure, and abnormal kidney structure. In the kidney, these animals develop thickening of arterial walls, focal areas of renal cortical inflammation, and hypoplasia of the inner medulla, which is associated with a defect in urinary concentrating function (10–13). The reduced survival and kidney abnormalities of *Agt* −/− mice can be rescued by human renin and angiotensinogen transgenes (14).

Thus, the absence of angiotensin II results in a severe phenotype. Two major features of this phenotype, reduced survival and abnormal kidney morphology, are not recapitulated by targeted deletions of the individual angiotensin receptors, AT_{1A}, AT_{1B}, or AT_2 (15–20). Furthermore, the relative roles of each of these receptors in the regulation of blood pressure and circulatory homeostasis are not clear. To define the contribution of the AT_1 receptor subtypes to these phenotypes, we have generated mice lacking AT_{1B} receptors (*Agtr1b* −/−) and mice lacking both AT_{1A} and AT_{1B} receptors (*Agtr1a* −/−*Agtr1b* −/−). These animals have reduced growth, abnormal kidney structure, and reduced blood pressure. In the mice that completely lack AT_1 receptors, we find evidence for regulation of blood pressure by AT_2 receptors.

MATERIALS AND METHODS

Generation of AT_{1B} Receptor-Deficient Mice. To clone the *Agtr1b* gene, we used a probe that we had previously used to clone the AT_{1A} receptor (15). This probe was used to screen a genomic library constructed with genomic DNA isolated from the mouse embryonic stem (ES) cell line E14tg2a that had been partially digested with the restriction endonuclease *Mbo*I. The phage clone containing the *Agtr1b* gene was identified by mapping using known sequences from the AT_{1B} receptor cDNA (3). Hybridizing phage was purified and a restriction map of the genomic DNA fragment was prepared, as depicted in Fig. 1*A*. To construct the targeting vector shown in Fig. 1*B*, a 10.5-kb *Hin*dIII fragment containing all of the

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Abbreviations: RAS, renin–angiotensin system; AT_1, type 1 angiotensin receptor; ES, embryonic stem; ACE, angiotensin converting enzyme; MAP, mean arterial blood pressure; RT-PCR, reverse transcription—PCR.

§To whom reprint requests should be addressed at: Room B3002/ Nephrology (111I), VA Medical Center, 508 Fulton Street, Durham, NC 27705. e-mail: tcoffman@acpub.duke.edu.

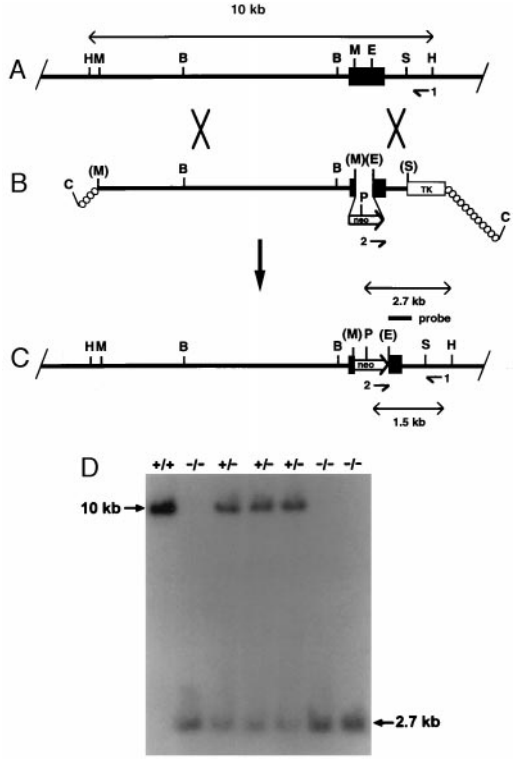

FIG. 1. Targeted disruption of the *Agtr1b* gene. (*A*) The endogenous *Agtr1b* gene locus. The intronless coding region is depicted by the black box. (*B*) Targeting construct. neo, neomycin resistance cassette; TK, thymidine kinase. (*C*) Structure of the disrupted gene. The PCR primers (indicated by arrowheads and numbers), the lengths of diagnostic restriction fragments, and the probe used for Southern analysis are shown. H, *Hin*dIII; M, Msc1; E, *Eag*I; S, Spe1; P, *Pst*I; C, *Cla*I B, *Bam*HI. Enzyme sites in brackets are destroyed during ligation. (*D*) Southern blot of offspring of an *Agtr1b* +/− × *Agtr1b* +/− cross. A 10.2-kb *Pst*I/*Hin*dIII fragment indicates the wild-type allele, and a 2.7-kb fragment identifies the targeted locus.

protein coding sequences was subcloned into pBluescript KS (Stratagene). A 0.4-kb *MscI*/*Eag*I fragment containing coding sequences was replaced by a neomycin resistance cassette (neo) from pMC1neopolA. The thymidine kinase (TK) gene from pMC1TK was inserted downstream of the 3′ homology arm. ES cells were grown, transformed, and screened by using standard methodologies (21). Colonies in which the plasmid had integrated by homologous recombination were identified by PCR using the primer pair depicted in Fig. 1*C*. The sequences for these primers are: TTGGGGACAAGGGTATCATAGCCA and TGGCGGACCGCTATCAGGAC.

Targeted ES cells were introduced into C57BL/6 blastocysts. The resulting male chimeras were mated with C57BL/6 or 129/SvEv females to identify germ-line competent chimeras that were capable of transferring the ES cell genome to their offspring. The targeted *Agtr1b* allele was detected by Southern blot analysis of genomic DNA isolated from tail biopsies as shown in Fig. 1*D*. Offspring carrying the mutant allele were intercrossed to obtain animals that were homozygous for the targeted mutation (*Agtr1b* −/−).

Production of Double Homozygous *Agtr1a* −/−*Agtr1b* −/− Mice. Mice with targeted mutations of the AT_{1A} receptor gene (*Agtr1a*) have been produced in our laboratories as described previously (15). Several breeding strategies were used to produce double homozygous *Agtr1a* −/−*Agtr1b* −/− mice. First, *Agtr1a*−/− and *Agtr1b* −/− mice were crossed to produce double heterozygous *Agtr1a* +/−*Agtr1b* +/− animals that were then intercrossed. To increase the likelihood of obtaining double homozygotes, we also intercrossed *Agtr1a* −/−*Agtr1b* +/− or *Agtr1a* +/−*Agtr1b* −/− mice. Finally, once double homozygous mice were obtained, they were crossed with *Agtr1a* −/−*Agtr1b* +/− or *Agtr1a* +/−*Agtr1b* −/− mice.

RNA Isolation and Reverse Transcription—PCR (RT-PCR) Analysis of AT_{1B} Expression. Adrenal glands were harvested from *Agtr1b* +/+ and *Agtr1b* −/− mice and RNA was extracted from the tissues by using a commercially available reagent (Tri-reagent; Sigma). Total RNA was reverse transcribed to cDNA by using poly-dT (22). A fragment of the AT_{1B} receptor cDNA was than amplified by PCR by using a sense primer derived from a portion from the *Agtr1b* gene that was deleted in the targeted gene (5′-ATACCGCTATGGAATACCAG-3′) and an antisense primer from the 3′-region of the *Agtr1b* gene (5′-TTGATAACCCTGCATGCGACC-3′). The diagnostic 700-bp fragment was identified on ethidium-stained gels. The presence and identity of the fragment were then confirmed by Southern blotting with a probe from the *Agtr1b* gene that was internal to the primer sequences.

Analysis of Growth and Development and Kidney Histology. To determine the effects of the combined *Agtr1a*/*Agtr1b* gene disruptions on growth and development, we measured body weights along with wet weights of kidneys and heart in 4-month-old male wild-type and *Agtr1a* −/−*Agtr1b* −/− mice. The major organs from these animals were fixed in formalin, sectioned, and stained with hematoxylin and eosin. The histomorphology of these organs was then examined by light microscopy.

Assessment of Urinary Concentrating Capacity. To examine the relative importance of the individual AT_1 receptors in urinary concentration, we measured urine osmolality in groups of wild-type ($n = 9$), *Agtr1a* +/+*Agtr1b* −/− ($n = 4$), *Agtr1a* −/−*Agtr1b* +/+ ($n = 14$), and *Agtr1a* −/−*Agtr1b* −/− ($n = 5$) mice. Urine osmolalities were measured while mice had free access to drinking water and following 12 hours of complete water deprivation. Urine was collected after bladder massage and urine osmolality was measured immediately with a vapor pressure osmometer (Wescor, Logan, UT).

Vasoconstrictor Effects of Angiotensin II. In preparation for studies to assess the vascular effects of angiotensin II, the angiotensin converting enzyme (ACE) inhibitor enalapril was administered to groups of *Agtr1a* +/+*Agtr1b* +/+ ($n = 4$), *Agtr1a* +/+*Agtr1b* −/− ($n = 4$) and *Agtr1a* −/−*Agtr1b* −/− ($n = 4$) mice to inhibit endogenous production of angiotensin II (23). Thirty mg/kg per day of enalapril was administered in the drinking water for 5 days. On the day of study, animals were anesthetized with isoflurane and a flexible plastic catheter (0.020ID; Braintree Scientific) was placed in the carotid artery to monitor arterial pressure. A second catheter was placed in the jugular vein to infuse angiotensin II. Intraarterial blood pressure was recorded continuously through the carotid catheter with a pressure transducer and WINDAQ acquisition and playback software (Dataq Instruments, Akron, OH).

Beginning with an equilibration period of 5 min, blood pressure was recorded continuously for the duration of the experiment at 50 recordings per sec. Data collected from each animal were compressed to five recordings per sec, and the mean arterial blood pressure (MAP) recordings from animals in each experimental group were integrated and averaged. Immediately after the equilibration period, mice received a bolus injection of 0.9% sodium chloride solution (NS) in a volume equal to 0.1% of their body weight. This is the volume used for each of the injections throughout the remainder of the experiment. At 10-min intervals thereafter, increasing doses of 0.1, 1.0, and 10 μg/kg of angiotensin II (Peninsula Laboratories) were injected intravenously. Ten minutes after the 10 μg/kg dose of angiotensin II, 10 μg/kg of epinephrine was administered intravenously and MAP was monitored for an additional 5 min.

Systolic Blood Pressure Measurements in Conscious Mice. Systolic blood pressures were measured in groups of *Agtr1a*+/+*Agtr1b*+/+ ($n = 8$), *Agtr1a* +/+*Agtr1b* −/− ($n = 10$), and *Agtr1a* −/−*Agtr1b* −/− ($n = 13$) mice by using a computerized tail-cuff system (Visitech Systems, Cary, NC) as previously described (24). Before the study was initiated, mice were adapted to the apparatus for at least 5 days. Systolic blood pressures were then measured for 10 days.

To examine the effect of reducing angiotensin II levels on blood pressure, these same groups of mice were treated with enalapril in drinking water at a dose of 30 mg/kg per day for 10 days, during which their systolic blood pressures were measured.

Statistical Analysis. Data are presented as means ± SEM. Statistical significance between groups was assessed by an unpaired *t* test or by analysis of variance (Bonferroni/Dunn). Significance of comparisons within groups was determined by using a paired *t* test.

RESULTS AND DISCUSSION

***Agtr1b* Gene Targeting.** The gene encoding the AT_{1B} receptor was disrupted in the E14Tg2a ES cell line by homologous recombination with the targeting plasmid shown in Fig. 1. Targeted ES cell lines identified by PCR and confirmed by Southern blot analysis were introduced into blastocysts to generate chimeric mice. Male chimeras were crossed with female C57BL/6 mice and *Agtr1b* +/− offspring carrying the mutant allele were identified by Southern blot analysis as shown in Fig. 1*D*.

Mice homozygous for the targeted mutation (*Agtr1b* −/−) were generated by intercrossing *Agtr1b* +/− animals. Among the progeny of these crosses, 25% were *Agtr1b*+/+, 54% were *Agtr1b* +/−, and 21% were *Agtr1b* −/−, corresponding to the proportions predicted by Mendelian inheritance. This shows that the absence of the AT_{1B} receptors does not significantly affect intrauterine development or perinatal survival. Furthermore, the *Agtr1b* −/− animals could not be distinguished from wild-type littermates by simple observation. Histological examination of their major organ systems, including heart, kidney, and adrenal glands, did not reveal any pathological changes. Our findings confirm a previous report suggesting that the absence of AT_{1B} receptors is not associated with an abnormal phenotype (20).

AT_{1B} Receptor mRNA Expression in Mutant Mice. To verify that the targeted mutation introduced into the *Agtr1b* locus resulted in inactivation of the gene, we examined expression of AT_{1B} receptor mRNA in adrenal glands by RT-PCR. As shown in Fig. 2, AT_{1B} receptor mRNA could not be detected in *Agtr1b* −/− mice, confirming the efficacy of the gene targeting strategy.

Production of Mice Lacking Both AT_{1A} and AT_{1B} Receptors. Because the *Agtr1a* and *Agtr1b* genes are located on different chromosomes (4), the mutations could be combined through simple breeding. *Agtr1a* −/−mice were crossed with *Agtr1b* −/− mice to produce progeny that were heterozygous for both the *Agtr1a* and *Agtr1b* disruptions. These *Agtr1a* +/−*Agtr1b* +/− animals were then intercrossed to produce mice that were double homozygotes for the null *Agtr1a* and *Agtr1b* alleles. To increase the frequency of generating *Agtr1a* −/−*Agtr1b* −/− animals, we also crossed mice that were heterozygous for one disruption and homozygous for the other (e.g., *Agtr1a* +/−*Agtr1b* −/− × *Agtr1a* −/− *Agtr1b* +/−). Among 343 consecutive 21-day-old weanlings from these different crosses, there were significantly fewer *Agtr1a* −/−*Agtr1b* −/− pups than predicted by simple Mendelian inheritance (29 observed vs. 50 expected; $P = 0.024$ by χ^2 analysis). Thus, in these mixed breedings, the complete absence of AT_1 receptors is detrimental to survival, with an apparent perinatal mortality rate of approximately 40%. By comparison, the perinatal mortality rate of angiotensinogen-deficient (*Agt* −/−) weanlings in our colony is approximately 60% (13 observed vs. 37 expected; $P = 0.0016$ by χ^2 analysis).

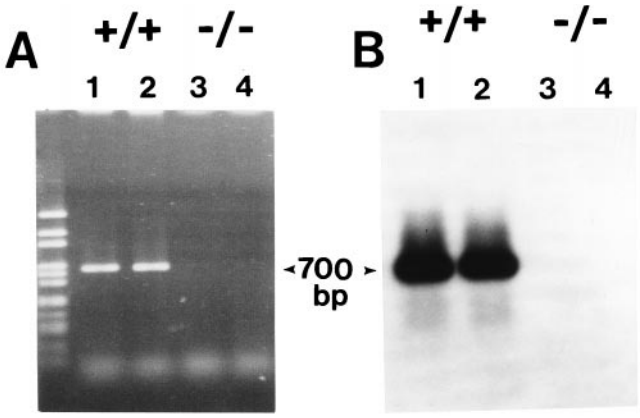

FIG. 2. Assessment of AT_{1B} mRNA expression by RT-PCR. Total RNA was isolated from adrenal glands of two wild-type and two *Agtr1b* −/− mice and each specimen was subjected to RT-PCR to amplify AT_{1B} receptor mRNA. In tissues from the wild-type mice (lanes 1 and 2), a PCR product of the expected size (700 bp) was detected on an ethidium bromide stained gel (*A*) and was confirmed to be AT_{1B} receptor mRNA by hybridization (*B*). This band was not detected in RNA from the *Agtr1b* −/− mice (lanes 3 and 4).

Once double homozygous *Agtr1a* −/−*Agtr1b* −/− mice were obtained, some of these animals were crossed with *Agtr1a* −/−*Agtr1b* +/− or *Agtr1a* +/−*Agtr1b* −/− mice to further increase the frequency of producing *Agtr1a* −/−*Agtr1b* −/− animals. When the genotypes of pups from these litters were analyzed, the observed frequency of *Agtr1a* −/−*Agtr1b* −/− weanlings was virtually identical to predicted (22 observed vs. 23 expected; $P = 0.9$ by χ^2. Thus, the survival disadvantage associated with the absence of AT_1 receptors can be overcome by selective breeding. This observation suggests that this trait can be modified significantly by background genes that are present in the C57BL/6 × 129 mixed breedings. In mice lacking only AT_{1A} receptors, we have identified similar modifying effects of background genes (25).

Both male and female *Agtr1a* −/−*Agtr1b* −/− mice are fertile. The frequency of productive matings, gestation, delivery, and care of litters was not different between *Agtr1a* −/−*Agtr1b* −/− and wild-type females. Male *Agtr1a* −/−*Agtr1b* −/− mice produced litters of normal size. Our group (12) and others (10) have previously identified fertility problems in male ACE-deficient mice, but not in male angiotensinogen-deficient mice (11). In view of our current results, the defective fertility observed in ACE-deficient mice is not because of a lack of AT_1-receptor signaling and may be caused by the loss of unique functions of testicular ACE probably unrelated to angiotensin II production. For example, other potential ACE substrates (bradykinin, enkephalins, and substance P) can alter functions of sperm (26, 27).

Growth Is Impaired in Mice with Combined AT_{1A}/AT_{1B} Receptor Deficiency. In neonatal rats, AT_1 receptor antagonists impair somatic and renal growth (28). However, in a previous study, we found that mice lacking only the AT_{1A} receptor grow and develop normally (29). To determine whether the combined absence of AT_{1A} and AT_{1B} receptors adversely affects growth, we compared body, heart, and kidney weights between 4-month-old male wild-type and double homozygous *Agtr1a* −/−*Agtr1b* −/− mice. The surviving *Agtr1a* −/−*Agtr1b* −/− mice appeared healthy and could not be distinguished from wild-type littermates based on their external appearance. However, their body weights were significantly lower than wild-type mice (25.7 ± 1.4 vs. 29.6 ± 0.6g; $P = 0.05$). In addition, both kidney weights (0.25 ± 0.02 vs. 0.32 ± 0.02 g; $P = 0.03$) and heart weights (0.09 ± 0.01

vs. 0.13 ± 0.01 g; $P = 0.02$) were also significantly reduced in the AT_1-deficient mice compared with wild-type controls. Because the ratios of heart weight to body weight (0.37 ± 0.02% vs. 0.45 ± 0.04%; $P = 0.1$) and kidney weight to body weight (0.96 ± 0.06% vs. 1.08 ± 0.06%; $P = 0.2$) are similar in *Agtr1a −/−Agtr1b −/−* and wild-type control mice, the combined absence of AT_{1A} and AT_{1B} receptors appears to cause a generalized impairment of growth.

Abnormalities in Kidney Structure in Double Homozygous *Agtr1a −/−Agtr1b −/−* Mice. *Agt −/−* or *Ace −/−* mice that are unable to synthesize angiotensin II develop structural abnormalities in the kidney. These include thickening of arterial walls, focal areas of interstitial fibrosis and tubular atrophy, and atrophy of the renal papilla (10, 11, 13). The absence of any single known angiotensin II receptor, AT_{1A}, AT_{1B}, or AT_2, does not completely recapitulate this phenotype (15–20). To determine whether the pathogenesis of these renal phenotypes requires the simultaneous absence of signaling through both AT_{1A} and AT_{1B} receptors, we examined renal histomorphology in *Agtr1a −/−Agtr1b −/−* mice and compared them to wild-type controls. As depicted in Fig. 3*B*, *Agtr1a −/−Agtr1b −/−* mice develop atrophy of the inner medulla that is easily detected by light microscopy. These mice also develop severe thickening of arterial walls within the kidney (Fig. 3*D*) that resembles the vascular abnormalities seen in *Agt −/−* and *Ace −/−* mice (10–13). We also found focal areas of cortical interstitial inflammation, fibrosis, and tubular atrophy in the *Agtr1a −/−Agtr1b −/−* mice (Fig. 3*F*), similar to lesions that we observed in *Agt −/−* mice (11). These findings are in general agreement with those of Tsuchida *et al.,* who recently reported that combined disruption of both AT_1 receptor genes produces a severe kidney phenotype (30). There were no abnormalities noted in the histology of hearts and adrenal glands of *Agtr1a −/−Agtr1b −/−* mice. In particular, there was no evidence of arterial or arteriolar thickening at these sites.

These data suggest that both AT_1 receptor isoforms contribute to the regulation of vascular structures in the kidney and to the maintenance of the renal papilla. Furthermore, as with the growth responses, the absence of a single AT_1 receptor isoform can be compensated by the other. However, the AT_{1A} receptors have a greater capacity for compensation than the AT_{1B} receptors, perhaps reflecting their relative levels of expression in kidney (15, 31). Accordingly, renal morphology is completely normal in the *Agtr1b −/−* mice that possess the full complement of AT_{1A} receptors. In contrast, subtle abnormalities can be detected in kidneys of AT_{1A}-deficient mice. By using magnetic resonance microscopy, modest reductions in the size of the renal papilla are revealed in *Agtr1a −/−* mice that were not detected by routine pathological evaluation

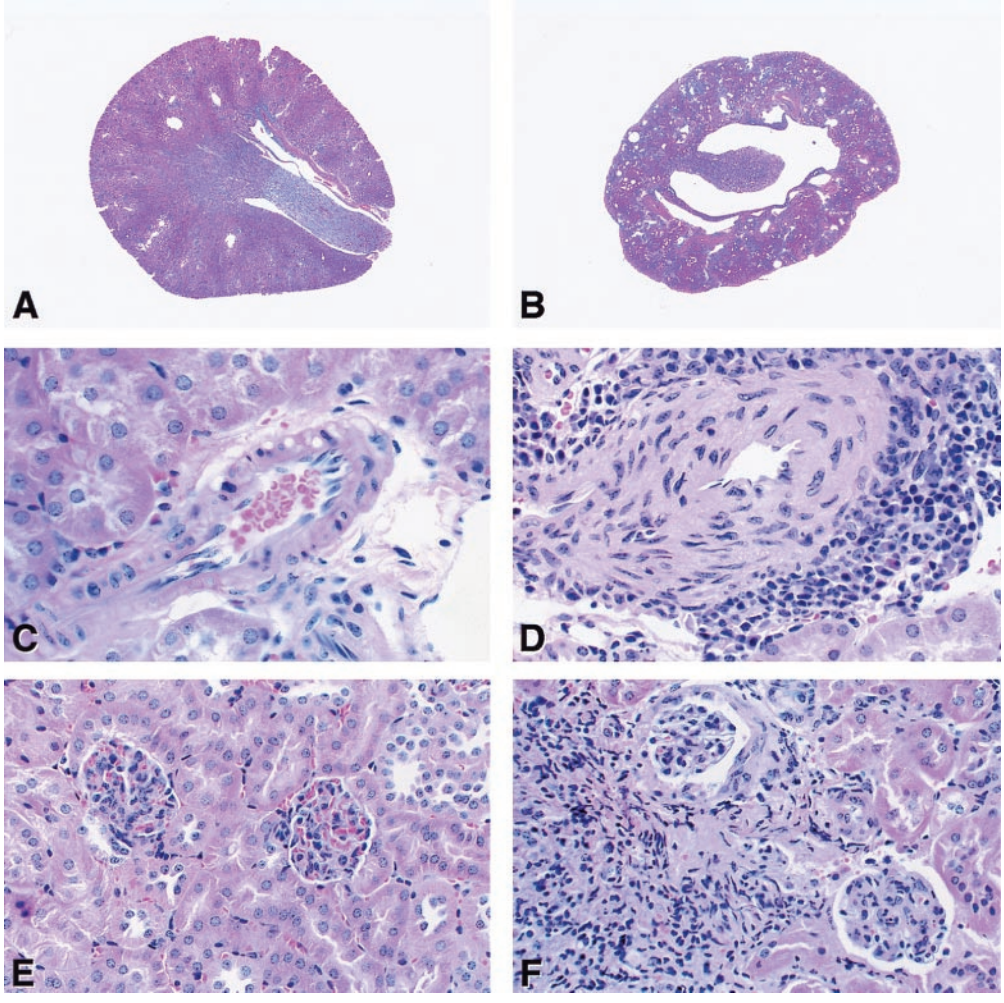

FIG. 3. Kidney histomorphology of wild-type and *Agtr1a −/−Agtr1b −/−* mice. (*A* and *B*): Axial sections from wild-type (*A*) and *Agtr1a−/−Agtr1b −/−* mice (*B*). (×5.) There is marked atrophy of the papilla in the AT_1-deficient kidney compared with the control. (*C* and *D*): Cross sections of representative small arteries in kidneys from wild-type (*C*) and *Agtr1a −/−Agtr1b −/−* (*D*) mice. The media of the vessels from the mutant animals was markedly thickened and many of the vessels were surrounded by inflammatory cells as shown. (×150.) (*E* and *F*): Sections of renal cortex from wild-type (*E*) and *Agtr1a−/−Agtr1b −/−* (*F*) mice. In the AT_1-deficient mice there were focal areas with tubular dropout and inflammatory cell infiltration. (×100.)

[Oliverio *et al.*, unpublished work]. In addition, minimal but significant thickening of renal interlobular arteries has been detected in *Agtr1a* −/− mice by using a careful morphometric analysis (18). Nonetheless, the difference between the modest abnormalities seen in *Agtr1a* −/− mice and the florid pathology seen in double homozygous *Agtr1a* −/−*Agtr1b* −/− mice must be due to compensation by the AT_{1B} receptor. This level of compensation seems remarkable because our previous studies have shown that AT_{1B} receptor binding is virtually undetectable in kidneys of *Agtr1a* −/− mice (15). Alternatively, it has been hypothesized that atrophy of the papilla may not depend directly on expression of AT_1 receptors in the kidney, but instead is related to loss of AT_1 receptor functions in the urinary collecting system (32).

AT_1 Receptors and Urinary Concentration. Among its many biological actions, the RAS regulates water metabolism, in part through diverse effects on urinary concentrating mechanisms (33, 34). Whereas a range of abnormalities in water homeostasis has been described in mice lacking angiotensin II or AT_{1A} receptors, impairment of urinary concentrating capacity is a feature common to all of these mutants (10, 35–37). To clarify the role of AT_{1A} and AT_{1B} receptors in these processes, we measured urinary concentrating capacities of wild-type, *Agtr1a* −/−, *Agtr1b* −/−, and *Agtr1a* −/−*Agtr1b* −/− mice. As shown in Fig. 4, after 12 hours of water deprivation, urine osmolalities in *Agtr1b* −/− mice increased to the same extent as in wild-type controls. In contrast, *Agtr1a* −/− mice have a defect in urinary concentration that is clearly apparent after 12 hours of water deprivation. Furthermore, the combined absence of both AT_{1A} and AT_{1B} receptors causes a urinary concentrating defect that is even more severe. Their urine osmolality remains virtually unchanged after 12 hours of water deprivation. Whereas this severe defect in the *Agtr1a* −/−*Agtr1b* −/− mice is not unexpected given their inner medullary atrophy (see Fig. 3*B*), the incremental differences in maximal urine osmolality between the three mutant groups further illustrate the relative abilities of the AT_{1A} and AT_{1B} receptor isoforms to compensate for one another.

Vasoconstrictor Actions of Angiotensin II. The vasoconstrictor actions of angiotensin II are mediated by AT_1 receptors (2). We have previously shown that the AT_{1A} receptor mediates a substantial portion of the pressor effects of angiotensin II based on the marked attenuation of these responses in *Agtr1a* −/− mice (15). However, when endogenous angiotensin II production is suppressed, a modest pressor and renal vasoconstrictor effect of angiotensin II can be detected in AT_{1A}-deficient mice. This response is blocked by AT_1 receptor antagonists, but not by sympatholytic agents, suggesting that it is a direct effect of AT_{1B} receptors (23, 38).

To directly test this hypothesis and to clarify the role of other angiotensin receptors in the vasoconstrictor actions of angiotensin II, we examined pressor responses to angiotensin II in wild-type, *Agtr1b* −/− and *Agtr1a* −/−*Agtr1b* −/− mice. As shown in Fig. 5, angiotensin II caused similar dose-proportional increases in mean arterial pressure in the wild-type and *Agtr1b* −/− mice. In contrast, angiotensin II had no discernable effect on MAP in the double homozygous *Agtr1a* −/−*Agtr1b* −/− mice. To determine whether responses to other pressor agents might also be impaired in these animals, each group received an infusion of epinephrine after the angiotensin II infusions were completed. As shown in Fig. 5, epinephrine caused vasoconstriction with significant and equivalent increases in MAP in all of the groups, including the *Agtr1a* −/−*Agtr1b* −/− mice. These studies verify the importance of AT_1 receptors in the vasoconstrictor effects of angiotensin II. In addition, they confirm the conclusions from our previous study that the vasoconstrictor actions of angiotensin II in *Agtr1a* −/− mice are mediated by AT_{1B} receptors (23). Finally, the absence of any hemodynamic response to angiotensin II in *Agtr1a* −/− *Agtr1b* −/− mice suggests that there are no other angiotensin II receptors with significant vasoactive functions.

Systolic Blood Pressure Regulation in Mice Lacking AT_{1A} and AT_{1B} Receptors. Systolic blood pressures in *Agtr1b* −/− and wild-type mice were similar (114 ± 3 vs. 123 ± 3 mmHg (1 mmHg = 133 Pa). In contrast, blood pressure was significantly reduced in mice lacking both AT_{1A} and AT_{1B} receptors (87 ± 3 mmHg; $P < 0.0001$ vs. wild-type or *Agtr1b* −/−), as shown in Fig. 6. This magnitude of blood pressure reduction is similar to levels that we have observed previously in *Agtr1a* −/− (15) and *Agt* −/− mice (11).

To determine the contribution of endogenous angiotensin II to the maintenance of blood pressure in these animals, each group was subsequently treated with the ACE inhibitor enalapril for 2 weeks, during which time systolic blood pressure was monitored. As shown in Fig. 6, the ACE inhibitor significantly lowered blood pressures in the wild-type animals. In contrast, ACE inhibition had a paradoxical effect on blood pressure in the mice lacking both AT_{1A} and AT_{1B} receptors. In these mice, administration of the ACE inhibitor caused a significant rise in blood pressure from 87 ± 3 to 94 ± 3 mmHg ($P = 0.03$). Following ACE inhibition, systolic blood pressures in the *Agtr1a* −/−*Agtr1b* −/− mice were not significantly

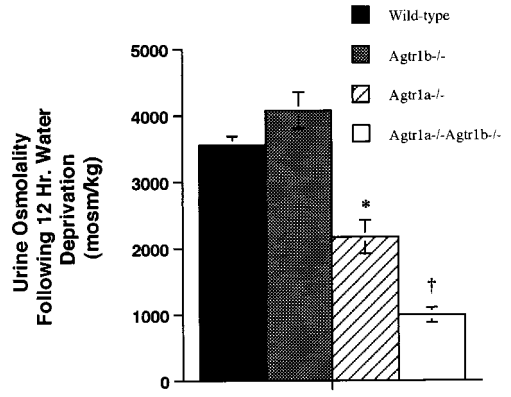

FIG. 4. Urine osmolality following 12 hours of water deprivation. The experimental groups are represented as follows: open bar is *Agtr1a*−/−*Agtr1b* −/− group, gray bar is the*Agtr1b*$^-$ −/− group, hatched bar is the Agtr1a$^-$ −/−group, and the black bar is the wild-type group. (*$P < 0.0003$ vs. wild-type or *Agtr1b* −/−; †$P < 0.0001$ vs. wild-type or *Agtr1b* −/−, $P = 0.004$ vs. *Agtr1a* −/−.

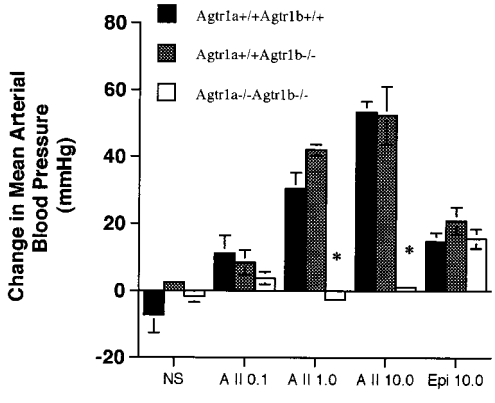

FIG. 5. Hemodynamic effects of angiotensin II in enalapril-pretreated, anesthetized wild-type, Agtr1b −/−, and *Agtr1a* −/− *Agtr1b* −/− mice. The changes in MAP at 120 seconds after injection of vehicle, 0.1, 1.0, and 10 μg/kg of angiotensin II, or 10 μg/kg epinephrine are shown. The experimental groups are represented as follows: open bars are the *Agtr1a* −/−*Agtr1b* −/− group, gray bars are the *Agtr1b* −/− group, and black bars are the wild-type group (*$P < 0.0003$ vs. wild-type or *Agtr1b*−/− groups).

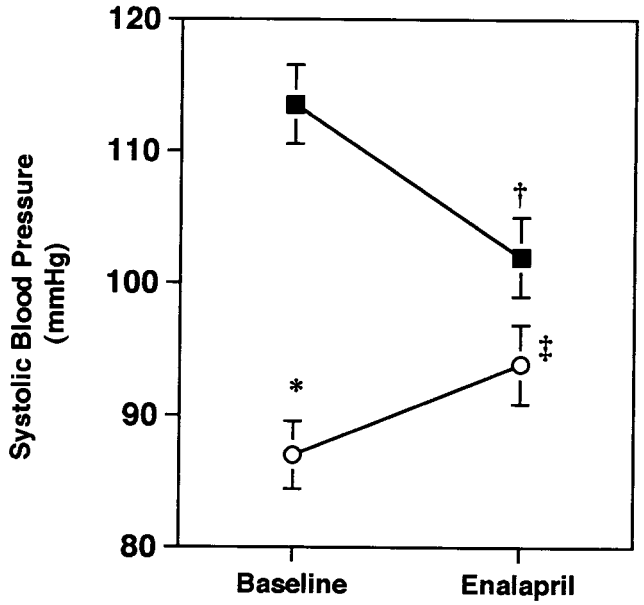

FIG. 6. The effect of chronic ACE inhibition on systolic blood pressure in conscious mice. Systolic blood pressures were measured daily for 10 days (Baseline) followed by an additional 10-day period while mice were treated with enalapril 30 mg/kg per day in drinking water (Enalapril). Data are mean ± SEM. The black squares represent the wild-type group and the open circles represent the *Agtr1a −/−Agtr1b −/−* group. (*$P < 0.0001$ vs. wild-type at baseline; †$P < 0.002$ vs. baseline for wild-type; ‡$P < 0.03$ vs. baseline for *Agtr1a −/−Agtr1b −/−*).

different from the wild-type mice on enalapril. We speculate that the paradoxical effect of ACE inhibition to increase blood pressure in *Agtr1a −/−Agtr1b −/−* mice represents inhibition of AT_2 receptor signaling. This would be consistent with studies suggesting that AT_2 receptors oppose the actions of AT_1 receptors on blood pressure (16, 17) and other cellular functions (39). Because angiotensin II infusions do not alter blood pressure in *Agtr1a −/−Agtr1b −/−* mice (see Fig. 4), AT_2 receptors may regulate blood pressure by modulating renal sodium excretion rather than by affecting vascular tone.

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