



Distinct roles for the kidney and systemic tissues in blood pressure regulation by the renin-angiotensin system

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Angiotensin II, acting through type 1 angiotensin (AT₁) receptors, has potent effects that alter renal excretory mechanisms. Control of sodium excretion by the kidney has been suggested to be the critical mechanism for blood pressure regulation by the renin-angiotensin system (RAS). However, since AT₁ receptors are ubiquitously expressed, precisely dissecting their physiological actions in individual tissue compartments including the kidney with conventional pharmacological or gene targeting experiments has been difficult. Here, we used a cross-transplantation strategy and AT_{1A} receptor-deficient mice to demonstrate distinct and virtually equivalent contributions of AT₁ receptor actions in the kidney and in extrarenal tissues to determining the level of blood pressure. We demonstrate that regulation of blood pressure by extrarenal AT_{1A} receptors cannot be explained by altered aldosterone generation, which suggests that AT₁ receptor actions in systemic tissues such as the vascular and/or the central nervous systems make nonredundant contributions to blood pressure regulation. We also show that interruption of the AT₁ receptor-mediated short-loop feedback in the kidney is not sufficient to explain the marked stimulation of renin production induced by global AT₁ receptor deficiency or by receptor blockade. Instead, the renin response seems to be primarily determined by renal baroreceptor mechanisms triggered by reduced blood pressure. Thus, the regulation of blood pressure by the RAS is mediated by AT₁ receptors both within and outside the kidney.

Introduction

The renin-angiotensin system (RAS), acting through type 1 angiotensin (AT₁) receptors, is a master regulator of fluid homeostasis (1). The critical role of this pathway in regulation of blood pressure is highlighted by the impressive efficacy of angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) in patients with hypertension and in mice lacking the AT_{1A} receptor, the major murine AT₁ receptor isoform. These animals have very low blood pressure and profound salt sensitivity (2, 3). Control of sodium excretion by the kidney has been suggested to be the critical mechanism for blood pressure regulation by the RAS (4). However, as AT₁ receptors are ubiquitously expressed, precisely dissecting and quantifying their physiological actions in individual tissue compartments including the kidney has been difficult. Accordingly, there is no direct proof of the primacy of the kidney in regulation of blood pressure by the RAS.

At the cellular level, responsiveness to angiotensin II is conferred by expression of angiotensin receptors. Angiotensin receptors can be divided into 2 pharmacological classes, type 1 (AT₁) and type 2 (AT₂), based on their differential affinities for various nonpeptide antagonists (5). Studies using these antagonists suggested that most of the classically recognized functions of the RAS are medi-

ated by AT₁ receptors (6). Gene targeting studies confirmed these conclusions (7). AT₁ receptors from a number of species have been cloned (8, 9), and 2 subtypes, designated AT_{1A} and AT_{1B}, have been identified in rat and mouse (10). The murine AT₁ receptors are products of separate genes and share substantial sequence homology (10). AT_{1A} receptors predominate in most organs, except the adrenal gland and regions of the CNS, where AT_{1B} expression may be more prominent (10). A single report has suggested that AT_{1B} receptors might also exist in humans (11), but this has not been confirmed in the unpublished work of several independent groups, and the consensus view is that there is no human counterpart to the murine AT_{1B} receptor. Thus, the AT_{1A} receptor is considered the closest murine homolog to the single human AT₁ receptor.

AT₁ receptors can be found in organ systems that play key roles in blood pressure homeostasis, including the heart, kidney, blood vessels, adrenal glands, and cardiovascular control centers in the brain (5). In the vascular system, stimulation of AT₁ receptors causes potent vasoconstriction (2). In the adrenal cortex, their activation stimulates the release of aldosterone (12), thereby promoting sodium reabsorption in the mineralocorticoid-responsive segments of the distal nephron (13). In the brain, intraventricular injection of angiotensin II causes a dramatic pressor response mediated by AT₁ receptors (14). In the kidney, activation of AT₁ receptors is associated with renal vasoconstriction and antinatriuresis (15, 16). Furthermore, it has been suggested that activation of AT₁ receptors at the juxtaglomerular apparatus suppresses renin release through the so-called short-loop feedback mechanism (17, 18). While AT₁ receptors have actions in myriad tissues that could potentially influence blood pressure, a prevailing view is that the

Nonstandard abbreviations used: ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; AT₁, type 1 angiotensin; JGA, juxtaglomerular apparatus; RAS, renin-angiotensin system.

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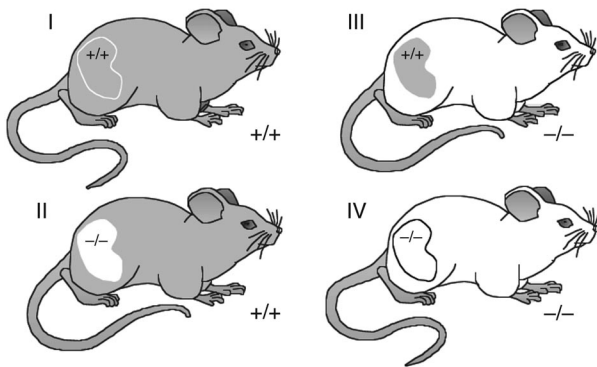


Figure 1

Kidney cross-transplantation groups. Wild-type (+/+) or AT_{1A} receptor-deficient (-/-) mice were transplanted with a wild-type or AT_{1A} receptor-deficient kidney. Nephrectomies were performed as described in Methods. Group I animals (D⁺R⁺) had a full complement of AT_{1A} receptors. Group II animals (D⁻R⁺) expressed AT_{1A} receptors only outside the kidney. Group III animals (D⁺R⁻) expressed AT_{1A} receptors only within the kidney. Group IV animals (D⁻R⁻) completely lacked AT_{1A} receptors. Recipients retained their own adrenal glands. $n \geq 6$ per group.

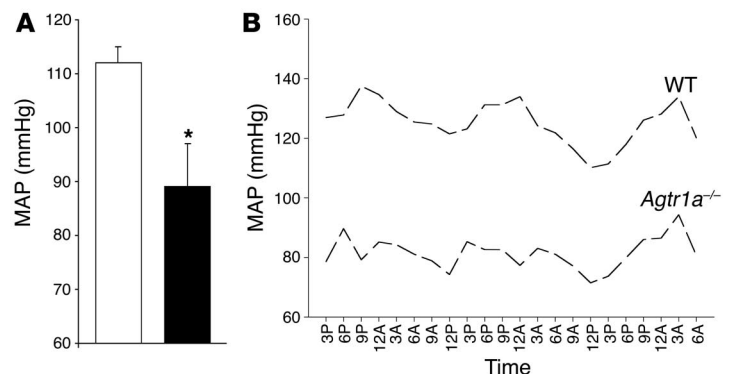
actions of the RAS to influence kidney function are dominant in chronic control of blood pressure (4, 19). Identifying the tissues and cell lineages responsible for blood pressure regulation by the RAS will provide novel insights into the integrated control of blood pressure and will more precisely define the mechanisms of the antihypertensive actions of ACE inhibitors and ARBs. Here, we used a cross-transplantation strategy and AT_{1A} receptor-deficient mice to demonstrate distinct and virtually equivalent contributions of AT₁ receptor actions in the kidney and in extrarenal tissues to determining the level of blood pressure.

Results

Cross-transplantation strategy. In order to define the relative importance of renal versus nonrenal AT₁ receptor pools in blood pressure regulation by the RAS, we carried out cross-transplantation experiments, performing fully vascularized kidney transplants between genetically matched F₁(C57BL/6 × 129) wild-type mice and mice homozygous for a targeted disruption of the *Agtr1a* gene locus encoding the AT_{1A} receptor. In the resulting recipient animals, both adrenal glands were retained. As shown in Figure 1, by varying the genotype of the transplant donors and recipients, we generated 4 groups of animals in which renal function was provided entirely by the single transplanted kidney. Group I animals were *Agtr1a*^{+/+} recipients (R⁺) that were transplanted with kidneys from *Agtr1a*^{+/+} donors (D⁺) and thus had normal expression of AT_{1A} receptors in the donor renal and recipient nonrenal tissues (D⁺R⁺). We will refer to group I as the D⁺R⁺ group, since AT_{1A} receptors were present in both kidney and somatic tissues. Group II animals lacked expression of AT_{1A} receptors

Figure 2

Radiotelemetry blood pressure in untransplanted wild-type and AT_{1A} receptor-deficient (*Agtr1a*^{-/-}) mice. (A) Blood pressure is reduced in *Agtr1a*^{-/-} mice (black bar) vs. wild-type controls (white bar); * $P = 0.03$; $n \geq 4$ per group. (B) Preservation of diurnal blood pressure variation in *Agtr1a*^{-/-} mice. MAP, mean arterial pressure. 3P, 3 pm; 12A, 12 am.



in the kidney, but had normal expression of receptors in systemic tissues (D⁺R⁺). In group III, AT_{1A} receptors were present in the kidney but absent from all nonrenal tissues (D⁺R⁻). Finally, group IV mice were completely deficient in AT_{1A} receptors (D⁻R⁻).

Blood pressure measurements in *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice. In order to accurately measure blood pressures in conscious, unrestrained animals, we implanted radiotelemetry transmitters. Using this approach, we first measured blood pressures in otherwise unmanipulated *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice. Figure 2A shows that blood pressures were significantly reduced in F₁(C57BL/6 × 129) *Agtr1a*^{-/-} mice (89 ± 8 mmHg) compared with wild-type controls (112 ± 3 mmHg; $P = 0.03$). The magnitude of the blood pressure reduction in *Agtr1a*^{-/-} animals was very similar to that observed in our previous experiments using tail cuff manometry (2). We also analyzed a series of 24-hour blood pressure recordings and found that the general nature of blood pressure fluctuations was similar in *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice (Figure 2B), which indicated that the absence of AT_{1A} receptors does not affect qualitative patterns of diurnal variation in blood pressure.

Distinct contributions of renal and systemic AT₁ receptors to blood pressure homeostasis. Among the transplanted animals, blood pressures in group I (D⁺R⁺) were very similar to those of nontransplanted, *Agtr1a*^{+/+} control mice (118 ± 5 vs. 112 ± 3 mmHg; $P = 0.44$), which indicated that the transplant procedure does not significantly alter blood pressure. Figure 3 shows that the absence of AT_{1A} receptors in the kidney alone in group II (D⁻R⁺) animals was associated with a significant reduction in blood pressure compared with the D⁺R⁺ group (99 ± 4 vs. 118 ± 5 mmHg; $P = 0.006$ vs. D⁺R⁺). This finding is consistent with the prevailing view that regulation of sodium handling by the kidney is a major determinant of the chronic level of blood pressure and indicates that the absence of AT_{1A} receptor pathways in the kidney alone is sufficient to lower blood pressure. However, blood pressures were similarly reduced in group III (D⁺R⁻) mice, which lack AT_{1A} receptors in systemic tissues but have normal renal expression of AT_{1A} receptors (99 ± 2 vs. 118 ± 5 mmHg; $P = 0.006$). Thus, AT_{1A} receptors in nonrenal tissues also make a nonredundant contribution to the determination of the blood pressure level. Moreover, the presence of intact AT_{1A} receptor pathways in the kidneys of these animals does not compensate for the absence of receptors outside of the kidney. With respect to blood pressure homeostasis, the finding that blood pressures were virtually identical in the D⁺R⁺ and D⁺R⁻ groups indicates that the contributions of AT_{1A} receptors in the kidney and systemic tissues are very similar in magnitude.

Blood pressures were further reduced in group IV (D⁻R⁻) animals, which completely lack AT_{1A} receptors (86 ± 3 mmHg; $P = 0.02$

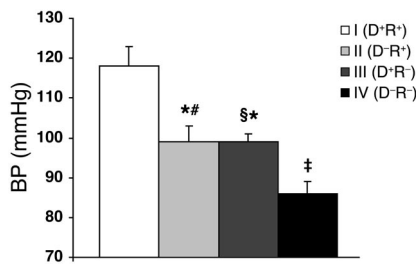


Figure 3 Blood pressures in the cross-transplantation groups measured by radio-telemetry. **P* = 0.006 vs. group I; #*P* = 0.02 vs. group IV; §*P* = 0.002 vs. group IV; †*P* = 0.00007 vs. group I (*n* ≥ 6 per group).

vs. D⁻R⁺; *P* = 0.002 vs. D⁺R⁻). This suggests that the mechanisms for control of blood pressure by systemic and renal AT_{1A} receptors are nonoverlapping. Finally, blood pressures in D⁻R⁻ animals were very similar to those measured in intact, nontransplanted *Agtr1a*^{-/-} mice (89 ± 8 mmHg), which further illustrates the lack of any significant impact of the transplant procedure on blood pressure.

Effects of oral sodium loading on blood pressures. To assess the impact of altered sodium homeostasis on blood pressure regulation in the experimental groups, we examined the effects of high-salt feeding. After baseline blood pressures of mice on the normal (0.4% NaCl) diet were established, mice in each experimental group were fed a high-salt (6% NaCl) diet, and blood pressures were simultaneously monitored. As shown in Figure 4, the high-salt diet caused significant increases in blood pressures only in the D⁻R⁺ (14.2% ± 5% [from 99 ± 4 to 114 ± 9 mmHg]; *P* = 0.03 vs. normal salt) and the D⁻R⁻ (20.1% ± 4.9% [from 85 ± 3 to 102 ± 5 mmHg]; *P* = 0.01 vs. normal salt) groups, lacking AT_{1A} receptors in the kidney. By contrast, in the D⁺R⁺ or D⁺R⁻ groups with a normal complement of renal AT_{1A} receptors, high-salt feeding did not significantly affect blood pressures (118 ± 5 to 122 ± 7 mmHg for D⁺R⁺, 99 ± 2 to 105 ± 3 mmHg for D⁺R⁻; *P* = not significant vs. normal salt in both groups). Thus, the absence of AT_{1A} receptors in the kidney was associated with sodium sensitivity, whereas groups with normal renal AT_{1A} receptor expression were sodium resistant.

Extrarenal AT_{1A} receptors, aldosterone, and blood pressure. As discussed above, there are a number of direct actions of AT₁ receptors that impact on blood pressure (19). In addition, AT₁ receptors in the zona glomerulosa of the adrenal gland indirectly affect blood pressure by stimulating aldosterone release and, in turn, promoting sodium reabsorption by the distal nephron (12, 13). We therefore considered the possibility that impaired aldosterone release might explain the blood pressure reduction that we observed in D⁻R⁻ mice, which lack AT_{1A} receptors in systemic tissues. To test this, we collected urine from animals in each of the experimental groups and measured urinary aldosterone levels by ELISA.

As shown in Figure 5, aldosterone excretion was equivalent in the D⁻R⁺ and D⁻R⁻ groups (2,395 ± 260 vs. 3,177 ± 649 pg/24 h), which is consistent with the fact that they have normal complements of adrenal AT_{1A} receptors. In contrast, aldosterone excretion was reduced by approximately 50% in D⁺R⁻ animals, lacking AT_{1A} receptors in nonrenal tissues including the adrenal glands (1,158 ± 207 pg/24 h; *P* = 0.005 vs. D⁺R⁺; *P* = 0.01 vs. D⁻R⁻), which suggests that the impaired aldosterone response in these animals is the cause of their reduced blood pressure. The aldosterone excretion in the D⁻R⁻ mice, which completely lack AT_{1A} receptors,

was significantly higher than that in the D⁺R⁻ group (2,643 ± 441 pg/24 h; *P* < 0.05). Since the adrenal glands in D⁻R⁻ mice also lack AT_{1A} receptors, this augmented aldosterone excretion is likely due to marked renin stimulation (see below) generating a high level of angiotensin II that stimulates the AT_{1B} receptors in the adrenal cortex. Other pathways for aldosterone stimulation may also play a role (20). Yet blood pressures are significantly lower in D⁻R⁻ mice completely lacking AT_{1A} receptors compared with those in the D⁺R⁻ group, despite their higher aldosterone levels.

To determine whether lower aldosterone production in the D⁺R⁻ animals accounts for their lower blood pressures versus the D⁺R⁺ group, we performed an additional experiment using adrenalectomized D⁺R⁺ and D⁺R⁻ animals in which aldosterone levels were clamped by the infusion of equivalent amounts of aldosterone via an osmotic minipump. As shown in Figure 6A, infusing aldosterone at 1 µg/day provided supraphysiological aldosterone replacement, since urinary aldosterone excretion was increased almost 4-fold in the infused mice compared with mice with intact adrenal glands (*P* = 0.005). Nevertheless, this enhanced level of aldosterone had no effect on blood pressure in the D⁺R⁺ groups (118 ± 1 vs. 118 ± 5 mmHg; *P* = not significant). Moreover, urinary aldosterone excretion was not significantly different between the adrenalectomized D⁺R⁺ and adrenalectomized D⁺R⁻ groups that received aldosterone infusions (8,020 ± 2,627 vs. 8,828 ± 1,364 pg/24 h, respectively; *P* = not significant). However, despite supraphysiological and equivalent aldosterone levels, blood pressures were significantly reduced in the aldosterone-infused D⁺R⁻ group compared with the D⁺R⁺ group (106 ± 1 vs. 118 ± 1 mmHg; *P* < 0.0001) as shown in Figure 6B. These data demonstrate that regulation of blood pressure by extrarenal AT_{1A} receptors cannot be explained by changes in aldosterone generation, which indicates that AT₁ receptor actions in other systemic tissues make nonredundant contributions to blood pressure regulation.

Extrarenal AT_{1A} receptors and renal sympathetic innervation. The sympathetic nervous system, acting through renal nerves can modulate sodium handling by the kidney (21). As AT₁ receptors in the CNS can stimulate sympathetic tone (22, 23), we considered the possibility that altered neural input to the kidney might represent a mechanism for the altered blood pressures in the D⁺R⁻ group. Furthermore, since the renal pedicle is transected in the course of harvesting the donor kidney, renal innervation may be impaired in this model. To examine these possibilities, we compared renal norepinephrine content in the D⁺R⁺ and D⁺R⁻ groups with that of kidneys from *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice. Norepinephrine content

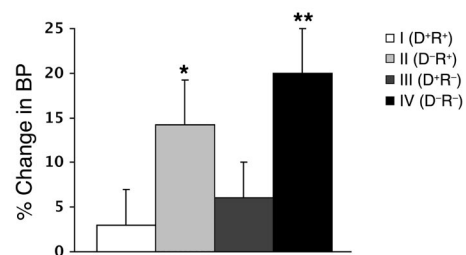
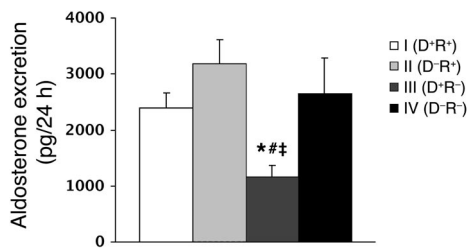


Figure 4 Effects of oral salt loading on blood pressure. Mice were fed a diet with high (6% NaCl) salt content. The percent change in blood pressure relative to the baseline blood pressure on a normal (0.4% NaCl) salt diet is shown. **P* = 0.03 vs. normal salt; ***P* = 0.01 vs. normal salt (*n* ≥ 5 per group).

**Figure 5**

Urinary aldosterone excretion in the cross-transplantation groups. Urine samples were collected over 24 hours from mice in metabolic cages. Aldosterone was quantitated by ELISA (pg/24 h). * $P = 0.005$ vs. group I; # $P = 0.01$ vs. group II; † $P < 0.05$ vs. group IV ($n \geq 6$ per group).

is an accepted measure of renal sympathetic nerve activity (24). As shown in Figure 7, renal norepinephrine content was reduced significantly in both groups of transplanted kidneys compared with those in *Agtr1a*^{+/+} controls ($P < 0.001$), which suggested that significant re-innervation of the transplanted kidneys had not occurred during the period of study. As there was no difference in norepinephrine levels between kidneys from the *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice ($1,094 \pm 132$ vs. $1,286 \pm 79$ ng/g), the absence of AT_{1A} receptors does not substantially affect renal norepinephrine content in the presence of intact renal nerves. Furthermore, norepinephrine levels were virtually identical in kidneys of the D⁺R⁺ and D⁺R⁻ mice (173 ± 53 vs. 163 ± 75 ng/g), which indicates that altered sympathetic nerve input in the kidney cannot explain the reduced blood pressures in the D⁺R⁻ animals.

Renal AT_{1A} receptors and regulation of renin. Angiotensin II may regulate its own synthesis by activation of AT₁ receptors at the juxtaglomerular apparatus (JGA), suppressing renin release (18). As this is one of several intrarenal actions of AT_{1A} receptors that could affect blood pressure, we examined the impact of renal and systemic AT_{1A} receptors on renin mRNA expression in the kidney.

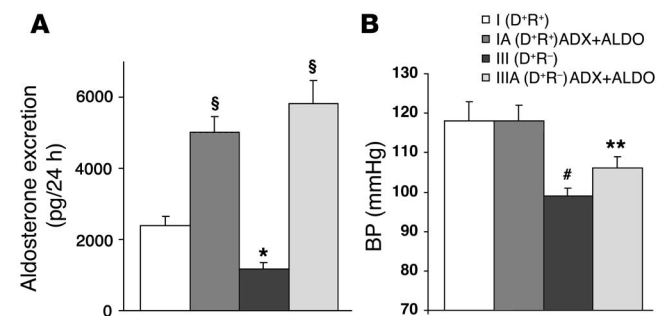
We found that renin mRNA levels in transplanted kidneys of D⁺R⁺ animals (29 ± 8 pg of renin mRNA/ μ g total mRNA) were nearly identical to those of unmanipulated kidneys from wild-type mice (25 ± 4 pg of renin mRNA/ μ g total mRNA). In previous studies, we and others have described markedly enhanced renin expression and JGA hypertrophy in *Agtr1a*^{-/-} mice with generalized deficiency of AT_{1A} receptors (25, 26). However, as shown in Figure 8, this phenotype was not recapitulated in D⁻R⁺ animals, which lack AT_{1A} receptors only in the kidney. While kidney renin mRNA levels tended to be higher in D⁻R⁺ mice (59 ± 19 pg of renin mRNA/ μ g total mRNA) than in the D⁺R⁺ group, this difference was not statistically significant ($P = 0.18$). Thus, the absence of AT_{1A} receptors only in the kidney and the consequent lack of short-loop feedback did not markedly alter renin mRNA levels. Similarly, the absence of AT_{1A} receptors only in nonrenal tissues of D⁺R⁻ animals had no effect on renin expression (22 ± 14 pg of renin mRNA/ μ g total mRNA) compared with the D⁺R⁺ group (Figure 8). By contrast, the simultaneous absence of AT_{1A} receptors in both renal and nonrenal tissues of D⁻R⁻ animals was associated with a marked upregulation of renin expression to 708 ± 161 pg of renin mRNA/ μ g total mRNA ($P < 0.003$ vs. all other groups). Based on the findings from the D⁻R⁺ group, interruption of short-loop feedback in the kidney cannot explain the more than 10-fold increase in renin mRNA levels in the D⁻R⁻ group. Accordingly, we postulate that this marked augmentation in renin expression is due to the lower blood pressures in

this group. Moreover, since blood pressure reductions of almost 20 mmHg in the D⁻R⁺ and D⁺R⁻ groups were not sufficient to trigger this response, our data suggest that a threshold of blood pressure lowering must be achieved for activation of baroreceptor mechanisms that stimulate renin (27, 28).

We performed additional experiments to examine the contribution of blood pressure to the stimulation of renin seen in the complete absence of AT_{1A} receptors. To this end, we measured blood pressure and renin mRNA expression in D⁻R⁻ mice fed a high-salt (6% NaCl) diet. As in our previous experiments on unmanipulated *Agtr1a*^{-/-} mice (3), high-salt feeding caused a significant increase in blood pressure in the D⁻R⁻ mice (102 ± 5 mmHg) compared with D⁻R⁻ mice fed a normal (0.4% NaCl) diet (86 ± 3 mmHg; $P = 0.01$). The blood pressures in the D⁻R⁻ animals fed a high-salt diet increased sufficiently, becoming not significantly different from those of the D⁻R⁺ and D⁺R⁻ groups on a normal diet (99 ± 4 and 99 ± 2 mmHg in D⁻R⁺ and D⁺R⁻ groups, respectively). This normalized blood pressure in the high-salt D⁻R⁻ group was associated with a dramatic suppression of renin mRNA levels (130 ± 58 pg of renin mRNA/ μ g total mRNA) compared with the D⁻R⁻ animals on the normal diet (708 ± 161 pg of renin mRNA/ μ g total mRNA; $P = 0.003$).

Discussion

The important role of the kidney in regulation of blood pressure has been long recognized (29), and the relationship between alterations in systemic blood pressure and changes in renal sodium excretion is well documented (30). For example, an elevation in perfusion pressure in the renal artery results in a rapid increase in sodium and water excretion by the kidney, so-called pressure natriuresis (30). Based on such observations, Guyton and coworkers suggested that whenever arterial pressure is elevated, activation of this pressure-natriuresis mechanism will cause sufficient excretion of sodium and water to return systemic pressures to normal (31). Because of its potent actions to modulate renal sodium excretion, angiotensin II has been implicated as a major determinant of these pressure-natriuresis relationships (19, 32). These actions may be mediated by direct effects of AT₁ receptors on renal resistance vessels and epithelial cells or indirectly through stimulation of aldosterone production via AT₁ receptors in the adrenal gland (32).

**Figure 6**

Aldosterone clamp experiment. Adrenalectomies (ADX) were performed at the time of native nephrectomies. Osmotic minipumps containing aldosterone (ALDO) were implanted at the time of second nephrectomy/adrenalectomy. (A) Urine samples were collected over 24 hours for aldosterone in ADX+ALDO groups (groups IA and IIIA) versus groups I and III from the initial experiment in Figure 5. § $P \leq 0.005$ vs. group I; * $P = 0.005$ vs. group I. $n \geq 5$ per group. (B) Corresponding blood pressures. ** $P < 0.0001$ vs. group IA; # $P = 0.006$ vs. group I.

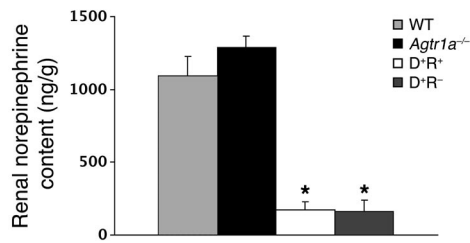


Figure 7
Renal norepinephrine content. Norepinephrine levels were measured in kidneys harvested from unmanipulated *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice as well as in kidney grafts harvested from D⁺R⁺ and D⁺R⁻ mice. **P* < 0.001 vs. WT (*n* = 5 per group).

The importance of the RAS in clinical medicine is highlighted by the impressive cardiovascular efficacy of pharmacological agents that inhibit the synthesis or activity of angiotensin II. ACE inhibitors and ARBs are very effective and well tolerated antihypertensive agents (1). Along with their ability to lower blood pressure, these agents also ameliorate morbidity and mortality associated with cardiovascular diseases including congestive heart failure, coronary artery disease, and stroke (33, 34). The remarkably similar profiles of ACE inhibitors and ARBs in these disorders suggest a common mechanism underlying their efficacy: reducing AT₁ receptor signaling in critical target tissues. Along with their expression in the kidney, AT₁ receptors are present not only in the kidney but in myriad sites that may influence blood pressure including the vasculature, adrenal glands, and brain. Yet the key sites that determine the level of blood pressure cannot be precisely localized using pharmacological antagonists that block AT₁ receptors in all tissues or in conventional gene targeting experiments. Accordingly, in the studies described here, we have used a renal cross-transplantation strategy to define the relative contributions of AT_{1A} receptor actions in the kidney and in extrarenal tissues.

We found that absence of AT_{1A} receptors in the kidney in D⁻R⁺ mice is sufficient to reduce blood pressure by almost 20 mmHg (Figure 3), despite normal expression of receptors in all other tissues. These findings indicate that renal AT_{1A} receptors have unique and nonredundant actions in blood pressure homeostasis. As aldosterone levels are unaffected in these D⁻R⁺ animals, these observations show that blood pressure is regulated by direct effects of AT₁ receptors on kidney cells, independent of any impact of mineralocorticoids. We suggest that this reduction in blood pressure is a direct consequence of interruption of AT₁ receptor actions in the renal vasculature and/or renal epithelia that would otherwise reduce urinary sodium excretion. Our finding of sodium-sensitive blood pressure changes in animals lacking renal AT_{1A} receptors is consistent with this conclusion. While the baseline blood pressures were significantly lower in the D⁻R⁻ compared with the D⁻R⁺ group, the absolute magnitude of the blood pressure increases with high-salt feeding were similar, 17 and 15 mmHg, respectively, representing the component of blood pressure reduction due to sodium and volume deficits. Furthermore, it is likely that inhibition of these intrarenal pathways is an important mechanism for blood pressure lowering by ACE inhibitors and ARBs.

Although our studies support a vital role for the kidney in regulation of blood pressure, we find that AT₁ receptors *outside* the kidney also make a unique contribution to blood pressure homeostasis that is virtually equivalent to and independent of the intrarenal

actions of angiotensin II. Thus, we found that D⁺R⁻ mice, which lack AT_{1A} receptors in extrarenal tissues but have the normal complement of receptors in the kidney, also had blood pressure reductions of approximately 20 mmHg (Figure 3). We considered the possibility that alterations of aldosterone release due to the absence of AT₁ receptors in the zona glomerulosa of the adrenal gland might be responsible for low blood pressures in D⁺R⁻ mice; this would be consistent with the hypothesis that control of renal sodium handling is the final common pathway for chronic blood pressure homeostasis. To test this possibility, we measured aldosterone excretion in D⁺R⁻ mice and found that absence of AT_{1A} receptors only in extrarenal tissues was associated with a reduction in aldosterone excretion of approximately 50% compared with controls (Figure 5). Preservation of a partial aldosterone response in these mice is probably due to the presence of AT_{1B} receptors, the minor murine AT₁ receptor isoform. The adrenal cortex is one of the few tissues with appreciable expression of AT_{1B} receptors (10).

In order to determine whether the reduced aldosterone generation in the D⁺R⁻ mice was the cause of their low blood pressures, we clamped aldosterone at supraphysiological levels by chronic infusion into adrenalectomized D⁺R⁻ and D⁺R⁺ animals using osmotic pumps. The aldosterone levels in the 2 groups were similar, yet the blood pressures remained lower in the D⁺R⁻ group lacking extrarenal AT_{1A} receptors, compared with those in D⁺R⁺ controls (Figure 6B). These findings corroborate other studies in which infusion of aldosterone alone failed to raise blood pressure (35) and suggest that the adrenal gland is not a critical site for blood pressure control by extrarenal AT_{1A} receptors. We speculate instead that AT_{1A} receptors in the CNS and/or in the vasculature are more likely to mediate the component of blood pressure regulation that is independent of the kidney. As our data on renal norepinephrine content indicate a persistent lack of sympathetic innervation in the transplanted kidneys (Figure 7), the contribution of extrarenal AT₁ receptors to enhancing or modulating renal nerve activity and thereby altering renal excretory functions may be underestimated in these experiments. Therefore, the major actions of extrarenal AT₁ receptors to regulate blood pressure in this model are likely to be due to effects on the vasculature. These could be direct actions of AT_{1A} receptors on vascular smooth muscle cells (2) or indirect effects of AT_{1A} receptors in the CNS to modulate vascular resistance (14). In this regard, recent studies have suggested that G-protein-coupled receptor actions in vascular smooth muscle make important contributions to chronic blood pressure homeostasis (36).

The availability of renin affects the generation of angiotensin II by the RAS, and renin expression is tightly controlled at the JGA

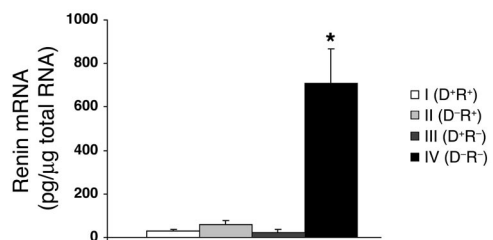


Figure 8
Kidney renin levels in the cross-transplantation groups. Kidneys were harvested at time of sacrifice. RNA was extracted using RNeasy protocol. Renin mRNA was quantitated by real-time PCR as described in Methods. **P* < 0.003 vs. other groups (*n* = 5 per group).



in the kidney (37). Angiotensin II is an important component in the regulatory pathways for renin and may control its own synthesis by activating AT₁ receptors at the JGA, thereby suppressing renin release (18, 25). The actions of ACE inhibitors and ARBs to increase renin mRNA expression and to cause JGA hypertrophy (38) have been cited as evidence supporting the existence of this so-called short-loop feedback mechanism. Although we find that renal AT_{1A} receptors are key determinants of blood pressure, our studies do not indicate a major role for these receptors in regulating renin expression in the kidney.

In D-R⁺ animals, which lack AT_{1A} receptors only in the kidney, we found that renin mRNA levels are not significantly elevated despite the absence of AT₁ receptors at the JGA and the significant reduction in blood pressure (Figure 8). Yet in the D-R⁻ group completely lacking AT_{1A} receptors, renin mRNA expression is increased more than 10-fold above baseline. Because blood pressures are reduced even further in D-R⁻ compared with D-R⁺ animals, we posit that renal baroreceptor mechanisms are primarily responsible for the marked stimulation of renin associated with interruption of AT₁ receptor signaling (28). Moreover, since blood pressure reductions of almost 20 mmHg in D-R⁺ or D-R⁻ groups were not sufficient to trigger an increase in renin expression, our data also suggest that a threshold of blood pressure lowering must be achieved before the baroreceptor mechanisms are activated to stimulate renin production (27). The conclusion that blood pressure as such affects renin production is further supported by our observation that increasing blood pressure by dietary sodium loading significantly suppresses renin expression in D-R⁻ mice. Taken together, our findings are consistent with previous studies by Matsusaka and colleagues showing that JGA hypertrophy is correlated with reduced blood pressure rather than with the absence of AT₁ receptors at the JGA in chimeric AT_{1A}-null mice (39).

In summary, AT₁ receptors in the kidney have unique actions to determine the normal level of blood pressure. These are direct effects of AT₁ receptors on kidney cells that are independent of aldosterone and likely target renal vasculature and/or epithelia to modulate sodium excretion. While the AT_{1A} receptors in the kidney are key determinants of blood pressure, they do not have a major role in regulating renin expression. Instead, our data indicate that baroreceptor mechanisms are dominant in the stimulation of renin production that is associated with absence of AT₁ receptor signaling. We have also demonstrated that AT₁ receptors outside the kidney make unequivocal and unique contributions to blood pressure homeostasis. These actions of extrarenal AT_{1A} receptors cannot be explained by altered aldosterone generation alone, and they affect the basal level of blood pressure despite the presence of intact AT_{1A} receptor pathways in the kidney. These studies illustrate the complexity of blood pressure regulation by the RAS and suggest that maximal efficacy of ACE inhibitors and ARBs requires complete blockade of renal and extrarenal pathways. Because these pharmacological inhibitors only ameliorate but do not prevent progressive renal disease and other complications, a more precise understanding of their mechanisms of blood pressure lowering and end-organ protection should lead to new opportunities for optimizing treatments for hypertension and its complications.

Methods

Animals. Mice lacking AT_{1A} receptors for angiotensin II were generated by homologous recombination in embryonic stem cells as previously described (2). Animals were bred and maintained in the animal facility of

the Durham Veterans Affairs Medical Center according to NIH guidelines with the approval of the Institutional Animal Care and Use Committees of the Duke University and Durham VA Medical Centers. *Agtr1a* genotypes, designated “+” for the wild-type allele and “-” for the targeted allele, were determined by PCR analysis of DNA isolated from tail biopsies using the following primers: *Agtr1a*^{+/+} upstream (5'-GCATCATCTTTGTGGTGGG-3') with a sequence common to both alleles, *Agtr1a*^{+/+} downstream (5'-ATCAGCACATCCAGGAATG-3'), and a sequence in the neomycin resistance cassette present only in the null allele (5'-TGCCGAGAAAGTATCCATCATGGCTGATGC-3'). F₁(C57BL/6 × 129)*Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice were generated from intercrosses of inbred 129/SvEv and C57BL/6 *Agtr1a*^{-/-} mice in which the *Agtr1a* mutation had been back-crossed onto each respective background for more than 12 generations. We studied male F₁ mice that were 2–4 months old.

Mouse kidney transplantation. Transplantation of a single mouse kidney with bilateral native nephrectomy was performed as we have described previously (40). Briefly, animals were anesthetized with isoflurane, and the donor kidney, ureter, and bladder were harvested en bloc, including the renal artery with a small aortic cuff and the renal vein with a small caval cuff. These vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. Total ischemic time averaged 35–40 minutes. Donor and recipient bladders were attached dome to dome. The right native kidney was removed at the time of transplant, and the left native kidney was removed through a flank incision 1–3 days later; care was taken to preserve the recipient adrenal glands intact with their normal blood supply. Overall surgical mortality was approximately 5%. Each experimental group included at least 6 animals.

Data collection was initiated 2 weeks after transplantation to allow resolution of any ischemic injury associated with the transplant procedure. In addition, at the end of the experiments, sections of kidney grafts were examined to confirm normal renal structure, including the absence of ischemic tubular damage. In previous studies, we have found that glomerular filtration rates in syngeneic mouse renal transplants are similar to those observed in uninephrectomized mice (40).

Radiotelemetry transmitter implantation. We used a radiotelemetry system (Transoma Medical) to monitor blood pressure in conscious mice, as described previously (41). Six to 8 days after the kidney transplantation procedure, mice were anesthetized with isoflurane, and a pressure-sensing catheter was implanted into the left carotid artery, as described previously (42). The transducer unit was then inserted into a subcutaneous pouch along the right flank generated by blunt dissection inferiorly from the original neck incision. Mice were allowed to recover for 7 days after surgery to regain their normal circadian rhythms before experiments were initiated. During the time that their blood pressures were being monitored, mice were housed in a monitoring room in the animal facility where quiet is maintained and no other activities are permitted. In separate experiments using identical procedures, catheters were also implanted into otherwise unmanipulated *Agtr1a*^{+/+} and *Agtr1a*^{-/-} mice.

Blood pressure analysis. Arterial pressure recordings from unanesthetized, unrestrained animals were collected, stored, and analyzed using Dataquest A.R.T. software (version 2.2; Transoma Medical). The blood pressure data were collected continuously with sampling every 5 minutes for 10-second intervals (41) during the prescribed time periods. In some experiments, pressures were recorded continuously for at least 48 hours. Otherwise, in order to conserve battery function, we carried out blood pressure measurements between 10:00 am and 2:00 pm during the appointed days of the protocol.

Experimental protocol. Baseline blood pressures were measured on 2 consecutive days while the animals ingested a conventional diet containing 0.4% sodium chloride. Mean blood pressure values for each animal were computed as the average of these measurements. After baseline blood pres-



sure levels were established, mice from each of the experimental groups were placed on high-salt (6% NaCl) diet (Harlan Teklad) for 1 week while continuously monitoring blood pressures. After the blood pressure measurements were taken, the mice were placed in metabolic cages, and urine was collected for 24 hours. Urinary aldosterone concentrations were determined by ELISA (Aldosterone Enzyme Immunoassay Kit) according to the manufacturer's instructions (Cayman Chemical). At the end of the experiment, the animals were anesthetized, blood was collected via intracardiac puncture, and the transplanted kidney was harvested.

Aldosterone infusions. In separate experiments, we performed bilateral adrenalectomy on selected groups of transplanted animals and infused them chronically with aldosterone in order to clamp aldosterone levels within a uniform range. Adrenalectomies were performed coincident with the native nephrectomies. At the time of the second nephrectomy and adrenalectomy, an osmotic minipump (ALZET model 2004; DURECT Corporation) containing aldosterone (A12930; Pfaltz & Bauer) and dexamethasone (D-1756; Sigma-Aldrich) was placed in a subcutaneous pouch. The doses of aldosterone (1 µg/day) and dexamethasone (1.2 µg/100 g body wt/d) were chosen to provide near-physiological replacement based on published studies (43, 44). The aldosterone and dexamethasone were dissolved in polyethylene glycol 400 (Sigma-Aldrich) (45) and further diluted in normal saline. All other experimental procedures were carried out as described above.

Measurement of renal norepinephrine levels. Kidney specimens were placed in cold 0.1 N HCl and were homogenized (24). To remove lipids from the homogenate, 0.1% Triton X-100 (Sigma-Aldrich) was added. The samples were then incubated at 37°C for 30 minutes and centrifuged (model 5417C; Eppendorf North America) at 9,600 g for 5 minutes. Renal norepinephrine was extracted from the supernatants and quantitated by enzyme immunoassay (NorAdrenaline EIA) according to the manufacturer's instructions (ALPCO Diagnostics).

Measurement of renin mRNA levels. Relative levels of mRNA for renin were determined by real time RT-PCR with the ABI Prism 7700 Sequence Detection System (PE Biosystems Inc.) as described previously (46). Kidneys were harvested, and total RNA was isolated using an RNeasy Mini Kit per the manufacturer's instructions (QIAGEN). The nucleotide sequences of the

PCR primers and their fluorogenic probes have been published previously (46). Real time RT-PCR amplifications were performed in a 96-well plate in the ABI Prism 7700 sequence detector (PE Biosystems Inc.) as previously described (46). During the amplification, the fluorescence of FAM (or TET), TAMRA, and ROX (a passive reference dye) was measured by the 7700 sequence detector in each well of the 96-well plate. The numbers of copies of the PCR template in the starting sample were calculated using the Sequence Detector Software (SDS) included in the ABI Prism 7700 Sequence Detector System. A standard plasmid containing a DNA fragment for the renin gene was used as an external control, and amplification of the β-actin gene was used as an endogenous control. For each experimental sample, the amounts of the target and of the endogenous control were determined according to the appropriate standard curves.

Histopathology. To aid in the assessment of transplanted kidney viability, we fixed kidneys in formalin and sectioned and stained them with H&E. The histomorphology of these organs was then examined by light microscopy.

Statistical analysis. The values for each parameter within a group are expressed as the mean ± SEM. For comparisons between groups, statistical significance was assessed using ANOVA followed by unpaired Student's *t* test adjusted for multiple comparisons. A paired Student's *t* test was used for comparisons within groups (normal salt vs. high salt).

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