Enhanced Angiotensin Receptor-Associated Protein in Renal Tubule Suppresses Angiotensin-Dependent Hypertension


Abstract—We have previously shown that angiotensin II type 1 receptor-associated protein (ATRAP/Agtrap) interacts with the angiotensin II type 1 receptor and promotes constitutive internalization of the receptor so as to inhibit the pathological activation of its downstream signaling but preserve baseline physiological signaling activity. The present study was designed to investigate the role of renal ATRAP in angiotensin II–dependent hypertension. We generated transgenic mice dominantly expressing ATRAP in the renal tubules, including renal distal tubules. The renal ATRAP transgenic mice exhibited no significant change in blood pressure at baseline on normal salt diet. However, in the renal ATRAP transgenic mice compared with wild-type mice, the following took place: (1) the development of high blood pressure in response to angiotensin II infusion was significantly suppressed based on radiotelemetry, (2) the extent of daily positive sodium balance was significantly reduced during angiotensin II infusion in metabolic cage analysis, and (3) the renal Na⁺-Cl⁻cotransporter activation and α-subunit of the epithelial sodium channel induction by angiotensin II infusion were inhibited. Furthermore, adenoviral overexpression of ATRAP suppressed the angiotensin II–mediated increase in the expression of α-subunit of the epithelial sodium channel in mouse distal convoluted tubule cells. These results indicate that renal tubule–dominant ATRAP activation provokes no evident effects on blood pressure at baseline but exerts an inhibitory effect on the pathological elevation of blood pressure in response to angiotensin II stimulation, thereby suggesting that ATRAP is a potential target of interest in blood pressure modulation under pathological conditions. (Hypertension. 2013;61:1203-1210.)

Key Words: angiotensin II ■ angiotensin receptors ■ basic science ■ gene expression/regulation ■ hypertension (kidney) ■ membrane transport/ion channels ■ receptors

Activation of angiotensin II (Ang II) type 1 receptor (AT₁R) through the tissue renin–angiotensin system plays a pivotal role in the pathogenesis of hypertension and associated end-organ injury. In addition, the activation of renal AT₁R signaling plays a key role in the altered renal sodium handling, which occurs in angiotensin-dependent hypertension.¹⁻³ This is consistent with Guyton’s hypothesis that defective handling of sodium by the kidney with a consequent dysregulation of body fluid volume is the requisite final common pathway in the pathogenesis of hypertension.⁴ The carboxyl (C)-terminal domain of AT₁R is involved in the control of AT₁R internalization independent of G protein coupling.⁵⁻⁸ It plays an important role in linking receptor-mediated signal transduction with the specific biological response to Ang II. The AT₁R-associated protein (ATRAP/Agtrap) has been identified as the specific binding protein of the C-terminal domain of AT₁R.³⁻⁴ ATRAP is expressed in many tissues, including the kidney, as is AT₁R. Our preceding studies suggest that ATRAP selectively suppresses Ang II–mediated pathological activation of AT₁R signaling in cardiovascular cells, and that cardiac ATRAP enhancement ameliorates cardiac hypertrophy in chronic Ang II–infused mice without affecting baseline cardiovascular function including blood pressure (BP).⁹⁻¹³

With respect to the intrarenal distribution of ATRAP, its protein was found to be widely expressed along the renal tubules,
with a weak level in the vascular smooth muscle cells of the vasculature, including the interlobular arteries, Bowman capsule, podocytes, and mesangial cells in the glomerulus.\(^{13,14}\)

However, despite there being abundant kidney ATRAP expression and that various pathological stimuli, including Ang II, are reported to downregulate renal ATRAP expression, little is known about actual function of renal ATRAP.\(^{15,16}\) The present study was designed to obtain in vivo evidence of renal ATRAP, with a special focus on Ang II–dependent hypertension by using transgenic (Tg) mice with a pattern of kidney-dominant ATRAP overexpression.

**Materials and Methods**

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were reviewed and approved by the Animal Studies Committee of Yokohama City University. Methods are described in detail in the online-only Data Supplement.

**Results**

**Generation of Renal ATRAP Tg Mice**

We generated Tg mice with a pattern of kidney-dominant overexpression of ATRAP (Figure 1S1B and S1C in the online-only Data Supplement). One of 6 lines of ATRAP Tg mice exhibited renal overexpression of the transgene hemagglutinin-tagged mouse ATRAP (HA-ATRAP) in comparison with wild-type (Wt) littermate mice (Figure 1B and Figure 1S1C) with scant levels of the transgene HA-ATRAP protein in the other tissues examined (Figure 1A). As shown in Figure 1B, the level of renal total ATRAP protein expression detected by the anti-ATRAP antibody was ≈10-fold higher in Tg mice (endogenous ATRAP and transgene HA-ATRAP) than in Wt mice (endogenous ATRAP).

To determine the expression and distribution of the ATRAP protein in the kidney of Tg mice, we performed an immunohistochemical examination using anti-HA antibody, anti-ATRAP antibody, and antibodies to specific nephron markers (Figure 1C). Although the renal expression of the HA-ATRAP protein prevailed over the endogenous ATRAP protein, histological analysis revealed a similar intrarendal distribution of HA immunostaining (transgene HA-ATRAP protein detected by the anti-HA antibody) and ATRAP immunostaining (transgene HA-ATRAP and endogenous ATRAP proteins detected by the anti-ATRAP antibody), mainly in the cortex.

We next stained consecutive sections with markers specific to the tubular segments. We used a polyclonal antibody against aquaporin-2, which is specifically expressed in the collecting ducts; a monoclonal antibody against calbindin-D, a calcium-binding protein expressed primarily in the distal convoluted tubules (DCT) and connecting tubules; and a monoclonal antibody against megalin, which is specifically expressed in the proximal convoluted tubules; and found that a high level of ATRAP immunostaining was predominantly detected along the renal distal tubules from the DCT to connecting tubules in the renal cortex. As shown in Figure 1D, the distal tubule–dominant expression of HA-ATRAP transgene was quantified by a laser capture microdissection method. The ATRAP mRNA expression in the distal tubules of renal cortex was ≈33.7-fold higher in Tg mice than in Wt mice. However, the ATRAP mRNA expression in the proximal tubules of renal cortex was only 3.5-fold higher in Tg mice than in Wt mice.

**Suppression of Ang II–Dependent Hypertension in Renal ATRAP Tg Mice**

The baseline 24-hour mean systolic BP (SBP), measured by a radiotelemetry method, was comparable between Wt and Tg mice (male, 14–18 weeks of age; 126±2 versus 122±1 mm Hg, unpaired t test; \(P=0.12\); Figure 2A and 2C). However, the SBP elevation by Ang II infusion (1000 ng/kg per min) was significantly suppressed in Tg mice compared with Wt mice (Figure 2A; 2-way repeated measures ANOVA \(F=7.476; P=0.0257\), Figure 2B; unpaired t test; \(P=0.0023\)). We also examined the effect of a higher dose of Ang II infusion (2000 ng/kg per min) on the SBP of Wt and Tg mice, and the difference in the Ang II–induced SBP elevation between Wt and Tg mice was more prominent at the higher dose (2000 ng/kg per min) of Ang II (Figure 2C; 2-way repeated measures ANOVA \(F=9.035; P=0.0012\)) (Figure 2D; unpaired t test; \(P=0.0017\)).

**Increase in Urinary Sodium Excretion in Renal ATRAP Tg Mice**

We hypothesized that renal enhancement of ATRAP might suppress angiotensin-dependent hypertension by influencing the handling of renal sodium and performed metabolic cage analysis (Figure S2A–S2D). Because urinary sodium excretion was significantly increased in Tg mice compared with Wt mice during the infusion period (Figure S2D; 2-way repeated measures ANOVA \(F=12.91; P=0.0029\), we analyzed daily sodium balance during Ang II infusion and cumulative sodium balance during the early phase (day 1–6) of Ang II infusion to more exactly compare the status of renal sodium handling between Tg and Wt mice.

As shown in Figure 3A, although sodium balance was comparable in Tg and Wt mice at baseline, the extent of daily positive sodium balance was significantly reduced in Tg mice compared with Wt mice during Ang II infusion (2-way repeated measures ANOVA \(F=11.37; P=0.0046\)). Furthermore, the extent of cumulative positive sodium balance during the early phase (day 1–6) was also significantly decreased in Tg mice compared with Wt mice (Figure 3B; 2-way repeated measures ANOVA \(F=7.04; P=0.043\) consistently with facilitated natriuresis as a mechanism for the resistance to hypertension in Tg mice.

With respect to the role of increased natriuresis during the later phase (day 7–9) in the lower BP in Tg mice (Figure 3A and Figure 2S2D), the difference in SBP between Tg and Wt mice became larger from day 8 to day 11 (Figure 2C; the SBP difference between Tg and Wt mice, 17 mm Hg on day 8 and 31 mm Hg on day 11), which also is consistent with facilitated natriuresis as the mechanism for the resistance to hypertension in Tg mice. However, body weight changes tended to be larger in Tg mice than Wt mice, but the differences did not reach statistical significance (Figure S2E). Accordingly, these results indicate that renal distal tubule–dominant overexpression of ATRAP suppressed Ang II–dependent hypertension, probably via a suppression of sodium reabsorption in vivo.
Suppression of Phosphorylated Na\(^+\)–Cl\(^-\) Cotransporter and \(\alpha\)-Subunit of the Epithelial Sodium Channel Expression in the Kidneys of Tg Mice

To examine mechanisms involved in the suppression of sodium reabsorption in response to Ang II in Tg mice, we compared renal mRNA expression of the major sodium transporters (sodium-proton antiporter 3, NHE3; sodium-potassium–two-chloride cotransporter, NKCC2; Na\(^+\)–Cl\(^-\) cotransporter, NCC; and epithelial sodium channel, ENaC subunits). Age-matched Wt and Tg mice were divided into 4 groups: (1) vehicle-infused Wt mice, (2) Ang II–infused Wt mice, (3) vehicle-infused Tg mice, and (4) Ang II–infused Tg mice. The results of quantitative real-time polymerase chain reaction analysis showed that Ang II infusion for 11 days significantly increased the renal mRNA levels of \(\alpha\)ENaC by 2.3-fold, and the \(\beta\)ENaC and \(\gamma\)ENaC mRNA levels also tended to increase in response to Ang II infusion, but without statistical significance in Wt mice (Figure S3). On the contrary, the Ang II–mediated upregulation of \(\alpha\)ENaC mRNA was significantly suppressed in Tg mice.

With respect to protein expression of sodium transporters, the renal NHE3 protein levels were similar in Tg and Wt mice at baseline and decreased to a similar degree after Ang II infusion (Figure 4A). The phosphorylated NKCC2 levels were similar
in Tg and Wt mice at baseline and decreased in both groups by Ang II with a tendency to be lower in Tg mice than in Wt mice but without statistical significance (30±6 versus 46±6% \( P < 0.05 \) vs Wt mice. Figure 4B). However, although expression of phosphorylated NCC, which is the activated form of NCC and plays an important role in sodium reabsorption, was increased by Ang II with a tendency to be lower in Tg mice than in Wt mice (53±4 mm Hg) compared with Wt mice (36±2 mm Hg), Values are expressed as the mean±SE (n=6–7 in each group), \( *P<0.05 \) vs Wt mice. Figure 2. Effects of angiotensin II (Ang II) infusion on blood pressure (BP) analyzed by the radiotelemetric method in wild-type (Wt) and renal angiotensin II type 1 receptor-associated protein transgenic (Tg) mice. A, Daily and 24-hour systolic BP (SBP) in Wt and Tg mice before (pre) and during 11 days of Ang II (1000 ng/kg per min) infusion. Values are expressed as the mean±SE (n=5 in each group), \( *P<0.05 \) vs Wt mice. B, The increase in SBP during Ang II (1000 ng/kg per min) infusion was significantly less in Tg mice (17±4 mm Hg) compared with Wt mice (36±2 mm Hg). Values are expressed as the mean±SE (n=5 in each group), \( **P<0.01 \) vs Wt mice. C, Daily and 24-hour SBP in Wt and Tg mice before (pre) and during 11 days of Ang II (2000 ng/kg per min) infusion. Values are expressed as the mean±SE (n=6–7 in each group), \( **P<0.01 \) vs Wt mice. D, The increase in SBP during the Ang II (2000 ng/kg per min) infusion was significantly less in Tg mice (28±5 mm Hg) compared with Wt mice (53±4 mm Hg). Values are expressed as the mean±SE (n=6–7 in each group), \( **P<0.01 \) vs Wt mice.

Discussion

This is the first report, to the best of our knowledge, of an inhibitory function of renal tubular ATRAP in angiotensin-dependent hypertension without an influence on baseline BP. In this study, chronic Ang II infusion was performed at 1000 and 2000 ng/kg per min to examine the effects of distal tubule–dominant overexpression of ATRAP on the Ang II–mediated BP increase. Although the higher dose of Ang II (2000 ng/kg per min) is reported to provoke a reduction in food intake and to cause Ang II–induced wasting and skeletal muscle atrophy,17 the lower dose of Ang II (1000 ng/kg per min) has been used in many previously performed experiments in mice.18,19 and suppression of the Ang II–induced BP increase by the distal tubule–dominant overexpression of ATRAP was observed with both the lower and higher doses of Ang II in the present study.

In the present study, the BP at baseline was not affected by renal ATRAP overexpression. On the contrary, the genetic inactivation of other renin–angiotensin system components, such as angiotensinogen, renin, and AT1R, was reported to result in significant decreases in BP, as well as an alteration in renal morphology and function compared with Wt mice even under baseline conditions.20,21 Thus, ATRAP would be expected to act as a minor player among the renin–angiotensin system components, at least in terms of BP regulation and renal morphological development under physiological conditions. However, the results of present study seem to be consistent with those of our previous studies, which showed that ATRAP is not a general inhibitor of the AT1R signaling as are the clinically available AT1R-specific blockers, but rather specifically inhibits the pathological activation of its downstream signaling with preservation of baseline physiological signaling activity.9–13

With regard to the regulatory role of renal tubule AT1R in renal sodium handling, a previous study reported that Ang II did not affect proximal tubule fluid reabsorption or sodium delivery to distal nephron segments, but sodium reabsorption in distal nephron segments was increased in Ang II–infused

ENaC is activated by aldosterone through its binding to the mineralocorticoid receptor. Therefore, to analyze the direct effect of ATRAP on αENaC, we examined whether overexpression of ATRAP would suppress the Ang II–mediated ENaC subunit expression in mouse DCT cells by performing adenoviral transfer of recombinant ATRAP. Although Ang II (10^{-6} \text{ mol/L}) treatment of mouse DCT cells infected with control bacterial β-galactosidase cDNA (Ad. LacZ) increased the αENaC mRNA expression, mouse DCT cells infected with adenoviral vector containing ATRAP cDNA (Ad.HA-ATRAP) exhibited an inhibition of the Ang II–induced enhancement (Figure 5B), thereby indicating that ATRAP directly suppressed the Ang II–mediated activation of αENaC expression, independent of the aldosterone-mineralocorticoid receptor pathway.

**αENaC expression was significantly less in Tg mice (28±5 mm Hg) compared with Wt mice (53±4 mm Hg). Values are expressed as the mean±SE (n=6–7 in each group), **P<0.01 vs Wt mice.**
neered mice were different among the studies, further studies
However, because strain backgrounds of these genetic engi-
kg per min) concomitantly with promotion of natriuresis.
examined the effect of proximal tubule–specific AT 1R dele-
mice than in Wt mice (Tg versus Wt, 17 versus 36 mm
increase on telemetry) on the same dose of
mice.22 Hashimoto et al23 also observed that disruption of tis-
<0.05 vs Wt mice.
A, Daily and 24-hour sodium balance in Wt and Tg mice before (pre) and during Ang II (2000 ng/kg per min) infusion. Values are expressed as the means±SE (n=6 in each group), **P<0.01 vs Wt mice.
B, Cumulative sodium balance during the 6 days (day 1–6) of Ang II infusion in Wt and Tg mice. Values are expressed as the means±SE (n=6 in each group), *P<0.05 vs Wt mice.
mice.22 Hashimoto et al23 also observed that disruption of tis-
and ENaCs.22,29–33 The results of recent studies showed that
In the proximal tubules, NHE3 plays an important role in
the distal tubule (PTKO mice), on Ang II–mediated BP elevation and
It is necessary to further investigate the role of renal ATRAP in angiotensin-dependent hypertension in vivo using cellular-targeted models. Another limitation is the lack of functional data with regard to the activity of NCC and NKCC2, such as diuretic tests or clearance experiments. In addition, because 33.7-fold increase in ATRAP mRNA expression in the distal tubules of Tg mice compared with Wt mice could not completely inhibit the Ang II–mediated NCC activation, the effect of ATRAP seems to be, at best, minor in the present study.

Enhanced Renal Tubular ATRAP Inhibits Hypertension

In the distal nephron, the modulation of sodium reabsorption in response to stimuli, such as Ang II, is mediated by NCC and ENaCs.22,29–33 The results of recent studies showed that Ang II induces phosphorylation of the renal NCC through with-no-lysine kinase 4–dependent pathway, independent of aldosterone.30,32,33 However, the ENaCs consist of 3 homologous subunits (α, β, and γ), and αENaC is reported to play an essential role in the formation of a functional ion channel among the ENaC subunits.34,35 Previous studies also showed a regulatory role of AT, R signaling in the renal αENaC expression and an antihypertensive effect of ENaC blockade in angiotensin-dependent hypertension.36,37 In the present study, we demonstrated that the enhancement of ATRAP in the distal nephron significantly suppressed the activation of NCC and the upregulation of αENaC by Ang II stimulation in vivo and, further, that overexpression of ATRAP completely suppressed Ang II–mediated activation of αENaC expression using mouse DCT cells. These results suggest that inhibition of NCC activity and downregulation of αENaC expression are likely to be involved in the suppression of angiotensin-dependent hypertension in renal ATRAP Tg mice.

Nevertheless, a limitation of the present study is that the results do not allow us to completely distinguish ATRAP functions in the distal tubules of the kidney. Tg mice with distal tubule–dominant overexpression of ATRAP were unexpectedly and fortuitously obtained on screening for cellular expression in these Tg animals. This model is not a specifically targeted cellular overexpression model, but rather a model in which there is variation in ATRAP expression within the nephron. Although the distal tubule is a predominant ATRAP expression site, other nephron segments, including the proximal tubules, do overexpress ATRAP to some degree in Tg mice. Therefore, it is necessary to further investigate the role of renal ATRAP in angiotensin-dependent hypertension in vivo using cellular-targeted models. Another limitation is the lack of functional data with regard to the activity of NCC and NKCC2, such as diuretic tests or clearance experiments. In addition, because 33.7-fold increase in ATRAP mRNA expression in the distal tubules of Tg mice compared with Wt mice could not completely inhibit the Ang II–mediated NCC activation, the effect of ATRAP seems to be, at best, minor in the present study.
Hypertension is the most common chronic disease worldwide. It is a multifactorial disease in which genetic and environmental factors are intricately intertwined. Understanding the mechanism underlying hypertension is thus extremely complex, and caution should be used in interpreting the findings of this study in Tg mice in terms of the pathophysiology of human hypertension. Nevertheless, the findings of the present study do provide a useful basis for the further investigation of the in vivo functional roles of ATRAP in angiotensin-dependent hypertension and also suggest the potential benefit of an ATRAP activation strategy.

**Figure 4.** Suppression of the angiotensin II (Ang II)-mediated renal sodium chloride cotransporter (NCC) activation and α-subunit of the epithelial sodium channel (αENaC) upregulation in renal Ang II type 1 receptor-associated protein transgenic (Tg) mice. Effects of Ang II (2000 ng/kg per min) infusion on protein expression of the major sodium transporters, sodium-potassium-potassium-two-chloride cotransporter (NKCC2), NCC, αENaC, βENaC, and γENaC in the kidneys of wild-type (Wt) and Tg mice. Values are expressed as the mean±SE (n=6 in each group). *P<0.05 vs vehicle. †P<0.05 vs Wt mice.

**Perspectives**

Hypertension is the most common chronic disease worldwide. It is a multifactorial disease in which genetic and environmental factors are intricately intertwined. Understanding the mechanism underlying hypertension is thus extremely complex, and caution should be used in interpreting the findings of this study in Tg mice in terms of the pathophysiology of human hypertension. Nevertheless, the findings of the present study do provide a useful basis for the further investigation of the in vivo functional roles of ATRAP in angiotensin-dependent hypertension and also suggest the potential benefit of an ATRAP activation strategy.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Angiotensin II (Ang II) type 1 receptor–associated protein (ATRAP), a specific binding molecule to Ang II type 1 receptor, inhibits pathological activation of Ang II type 1 receptor in local tissues but is downregulated in the kidney by Ang II. In transgenic mice dominantly expressing ATRAP in renal distal tubules, Ang II–induced hypertension was found to be attenuated with a concomitant increase in natriuresis via a suppression of the epithelial sodium channel.

**What Is Relevant?**

- A potential therapeutic effect of ATRAP activation in the renal distal tubule on Ang II–mediated salt-sensitive hypertension was implicated. This observation suggests that ATRAP is a target of interest in hypertension.

**Summary**

The findings in this study suggest a possible role for renal distal tubule ATRAP in blood pressure regulation.
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**Supplemental Materials and Methods**

**Generation of ATRAP Transgenic (Tg) Mice**

A speed congenic method was used to generate the ATRAP Tg mice. C3H/He and C57BL/6J were selected as the parental inbred lines and backcrossed to a pure C57BL/6J background. Hemagglutinin-tagged mouse ATRAP (HA-ATRAP) cDNA was subcloned into a Tg vector between the 1.2-kb fragment of the cytomegalovirus enhancer-promoter and the bovine growth hormone polyadenylation sequence, as illustrated in **Supplemental Figure S1A**. This transgene was microinjected into the pronucleus of fertilized mouse embryos. The resulting pups were screened for the presence of the transgene by PCR, using forward (5’-TGCTTGGGGCAACTTCACTATC-3’) and reverse (5’-ACGGTGCTGTGTGGTAGACGAG-3’) primers. PCR was performed as follows: 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min for 35 cycles, with a final extension step at 72°C for 10 min.

**BP Measurements and Metabolic Cage Analysis**

All of the mice were fed a normal (0.3%) sodium diet throughout the present study. BP and Heart rate (HR) were measured in the conscious state using a radiotelemetry system, as described previously.\(^1\) Briefly, under anesthesia with isoflurane, an incision was made from the chin to the superior sternum and the left common carotid artery was surgically exposed. A small incision was made in the artery adjacent to the bifurcation, and the tip of a BP transducer (PA-C10, Data Science International [DSI]) was placed in the artery. The catheter was then tied and the transducer secured in place under the skin of the right flank with tissue adhesive. All skin wounds were closed with 5-0 nylon (Sigma Rex). Fourteen days after transplantation, when the circadian rhythm had been restored, mice were acclimated for the following week to metabolic cages (Techniplast). After an additional 3 days of baseline, Ang II (1000 or 2000 ng/kg/min) was continuously infused subcutaneously into the mice via an osmotic minipump (ALZA) for 11 days and hemodynamic measurements were recorded every 5 min using the software Dataquest A.R.T. 4.1 (DSI). The BP values at baseline were the average of data obtained on three consecutive days. Daily BW, food intake and water intake were measured, and urine was collected. The mice were given free access to tap water and fed the indicated diet. The urinary excretion of angiotensinogen was measured using ELISA, as described previously.\(^2\)
Analysis of ATRAP and HA-ATRAP Protein Expression
The characterization and specificity of the anti-mouse ATRAP antibody was described previously in detail.\textsuperscript{1,3,4} The anti-HA polyclonal antibody was obtained from Bethyl Laboratories (A190-107A). Western blot analysis was performed as described previously.\textsuperscript{1,3,4} Briefly, the total protein was extracted from the tissues with SDS-containing sample buffer, and the protein concentration of each sample was measured with a DC protein assay kit (Bio-Rad) using bovine serum albumin as the standard. Equal amounts of protein extract from the tissue samples were fractionated on a 5-20\% polyacrylamide gel (ATTO), then transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked for 1 h at room temperature with phosphate-buffered saline containing 5 \% skim milk powder, and probed overnight at 4\°C with specific primary antibodies. Then the membranes were washed and incubated with secondary antibodies for 40 min at room temperature. After they were washed, the sites of the antibody-antigen reaction were visualized by enhanced chemiluminescence substrate (GE healthcare). The images were quantitated using a FUJI LAS3000 Image Analyzer (FUJI Film).

Membranous Protein Extraction and Immunoblot Analysis for Sodium Channels
Membranous proteins were extracted from kidney tissues using the Plasma Membrane Extraction Kit (Biovision; K268-50) according to the manufacturer’s protocol and then used for SDS-PAGE. Membranes were incubated with affinity-purified primary antibodies to NHE3, phosphorylated NKCC2, NKCC2, phosphorylated NCC, NCC, and the \(\alpha\), \(\beta\), and \(\gamma\) subunits of the ENaC. The antibody against NHE3 was obtained from Alpha Diagnostic Intl. Inc. (NHE31-A); The antibody against phospho-NKCC2 on Thr96 was kindly provided by Shih-Hua Lin (Tri-Service General Hospital, Taipei, Taiwan);\textsuperscript{5} The antibody against NKCC2 was obtained from Abcam (ab60301); The antibody against phospho-NCC on Ser71 was characterized previously.\textsuperscript{6} The antibody against NCC was obtained from Chemicon (AB3553); the antibody against \(\alpha\)ENaC was obtained from Affinity Bioreagents (PA1-920A); the antibody against \(\beta\)ENaC was obtained from Santa Cruz Biotechnology (sc-48428); the antibody against \(\gamma\)ENaC was obtained from Abcam (ab3468).
Real-Time Quantitative RT-PCR Analysis
Total RNA was extracted from the kidney with ISOGEN (Nippon Gene) and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed by incubating the RT product with the TaqMan Universal PCR Master Mix and designed TaqMan probe (NHE3: Mm01352473_m1, NKCC2: Mm01275821_m1, NCC: Mm00490213_m1, αENaC: Mm00803386_m1, βENaC: Mm00441215_m1, γENaC: Mm00441228_m1) (Applied Biosystems), as described previously. The RNA quantity was expressed relative to the 18S rRNA endogenous control.

Immunohistochemical Analysis
Immunohistochemistry was performed as described previously. Mice kidneys were perfusion-fixed with 4% paraformaldehyde and subsequently embedded in paraffin. The 4 µm-thick sections were dewaxed and rehydrated, and antigen retrieval was performed by microwave heating. The sections were blocked for endogenous biotin activity using Peroxidase Blocking Reagent (DAKO) and treated for 60 min with 10% normal goat serum in phosphate-buffered saline. The sections were then incubated with one of the following: 1) anti-HA antibody diluted at 1:100, 2) anti-ATRAP antibody diluted at 1:100, 3) anti-aquaporin 2 antibody (254-271, CALBIOCHEM) diluted at 1:200, 4) anti-calbindin D-28K antibody (C9848, Sigma-Aldrich) diluted at 1:3000, or 5) anti-megalin antibody (NB110-96417, Novus Biologicals) diluted at 1:1000.

Confocal Microscopy Analysis
Cryostat sections (5 µm) of snap-frozen kidneys from Wt and Tg mice were fixed with acetone for 5 minutes at room temperature and subsequent blocking with 2% BSA in PBST. Then, the sections were subjected to staining with anti-αENaC antibody, which was characterized previously, diluted at 1:50 overnight at 4°C, followed by an incubation with the Alexa Fluor-conjugated secondary antibody. Phalloidin was added for F-actin staining. DAPI was added for nuclear staining. The images were observed with a FV300 confocal laser microscopy (Olympus) immediately after immunostaining.

Laser Capture Microdissection (LMD) and Subsequent qRT-PCR Analysis
LMD was performed on a Leica LMD System (LMD6000) according to the
manufacturer's standard manual. Briefly, formalin-fixed paraffin-embedded tissues were cut into 10 µm-thick sections and mounted on polyethylene terephthalate (PET) membrane slides and stained with hematoxylin/eosin. Then, proximal or distal tubules in the renal cortex were microdissected under LMD 6000 lazer microdissection microscopy. A hundred areas (approximately 700,000 µm²) of the proximal or distal tubules were microdissected from the renal cortex per mouse. The total microdissected area was approximately 2,800,000 µm² in each group (N=4 in each group). Total RNA was extracted from the microdissected tissues using the RNeasy FFPE Kit (Qiagen) and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen), and applied to Taqman qRT-PCR analysis.

Distal Convoluted Tubule Cell Analysis

Mouse distal convoluted tubule (mDCT) cells were kindly provided by Dr. Peter A. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA). The cells had been previously isolated and functionally characterized.8-10 Cells were grown on 100-mm dishes (Corning) in DMEM/HAM F-12 media (1:1, Sigma-Aldrich) supplemented with 5% heat-inactivated fetal calf serum (MBL), 2 mM L-glutamine (GIBCO), 0.5 mg/ml streptomycin, 0.5 mg/ml penicillin, and 1 mg/ml neomycin (GIBCO), in a humidified atmosphere of 5% CO2-95% air. Adenoviral vectors were prepared using cDNAs coding for the NH2-terminal HA epitope-tagged ATRAP (Ad.HA-ATRAP) and bacterial β-galactosidase (Ad.LacZ) using a commercially available system (Adeno X Expression System, Clontech), and the virus titer was determined with a plaque assay.11 For the adenoviral gene transfer experiments, mDCT cells were subcultured in 6 cm-diameter dishes (5×10⁴/ml), incubated overnight, infected with recombinant adenovirus (Ad.HA-ATRAP or Ad.LacZ) at 50 multiplicity of infection for 24 h, and further incubated in a serum-free medium for an additional 24 h. The cells were then treated with Ang II (10⁻⁶ M) for the indicated time and subsequently harvested for analysis, as described previously.10, 12, 13

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. All the quantitative data are expressed as the means±SE. Differences were analyzed by Student's unpaired t-test or analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. Values of P<0.05 were considered
statistically significant.

**Supplemental References**


Supplemental Figure S1

A

CMV promoter → HA → ATRAP → BGH polyA

F → R

B

Endogenous ATRAP → HA-ATRAP

C

Kidney

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Supplemental Figure S1
Generation of Renal ATRAP Tg Mice
A, Schematic representation of the Tg vector. F and R indicate the locations of the forward and reverse primers used for the genotyping by PCR. CMV indicates cytomegalovirus, HA; hemagglutinin-tag, BGH polyA; bovine growth hormone polyadenylation. B, Agarose gel electrophoresis of PCR products after DNA amplification. The 755-bp endogenous ATRAP band appeared in both the Wt and ATRAP Tg mice founders. The 243-bp HA-ATRAP transgene band appeared only in the ATRAP transgenic mice founders. P indicates the positive control. C, Representative Western blot analysis of HA-ATRAP with the polyclonal anti-HA antibody in the kidney of Wt and ATRAP Tg mice founders.
Supplemental Figure S2

A

Sodium intake (mmEq)

Day of Ang II infusion

B

Water intake (ml)

Day of Ang II infusion

C

Urine volume (ml)

Day of Ang II infusion

D

Sodium excretion (mmEq)

Day of Ang II infusion

E

Body weight changes (%)

Day of Ang II infusion

Supplemental Figure S2
**Effects of Ang II Infusion on Metabolic Parameters and Urinary Patterns of Angiotensinogen in Renal ATRAP Tg Mice**

A, Daily sodium intake; B, water intake; C, urine volume; and D, urinary sodium excretion of the Wt and Tg mice during the Ang II (2000 ng/kg/min) infusion period. Sodium intake was comparable in the Wt and Tg mice during the infusion period (2-way repeated measures ANOVA $F=0.1047, P=0.75$). Water intake was significantly increased in the Tg mice compared with the Wt mice (2-way repeated measures ANOVA $F=5.288, P=0.037$). Urine volume tended to be increased in the Tg mice compared with the Wt mice (2-way repeated measures ANOVA $F=3.585, P=0.079$). Urine sodium excretion was significantly increased in the Tg mice compared with the Wt mice during the infusion period (2-way repeated measures ANOVA $F=12.91, P=0.0029$). Values are expressed as the mean±SE ($N=6$ in each group). *$P<0.05$, versus Wt mice; **$P<0.01$, versus Wt mice. E, BW changes calculated as follows: BW change = [(BW at day 11) - (BW at baseline)] / (BW at baseline)×100. Values are expressed as the mean±SE ($N=6$ in each group).

**Supplemental Figure S3**
Supplemental Figure S3
Suppression of Ang II-mediated Up-regulation of Renal $\alpha$ENaC mRNA in Renal ATRAP Tg Mice
Effects of Ang II infusion on the mRNA expression of major sodium transporters (NHE3, NKCC2, NCC and ENaC subunits) in the kidney of Wt and ATRAP Tg mice. Values are expressed as the mean±SE ($N=6$ in each group). *$P<0.05$, versus vehicle.