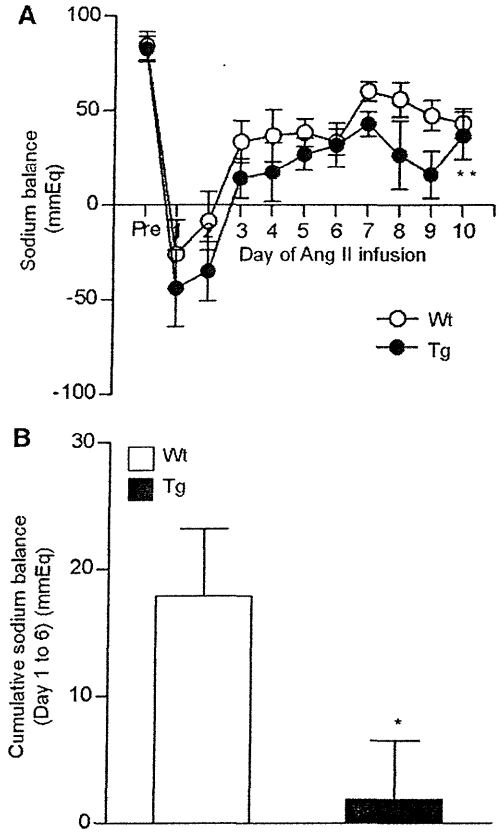

Figure 3. Effects of angiotensin II (Ang II) infusion on sodium balance in wild-type (Wt) and renal Ang II type 1 receptor-associated protein transgenic (Tg) mice. **A**, Daily and 24-hour sodium balance in Wt and Tg mice before (pre) and during Ang II (2000 ng/kg per min) infusion. Values are expressed as the mean±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 mean±SE (n=6 in each group), $^{*}P<0.05$ vs Wt mice.

mice.[22] Hashimoto et al[23] also observed that disruption of tissue angiotensin-converting enzyme did not alter proximal tubule fluid reabsorption. These results suggest that the distal nephron segments play a role in AT_1R signal–mediated renal sodium reabsorption in vivo.

On the contrary, Li et al[24] have shown that a reduction in baseline BP occurred when the proximal tubule AT_1R was selectively targeted in the kidney. In addition, Gurley et al[25] examined the effect of proximal tubule–specific AT_1R deletion, using mice lacking AT_1R only in the renal proximal tubule (PTKO mice), on Ang II–mediated BP elevation and showed that SBP elevation by Ang II (1000 ng/kg per min) was 15 mmHg lower in PTKO mice than in Wt mice (control) (PTKO versus control, 23 versus 38 mmHg increase on telemetry) with suppression of antinatriuresis, thereby indicating an important role of proximal tubule AT_1R in angiotensin-dependent sodium retention and hypertension. In the present study, SBP elevation by Ang II was 19 mmHg lower in Tg mice than in Wt mice (Tg versus Wt, 17 versus 36 mmHg increase on telemetry) on the same dose of Ang II (1000 ng/kg per min) concomitantly with promotion of natriuresis. However, because strain backgrounds of these genetic engineered mice were different among the studies, further studies are needed to examine whether the inhibitory effect of distal tubule–dominant ATRAP activation on angiotensin-dependent sodium retention and BP elevation is comparable with that of proximal tubule–specific AT_1R blockade.

In the proximal tubules, NHE3 plays an important role in sodium reabsorption, and previous in vitro studies reported that Ang II stimulation increases NHE3 expression to increase sodium reabsorption.[26,27] In addition, in the medullary thick ascending limb, NKCC2 is a major sodium transporter and is involved in sodium reabsorption. The present study showed that abundance of NHE3 and activation of NKCC2 were equivalent at baseline in the 2 groups and fell to a similar extent by Ang II infusion. The downregulation of these renal sodium transporters in response to Ang II–mediated hypertension may be 1 mechanism facilitating natriuresis as pressure increases,[28] which is consistent with the results observed by other group in the same Ang II–mediated hypertensive mice.[25]

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_1R 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.

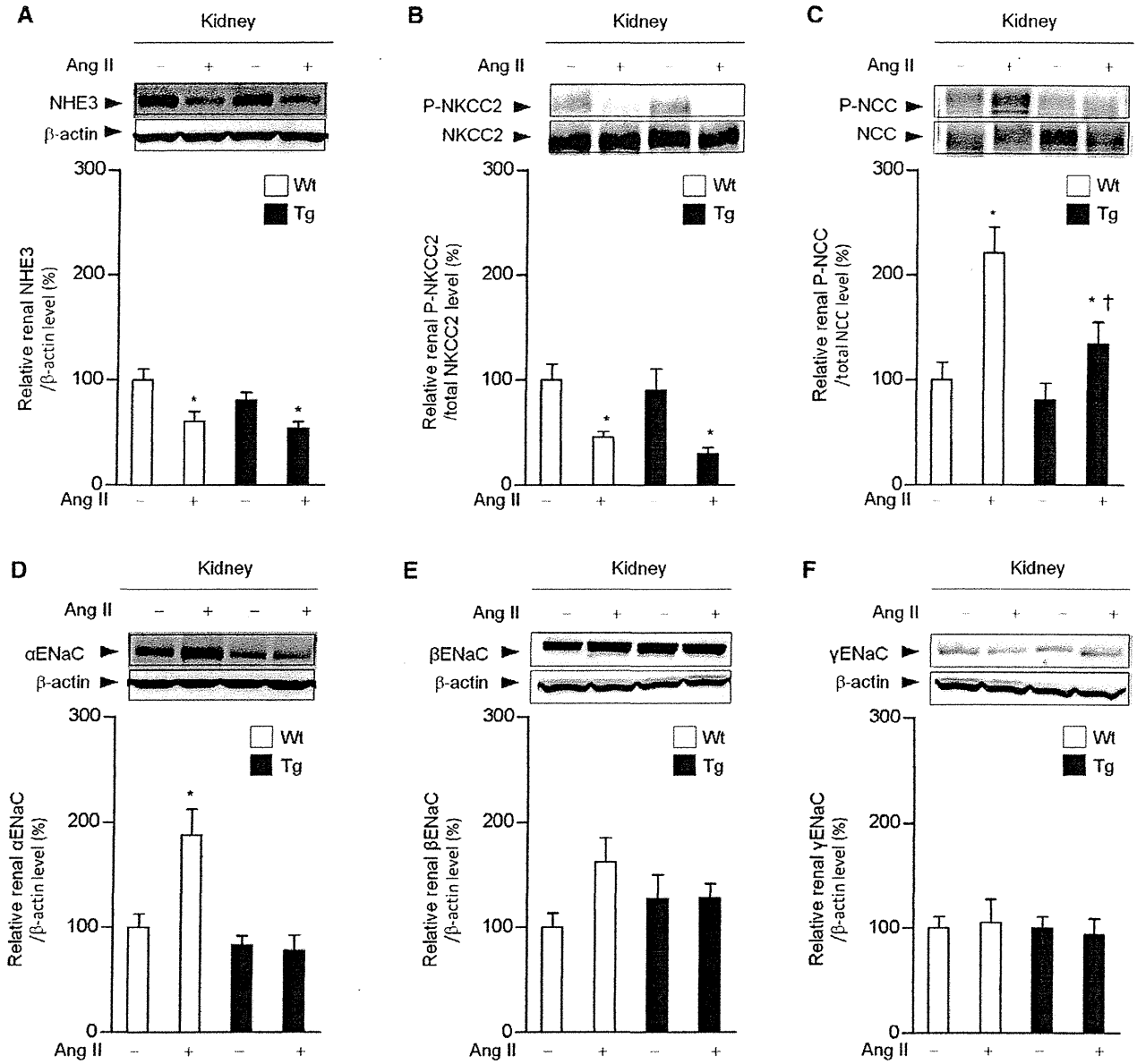

Figure 4. Suppression of the angiotensin II (Ang II)-mediated renal sodium chloride cotransporter (NCC) activation and α-subunit of the epithelial sodium channel (αENaC) upregulation in renal Ang II type 1 receptor-associated protein transgenic (Tg) mice. Effects of Ang II (2000 ng/kg per min) infusion on protein expression of the major sodium transporters, sodium-proton antiporter 3 (NHE3, **A**), sodium-potassium-two-chloride cotransporter (NKCC2, **B**), NCC (**C**), αENaC (**D**), βENaC (**E**), and γENaC (**F**) 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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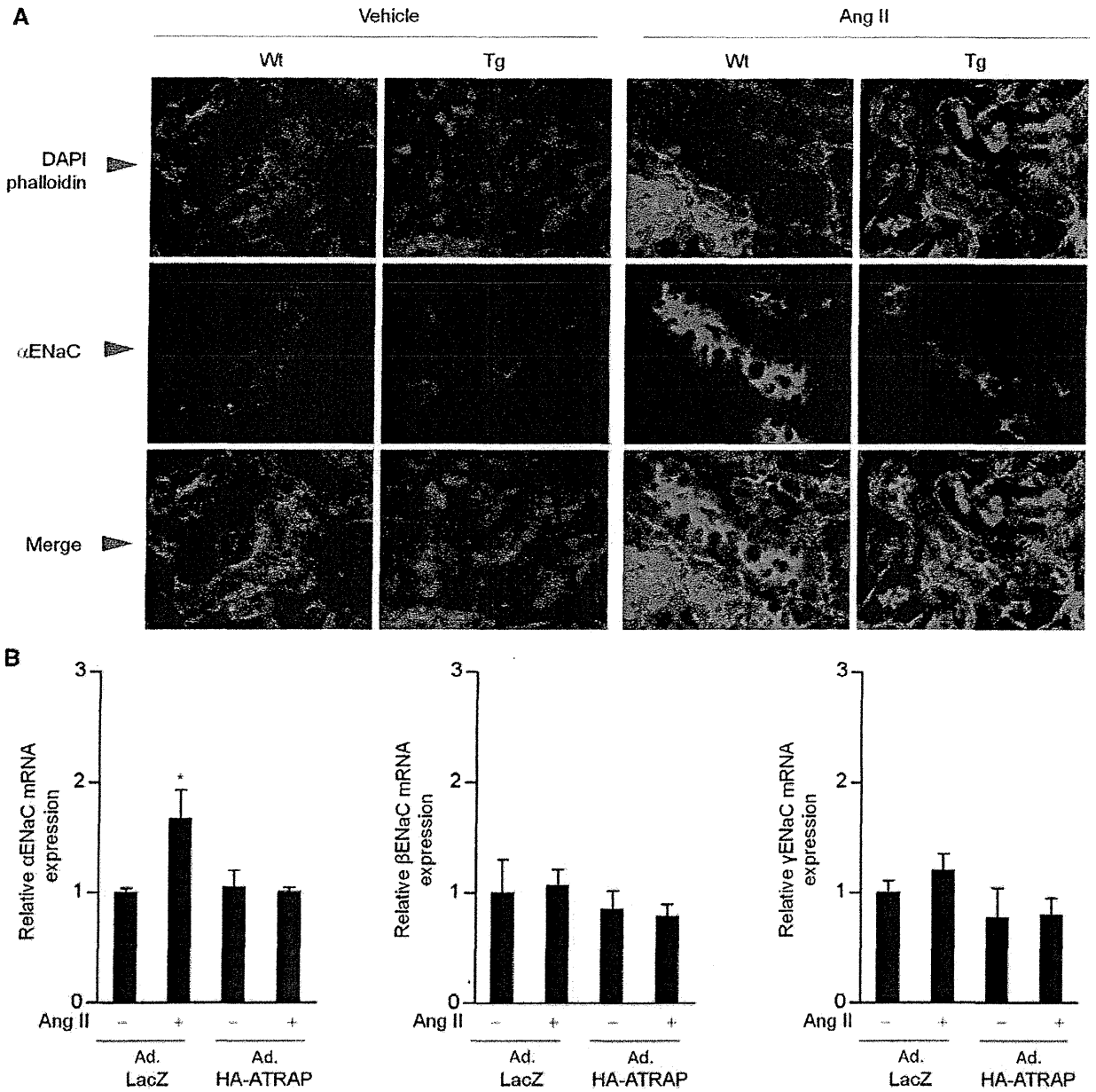

Figure 5. Suppression of α-subunit of the epithelial sodium channel (αENaC) by renal angiotensin II type 1 receptor-associated protein (ATRAP) is not caused by an effect on the trafficking of αENaC to the apical membrane but through the regulation of αENaC expression levels. **A**, Representative confocal laser-scanning microscopy image (×80) of the renal cortex from wild-type (Wt) and transgenic (Tg) mice. Phalloidin is green color; DAPI, blue; αENaC, red. Ang II, on day 11 after the start of Ang II infusion. **B**, Effects of Ang II and adenoviral transfer of recombinant ATRAP on mRNA expression of the α-, β-, and γ-subunits of ENaC in mouse distal convoluted tubule (mDCT) cells. Forty-eight hours after infection with adenoviral vector containing ATRAP cDNA (Ad.HA-ATRAP) or control bacterial β-galactosidase cDNA (Ad.LacZ), cells were stimulated with vehicle or Ang II at 10^{-6} mol/L for 24 hours. Values are calculated relative to those achieved with extracts from mDCT cells infected with Ad.LacZ and stimulated with vehicle and are expressed as means±SE (n=10–12 in each group). *P<0.05 vs vehicle.

Disclosures

None.

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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.

Online Supplement

Original research article:
Enhanced angiotensin receptor-associated protein in renal tubule suppresses angiotensin dependent hypertension (HYPE201200572.R2)

Running title:
Enhanced renal tubular ATRAP inhibits hypertension

Hiromichi Wakui, Kouichi Tamura, Shin-ichiro Masuda, Yuko Tsurumi-Ikeya, Megumi Fujita, Akinobu Maeda, Masato Ohsawa, Kengo Azushima, Kazushi Uneda, Miyuki Matsuda, Kenichiro Kitamura, Shinichi Uchida, Yoshiyuki Toya, Hiroyuki Kobori, Kiyotaka Nagahama, Akio Yamashita, Satoshi Umemura

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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'-ACGGTGCATGTGGTAGACGAG-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.[1, 3, 4] The anti-HA polyclonal antibody was obtained from Bethyl Laboratories (A190-107A). Western blot analysis was performed as described previously.[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 α, β and γ 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);[5] The antibody against NKCC2 was obtained from Abcam (ab60301); The antibody against phospho-NCC on Ser71 was characterized previously.[6] The antibody against NCC was obtained from Chemicon (AB3553); the antibody against αENaC was obtained from Affinity Bioreagents (PA1-920A); the antibody against βENaC was obtained from Santa Cruz Biotechnology (sc-48428); the antibody against γENaC 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.[1] the RNA quantity was expressed relative to the 18S rRNA endogenous control.

Immunohistochemical Analysis

Immunohistochemistry was performed as described previously.[3, 4] 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,[7] 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 terepthalate (PET) membrane slides and stained with hematoxlin/eosin. Then, proximal or distal tubules in the renal cortex were microdissected under LMD 6000 lazer microdissection microscopy. A hundred areas (approximately 700,000 μm^2) of the proximal or distal tubules were microdissected from the renal cortex per mouse. The total microdissected area was approximately 2,800,000 μm^2 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 (5×10^4/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.

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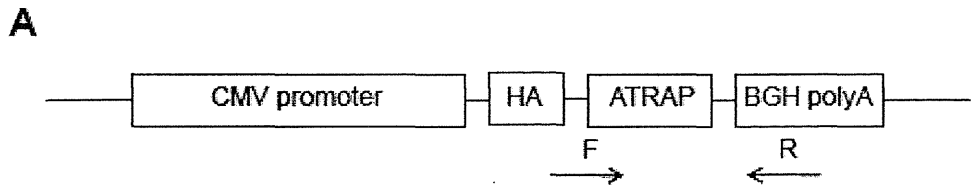
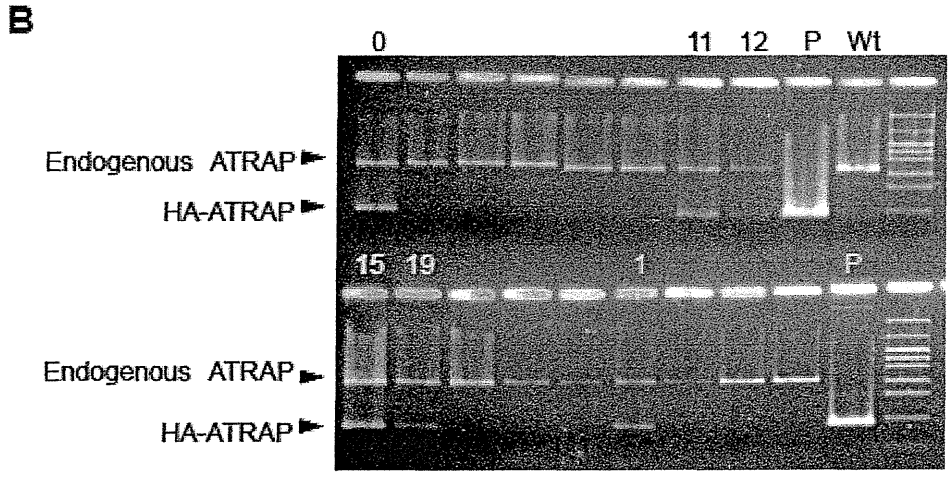
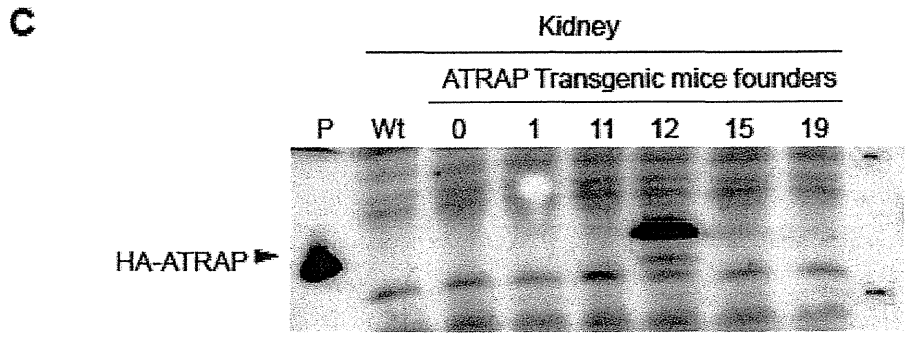
Supplemental Figure S1





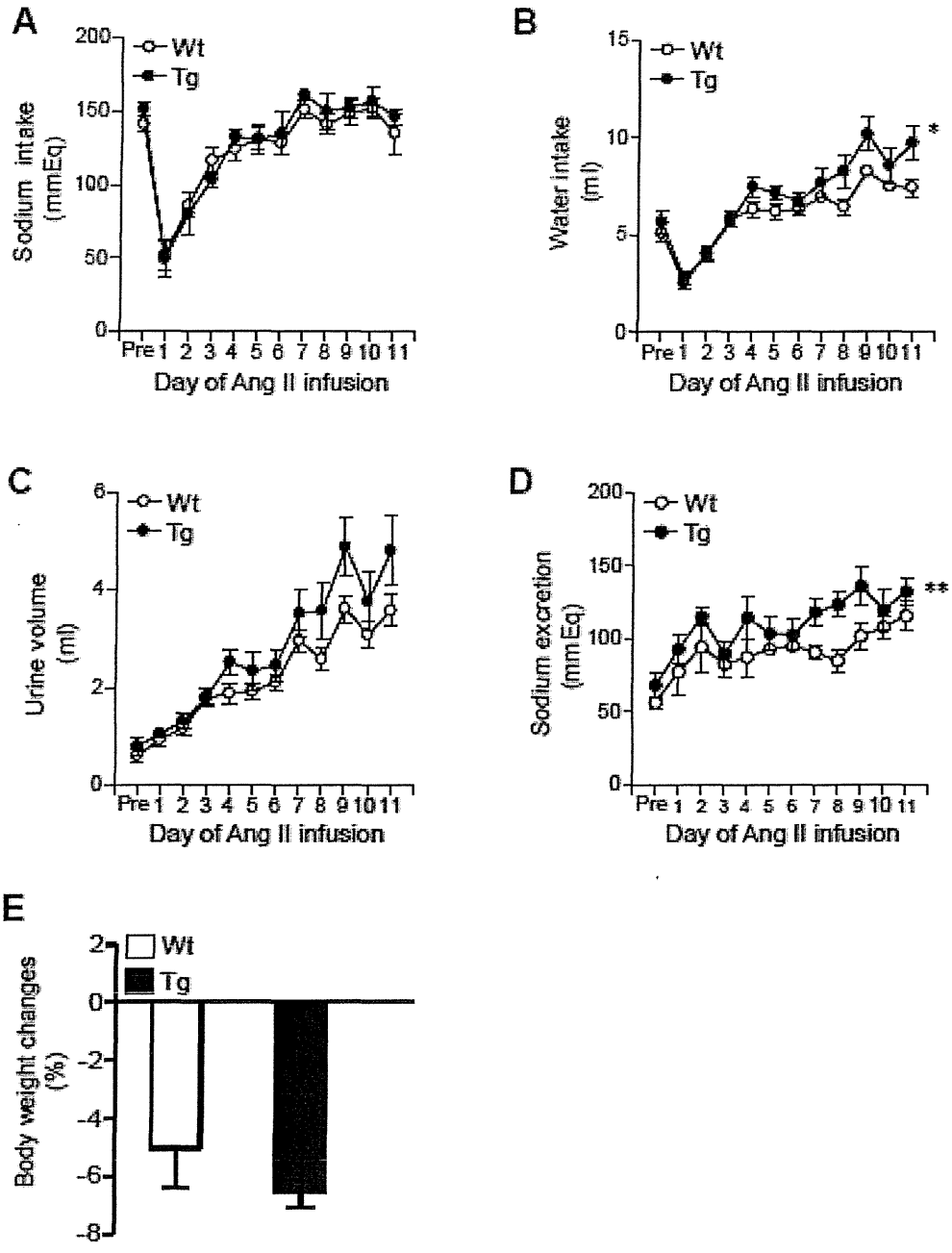


Supplemental Figure S1

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

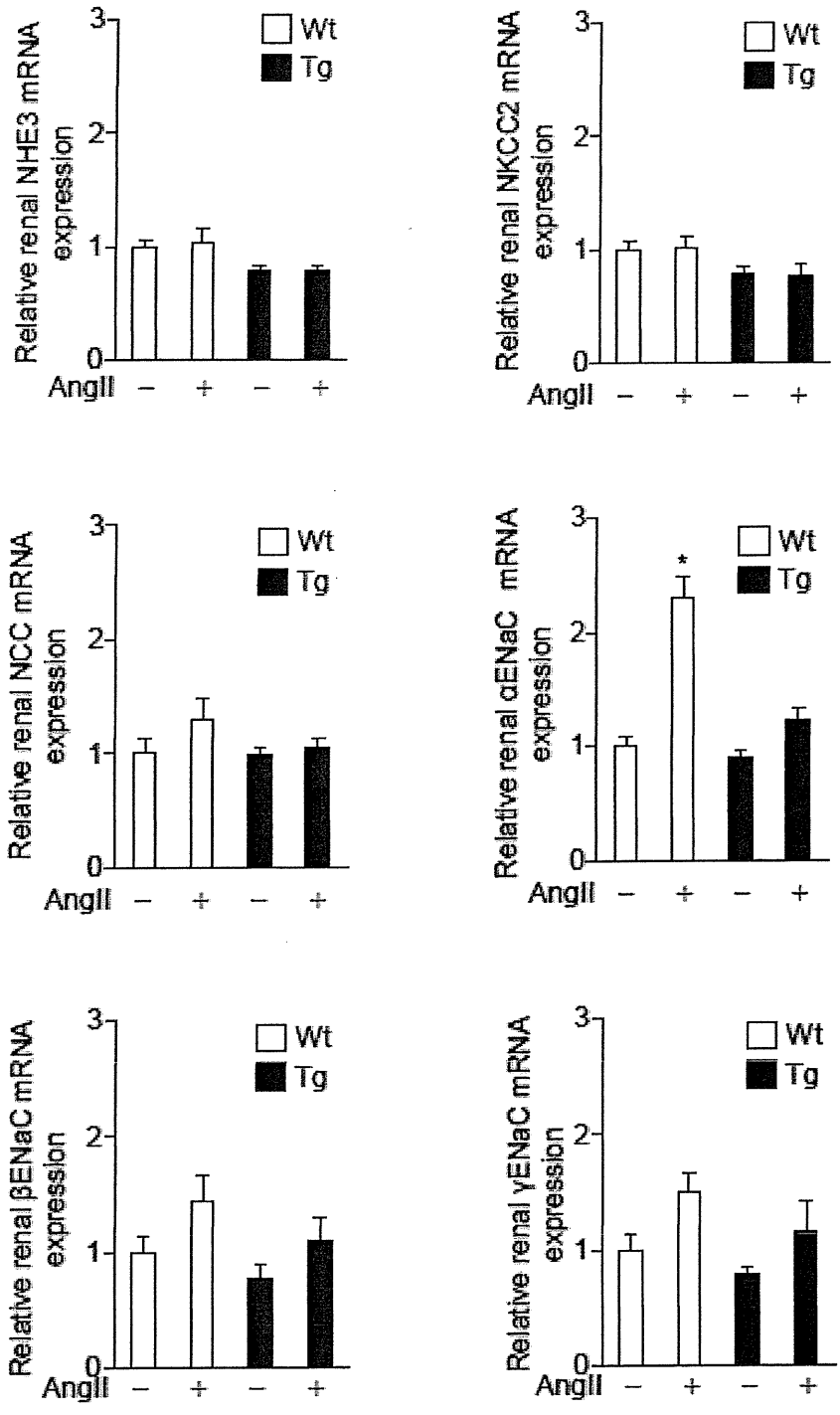


Supplemental Figure S2

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 αENaC 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.

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The Physiology and Pathophysiology of a Novel Angiotensin Receptor-binding Protein ATRAP/*Agtrap*

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Abstract: The Ang II type 1 receptor (AT1R)-associated protein (ATRAP/*Agtrap*) is a molecule specifically interacting with the carboxyl-terminal domain of AT1R. The results of *in vitro* studies showed that ATRAP suppresses Ang II-mediated pathological responses in cardiovascular cells by promoting AT1R internalization. With respect to the tissue distribution and regulation of ATRAP expression *in vivo*, ATRAP is broadly expressed in many tissues as is AT1R. Accumulating evidence indicates that a tissue-specific regulatory balancing of ATRAP and AT1R expression may be involved in the modulation of AT1R signaling at local tissue sites and also in the pathophysiology of hypertension and its associated end-organ injury. Furthermore, the activation of ATRAP in transgenic-models inhibited inflammatory vascular remodeling and cardiac hypertrophy in response to Ang II stimulation. These results suggest the clinical potential benefit of an ATRAP activation strategy in the treatment of hypertension and related organ injury.

Keywords: Gene expression/regulation, hypertension, receptor internalization, receptor signaling, renin-angiotensin system, target organ injury.

1. INTRODUCTION

Recent progress in molecular research in the fields of cardiovascular and renal medicine has identified several interesting molecules which interact with Ang II type 1 receptor (AT1R) or Ang II type 2 receptor (AT2R) to modulate respective receptor functions [1-3]. Particularly, a lot of preceding investigation aimed to identify molecules which directly bind to AT1R or AT2R and regulate activity of downstream signaling pathways, and a novel molecule which interacts with the carboxyl-terminal domain of AT1R was identified for the first time and named AT1R-associated protein (ATRAP/*Agtrap*) [4].

2. IDENTIFICATION OF ATRAP AS A NOVEL INTERACTING MOLECULE WITH AT1R

The G protein-coupled receptors (GPCRs) interact with different classes of intracellular proteins, including heterotrimeric G proteins, kinases, and arrestins [5-7]. Although the intracellular third loop of a number of GPCRs plays an important role as a structural determinant of coupling of the receptor to heterotrimeric G proteins, accumulated experimental results also highlighted the functional importance of the carboxyl-terminal cytoplasmic domain in receptor signaling and internalization [8-10]. Employing yeast two-hybrid screening of a mouse kidney cDNA library, with the carboxyl-terminal cytoplasmic domain of the mouse AT1R as a bait, a novel protein, with an open reading frame of 483 base pairs in its cDNA and with a predicted molecular mass of 18 kDa, was isolated and named ATRAP/*Agtrap* (for AT1R–associated protein) [4] (Fig. 1). The ATRAP did not interact with the carboxyl-terminal cytoplasmic domains of the AT2R and those of several Gq-coupled receptors such as m_3 muscarinic, bradykinin B_2, and endothelin B receptors, nor did it associate with the Gs-coupled β_2-adrenergic receptor. Thus, ATRAP is likely to be an AT1R-specific binding molecule to date. The human ATRAP/*Agtrap* cDNA was also cloned and the deduced polypeptide product of the cDNA was 22 kDa in size [11]. The human ATRAP/*Agtrap* cDNA and amino acid sequences were 85 and 77% identical to those of the mouse ATRAP/*Agtrap* gene, respectively.

3. PREDICTED DOMAIN STRUCTURE OF ATRAP

Characterization using cultured cells revealed ATRAP as a transmembrane protein localized in intracellular trafficking vesicles and plasma membrane [4, 12]. With respect to the domain structure, ATRAP is predicted *in silico* to contain three hydrophobic domains at the amino-terminal end of the protein, encompassing the amino acid residues 14-36, 55-77, and 88-108 and a hydrophilic cytoplasmic carboxyl-terminal tail from residues 109-161. The first transmembrane domain consists of a mixture of apolar and polar amino acid residues; the second and third transmembrane domains are composed mainly of hydrophobic residues with some polar amino acid residues.

4. WIDE TISSUE DISTRIBUTION OF ATRAP

To date several polyclonal anti-ATRAP antibodies, which are able to recognize the ATRAP protein specifically, were produced to examine the tissue distribution of ATRAP *in vivo* [13-17]. The results of Western blot analysis showed that the ATRAP antibody detected a major protein band at 18 kDa in the mouse tissues and that ATRAP was widely distributed in the mouse tissues as was AT1R [15]. The mouse ATRAP protein was expressed at relatively high levels in the kidney, lung, and testis, followed by the spleen, but at lower levels in the heart, brain, liver, skeletal muscles, and aorta. Similarly, the human ATRAP mRNA was most abundantly expressed in kidney, heart, pancreas and thyroid [11].

The results of immunohistochemistry using the polyclonal anti-ATRAP antibody within kidney sections from normal adult mouse and humans showed a relatively high level of ATRAP immunoreac-

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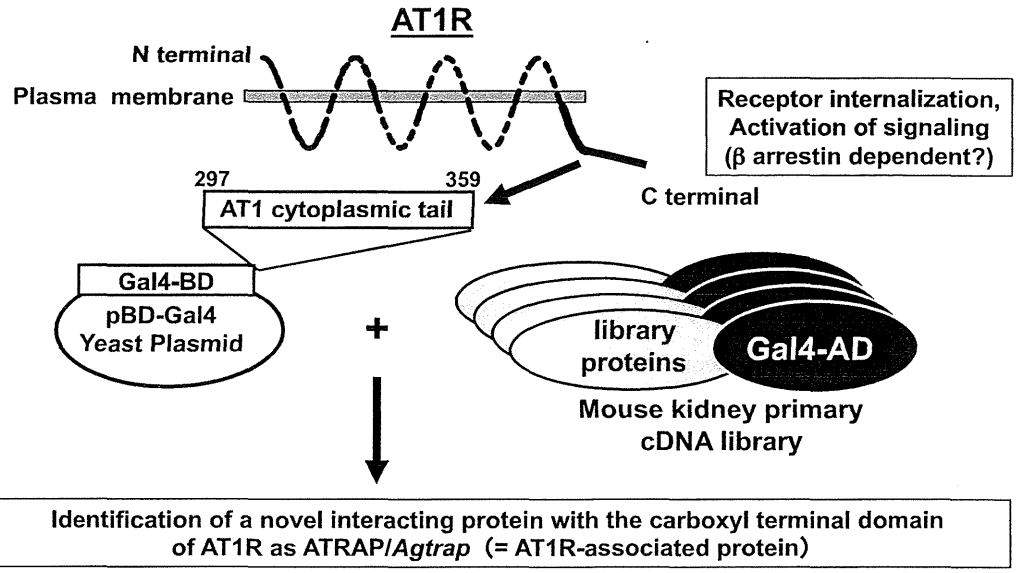

Fig. (1). Employing a yeast two-hybrid screening system, we previously cloned a novel AT1R-associated protein (ATRAP/*Agtrap*) that is predicted to have three transmembrane domains and specifically interacts with the carboxyl-terminal cytoplasmic domain of the AT1R.

tivity in Bowman's capsules, the proximal convoluted tubules (PCT), the proximal straight tubules (PST), and the distal convoluted tubules (DCT). A lower level of ATRAP immunostaining was also detected in other nephron segments. However, significant staining was not found in the glomeruli, in the vasculature, (including arcuate artery, interlobular arteries, and arterioles), or in the interstitial cells [15, 18].

5. PROMOTING EFFECT OF ATRAP ON AT1R INTERNALIZATION

The results of analysis of intracellular distribution of ATRAP showed a particulate distribution; electron microscopy reveals the presence of ATRAP in prominent perinuclear vesicular membranes; and co-localization analysis by immunofluorescence shows that ATRAP co-localizes in an intracellular vesicular compartment corresponding to endoplasmic reticulum, Golgi, and endocytic vesicles [12].

With respect to the interaction of ATRAP with AT1R and effects of ATRAP on AT1R internalization in cells, the results of immunoprecipitation assay, BRET analysis, and immunofluorescence staining in cultured cells including cardiovascular cells indicate that ATRAP is able to interact with AT1R even without Ang II stimulation and that Ang II stimulation significantly facilitated the interaction of these proteins [19]. The results of real-time trafficking analysis of ATRAP vesicles also showed a constitutive translocation of ATRAP from intracellular vesicle compartments to the periphery of the cell, which was not affected by the treatment with Ang II [12].

Taken together, these results suggest that ATRAP is actually able to bind to the AT1R under baseline conditions but that ATRAP interacts mainly with the AT1R that is internalized from the cell surface into the endocytic vesicles on Ang II stimulation to keep the receptor internalized even after the removal of Ang II. Furthermore, the quantitative analysis of immunofluorescence staining indicated that almost all of the internalized AT1R were associated with ATRAP, indicating that a major function of ATRAP in cultured cells including cardiovascular cells is to promote the constitutive internalization of AT1R [13, 19, 20]. Furthermore, a transgenic model increase in renal ATRAP expression beyond baseline *in vivo* was accompanied by a constitutive reduction of renal plasma membrane AT1R expression and by the promotion of renal AT1R internalization in response to Ang II [21]. Furthermore, another study also showed that a genetic deficiency of ATRAP in mice caused an enhanced surface expression of AT1R in the kidney, which is consistent with these results [17].

6. PUTATIVE FUNCTIONAL ROLE OF ATRAP IN CULTURED CELLS

Initially, this protein has been found to modulate AT1R function in transformed African green monkey kidney fibroblast (COS-7) cells and human embryonic kidney (HEK) 293 cells [4, 12]. Overexpression of ATRAP in COS-7 cells caused a marked inhibition of AT1R-mediated activation of phospholipase C, and functional analysis of the effects of ATRAP on Ang II-induced AT1-receptor signaling in HEK293 cells reveals a moderate decrease in the generation of inositol lipids, a marked decrease in Ang II–stimulated transcriptional activity of the c-fos promoter luciferase reporter gene, and a decrease in cell proliferation.

In cardiomyocytes, overexpression of ATRAP by adenoviral gene transfer significantly decreases the number of AT1R on the surface of cardiomyocytes, and it also decreases the degree of p38 mitogen–activated protein kinase phosphorylation, the activity of the c-fos promoter, and protein synthesis upon Ang II stimulation in cardiomyocytes. In addition, in vascular smooth muscle cells (VSMC) and in distal convoluted tubule cells (mDCT), overexpression of ATRAP inhibited Ang II–mediated increases in TGF-β mRNA expression and TGF-β production into the medium [18-20]. On the other hand, ATRAP knockdown by small-interference RNA in VSMC activated Ang II-induced c-fos gene expression, which was effectively inhibited by valsartan, an AT1R-specific antagonist [19].

The nuclear factor of activated T cells (NFAT) transcription factor, which is dephosphorylated by the phosphatase calcineurin activated by the calcium signaling regulator and cyclophilin-binding protein, calcium-modulating cyclophilin ligand (CAML), has received broader interest in relation to various signaling events, in addition to regulating T cell receptor signaling [22]. It is expressed in cardiomyocytes, endothelial, and VSMC and is implicated in Ang II signaling through the AT1R [23]. Several findings have shown that the calcineurin/NFATsignaling pathway induced

by Ang II regulates cell growth and cardiovascular hypertrophy, contributing to pathological cardiovascular remodeling [24]. The CAML has been shown as an ATRAP partner, and the N-terminal hydrophilic domain of CAML (the amino acid residues 1-189) mediates a specific interaction between ATRAP and CAML. The amino acid residues 40-82 of ATRAP contribute to this interaction. Functionally, overexpression of ATRAP decreased Ang II–mediated and CAML–induced activation of calcineurin-NFAT pathway and inhibited cardiomyocyte hypertrophic response and VSMC senescence process [25, 26]. These results indicate that ATRAP significantly promotes the constitutive internalization of the AT1R and further attenuates certain Ang II–mediated pathological responses in cardiovascular and renal cells (Fig. 1).

7. REGULATION OF ATRAP EXPRESSION IN PATHOPHYSIOLOGICAL CONDITIONS

To understand the pathophysiological roles of ATRAP in hypertension and its related cardiovascular and renal disease, it should be important to investigate the regulation of endogenous expression of ATRAP gene in response to pathological stimuli and in the diseased conditions. Although the ATRAP mRNA and protein are abundantly and widely distributed along the renal tubules, including the distal and proximal tubules, a subpressor or pressor infusion of Ang II in mice causes a significant suppression of intrarenal ATRAP expression and that this response is dependent on the activation of AT1R [21]. Unilateral ureteral obstruction (UUO) is a well-established experimental model of progressive tubulointerstitial fibrosis. UUO leads to changes in renal hemodynamics, inflammatory responses in the kidney, and tubular hypertrophy and interstitial fibrosis of the affected kidney. The renin-angiotensin system is also known to be activated in UUO, and a recent study showed a significant down-regulation of ATRAP expression, with a concomitant decrease in Runx3 which is an activator of ATRAP gene transcription, in the affected kidney [27].

In the normal human kidney, both ATRAP mRNA and protein were widely and abundantly distributed along the renal tubules from Bowman's capsule to the medullary collecting ducts. In all renal tubular epithelial cells, the

ATRAP protein co-localized with the AT1R. In renal biopsy specimens with IgA nephropathy, a significant positive correlation between ATRAP and AT1R gene expression was observed. Furthermore, there was also a positive relationship between tubulointerstitial ATRAP expression and the estimated glomerular filtration rate in patients with IgA nephropathy, indicating that renal ATRAP expression appears to be influenced by renal functional status without significant compensatory up-regulation of endogenous ATRAP expression under the renal pathological condition [18].

In addition to the kidney, ATRAP is expressed in the cardiovascular tissues. As in the kidney, a subpressor or pressor infusion of Ang II in mice caused a significant suppression of cardiac ATRAP expression with a concomitant development of cardiac hypertrophy [21, 28], and in the vasculature, the cuff-mediated vascular injury in mice was shown to down-regulate the vascular ATRAP expression with atherosclerotic lesion development [14].

8. POSSIBLE INVOLVEMENT OF AT1R IN THE MODULATION OF TISSUE ATRAP EXPRESSION

From the accumulated preceding results *in vitro* and *in vivo*, we hypothesized that the tissue-specific balancing of ATRAP and AT1R expression may be an important regulator in the pathogenesis of hypertension and its related cardiovascular and renal disease (Fig. 2). In spontaneously hypertensive rats (SHR), concomitant with blood pressure increase and cardiac hypertrophy, there was a constitutive decrease in the ratio of cardiac expression of ATRAP to AT1R [16, 29]. However, treatment with AT1R–specific blocker

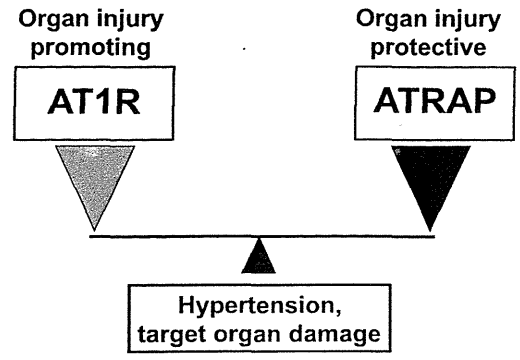

Fig. (2). ATRAP is widely expressed in many tissues as is AT1R, and tissue expression balance between ATRAP and AT1R may play a role in the determination of progression or suppression of hypertension and/or its related target organ damage.

(ARB), either at a depressor or subdepressor dose, recovered the suppressed cardiac ATRAP to AT1R ratio, which was accompanied by a decrease in AT1R density, an inhibition of p38 mitogen-activated protein kinase activity, and a regression of cardiac hypertrophy in SHR.

We also examined the regulation of endogenous ATRAP expression and effects of ARB in a genetic model of salt-sensitive hypertension. In Dahl Iwai salt-sensitive rats, renal ATRAP expression was suppressed concomitant with up-regulation of renal oxidative stress, inflammation and fibrosis-related markers such as p22phox, TGF-β, fibronectin, MCP-1 and type 1 collagen. However, prepubertal as well as continuous ARB treatment recovered the suppressed renal ATRAP expression and inhibited the renal activation of p22phox, TGF-β, fibronectin, MCP-1 and type 1 collagen [30]. These results showed that activation of AT1R signaling is one of major depressant of tissue ATRAP expression but indicated that prepubertal transient blockade of AT1R signaling exerts a long-term therapeutic effect on salt-induced hypertension and renal injury in Dahl Iwai salt-sensitive rats, partly through a ARB-mediated sustained enhancement of renal ATRAP expression (Fig. 3). Furthermore, these results demonstrated that there is a tissue-specific regulatory balancing of the expression of ATRAP and AT1R during the development of hypertension and related cardiovascular and renal disease.

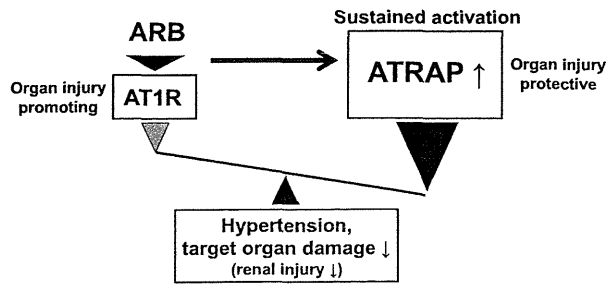

Fig. (3). Prepubertal transient blockade of renal AT1R signaling by ARB may exert a sustained activation of renal ATRAP expression to exhibit a long-term therapeutic effect even after withdrawal of ARB.

9. PUTATIVE FUNCTIONAL ROLE OF ATRAP *IN VIVO*

To examine the ATRAP-mediated effect on tissue AT1R internalization and AT1R signaling by a different strategy *in vivo*, several kinds of ATRAP transgenic mice were produced and analyzed to date. A transgenic model increase in renal ATRAP expression