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Genetic deletion of AT_{1a} receptors attenuates intracellular accumulation of ANG II in the kidney of AT_{1a} receptor-deficient mice

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Abstract

We and others have previously shown that high levels of ANG II are accumulated in the rat kidney via a type 1 (AT_1) receptor-mediated mechanism, but it is not known which AT_1 receptor is involved in this process in rodents. We tested the hypothesis that AT_{1a} receptor-deficient mice (Agtr1a−/−) are unable to accumulate ANG II intracellularly in the kidney because of the absence of AT_{1a} receptor-mediated endocytosis. Adult male wild-type (Agtr1a+/+), heterozygous (Agtr1a +/−), and Agtr1a−/− were treated with vehicle, ANG II (40 ng/min ip via osmotic minipump), or ANG II plus the AT_1 antagonist losartan (10 mg · kg^{-1} · day^{-1} po) for 2 wk. In wild-type mice, ANG II induced hypertension (168 ± 4 vs. 113 ± 3 mmHg, $P < 0.001$), increased kidney-to-body weight ratio ($P < 0.01$), caused pressure natriuresis ($P < 0.05$), and elevated plasma and whole kidney ANG II levels ($P < 0.001$). Concurrent administration of ANG II with losartan attenuated these responses to ANG II. In contrast, Agtr1a−/− mice had lower basal systolic pressures ($P < 0.001$), smaller kidneys with much fewer AT_{1b} receptors ($P < 0.001$), higher basal 24-h urinary sodium excretion ($P < 0.01$), as well as basal plasma and whole kidney ANG II levels ($P < 0.01$). However, intracellular ANG II levels in the kidney were lower in Agtr1a−/− mice. In Agtr1a−/− mice, ANG II slightly increased systolic pressure ($P < 0.05$) but had no effect on the kidney weight, urinary sodium excretion, and whole kidney ANG II levels. Losartan restored systolic pressure to basal levels and decreased whole kidney ANG II levels by ~20% ($P < 0.05$). These results demonstrate a predominant role of AT_{1a} receptors in blood pressure regulation and in the renal responses to long-term ANG II administration, that AT_{1b} receptors may play a limited role in blood pressure control and mediating intrarenal ANG II accumulation in the absence of AT_{1a} receptors.

Keywords

Kidney; losartan; receptor endocytosis; urinary sodium excretion

The kidney plays a major role in blood pressure regulation and body salt and fluid balance in part by producing the vasoconstrictor and sodium retaining-hormone ANG II. All major components of the renin-angiotensin system necessary for ANG II formation are present in the kidney, with renin expressed in juxtaglomerular apparatus, angiotensin-converting

enzyme (ACE) in the brush-border of proximal tubule cells, and ANG II receptors in various renal cells (1,6,9,14,25,26). Kidney-derived renin and ANG II are released in the circulation, acting as an endocrine hormone system to maintain blood pressure, enhance cardiovascular performance, and stimulate aldosterone synthesis. At the local level, ANG II exerts paracrine and/or autocrine regulation of glomerular hemodynamics, renal microcirculation, and tubular transport (15,20,29). Although the ability of the kidney to produce ANG II contributes to high intrarenal ANG II levels in normal and ANG II-induced hypertensive rats, the augmentation is also the result of the accumulation of circulating/extracellular ANG II via angiotensin type 1 (AT_1) receptors. Both local synthesis and ANG II uptake account for the high ANG II environment in the kidney (13,14,22,23,27,30).

Previous studies have shown that kidney cells take up circulating and locally produced ANG II, thereby accumulating ANG II in the interstitial and intracellular compartments (22,23,27,30). Von Thun et al. (23) reported that hypertension induced by chronic infusion of ANG II augmented renal ANG II levels to a greater extent than could be explained by plasma renin or ANG II concentrations. Zou et al. (30) later showed that the renal accumulation of infused ANG II was AT_1 receptor mediated. These findings were later supported by studies in pigs infused with ^{125}I-labeled ANG II and in renal cortical endosomes of ANG II-infused hypertensive rats (22,27). However, rodent kidneys express two isoforms of AT_1 receptors, AT_{1a} and AT_{1b}, and pharmacological blockers cannot distinguish the relative contribution of AT_{1a} vs. AT_{1b} to this mechanism (7). Mutational studies using Chinese hamster ovary cells expressing either AT_{1a} or AT_{1b} showed that both receptors internalized after exposure to ANG II to similar efficiencies (5,7). We have recently shown AT_1 receptor (equivalent to rodent AT_{1a})-mediated endocytosis of extracellular ANG II in rabbit proximal tubule cells (13). It remains unclear how these mutational and cell biology studies apply to integrative renal physiology. Cervenka et al. (3) demonstrated that AT_{1a} knockout mice subjected to acute volume expansion fail to alter intrarenal ANG II levels. In the present study, we used AT_{1a} receptor-deficient mice to test the hypothesis that mice lacking the AT_{1a} receptor are unable to accumulate ANG II intracellularly in the kidney in response to chronic ANG II infusion because of the absence of AT_{1a} receptor-mediated endocytosis and a relatively smaller contribution of AT_{1b} receptors after AT_{1a} is deleted.

METHODS

Animals and genotyping

Adult male wild-type C57BL/6J (~25 g, 10 wk old; Agtr1a+/+) were purchased from Jackson Laboratories and maintained on a normal rodent chow and had free access to tap water. Upon arrival, mice were trained for 1 wk for measurement of systolic blood pressure via a computerized tail-cuff method (Visitech, Cary, NC). A breeding pair of heterozygous AT_{1a} mice (Agtr1a+/−) was purchased from Jackson Laboratories, originally deposited by Dr. Oliver Smithies of the University of North Carolina (B6.129P2-*Agtr1^{tm1Unc}*/J; see Refs. 17–19). Male heterozygous Agtr1a+/− and homozygous AT_{1a} receptor knockout mice (Agtr1a−/−) were bred from this colony in our laboratory according to a standard protocol provided by the vendor (Stock no. 002682). All animal protocols were approved by the Institutional Animal Care and Use Committee of Henry Ford Health System. Genotyping of Agtr1a−/− mice was performed by PCR duplicate on tail DNA samples using Jackson Laboratories' standard protocols (17–19). PCR primers for genotyping the AT_{1a} receptor were purchased from Invitrogen, and their respective sequences are neo generic primers, which amplify a 280-bp DNA product from the bacterial neomycin resistance gene (oIMR0013: 5′-CTT ggg Tgg AgA ggC TAT TC-3′; oIMR0014: 5′-Agg TgA gAT gAC Agg AgA TC-3′), and Agtr1a wild-type primers, which together amplify a 483-bp DNA product from the wild-type allele (0IMR0738: 5′-TgA gAA CAC CAA TAT CAC Tg-3′:

oIMR0739: 5′-TTC gTA gAC Agg CTT gAg-3′). Cycling conditions are set for denaturation at 94°C for 3 min, annealing at 55°C for 45 s, and extension at 72°C for 45 s over 35 cycles. Adult male Agtr1a+/− and Agtr1a−/− mice (~25 g, 10–12 wk old) were trained for blood pressure measurements and urine collection for determinations of urinary sodium and potassium excretion via a metabolic cage before commencing the infusion studies.

Long-term infusion of Val[5]-ANG II via osmotic minipump

After basal systolic pressures and 24-h urinary sodium and potassium excretion were measured from wild-type, Agtr1a+/−, and Agtr1a−/− mice, osmotic minipumps (model 2002; Alza) were implanted intraperitoneally for long-term infusion of Val[5]-ANG II as described previously (27,30). Val[5]-ANG II was infused at 40 ng/min for 2 wk and the infusion rate was determined from preliminary studies in which systolic pressure was increased to ~160 mmHg (27). Three groups each of wild-type, Agtr1a+/−, and Agtr1a−/− mice ($n = 6$–10) were treated with: *1*) vehicle as control; *2*) ANG II; and *3*) ANG II plus losartan (10 mg · kg^{-1} · day^{-1} po). Systolic blood pressure was measured weekly by the computerized tail-cuff methods (Visitech).

Measurements of plasma, whole kidney, and intracellular ANG II

Plasma and whole kidney ANG II levels were measured by a sensitive ANG II enzyme immunoassay as described previously (13,29). At 2 wk of ANG II infusion, mice were decapitated without anesthesia to prevent anesthetic-induced ANG II formation, and trunk blood was collected in chilled tubes containing a mixed inhibitor cocktail (5 mM EDTA, 10 μM pepstatin A/*o*-phenanthroline, 10 μM captopril, 10 mg/ml bestatin, and 1 mg/ml aprotinin) for measurement of plasma ANG II as described previously (11,13,25). For measurement of whole kidney ANG II, one kidney from each mouse was sliced into two parts with one saved for ANG II receptor autoradiography (see below). The kidney tissues were weighed and homogenized in an ANG II extraction buffer containing 20 mM Tris base, 10 mM EDTA, 5 mM EGTA, 5 mM β-mercaptoethanol, 50 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, as described previously (11,23,27). To exclude the confounding influence of high plasma or circulating ANG II levels on intrarenal or intracellular ANG II in Agtr1a−/− mice, one kidney from wild-type and Agtr1a−/− mice was perfusion-washed with 5 ml ice-cold acid buffer, which dissociates membrane receptor-bound ANG II from cells before the kidneys were processed for ANG II measurements (13). ANG II was extracted in a phenyl-bonded solid-phase peptide extraction column (Bond Elut-C18; Varian), vacuum-dried overnight, and reconstituted in ANG II assay buffer before ELISA of ANG II (13,29).

Autoradiographic mapping of AT$_{1a}$ and AT$_{1b}$ receptors in the kidney

In addition to PCR genotyping as described above, ANG II receptor phenotypes in the kidneys of wild-type, Agtr1a+/−, and Agtr1a−/− mice were also mapped by quantitative in vitro autoradiography using either [^{125}I]Val[5]-ANG II or [^{125}I]Sar[1],Ile[8]-ANG II, an analog antagonist, as we described previously (9,26,28). [^{125}I]Sar[1],Ile[8]-ANG II binds both AT$_1$ and AT$_2$ receptors. Additionally, in vitro autoradiography was also used to determine the long-term effects of ANG II infusion and losartan administration in the kidneys of wild-type and Agtr1a−/− mice because ANG II has been shown to downregulate AT$_1$ receptor density in the rat kidney (7,13,26). Briefly, one-half of one kidney from each mouse was snap-frozen in isopentane on dry ice at the time of death. Frozen kidney sections, 20 μm thick, were cut on a cryostat and preincubated in a buffer containing 10 mM Na$_2$HPO$_4$, 150 mM NaCl, 5 mM Na$_2$EDTA, and 0.02% NaN$_3$ (*p*H 7.4) for 15 min to dissociate endogenous ANG II bound to the receptor. Sections were then incubated with the same fresh buffer containing 100 pmol of [^{125}I]Sar[1],Ile[8]-ANG II, 0.2% BSA, and 0.5 mg/ml bacitracin at 22°C for 60 min without (i.e., for total binding) or in the presence of an excess of unlabeled ANG II (1

μM for nonspecific binding), losartan (1 μM), or PD-123319 (1 μM) to compete for $[^{125}I]Sar^1,Ile^8$-ANG II binding (9,26,28). Total AT_1 receptors (AT_{1a} and AT_{1b}) were determined in the presence of an excess of AT_2 receptor blocker PD-123319 (1 μM). Because losartan has similar affinity to AT_{1a} and AT_{1b} receptors, the AT_{1b} receptor could not be determined in the kidneys of wild-type and Agtr1a+/− mice. By contrast, the AT_{1b} receptor could be measured in the kidneys of Agtr1a−/− mice as total binding of $[^{125}I]Sar^1,Ile^8$-ANG II because the AT_{1a} receptor has been deleted and losartan completely displaced this binding. After incubations, kidney sections were washed, dried, and exposed to X-ray films for 3 days, and resultant autoradiographs were analyzed using computerized densitometry (MCID; Imaging Research, Ontario, Canada; see Refs. 9,26,28).

Measurement of urinary sodium and potassium excretion

To determine drinking and urinary excretory responses to long-term administration of ANG II or concurrent administration of ANG II and the AT_1 blocker losartan, mice were housed individually in metabolic cages to ensure measurements of 24-h drinking and to collect urine samples as we described for rats (27). To minimize the effects of stress on these measurements, special care was taken to train each mouse individually in a mouse metabolic cage for 3 days before drinking, and urine collections were made. Twenty-four-hour drinking and urine output was measured gravimetrically, and plasma and urinary sodium and potassium concentrations were determined by flame photometry as previously described (27).

Statistical analysis

All data are presented as means ± SE. Differences in all parameters between groups of control, ANG II-infused, and ANG II plus losartan-treated in the same strains were analyzed using one-way ANOVA followed by Dunnett's comparisons between group means if $P < 0.05$. The differences in the same parameter between wild-type, Agtr1a+/−, or Agtr1a−/− mice were compared using an unpaired t-test. The significance was set at $P < 0.05$.

RESULTS

AT_{1a} and AT_{1b} receptor genotyping and renal AT_1 receptor mapping in wild-type, Agtr1a+/−, and Agtr1a−/− mice

The PCR products for genotyping three representative wild-type (C57BL/6J; Agtr1a+/+), heterozygous (Agtr1a+/−), or homozygous (Agtr1a−/−) mice are shown in Fig. 1. Wild-type mice yielded a single PCR product of 483 bp, whereas homozygous Agtr1a−/− mice produced a single PCR product of 280 bp. By contrast, heterozygous Agtr1a+/− mice had PCR products of both 483 and 280 bp (Fig. 1, *top*). The intrarenal localization and relative levels of total AT_1 receptors (AT_{1a} and AT_{1b}) in wild-type and Agtr1a+/− mice and AT_{1b} receptors in Agtr1a−/− mice are shown in autoradiographs (Fig. 1, *bottom*). In wild-type and Agtr1a+/− mice, AT_1 receptors predominated in the outer cortex and the inner stripes of outer medulla, but wild-type kidneys had a much higher level of AT_1 receptor binding than Agtr1a+/− mice (Fig. 1). However, the proportions of AT_{1a} and AT_{1b} receptors could not be determined by pharmacological means in these mice. In Agtr1a−/− mice, there was only a small proportion (~5–10%) of AT_1 receptors that could be displaced by losartan, therefore representing AT_{1b} receptors (Fig. 1). These data suggest that, in the mouse kidney, the majority of AT_1 receptors belong to AT_{1a}.

Basal systolic blood pressure and urinary excretory functions in wild-type, Agtr1a+/−, and Agtr1a−/− mice

Basal systolic blood pressure and renal excretory function in age- and body weight-matched wild-type, Agtr1a+/−, and Agtr1a−/− mice are summarized in Table 1. These functional measurements were made before long-term administration of ANG II to induce hypertension or losartan in wild-type and Agtr1a+/− mice. Although age and body weights were similar for all mice, the kidney weights and kidney weight-to-body weight ratios in Agtr1a−/− mice were 15% lower than wild-type or Agtr1a+/− mice ($P < 0.01$). Systolic blood pressure was ~24 ± 5 and 16 ± 3 mmHg lower in Agtr1a−/− mice than in wild-type or Agtr1a+/− mice, respectively ($P < 0.01$). Agtr1a−/− mice drank more water (171%, $P < 0.01$) and excreted more sodium (251%, $P < 0.01$) and potassium (245%, $P < 0.01$) during a 24-h period than wild-type mice (Table 1). Agtr1a+/− mice had basal systolic blood pressure, drinking, and urinary excretion rates of sodium and potassium between wild-type and AT_{1a} receptor knockout mice (Table 1).

Effects of long-term infusion of Val[5]-ANG II and administration of losartan on systolic blood pressure in wild-type, Agtr1a+/−, and Agtr1a−/− mice

Table 2 summarizes systolic blood pressure and drinking and renal excretory responses to ANG II infusion alone or concurrent administration of ANG II and losartan during the 1st wk in wild-type, Agtr1a+/−, and Agtr1a−/− mice. This represents the status of urinary sodium excretion during the early phase of development of hypertension in these mice. As expected, systolic blood pressure was markedly increased by ANG II infusion in both wild-type and Agtr1a+/− mice. With the exception that wild-type mice drank slightly more water and excreted less potassium during ANG II infusion, there were no significant differences in 24-h urinary sodium excretion (Table 2). Although losartan significantly attenuated the effect of ANG II on systolic blood pressure, it did not restore the pressure to basal levels at this stage. In Agtr1a−/− mice, ANG II also increased blood pressure moderately, but it did not significantly affect other functional indexes from their basal levels (Table 2). Concurrent administration of losartan did not alter these parameters either.

Figure 2 shows systolic blood pressure responses to ANG II infusion alone or concurrent administration of ANG II and losartan in wild-type, Agtr1a+/−, and Agtr1a−/− mice at the 2nd wk, when ANG II-induced hypertension was stabilized. Compared with the response at the 1st wk, blood pressure increased further to 168 ± 4 mmHg in wild-type ($P < 0.01$) and to 156 ± 4 mmHg in Agtr1a+/− mice ($P < 0.01$). By contrast, blood pressure was increased only to 101 ± 4 mmHg in Agtr1a−/− mice ($P < 0.01$). At this time point, concurrent administration of ANG II with losartan normalized blood pressure to control in wild type [118 ± 5 mmHg, not signifi-cant (NS) vs. control] and Agtr1a−/− mice (92 ± 4 mmHg, NS vs. control). Systolic blood pressure was reduced by losartan only by one-half in Agtr1a+/− mice (127 ± 4 mmHg, $P < 0.01$ vs. control or ANG II; Fig. 2).

Effects of long-term infusion of Val[5]-ANG II on 24-h urinary sodium and potassium excretion in wild-type, Agtr1a+/−, and Agtr1a−/− mice

As shown in Fig. 3, long-term ANG II infusion induced pressure natriuresis in wild-type mice, increasing 24-h urinary sodium excretion by 53% ($P < 0.01$ Fig. 3, *top*). Concurrent administration of ANG II with losartan normalized urinary sodium excretion to control (NS vs. control), in association with normalization of systolic pressure by losartan in these mice (Fig. 2). By comparison, there were no significant differences in 24-h urinary sodium excretion in Agtr1a−/− mice (Fig. 3, *top*). In Agtr1a+/− mice, 24-h urinary sodium excretion rate was higher than in wild-type mice under basal conditions and during concurrent administration of ANG II and losartan, but the response was not different from those of wild-type or Agtr1a−/− mice during ANG II infusion (Fig. 3, *top*). There were no

significant differences in 24-h urinary potassium excretion rates among the different groups within wild-type, Agtr1a+/−, and Agtr1a−/− mice (ANOVA). However, 24-h urinary potassium excretion was significantly higher in Agtr1a−/− mice than in wild-type mice (Fig. 3, *bottom*).

Effect of long-term infusion of Val[5]-ANG II on the kidney-to-body weight ratio in wild-type, Agtr1a+/−, and Agtr1a−/− mice

Long-term infusion of ANG II for 2 wk induced renal hypertrophy in wild-type and Agtr1a +/− mice, as reflected by increases in the kidney-to-body weight ratio (Fig. 4). However, losartan did not normalize the renal hypertrophic response to ANG II to control. In Agtr1a−/− mice, there were no significant differences in the kidney-to-body weight ratio between control and ANG II- or losartan-treated groups (Fig. 4).

Effect of long-term infusion of Val[5]-ANG II on plasma and kidney ANG II levels in wild-type, Agtr1a+/−, and Agtr1a−/− mice

Figure 5, *top*, shows that plasma ANG II levels were markedly increased from 104.6 ± 12.6 to 183.8 ± 40.4 fmol/ml by long-term infusion of ANG II in wild-type mice ($P < 0.01$). Concurrent administration of ANG II with losartan increased plasma ANG II levels further to 223.8 ± 21.3 fmol/ml ($P < 0.01$ vs. control). In Agtr1a−/− mice, basal plasma ANG II levels were 3.6-fold higher than those of wild-type mice (377 ± 66 fmol/ml, $P < 0.01$). ANG II infusion or concurrent administration with losartan increased plasma ANG II levels further (ANG II: 566.2 ± 114 fmol/ml, $P < 0.05$ vs. control; ANG II + losartan: 834.1 ± 55.9 fmol/ml, $P < 0.05$ vs. ANG II). In Agtr1a+/− mice, plasma ANG II levels were at levels between those of wild-type and Agtr1a−/− mice for their corresponding groups (Fig. 5, *top*). The whole kidney ANG II levels in wild-type, Agtr1a+/−, and Agtr1a−/− mice at basal conditions and in response to ANG II infusion or concurrent administration with losartan are shown in Fig. 5, *bottom*. Basal whole kidney ANG II levels were threefold higher in Agtr1a −/− mice [573.2 ± 80.2 pg/100 mg kidney weight (KW)] than wild-type mice (184.60 ± 110 pg/100 mg KW; $P < 0.01$), which was not significantly different from plasma ANG II levels. In wild-type mice, ANG II infusion markedly increased whole kidney ANG II levels to 509.3 ± 49.7 pg/100 mg KW ($P < 0.01$), which was reduced to 298.9 ± 58.4 pg/100 mg KW by losartan ($P < 0.01$). By comparison, there were no significant differences between control and ANG II-treated groups in whole kidney ANG II levels in Agtr1a−/− mice (Fig. 5, *bottom*). However, losartan decreased whole kidney ANG II levels by ~20% to a level significantly different from the ANG II-treated group in Agtr1a−/− mice (ANG II: 602.1 ± 30.8 pg/100 mg KW; ANG II + losartan: 406.1 ± 83.8 pg/100 mg KW; $P < 0.05$). The responses of renal ANG II levels to ANG II or losartan in Agtr1a+/− mice were not significantly different from those of wild types (Fig. 5, *bottom*).

Effect of long-term infusion of Val[5]-ANG II on intracellular ANG II levels in the kidneys of wild-type and Agtr1a−/− mice

To exclude the influence of high plasma or extracellular ANG II levels on whole kidney ANG II levels in Agtr1a−/− mice, the kidneys from wild-type and Agtr1a−/− mice treated without (control) or with ANG II or concurrent administration of ANG II and losartan ($n = 6$) were first perfusion washed with 5 ml ice-cold acid buffer, which clears membrane receptor-bound extracellular ANG II from cells before the kidneys were removed and processed for ANG II measurements. Figure 6 shows that intracellular ANG II levels in the kidneys of Agtr1a−/− mice were significantly lower than those of wild-type mice under basal conditions (wild type: 90.7 ± 7.4 pg/100 mg KW vs. Agtr1a−/−: 59.2 ± 14.8 pg/100 mg KW, $P < 0.01$), during ANG II infusion (wild type: 172 ± 25 pg/100 mg KW vs. Agtr1a −/−: 68 ± 12 pg/100 mg KW, $P < 0.01$), and during concurrent administration of ANG II

and losartan (wild type: 110.7 ± 15.6 pg/100 mg KW vs. Agtr1a$-$/$-$: 62.3 ± 8.9 pg/100 mg KW, $P < 0.01$).

Effects of long-term ANG II infusion or concurrent administration of ANG II and losartan on renal AT$_1$ receptor binding in wild-type and Agtr1a$-$/$-$ mice

AT$_1$ receptors are known to be downregulated by ANG II in vascular smooth muscle cells and in the kidney in acute experiments, but long-term effects of ANG II and losartan on AT$_1$ receptors have not been well studied in mice. Figure 7 shows in vitro autoradiographic mapping of AT$_1$ receptors in wild-type and Agtr1a$-$/$-$ mice using [^{125}I]Sar^1,Ile^8-ANG II, and the quantitative results are shown in Fig. 8. In wild-type mice, long-term ANG II administration decreased AT$_1$ receptor binding by ~33% [control: $1,056 \pm 14$ disintegrations $\cdot$ min^{-1} (dpm) $\cdot$ mm^{-2} vs. ANG II treated: 709 ± 32 dpm/mm^2, $P < 0.01$], and concurrent administration of losartan for 2 wk reduced the binding to ~16% of control (169 ± 33 dpm/mm^2; Fig. 8, *top*). In Agtr1a$-$/$-$ mice, basal AT$_1$ receptor binding (representing AT$_{1b}$) was 45 ± 6 dpm/mm^2, but ANG II had no significant effects on this binding (33 ± 8 dpm/mm^2, NS). By contrast, losartan reduced the remaining AT$_1$ binding in the kidney of Agtr1a$-$/$-$ mice significantly (25 ± 6 dpm/mm^2, $P < 0.01$ vs. basal; Fig. 8, *top*). However, there were no significant differences in binding dissociation constant (K_d) between control and ANG II-infused wild-type mice (Fig. 8, *bottom*).

DISCUSSION

Although renal histological and functional phenotypes of Agtr1a$-$/$-$ mice have been studied extensively since it was first reported in 1995 (12), most previous studies had focused on the role of AT$_{1a}$ receptors in mediating acute systemic or intrarenal arteriolar pressor responses to ANG II (9,12,17) and the regulation of acute tubuloglomerular feedback responses (10,20). However, blood pressure and renal excretory responses to long-term ANG II administration have not been examined, nor has the role of AT$_{1a}$ receptors in mediating uptake or accumulation of ANG II in the kidney been studied previously in Agtr1a$-$/$-$ mice.

The present study demonstrates several key findings. The Agtr1a$-$/$-$ mice exhibited much lower systolic blood pressure as expected (Fig. 2 and Ref. 12), expressed only a small fraction (5–10%) of AT$_{1b}$ receptors in the kidney (Figs. 1 and 6), had a smaller kidney-to-body weight ratio despite similar body weights (Fig. 4), and drank more water and had higher urinary sodium excretion rates (Tables 1 and 2 and Fig. 3) compared with wild-type and heterozygous Agtr1a$+$/$-$ mice. Second, long-term ANG II infusion raised blood pressure, induced renal hypertrophy, and increased 24-h urinary sodium excretion in wild-type mice. Concurrent administration of losartan significantly attenuated the responses to ANG II in wild-type mice. Third, long-term ANG II infusion increased systolic blood pressure to a much lesser extent in Agtr1a$-$/$-$ than in wide-type mice but had no effect on the kidney-to-body weight ratio and 24-h urinary sodium excretion rates. Interestingly, concurrent administration of losartan with ANG II restored systolic pressure to basal levels in Agtr1a$-$/$-$ mice (Fig. 2). Fourth, Agtr1a$-$/$-$ mice had markedly elevated basal plasma and whole kidney ANG II levels, but their intracellular ANG II levels were lower than wild-type mice (Fig. 6). Finally, comparison of systemic and renal effects of losartan in ANG II-treated wild-type and Agtr1a$-$/$-$ mice suggests a relatively minor role for the remaining AT$_{1b}$ receptors in blood pressure and renal regulation in the absence of AT$_{1a}$ receptors. Overall, these results confirm a predominant role of AT$_{1a}$ receptors in the regulation of blood pressure and renal excretory responses to ANG II and intracellular ANG II accumulation during ANG II-induced hypertension.

Previous studies have shown that, in the absence of AT$_{1a}$ receptors, AT$_{1b}$ receptors may assume the role of AT$_{1a}$ receptors to compensate for the loss of predominant AT$_{1a}$ influence

(4,9,17,24). Oliverio et al. (17) reported that acute bolus injections of ANG II increased blood pressure by an average of 10 mmHg in Agtr1a−/− mice, and this pressor effect was completely inhibited by AT_1 receptor blockers, therefore suggesting a role for AT_{1b} receptors in blood pressure control in the absence of AT_{1a} receptors. However, Chen et al. (4) showed that targeting deletion of AT_{1b} receptors did not alter renal morphology, blood pressure, and aldosterone responses to low or high salt, nor in AT_{1a} receptor mRNA expression in Agtr1b−/− mice. The findings of the latter study are unexpected because targeting deletion of AT_{1a} receptors had no appreciable effects on renal growth or histology (12,18), but double deletion of AT_{1a} and AT_{1b} led to typical pathological phenotypes similar to those in ACE knockout or angiotensinogen knockout mice (19,21). In the present study, we focused on a possible compensatory role of AT_{1b} receptors in blood pressure and renal hypertrophic and excretory responses to long-term ANG II administration. We found that ANG II infusion for 2 wk increased blood pressure significantly from ~90 to 101 mmHg in Agtr1a−/− mice compared with an ~55-mmHg increase in wild-type mice. Because losartan completely blocked this pressor response in Agtr1a−/− mice, AT_{1b} receptors may have mediated this response, as suggested by Oliverio et al. (17). However, we found no significant role of AT_{1b} receptors in mediating renal hypertrophic or urinary sodium excretory responses to long-term ANG II administration in Agtr1a−/− mice. There were also no differences in 24-h urinary excretion of sodium and potassium in Agtr1a−/− mice between basal, ANG II infusion, and concurrent administration of losartan (Table 2 and Fig. 3). These findings may not be surprising given that AT_{1b} receptors account for only 5–10% of total AT_1 receptors in the mouse kidney, as determined by quantitative in vitro autoradiography (Figs. 1, 7, and 8). Our study suggests that AT_{1a} receptors play the overwhelming role in mediating blood pressure and renal hypertrophic and urinary excretory responses to long-term ANG II infusion and that AT_{1b} receptors may not exert a perceptible role in the regulation of renal growth and urinary excretory responses in Agtr1a−/− mice.

AT_1 receptor-mediated endocytosis of extracellular ANG II plays an important role in regulating intracellular signaling in many target cells (7,13,29). This process also contributes to accumulation of high levels of ANG II in the rat kidney and in cultured proximal tubule cells (13,22,23,27,30). However, because the rodent kidney expresses two AT_1 receptors, AT_{1a} and AT_{1b}, and both receptors internalize upon exposure to ANG II (5), the conventional view is that both receptors mediate ANG II endocytosis and therefore may contribute to maintaining high intrarenal ANG II levels. In the current study, we demonstrated that infusion of ANG II for 2 wk increased whole kidney ANG II levels more than threefold in wild-type mice. This response was substantially attenuated by AT_1 receptor blockade, confirming the essential role of AT_1 receptor-mediated mechanisms in mice (22,27,30). Because losartan also reduced whole kidney ANG II levels in ANG II-infused Agtr1a−/− mice, AT_{1b} receptors may play a limited role in AT_1 receptor-mediated ANG II endocytosis and accumulation in the mouse kidney (Fig. 5).

The finding that Agtr1a−/− mice had higher basal whole kidney ANG II levels than wild-type mice requires an explanation. High circulating ANG II levels in Agtr1a−/− mice are expected (Fig. 5) because the AT_{1a} receptor predominates in most cardiovascular and renal tissues (7), and its deletion would lead to increased unbound ANG II in the circulation and extracellular fluid compartments. Higher plasma ANG II levels have been reported in rats or humans treated with AT_1 receptor blockers (2,27). However, the high whole kidney ANG II level in Agtr1a−/− mice under basal conditions was unexpected because it has been shown that AT_1 receptors mediate intrarenal accumulation of ANG II in rats and pigs (22,23,27,30). Moreover, lower renal ANG II levels have been reported in anesthetized, acutely volume-expanded Agtr1a−/− mice (3). There are several explanations for these differences. Oliverio et al. (17) have shown that renin mRNA expression was three times higher in Agtr1a−/− than wild-type mice. This suggests that ANG II may be continuously produced and released

because of the lack of AT_1 receptor-mediated negative feed-back regulation of renin. Second, markedly increased whole kidney ANG II levels have been reported in AT_{1a}/AT_{1b} receptor double-knockout mice (16,21). Third, although AT_{1a} receptor-mediated endocytosis of extracellular ANG II is the predominant mechanism responsible for ANG II uptake in the kidney of wild-type mice, AT_{1b} receptors may partly assume this role after the AT_{1a} receptor is deleted (Fig. 5). Furthermore, Gonzalez-Villalobos et al. (8) recently reported that the protein megalin also mediates endocytosis of ANG II in a proximal tubule epithelial cell line. Finally, high whole kidney ANG II levels in Agtr1a−/− mice may simply reflect the high plasma and extracellular fluid levels. This may mask the differences in ANG II levels existing between extracellular and intracellular fluid compartments. To exclude the influence of high plasma/extracellular ANG II on intrarenal ANG II levels, we perfusion washed the kidneys with an acid buffer to dissociate ANG II bound to membrane receptors before harvesting and processing the samples. Under this condition, we found intracellular ANG II levels were indeed lower in Agtr1a−/− mice than in wild-type mice under basal conditions, during ANG II infusion, or concurrent administration with losartan (Fig. 6). These results suggest that lower intracellular ANG II levels in Agtr1a−/− mice are likely because of the lack of AT_{1a} receptor-mediated endocytosis.

In summary, we demonstrate that Agtr1a−/− mice had markedly lower basal blood pressure, smaller kidney-to-body weight ratios, higher circulating and whole kidney ANG II levels, drank more water, and excreted more urinary sodium and potassium. By contrast, the intracellular ANG II level in the kidney was much lower in Agtr1a−/− mice because of the absence of AT_1 receptor-mediated endocytosis. This suggests that AT_{1a} receptors play a dominant role in blood pressure regulation and renal hypertrophic and excretory functional responses to long-term ANG II stimulation and receptor-mediated ANG II accumulation in the kidney. By contrast, AT_{1b} receptors only play a limited role when the AT_{1a} receptor is deleted because of its low levels of expression in the kidney. Because the AT_{1a} receptor in rodents is equivalent to human AT_1 receptors, our study helps to elucidate the mechanisms by which ANG II receptor blockers exert beneficial effects in treating hypertension and preventing target organ damage in humans.

Acknowledgments

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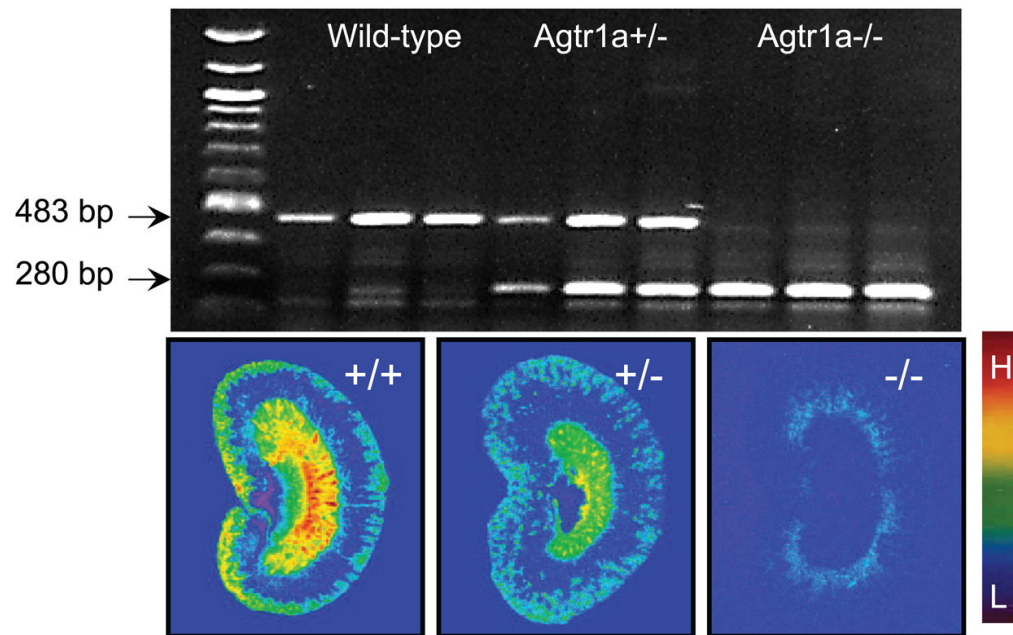
Fig. 1.
Representative PCR products for genotyping wild-type (Agtr1a+/+), heterozygous (Agtr1a+/−), and homozygous (Agtr1a−/−) mice on tail DNA samples (*top*) and autoradiographic mapping of angiotensin type 1 (AT$_1$) receptors in the kidney using [^{125}I]Val^5-ANG II as the radioligand (*bottom*). The PCR product of 483 bp represents wild-type mice, whereas that of 280 bp shows homozygous Agtr1a−/− mice. Heterozygous Agtr1a+/− mice have both 483- and 280-bp PCR products. The relative levels of AT$_1$ II receptor binding are shown with red representing the highest (H) and blue representing the background level of binding (L).
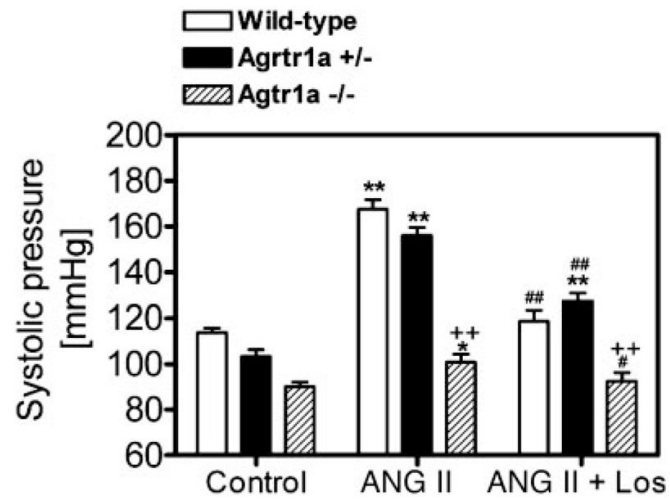
Fig. 2.
Systolic blood pressure responses to long-term administration of Val^5-ANG II and/or losartan (Los) for 2 wk in wild-type, Agtr1a+/−, and Agtr1a−/− mice. *$P < 0.05$ and **$P < 0.01$ vs. their respective basal blood pressure. #$P < 0.05$ and ##$P < 0.01$ vs. ANG II in the same group. ++$P < 0.01$ vs. wild-type or Agtr1a+/− mice in response to the same treatment.
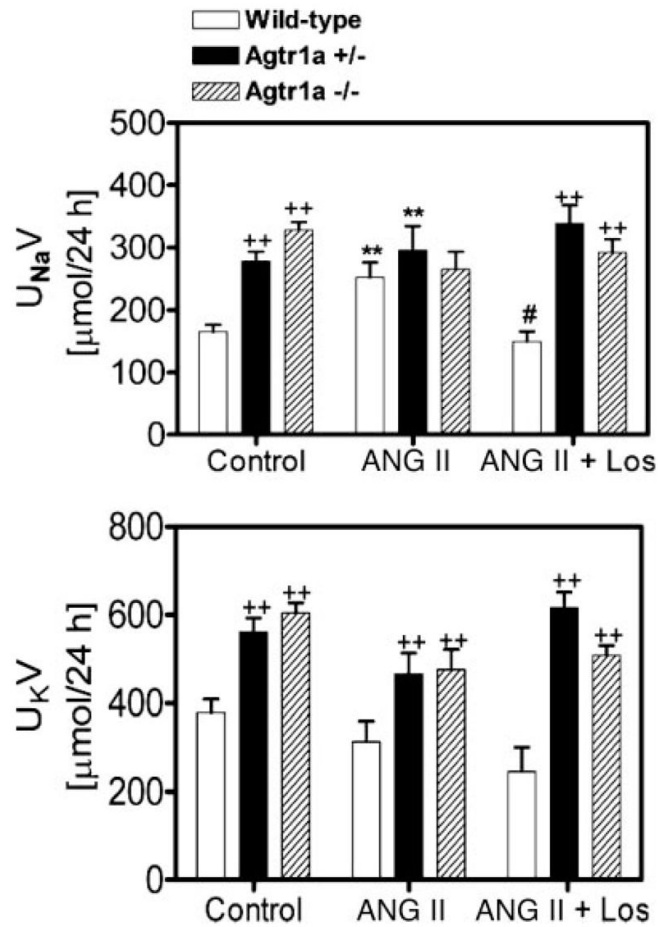
Fig. 3.
Twenty-four-hour urinary sodium ($U_{Na}V$) and potassium (U_KV) excretory responses to long-term administration of ANG II and/or losartan for 2 wk in wild-type, Agtr1a+/−, and Agtr1a−/− mice. **$P < 0.01$ vs. respective controls in the same group. #$P < 0.05$ vs. ANG II in the same group. ++$P < 0.01$ vs. wild-type or Agtr1a+/− mice in response to the same treatment.

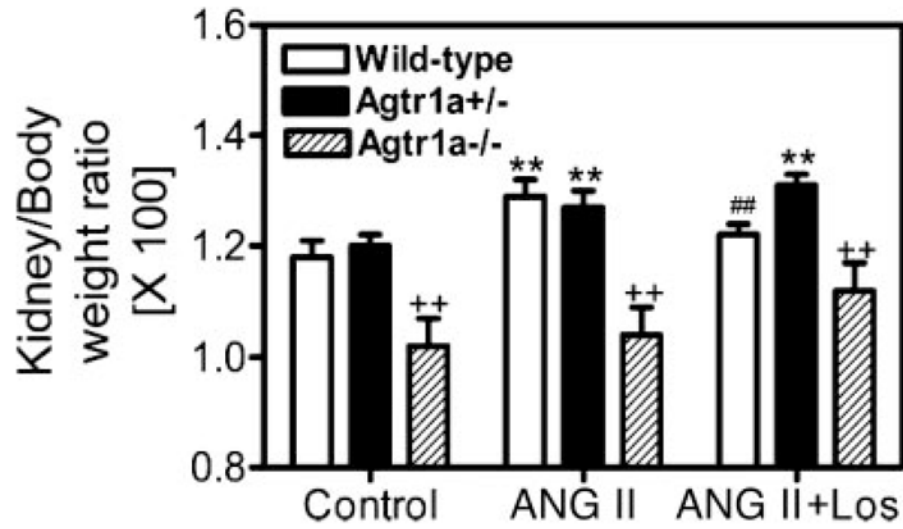**Fig. 4.**
Renal hypertrophic responses to long-term administration of ANG II or concurrent administration of ANG II and losartan for 2 wk in wild-type, Agtr1a+/−, and Agtr1a−/− mice. **$P < 0.01$ vs. respective controls in the same group. ##$P < 0.01$ vs. ANG II in the same group. ++$P < 0.01$ vs. wild-type or Agtr1a+/− mice in response to the same treatment.
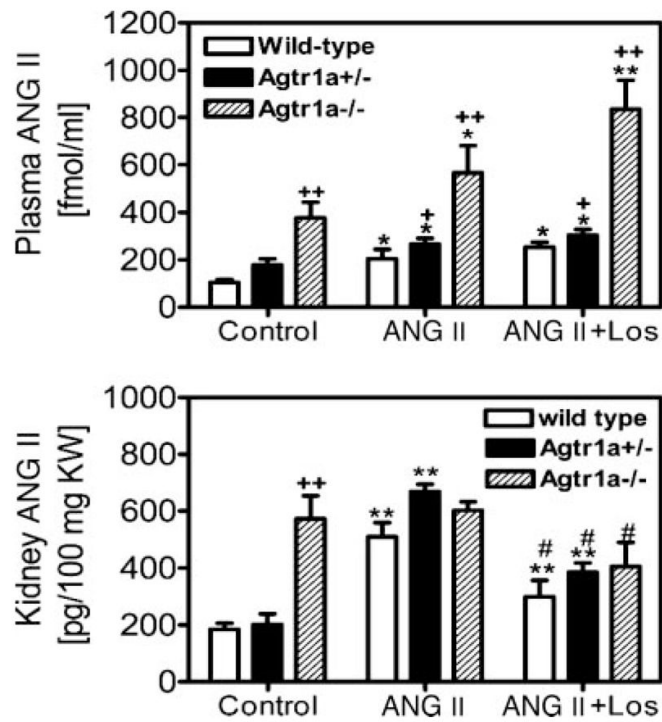
Fig. 5.
Circulating and whole kidney ANG II levels in response to long-term administration of ANG II or concurrent administration with losartan for 2 wk in wild-type, Agtr1a+/−, and Agtr1a−/− mice. *$P < 0.05$ and **$P < 0.01$ vs. respective controls in the same group. #$P < 0.05$ vs. ANG II in the same group. +$P < 0.05$ and ++$P < 0.01$ vs. wild-type or Agtr1a+/− mice in response to the same treatment.
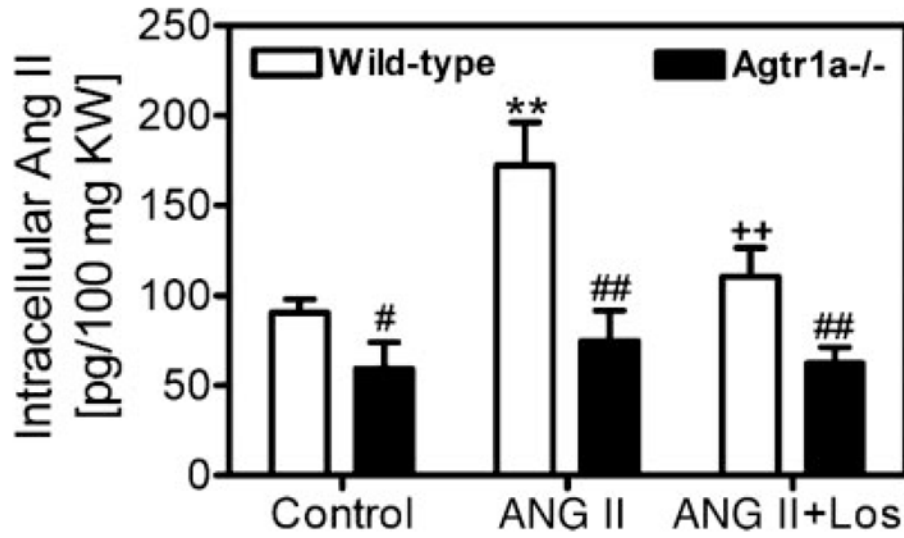
Fig. 6.
Renal intracellular ANG II levels in response to long-term ANG II infusion or concurrent administration of ANG II and losartan for 2 wk in wild-type and Agtr1a−/− mice. **$P < 0.001$ vs. control. ++$P < 0.01$ vs. ANG II infusion in wild-type mice. #$P < 0.05$ and ##$P < 0.01$ vs. corresponding wild-type mice.
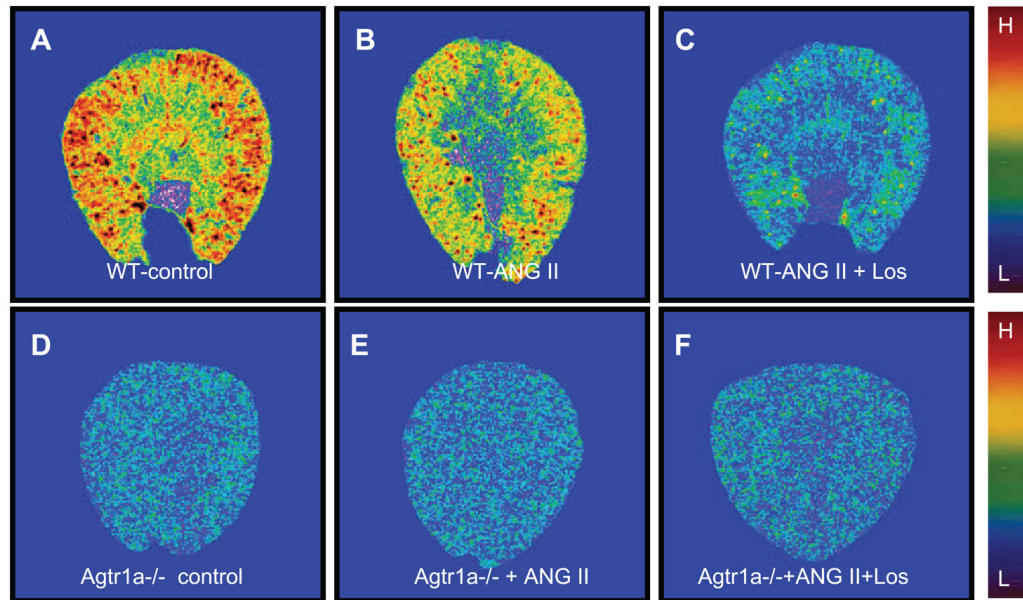
Fig. 7.
Quantitative in vitro autoradiographic mapping of AT_1 receptors in the kidneys of wild-type and Agtr1a−/− mice treated without (control) or with ANG II or with concurrent administration of ANG II and losartan for 2 wk. The levels of AT_1 receptor binding in the kidney are shown with red representing the highest and blue representing the background level of binding.
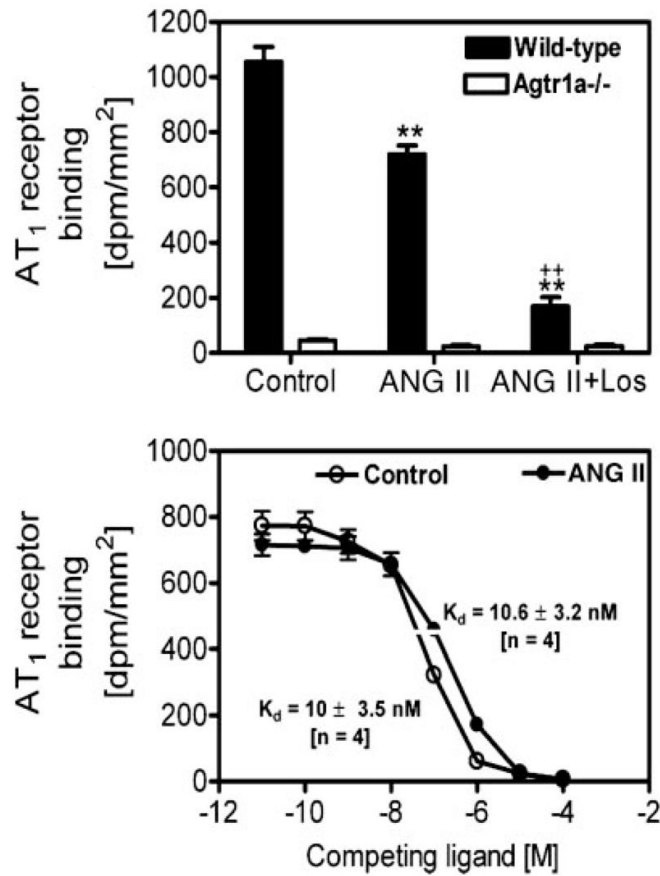
Fig. 8.
Quantitative results showing the effects of long-term administration of ANG II and/or losartan on the levels of AT_1 receptor binding in the kidneys of wild-type and Agtr1a−/− mice, or on the binding dissociation constant (K_d) in wild-type mice treated without (control) or with ANG II for 2 wk. **$P < 0.01$ vs. control. ++$P < 0.01$ vs. ANG II in the kidneys of wild-type mice.

Table 1

Basal systolic blood pressure and renal functional indexes in Agtr1a+/+, Agtr1a+/−, and Agtr1a−/− mice

Parameters	Agtr1a+/+	Agtr1a+/−	Agtr1a−/−
Body wt, g	26.9±1	28.2±0.8	27.5±1.1
Kidney wt, g	0.32±0.02	0.34±0.01	0.27±0.02^a
Kidney-to-body wt ratio, X100	1.2±0.03	1.2±0.01	1.0±0.04^a
Drinking, ml/24 h	4.2±0.2	4.9±0.4	7.2±0.8^a
SBP, mmHg	112±2	104±3^a	88±2^{a,c}
$U_{Na}V$, μmol/24 h	133.0±7.1	156.4±8.5^b	334.7±7.8^{a,c}
U_KV, μmol/24 h	257.8±22.5	313.6±23.3^b	648.8±8.5^{a,c}

Values are means ± SE. Agtr1a+/+, wild-type mice; Agtr1a+/−, heterozygous mice; Agtr1a−/−, homozygous mice; SBP, systolic blood pressure; $U_{Na}V$, urinary sodium excretion; U_KV, urinary potassium excretion.

[b] $P < 0.05$ or

[a] $P < 0.01$ vs. Agtr1a+/+; and

[c] $P < 0.01$ vs. Agtr1a+/−.

Table 2

SBP, drinking and renal excretory responses to ANG II infusion alone or concurrent administration of ANG II and the AT_1 receptor antagonist losartan during the 1st week in wild-type, Agtr1a+/−, and Agtr1a−/− mice

Responses	Wild-type			Agtr1a+/−			Agtr1a−/−		
	C	ANG II	ANG II+L	C	ANG II	ANG II+L	C	ANG II	ANG II+L
SBP, mmHg	113±4	153±7[b]	132±4[b,d]	104±3	145±4[b]	135±2[b,c]	89±5[f]	99±4[f]	95±3[f]
Drinking, ml/24 h	2.6±0.3	4.3±0.6[a]	2.9±0.2	5.2±0.2	5.5±0.4	4.4±0.4	9.3±0.7[f]	8.0±0.8[f]	7.6±0.5[f]
$U_{Na}V$, μmol/24 h	158±6.8	150±5.9	184±7.8	235±4.6	229±9.5	263±4.1	236±9.4[f]	252±14[f]	186±9.9
U_KV, μmol/24 h	397±18.1	263±10.5[a]	435±14.8[a]	539±5.6	354±24.7[b]	553±11.3[d]	527±19.3[e]	479±25[f]	426±16.7

Results are expressed as means ± SE. C, control or basal; L, losartan. The differences in the same response between different groups for the same strain of mice were analyzed by one-way ANOVA:

[a] $P < 0.05$ or

[b] $P < 0.01$ vs. control;

[c] $P < 0.05$ or

[d] $P < 0.01$ vs. ANG II. The differences in the same response between wild-type or Agtr1a+/− and Agtr1a−/− mice were analyzed by unpaired t-test:

[e] $P < 0.05$ or

[f] $P < 0.01$ versus wild-type or Agtr1a+/− mice.