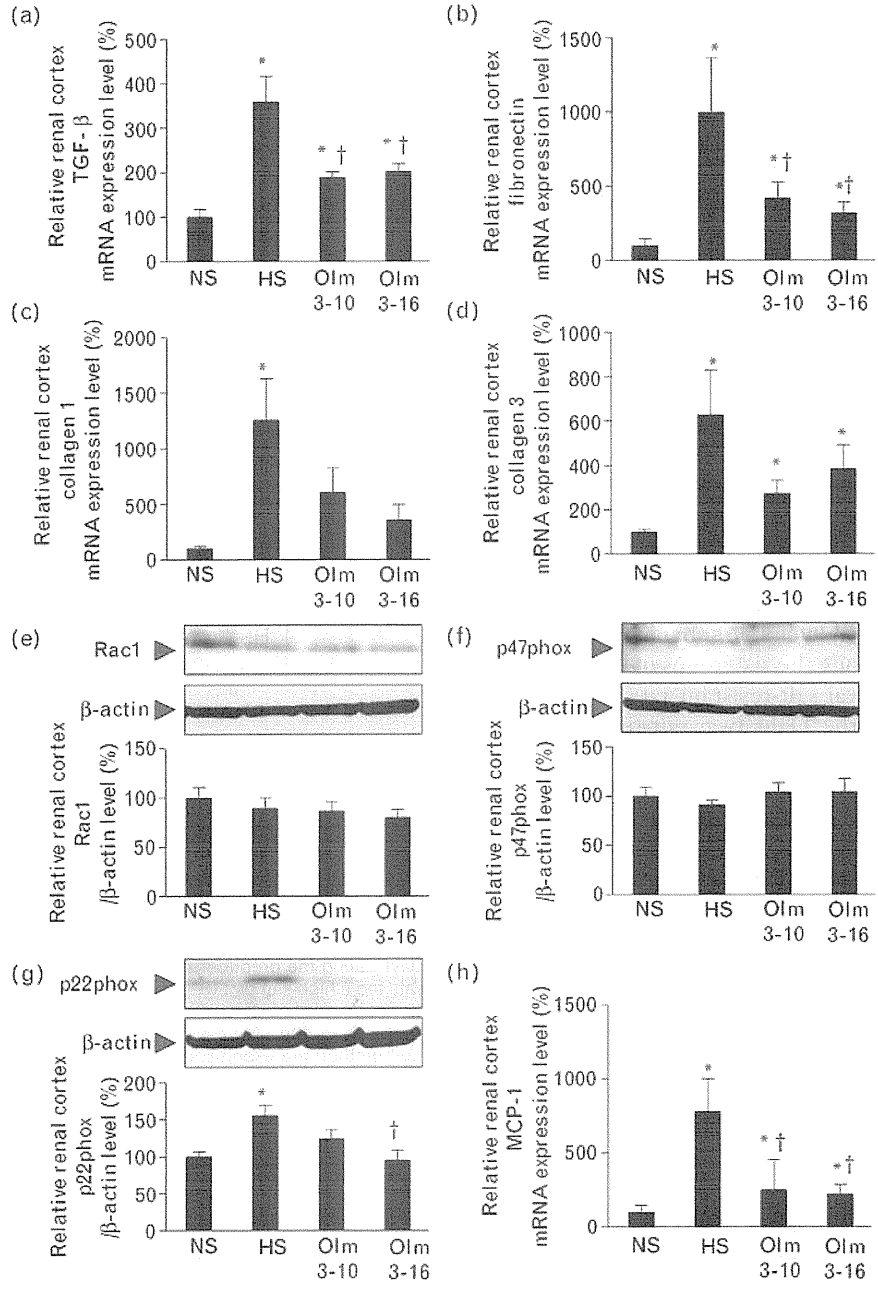
Fig. 2



Effects of prepubertal transient olmesartan treatment on the expression of renal oxidative stress, inflammatory response, and fibrosis-related genes in Dahl Iwai salt-sensitive rats. The effects of high salt loading and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on TGF-β (a), fibronectin (b), type 1 collagen (c), type 3 collagen (d) and MCP-1 (h) mRNA expression in the renal cortex of DS rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DS rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). $^{*}P < 0.05$, vs. NS, $^{\dagger}P < 0.05$, vs. HS. Representative western blot and quantitative analysis of the effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on Rac1 (e), p47phox (f) and p22phox (g) protein expression in the renal cortex of DS rats at 16 weeks are also shown. Values are calculated relative to those achieved with extracts in DS rats fed a NS and are expressed as the mean ± SEM ($n = 6$ in each group). $^{*}P < 0.05$, vs. NS, $^{\dagger}P < 0.05$, vs. HS. DS, Dahl Iwai salt-sensitive; MCP-1, monocyte chemotactic protein-1.

p22phox expression, and prepubertal transient olmesartan treatment (Olm3-10) had a moderate inhibitory effect on the renal cortical p22phox expression (Fig. 2 g). Furthermore, whereas high salt loading significantly increased the renal cortical monocyte chemotactic protein-1 (MCP-1) expression compared to normal salt loading, both continuous olmesartan treatment (Olm3-16) and prepubertal transient olmesartan treatment

(Olm3-10) significantly suppressed the high salt loading-mediated increase in the renal cortical MCP-1 expression (Fig. 2 h).

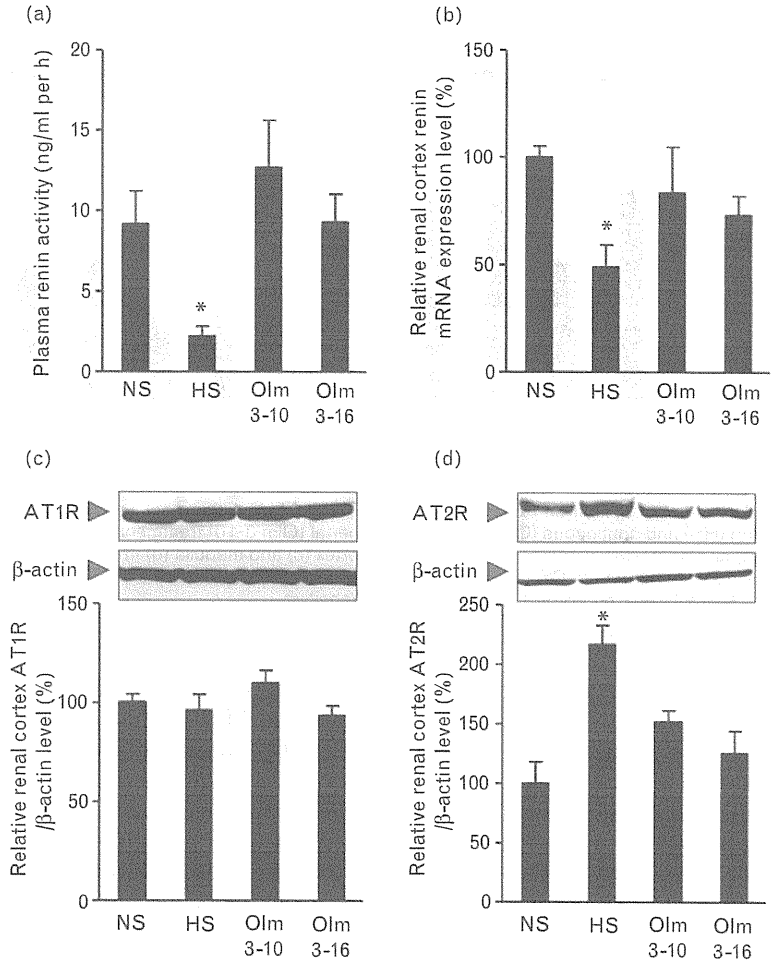
Effects of prepubertal olmesartan treatment on renal expression of renin, AT1R and AT2R in Dahl Iwai salt-sensitive rats

In Dahl Iwai salt-sensitive rats, the high salt loading significantly decreased plasma renin activity and renin mRNA expression at 16 weeks, which was reversed by either continuous (Olm 3-16) or prepubertal transient (Olm3-10) olmesartan treatment (Fig. 3a and b). The results of western blot analysis showed that high salt loading did not affect renal cortex AT1R protein levels, whereas AT2R protein levels were significantly increased by high salt loading and olmesartan treatment suppressed this increase (Fig. 3c and d).

Effects of prepubertal olmesartan treatment on renal expression of ATRAP in Dahl Iwai salt-sensitive rats

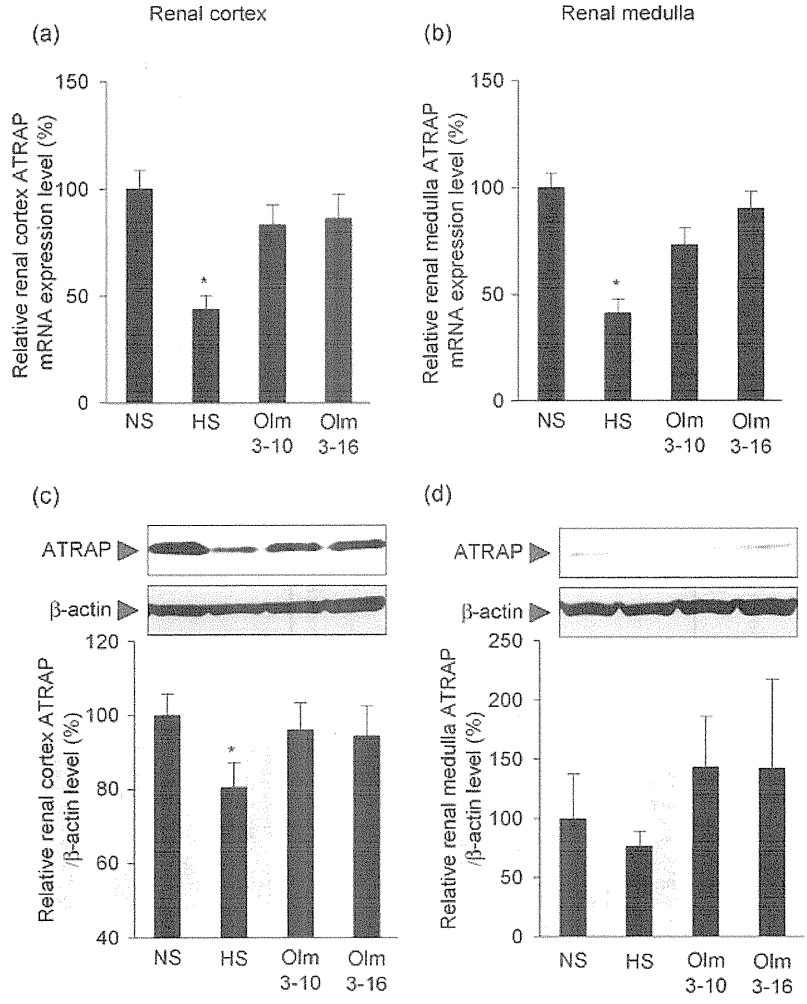
We also examined the effects of high salt loading and olmesartan treatment on endogenous ATRAP gene expression in the kidneys of Dahl Iwai salt-sensitive rats at 16 weeks. The results of real-time RT-PCR analysis showed that high salt loading significantly decreased ATRAP mRNA expression in the renal cortex and medulla, which was reversed by either continuous (Olm 3-16) or prepubertal transient (Olm3-10) olmesartan treatment (Fig. 4a and b). The results of western blot analysis also demonstrated a significant reduction of renal

Fig. 3



Effects of prepubertal transient olmesartan treatment on plasma renin activity and expression of renal renin, AT1R and AT2R expression in Dahl Iwai salt-sensitive rats. The effects of high salt loading (HS) and continuous (Olm3-16) or prepubertal (Olm 3-10) treatment with olmesartan on PRA (a), renal cortex renin mRNA (b) and AT1R (c) and AT2R (d) protein expression in DS rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DS rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). *$P < 0.05$, vs. NS. AT1R, Ang II type 1 receptor; DS, Dahl Iwai salt-sensitive; PRA, plasma renin activity.

Fig. 4



Effects of prepubertal transient olmesartan treatment on expression of ATRAP and AT1R in the renal cortex and medulla of Dahl Iwai salt-sensitive rats. The effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on ATRAP mRNA levels in the renal cortex (a) and medulla (b) and on ATRAP protein levels in the renal cortex (c) and medulla (d) in DS rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DS rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). $^*P < 0.05$, vs. NS. AT1R, Ang II type 1 receptor; DS, Dahl Iwai salt-sensitive.

cortical ATRAP protein level, which was comparably recovered to the baseline level under normal salt loading by either continuous (Olm 3-16) or prepubertal transient (Olm3-10) olmesartan treatment (Fig. 4c). On the contrary, a lower level of ATRAP protein expression was detected in the renal medulla and the inhibitory effect of high salt loading on renal medullary ATRAP protein levels did not reach the statistical significance (Fig. 4d).

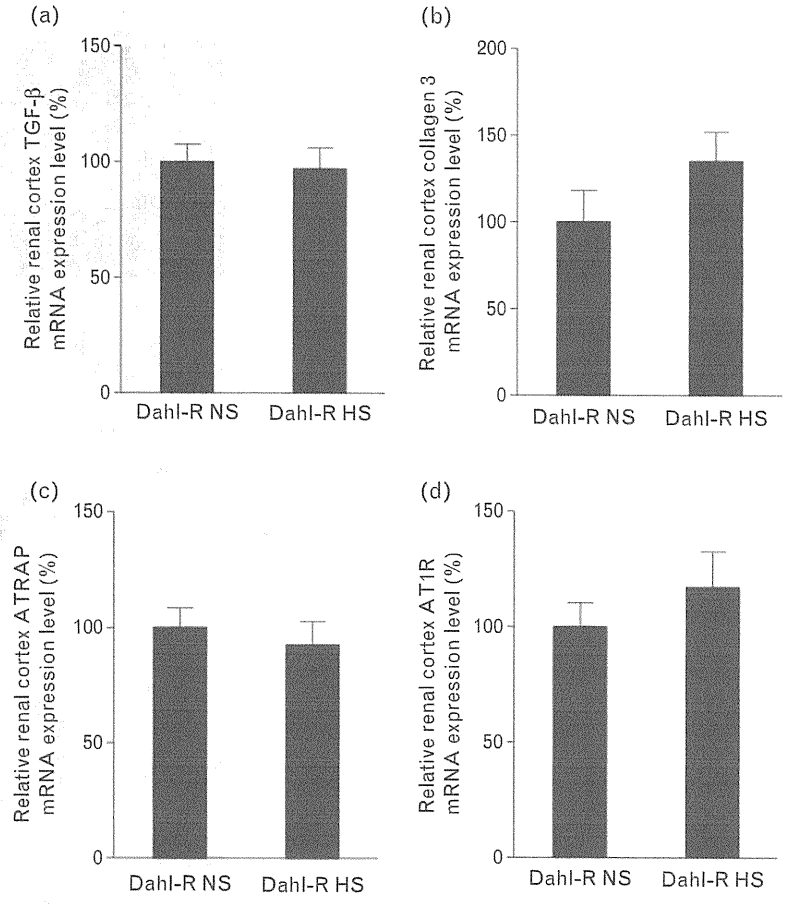
Effects of high salt loading on renal pathological responses and expression of ATRAP and AT1R in Dahl Iwai salt-resistant rats

In control Dahl Iwai salt-resistant rats, the high salt loading exhibited no apparent effects on the mRNA expression of TGF-β, type 3 collagen, ATRAP and AT1R in the renal cortex at 16 weeks (Fig. 5a–d).

Effects of prepubertal olmesartan treatment on intrarenal AT1R and p22phox immunostaining and interstitial fibrosis in Dahl Iwai salt-sensitive rats

The results of immunohistchemical analysis showed that neither high salt loading nor olmesartan treatment (Olm3-10, Olm3-16) affected the intensity or distribution of renal AT1R immunostaining in Dahl Iwai salt-sensitive rats at 16 weeks (Fig. 6). On the contrary, the results showed that high salt loading increased the renal cortical p22phox immunostaining, which was decreased to the same degree as in normal salt loading by either continuous (Olm3-16) or prepubertal transient (Olm 3-10) olmesartan treatment. With respect to the renal fibrotic response, the results of Masson's trichrome staining showed a prominent interstitial fibrosis in the high salt group compared to the normal salt group, which

Fig. 5



Effects of high salt loading on renal pathological responses and expression of ATRAP and AT1R in Dahl Iwai salt-resistant rats. The effects of high salt loading (HS) on TGF-β (a), type 3 collagen (b), ATRAP (c) and AT1R (d) mRNA expression in the renal cortex of DR rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DR rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). AT1R, Ang II type 1 receptor; DR, Dahl Iwai salt-resistant.

was attenuated in both the prepubertal olmesartan treatment group (Olm3-10) and continuous olmesartan treatment group (Olm3-16).

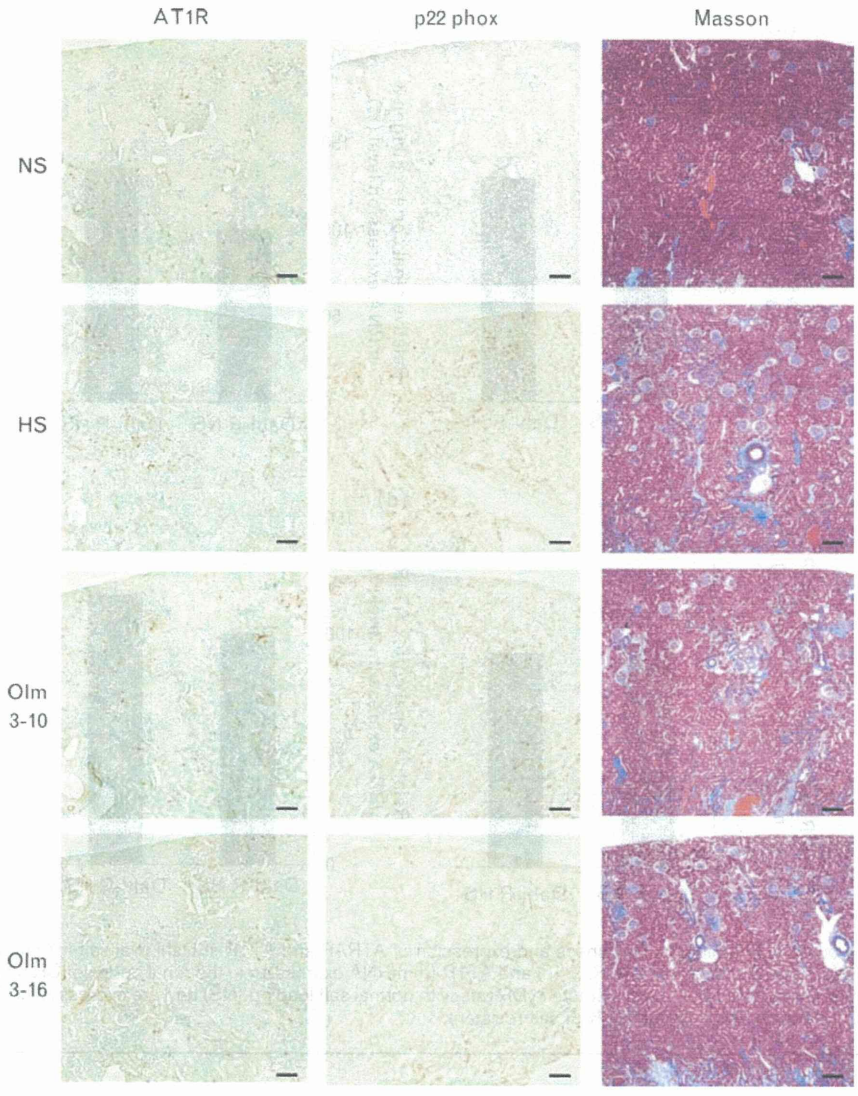
Effects of prepubertal olmesartan treatment on intrarenal distribution of ATRAP immunostaining in Dahl Iwai salt-sensitive rats

With respect to the intrarenal distribution of ATRAP, there was a relatively dominant ATRAP immunostaining in the inner cortex region of the kidney in Dahl Iwai salt-sensitive rats fed a normal salt diet, but ATRAP immunostaining in the inner cortex was decreased in Dahl Iwai salt-sensitive rats fed a high salt diet (Fig. 7a). However, either prepubertal (Olm3-10) or continuous (Olm3-16) olmesartan treatment recovered the suppressed ATRAP immunostaining to degree comparable with the Dahl Iwai salt-sensitive rats fed a normal salt diet. The results of further immunohistochemical analysis using consecutive sections stained for ATRAP and AQP1, which is specifically expressed in the proximal tubule [27], showed that the ATRAP immunostaining sites in the proximal tubules in the inner cortex were involved in the high salt loading-mediated decrease in the kidneys of Dahl Iwai salt-sensitive rats at 16 weeks (Fig. 7b).

Discussion

The main findings of this study are as follows: prepubertal transient treatment with the AT1R-specific blocker olmesartan continued to exert a long-term blood pressure-lowering effect after the treatment, an effect which was comparable with that obtained by continuous olmesartan treatment in Dahl Iwai salt-sensitive rats under high salt loading; prepubertal transient treatment with olmesartan continued to maintain the inhibitory effects on renal oxidative stress and inflammatory and fibrotic responses after the treatment; the long-term therapeutic effects of the prepubertal transient treatment with olmesartan were accompanied by a sustained

Fig. 6

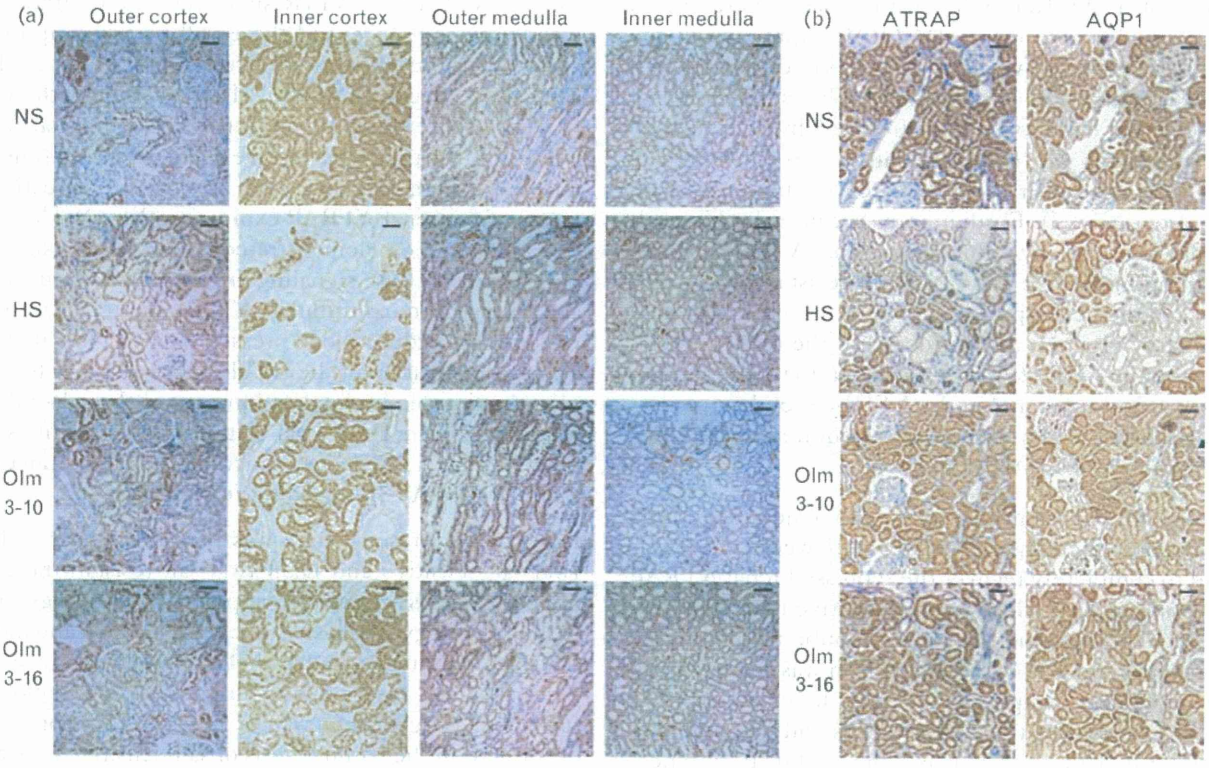


Representative kidney sections showing the effects of prepubertal transient olmesartan treatment on intrarenal AT1R and p22phox immunostaining and interstitial fibrosis in Dahl Iwai salt-sensitive rats. Representative immunohistochemical staining of the effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on AT1R and p22phox immunostaining in the renal cortex of DS rats at 16 weeks are shown. The positive areas for AT1R and p22phox are evident as brown dots in the sections. Representative Masson's trichrome staining of the effects of high salt loading and continuous or prepubertal treatment with olmesartan on renal interstitial fibrosis in the renal cortex of DS rats at 16 weeks is also shown. Original magnification: ×40. Bars = 100 μm. AT1R, Ang II type 1 receptor; DS, Dahl Iwai salt-sensitive; NS, normal salt loading.

recovery of repressed renal ATRAP expression in Dahl Iwai salt-sensitive rats under high salt loading.

In the present study, the high salt diet-induced elevation of blood pressure was attenuated by transient administration of olmesartan only during the prepubertal period (from 3 to 10 weeks of age) in Dahl Iwai salt-sensitive rats (Fig. 1), thereby supporting the existence of the 'renin–angiotensin system block memory phenomenon' reported previously [29]. In several types of genetically hypertensive rats, including SHR and Dahl Iwai salt-sensitive rats fed a high salt diet, the blood pressure increases gradually from the age of 3–10 weeks, and subsequently the hypertensive state is established. Thus, the age of 3–10 weeks is called the critical period and corresponds to prehypertension in humans. The activity of the renin–angiotensin system during this critical period is suggested to have a major influence on the pathogenesis of hypertension [29]. Previous studies showed that transient pharmacological blockade of the renin–angiotensin system during the critical period significantly inhibits the vascular hypertrophy of renal arteries and arterioles as well as increases in blood pressure, and thus efficiently blocks this vicious cycle by

Fig. 7



Representative kidney sections showing the effects of prepubertal transient olmesartan treatment on intrarenal localization of ATRAP immunostaining in Dahl Iwai salt-sensitive rats. Representative high magnification of the kidney sections for the effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on immunohistochemical localization of ATRAP expression in the outer cortex, inner cortex, outer medulla and inner medulla is shown (a). Consecutive sections also show the immunostaining of ATRAP and aquaporin 1 (AQP1), respectively (b). The positive areas for ATRAP or AQP1 are evident as brown dots in the sections. Original magnification: ×200. Bars = 50 μm. DS, Dahl Iwai salt-sensitive; NS, normal salt loading.

accelerating the 'reno-vascular amplifier' mechanism [29–31]. Furthermore, a persistent lowering of blood pressure and an attenuation of renal pathological changes were achieved by transient ARB treatment of Dahl Iwai salt-sensitive rats fed a high salt diet [23]. In this study, high salt loading caused glomerular sclerosis and vascular hypertrophy, and these pathological changes were attenuated, albeit not completely prevented, in the Dahl Iwai salt-sensitive rats treated either prepubertally or continuously with olmesartan (data not shown).

The increase in renal oxidative stress plays an important role in the pathogenesis of hypertension and the development of renal injury [12,32–35]. Ang II and high salt loading provoke organ injury in various tissues, including blood vessels, glomeruli and renal tubules, through an activation of NADPH oxidase and stimulation of oxidative stress at local tissue sites [9,12,33,36–39]. Previous studies showed that high salt loading-mediated paradoxical activation of the renal renin–angiotensin system, such as an enhancement of the renal angiotensinogen and (pro)renin receptors, in spite of the suppression of the circulating renin–angiotensin system, still contributed to hypertension and renal injury in Dahl Iwai salt-sensitive rats [7,8,40,41]. Because the biological actions of Ang II are influenced by the AT1R expression levels [42], and Ang II infusion in mice specifically lacking renal AT1R failed to develop hypertension [43], investigation of the renal activity of AT1R signaling in Dahl Iwai salt-sensitive rats fed a high salt diet is important in order to elucidate the mechanisms responsible for the salt-sensitive hypertension and renal injury observed in this hypertension model.

We previously cloned ATRAP as a molecule which interacts with AT1R, and showed that ATRAP suppressed the Ang II-induced hypertrophic and proliferative responses of cardiovascular cells by inducing AT1R internalization [17,18]. Thus, a tissue-specific regulatory balancing of ATRAP and AT1R expression may be involved in the modulation of AT1R signaling in each tissue. With respect to salt-induced hypertension, previous studies showed significant increases [8,44] or no evident changes [23,41] in AT1R mRNA and protein levels in response to high salt loading in Dahl Iwai salt-sensitive rats. In this study, whereas AT1R expression

did not appear to be modulated by high salt diet in the kidney of Dahl Iwai salt-sensitive rats, there was a significant decrease in renal ATRAP expression in the Dahl Iwai salt-sensitive rats fed a high salt diet along with the development of hypertension. This suppression of the renal ATRAP expression induced by salt-induced hypertension is AT1R-dependent, as it is prevented in Dahl Iwai salt-sensitive rats treated continuously with olmesartan (Fig. 3). Furthermore, because the high salt diet did not have any influence on renal ATRAP expression in normotensive Dahl Iwai salt-resistant rats in this study and the development of hypertension without high salt dietary regimen did not decrease the renal ATRAP expression in SHR [19], it is suggested that the high salt loading is not sufficient but the AT1R-dependent process during salt-induced hypertension is required for the renal suppression of ATRAP expression.

Interestingly, transient treatment with olmesartan during only the prepubertal period from 3 to 10 weeks of age was found to completely recover the repressed renal ATRAP expression in the Dahl Iwai salt-sensitive rats fed a high salt diet. This sustained effect of prepubertal olmesartan treatment on renal ATRAP expression was accompanied with significant suppression of the high salt-induced up-regulation of renal oxidative stress and inflammatory and fibrotic markers such as p22phox, TGF-β, fibronectin, MCP-1 and type 1 collagen (Fig. 2). Among the components of NADPH oxidase, p22phox is an essential subunit of NADPH oxidase by influencing the activity of this enzyme through the assembly of membrane-bound p22phox [12,45–47].

In the present study, there was a significant decrease in renal ATRAP expression, particularly in the AQP1-positive proximal tubules in the inner cortex, in the Dahl Iwai salt-sensitive rats fed a high salt diet along with the development of hypertension (Fig. 7), and the prepubertal transient AT1R blockade-mediated recovery of the repressed ATRAP expression in the inner cortex region of Dahl Iwai salt-sensitive rats on the high salt loading coincided with the sustained suppression of p22phox expression in the inner cortex region even after the discontinuation of AT1R blockade. Since our previous results showed that adenoviral overexpression of ATRAP suppressed pathological AT1R signaling such as Ang II-mediated TGF-β production in renal tubular cells [27], the results in this study suggest that the sustained recovery of renal tubular ATRAP exerts a long-term inhibitory effect on pathological AT1R signaling to maintain the suppression of p22phox expression in Dahl Iwai salt-sensitive rats on high salt loading.

A limitation of the present study is that this study is mainly an association study and no specific direct proofs are provided regarding the role of ATRAP on the consequence of the high salt loading-mediated process of hypertension in Dahl Iwai salt-sensitive rats and further studies are needed to elucidate the function of renal tubular ATRAP on AT1R signaling under pathological interventions *in vivo*, and these will be taken up in due course. Our previous in-vitro study showed that ATRAP associated specifically with AT1R but not with AT2R [13], and there was a significant increase in renal AT2R expression in the hypertensive Dahl Iwai salt-sensitive rats fed a high salt diet with an opposite direction of change from renal ATRAP expression (Fig. 3). However, previous studies demonstrated that AT2R signaling antagonizes AT1R signaling in the renal tubules and activation of renal tubular AT2R exerted natriuretic responses to reduce blood pressure *in vivo* [48,49]. Thus, although activation of the AT2R pathway is not likely to play a major role in the development of the salt-induced hypertension in Dahl Iwai salt-sensitive rats, it is still possible that ATRAP may function as a novel functional modulator of AT2R signaling *in vivo*.

Because the present study was performed only in Dahl Iwai salt-sensitive and Dahl Iwai salt-resistant rats, it is important to use caution in applying the findings to the pathophysiology of salt-sensitive hypertension and kidney damage in humans. Nevertheless, the findings of the present study may provide important information for further investigation *in vivo* of possible functional roles of ATRAP in the pathogenesis of salt-sensitive hypertension and kidney damage in humans, and suggest the potential benefit of an ATRAP activation strategy. Further studies to elucidate the molecular mechanisms of the antihypertensive and kidney-protecting properties of ATRAP may enable clinical applications of ATRAP in the near future, such as activating ligands for a more efficient inhibition of AT1R signaling in combination with inhibitors of the renin–angiotensin system. In conclusion, the present study shows that prepubertal temporary AT1R blockade attenuated salt-induced hypertension and renal injury, including oxidative stress overproduction, inflammation, and fibrosis, and this in turn was associated the sustained recovery of renal ATRAP expression.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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See Editorial Commentary, pp 542–544

Abstract—The angiotensin II (Ang II) type 1 (AT_1) receptor mainly mediates the physiological and pathological actions of Ang II, but recent studies have suggested that AT_1 receptor inherently shows spontaneous constitutive activity even in the absence of Ang II in culture cells. To elucidate the role of Ang II–independent AT_1 receptor activation in the pathogenesis of cardiac remodeling, we generated transgenic mice overexpressing AT_1 receptor under the control of α-myosin heavy chain promoter in angiotensinogen-knockout background (AT_1Tg-AgtKO mice). In AT_1Tg-AgtKO hearts, redistributions of the $G\alpha_{q11}$ subunit into cytosol and phosphorylation of extracellular signal-regulated kinases were significantly increased, compared with angiotensinogen-knockout mice hearts, suggesting that the AT_1 receptor is constitutively activated independent of Ang II. As a consequence, AT_1Tg-AgtKO mice showed spontaneous systolic dysfunction and chamber dilatation, accompanied by severe interstitial fibrosis. Progression of cardiac remodeling in AT_1Tg-AgtKO mice was prevented by treatment with candesartan, an inverse agonist for the AT_1 receptor, but not by its derivative candesartan-7H, deficient of inverse agonism attributed to a lack of the carboxyl group at the benzimidazole ring. Our results demonstrate that constitutive activity of the AT_1 receptor under basal conditions contributes to the cardiac remodeling even in the absence of Ang II, when the AT_1 receptor is upregulated in the heart. (***Hypertension***. **2012;59:627-633.)** • **Online Data Supplement**

Key Words: ARB ■ cardiac dysfunction ■ fibrosis ■ G protein-coupled receptor ■ inverse agonist

The angiotensin II (Ang II) type 1 (AT_1) receptor is a 7 transmembrane spanning G protein-coupled receptor (GPCR), and the activation of AT_1 receptor is involved in regulating pathophysiological processes of the cardiovascular system. In principle, the AT_1 receptor is activated on binding to Ang II, which is produced systemically or locally after sequential proteolytic processing. However, recent studies demonstrated that the AT_1 receptor inherently shows spontaneous constitutive activity even in the absence of Ang II in cultured cells.[1–3] GPCRs are structurally unstable and show significant levels of spontaneous activity in an agonist-independent manner.[4] In addition, we and others demonstrated that the AT_1 receptor can be activated by mechanical stress independent of Ang II[5–7] through conformational switch of the receptor.[1] These observations have highlighted the inverse agonist activity of AT_1 receptor blockers (ARBs) as a drug-specific property that can inhibit Ang II–independent constitutive activity and mechanical stress-induced receptor activation.[1,2,5,8] In a mouse model, mechanical stress-induced AT_1 receptor activation led to the development of cardiac hypertrophy independent of Ang II, and treatment with inverse agonists for the AT_1 receptor-attenuated cardiac hypertrophy thus formed.[5] However, the pathogenic role of Ang II–independent constitutive activity of the AT_1 receptor and clinical relevance of inverse agonist activity of ARBs against constitutive receptor activation remains to be elucidated in vivo. In several GPCRs, gain-of-function mutations are causative of diseases, but any activating mutations in the

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coding region of the AT_1 receptor gene have not been identified in hypertension or primary hyperaldosteronism.[9,10] Although knock-in mice with a constitutively activating mutation (substitution of Asn^{111} to Ser with a C-terminal deletion) showed low-renin hypertension and progressive fibrosis in kidney and heart,[11] it remains unclear whether constitutive activity of the native AT_1 receptor leads to some phenotypic abnormalities even under circumstances where the production of Ang II is genetically inhibited.

Therefore, we generated transgenic mice overexpressing AT_1 receptor under the control of *α-myosin heavy chain* promoter in the *angiotensinogen* (*Agt*)-knockout background. Here, we show that constitutive activity of the AT_1 receptor indeed contributes to cardiac remodeling independent of Ang II even in vivo, when the AT_1 receptor is upregulated in the heart.

Methods

An expanded Methods section is available in the online-only Data Supplement.

Mice, Transverse Aortic Constriction Operation, and Transthoracic Echocardiography

Mice expressing the human *AGTR1* gene under the control of *α-myosin heavy chain* promoter (on the C57BL/6J background) and mice deficient for the *Agt* gene (on the Institute of Cancer Research [ICR] background) were described previously.[12,13] Candesartan cilexetil and candesartan-7H were synthesized by Takeda Pharmaceutical Co, Ltd, and administered via drinking water. Sham or transverse aortic constriction operation was performed as described previously,[5] and transthoracic echocardiography was performed on conscious mice with a Vevo 770 Imaging System. All of the protocols were approved by the institutional animal care and use committee of Chiba University.

Ang II Infusion and BP Measurement

Eight-week–old C57BL/6J male mice were treated with Ang II (0.6 mg/kg per day) or vehicle for 2 weeks using an osmotic mini-pump (ALZET model 2002; Durent Corp). The BP and pulse rates were measured noninvasively by a programmable sphygmomanometer (BP-98A, Softron) using the tail-cuff method.

Real-Time RT-PCR Analysis

Total RNA was extracted by using the RNeasy kit (Qiagen), and single-stranded cDNA was transcribed by using QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturer's protocol. We conducted quantitative real-time PCR analysis with the Universal ProbeLibrary Assays (Roche Applied Science), according to the manufacturer's instructions.

Western Blot Analysis and Histological Analysis

Western blot analysis and histological were performed as described previously.[1,5]

Radioligand Receptor Binding Assay

Radioligand binding assays were performed as described previously.[1,14]

Statistics

All of the data are presented as mean±SEM. Two-group comparison was analyzed by unpaired 2-tailed Student *t* test, and multiple-group comparison was performed by 1-way ANOVA followed by the Fisher protected least significant difference test for comparison of means. A *P* value of $P<0.05$ was considered to be statistically significant.

Results

AT_1 Receptor Is Constitutively Activated Without the Involvement of Ang II in AT_1 Transgenic-Angiotensinogen Knockout Mice Hearts

To elucidate the pathogenic role of Ang II–independent AT_1 receptor activation in the hearts, we crossed transgenic mice overexpressing human AT_1 receptor under the control of cardiac-specific *α-myosin heavy chain* promoter (AT_1Tg) with angiotensinogen knockout mice (AgtKO) to generate AT_1Tg-AgtKO mice. First, we examined the expression levels of renin-angiotensin system components. Although the mRNA level of the AT_2 receptor (*Agtr2*) was significantly higher in AT_1Tg-AgtKO hearts than in AgtKO hearts, there was no significant difference in protein levels of the AT_2 receptor between AT_1Tg-AgtKO and AgtKO hearts (Figure S1 in the online-only Data Supplement). Furthermore, the mRNA levels of the AT_{1b} receptor (*Agtr1b*), angiotensin-converting enzyme (*Ace*), and renin (*Ren1* and *Ren2*) did not differ significantly between AT_1Tg-AgtKO and AgtKO hearts (Figure S1A).

We next determined the density of the AT_1 receptor (B_{max} values of receptor binding) in membranes isolated from the ventricles of AgtKO and AT_1Tg-AgtKO mice by radioligand binding assays using ^{125}I-[Sar^1, Ile^8] Ang II as ligand. Consistent with the previous report,[12] the B_{max} of AT_1 receptor was increased by >200-fold in AT_1Tg-AgtKO hearts compared with AgtKO hearts (AT_1Tg-AgtKO: 5.41±1.79 pmol/mg of protein; AgtKO: 24.0±13.9 fmol/mg of protein; n=4 per group; $P<0.01$). Next, to evaluate whether the AT_1 receptor is constitutively activated in the AT_1Tg-AgtKO hearts, we examined redistribution of $G\alpha_{q11}$ into the cytosolic fraction and phosphorylation of extracellular signal–regulated kinases (ERKs) in AgtKO and AT_1Tg-AgtKO hearts. On activation of the AT_1 receptor, the heterotrimeric G_q protein dissociates into α and $\beta\gamma$ subunits, and the GTP-bound $G\alpha_q$ subunit stimulates diverse intracellular signaling pathways, including the ERK pathway.[15,16] Redistribution of $G\alpha_{q11}$ subunits from the particulate to the cytosolic fraction was significantly increased in AT_1Tg-AgtKO hearts compared with AgtKO hearts (Figure 1A). In addition, the levels of phosphorylated ERKs in AT_1Tg-AgtKO hearts was significantly increased compared with AgtKO hearts (Figure 1B). These results suggest that the AT_1 receptor is upregulated and constitutively activated without the involvement of Ang II in the AT_1Tg-AgtKO hearts.

AT_1Tg-AgtKO Mice Display Progressive Cardiac Remodeling

Tail-cuff measurements of systolic and diastolic blood pressure (BPs) and pulse rates revealed that these parameters did not differ significantly between AgtKO and AT_1Tg-AgtKO mice at 20 weeks of age (Table). However, morphological and physiological analysis revealed progressive chamber dilatation, contractile dysfunction, and interstitial fibrosis in AT_1Tg-AgtKO mice, whereas cardiac structure and function were normal in AgtKO mice. At 20 weeks of age, AT_1Tg-AgtKO mice displayed ≈1.5-fold increase in heart:body

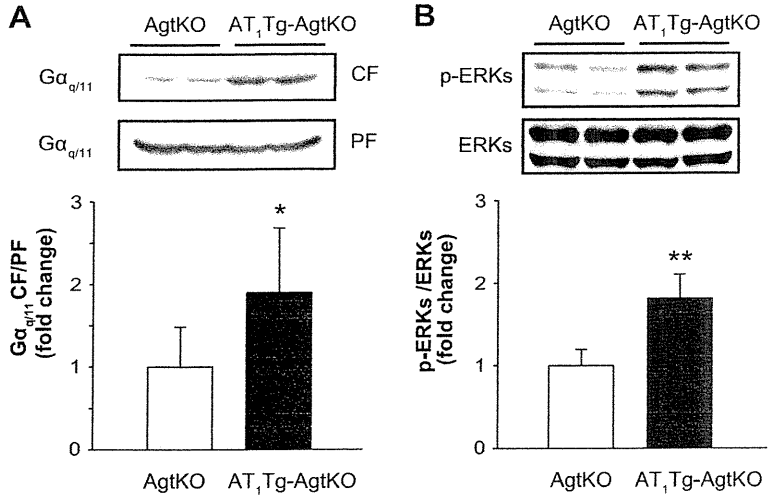

Figure 1. Constitutive activation of angiotensin II type 1 (AT_1) receptor in AT_1 transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) hearts. **A**, Immunoblot analysis of $G\alpha_{q/11}$ in cytosolic fraction (CF) and particulate fraction (PF) extracted from AgtKO (n=6) and AT_1Tg-AgtKO (n=6) hearts. The quantitation of the $G\alpha_{q/11}$ in CF/PF is shown as a bar graph. Data are presented as mean±SEM. *$P<0.05$ vs AgtKO mice. **B**, Immunoblot analysis of phosphorylated extracellular signal–regulated kinases (ERKs; p-ERKs) and total ERKs in AgtKO (n=8) and AT_1Tg-AgtKO (n=8) hearts. The quantitation of the p-ERKs/ERKs is shown as a bar graph. Data are presented as mean±SEM. **$P<0.01$ vs AgtKO mice.

weight ratio compared with AgtKO mice (Table). Echocardiographic examination revealed a progressive increase in left ventricular end-diastolic dimension and decrease in the percentage of fractional shortening (Figure 2A). Histologically, a significant increase in interstitial fibrosis was observed in AT_1Tg-AgtKO mice at 20 weeks of age and further exacerbated at 36 weeks of age (Figure 2B). Furthermore, real-time RT-PCR indicated that mRNA levels of fetal cardiac genes (*Nppa*, *Nppb*, and *Acta1*) and extracellular matrix genes (*Col3a1* and *Postn*) were significantly increased in AT_1Tg-AgtKO hearts compared with AgtKO hearts (Figure 2C). These results indicate that upregulation of the AT_1 receptor induced spontaneous and progressive cardiac remodeling in AT_1Tg-AgtKO mice in spite of systemic deficiency of Ang II.

Cardiac Remodeling in AT_1Tg-AgtKO Mice Is Prevented by Treatment With an Inverse Agonist for the AT_1 Receptor

We examined whether an AT_1 receptor blocker candesartan could prevent the progression of cardiac remodeling in AT_1Tg-AgtKO mice. In cultured cells, candesartan reduces the basal activity of both the wild-type AT_1 receptor and constitutively active AT_1 mutant receptors, suggesting that candesartan is an inverse agonist for the AT_1 receptor.[1] Candesartan also suppresses mechanical stretch-induced hemodynamic movement and thereby inhibits receptor activation[1] and prevents pressure-overload cardiac hypertrophy in mice.[5]

Table. Measurement of Heart Weight, Heart Rate, and BP in AgtKO and AT_1Tg-AgtKO Mice at 20 wk of Age

Parameters	AgtKO	No.	AT_1Tg-AgtKO	No.
BW, g	31.0±3.4	9	30.2±3.5	6
HW/BW, mg/g	3.48±0.25	9	5.08±0.19*	6
HR, bpm	556.0±85.3	6	540.1±55.0	6
Systolic BP, mm Hg	83.4±8.8	6	85.9±3.7	6
Diastolic BP, mm Hg	57.3±6.0	6	55.7±7.4	6
Mean BP, mm Hg	65.7±5.3	6	66.0±5.0	6

BW indicates body weight; HR, heart rate; HW/BW, heart:body weight ratio; BP, blood pressure; AgtKO, angiotensinogen-knockout; AT_1Tg, angiotensin II type 1 transgenic.
*$P<0.01$ vs sham.

Tail-cuff measurements revealed a significant increase in systolic BP in 8-week–old C57BL/6 male mice treated with Ang II (0.6 mg/kg per day) for 2 weeks using an osmotic minipump (Figure 3A). This BP elevation was abolished by treatment with candesartan cilexetil (1 mg/kg per day) in drinking water. Candesartan cilexetil is a prodrug that is converted rapidly and completely to candesartan during gastrointestinal absorption.[17] Interestingly, treatment with candesartan cilexetil prevented the progression of cardiac remodeling in AT_1Tg-AgtKO mice, when treatment was initiated at 6 weeks of age. The increases in heart:body weight ratio (Figure 3B), chamber dilatation and contractile dysfunction (Figure 3C), and interstitial fibrosis (Figure 3D) were significantly attenuated by candesartan cilexetil. Consistently, real-time RT-PCR indicated that the increases in mRNA levels of fetal cardiac genes (*Nppa*, *Nppb*, and *Acta1*) and extracellular matrix genes (*Col3a1* and *Postn*) in AT_1Tg-AgtKO hearts were significantly attenuated by treatment with candesartan cilexetil (Figure 3E).

We reported previously that tight binding between the carboxyl group of candesartan and specific residues of the AT_1 receptor was critical for the potent inverse agonism and that a derivative of candesartan (candesartan-7H), lacking the carboxyl group at the benzimidazole ring, could not suppress agonist-independent activities of the receptor.[1] Although treatment with candesartan-7H (1 mg/kg per day) had no effect, treatment with candesartan-7H (20 mg/kg per day) suppressed Ang II–induced BP elevation in C57BL/6 male mice, almost equally as treatment with candesartan cilexetil (1 mg/kg per day) did. (Figure 3A). However, treatment with candesartan-7H (20 mg/kg per day) did not prevent the increase in heart:body weight ratio (Figure 3B), progression of chamber dilatation, contractile dysfunction (Figure 3C), interstitial fibrosis (Figure 3D), or the increase in mRNA levels of fetal cardiac genes and extracellular matrix genes in AT_1Tg-AgtKO mice. Tail-cuff measurements revealed that treatment with candesartan cilexetil and candesartan-7H did not change systolic BP in AT_1Tg-AgtKO mice (Figure S2)

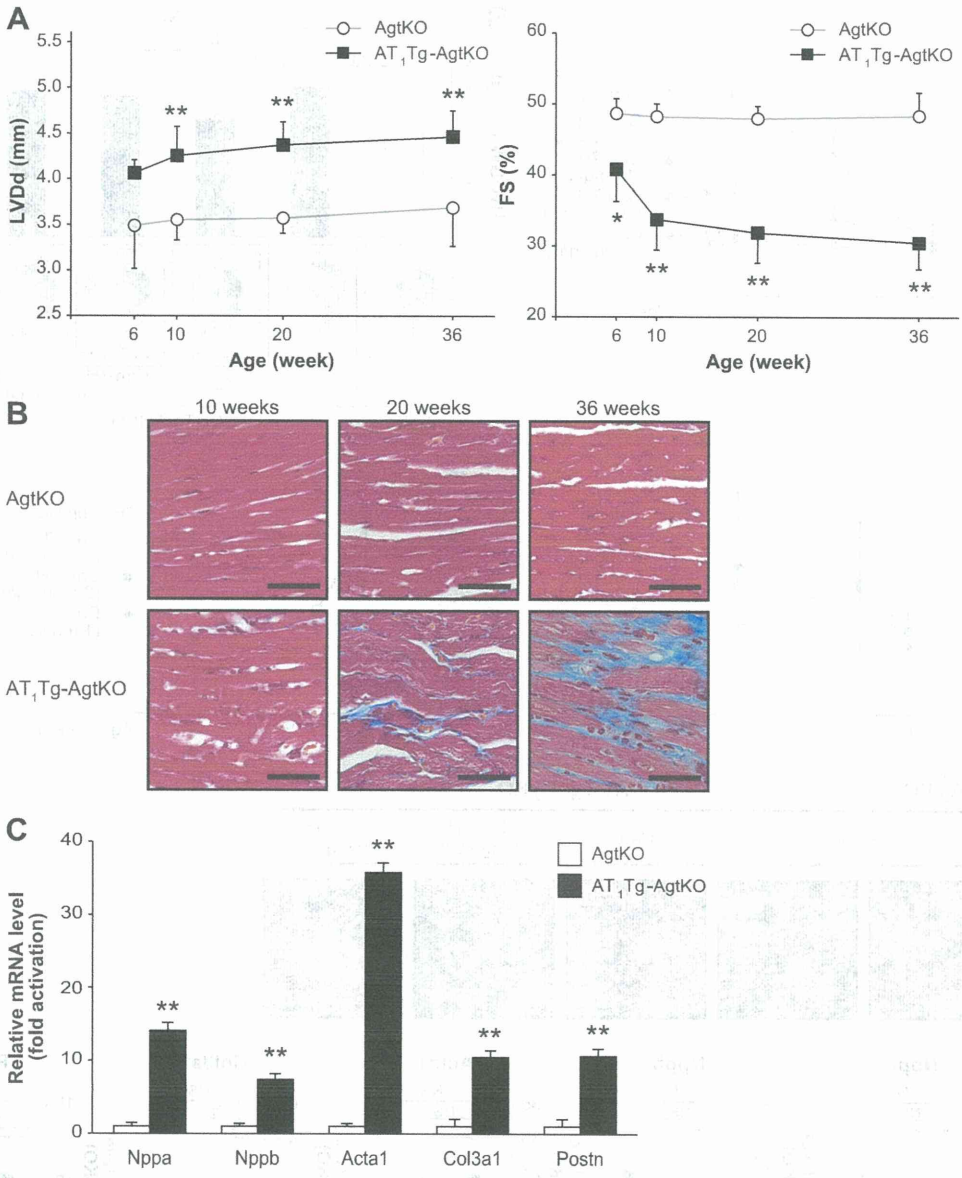

Figure 2. Spontaneous development of cardiac remodeling in angiotensin II type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice. **A**, Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AgtKO (n=7–9) and AT_1Tg-AgtKO (n=9–11) mice measured by echocardiogram at 6, 10, 20, and 36 weeks of age. Data are presented as mean±SEM. *P<0.05, **P<0.01 vs AgtKO mice. ○, AgtKO; ■, AT_1Tg-AgtKO. **B**, Histological sections with Masson trichrome staining of AgtKO and AT_1Tg-AgtKO hearts at 10, 20, and 36 weeks of age. Scale bars, 50 μm. **C**, The mRNA expressions of cardiac genes *Nppa*, *Nppb*, and *Acta1*, and extracellular matrix genes *Col3a1* and *Postn* in AgtKO (n=9) and AT_1Tg-AgtKO (n=9) hearts at 10 weeks of age. □, AgtKO; ■, AT_1Tg-AgtKO. Data are presented as mean±SEM. **P<0.01 vs AgtKO mice.

because Ang II is not produced in AT_1Tg-AgtKO mice. Collectively, these results suggest that cardiac remodeling in AT_1Tg-AgtKO mice was prevented by candesartan, an inverse agonist for the AT_1 receptor, but not by candesartan-7H, which cannot inhibit Ang II–independent AT_1 receptor activation because of a lack of inverse agonist activity.

Discussion

In several GPCRs, the constitutive activity is closely related to physiological function. For example, constitutive activity of the histamine H_3 receptor controls histaminergic neuron activity in rodents.[18] The melanocortin-4 receptor and growth hormone secretagogue receptor have high constitutive activity, and loss of constitutive activity in mutant melanocortin-4 receptors or growth hormone secretagogue receptors leads to obesity or short stature in humans, respectively.[19,20] In contrast, constitutively active mutations in several GPCRs give rise to diseases in humans. For example, somatic mutations of thyrotropin-stimulating hormone receptor or luteinizing hormone receptor lead to hyperfunctioning thyroid adenoma or male precocious puberty, respectively.[21,22]

In the present work, we provide experimental evidence that transgenic myocardial overexpression of the wild-type AT_1 receptor increases constitutive activity of the receptor, leading to cardiac enlargement, interstitial fibrosis, and contractile dysfunction, even in the absence of Ang II. To exclude a

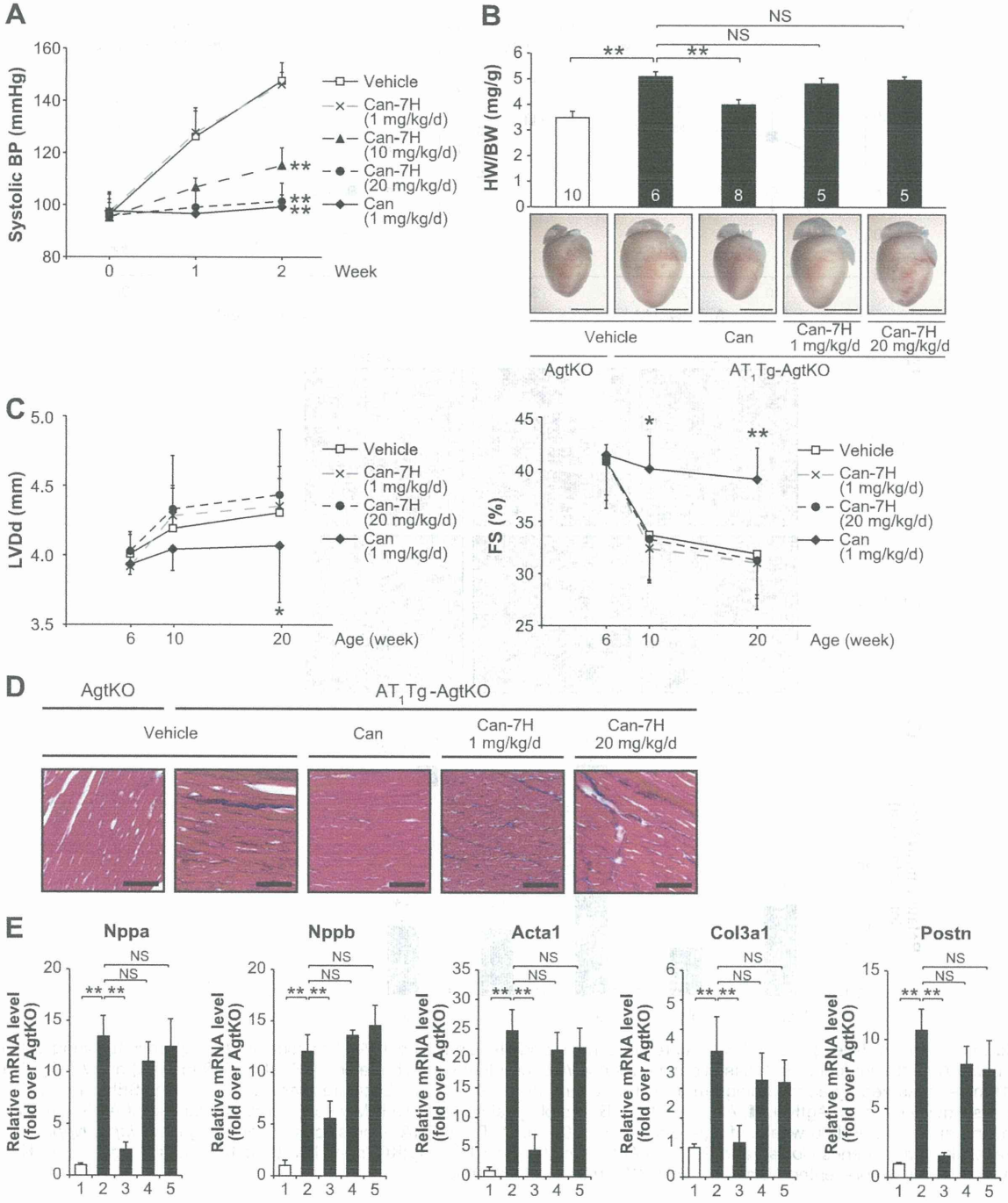

Figure 3. Prevention of cardiac remodeling in angiotensin II (Ang II) type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice by candesartan but not by candesartan-7H. **A**, Blood pressure–lowering effects of candesartan cilexetil (Can) and candesartan-7H (Can-7H) in Ang II–infused mice. Eight-week–old C57BL/6J male mice were continuously infused with Ang II (0.6 mg/kg per day) and treated with candesartan cilexetil (1 mg/kg per day), candesartan-7H (1, 10, and 20 mg/kg per day), or vehicle in drinking water (n=5, in each group). *$P<0.05$, **$P<0.01$ vs vehicle-treated group. **B**, Heart:body weight ratios and gross hearts in AgtKO and AT_1Tg-Agt KO mice (20 weeks of age) treated with Can (1 mg/kg per day), Can-7H (1, 20 mg/kg per day), or vehicle. Data are presented as mean±SEM. Number of mice for each experiment is indicated in the bars. **$P<0.01$. Scale bars, 5 mm. **C**, Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AT_1Tg-AgtKO mice treated with Can or Can-7H. Can (1 mg/kg per day, n=11), Can-7H (1, 20 mg/kg per day; n=7 in each group), or vehicle (n=7) was given for 14 weeks in 6-week–old AT_1Tg-AgtKO mice. Data are presented as mean±SEM. *$P<0.05$, **$P<0.01$ vs vehicle-treated group. **D**, Histological sections with Masson trichrome staining in AgtKO and AT_1Tg-Agt KO mice (20 weeks of age) treated with Can (1 mg/kg per day), Can-7H (1, 20 mg/kg per day), or vehicle. Scale bars, 50 μm. **E**, The mRNA expressions of cardiac genes *Nppa*, *Nppb*, and *Acta1* and extracellular matrix genes *Col3a1* and *Postn* in AgtKO (lane 1) and AT_1Tg-Agt KO mice (20 weeks of age) treated with Can (1 mg/kg per day; lane 3), Can-7H (1, 20 mg/kg per day; lane 4, 5, respectively), or vehicle (lane 2). Data are presented as mean±SEM. **$P<0.01$ vs AgtKO mice. NS indicates not significant ($P>0.05$). □, vehicle; ×, Can-7H (1 mg/kg per d); ▲, Can-7H (10 mg/kg per d); ●, Can-7H (20 mg/kg per d); ♦, Can (1 mg/kg per d).

contribution of endogenous Ang II to the activity of AT_1 receptor in native tissues, we used AgtKO mice, deficient in the production of Ang II.[13] Furthermore, AT_1Tg-AgtKO mice developed cardiac remodeling regardless of whether they were the offspring of $Agt^{+/-}$ females or $Agt^{-/-}$ females (Figure S3), suggesting that maternal or placental angiotensinogen had little influence on the postnatal development of cardiac remodeling in AT_1Tg-AgtKO mice. Among the renin-angiotensin system components, the mRNA level of the AT_2 receptor was significantly upregulated in AT_1Tg-AgtKO hearts compared with AgtKO hearts (Figure S1A), but the protein level of the AT_2 receptor was comparable between AT_1Tg-AgtKO and AgtKO hearts. Therefore, we believe that constitutive activity of the AT_1 receptor is sufficient for inducing structural and functional cardiac remodeling, when the AT_1 receptor is upregulated in the hearts.

Redistribution of $G\alpha_{q11}$ into the cytosolic fraction in AT_1Tg-AgtKO hearts (Figure 1A) indicates that constitutive activity of the AT_1 receptor is mediated through the $G\alpha_{q11}$-dependent signaling pathway. On binding to Ang II, the AT_1 receptor is phosphorylated by GPCR kinases and recruits β-arrestins, leading to clathrin-coated, pit-dependent internalization and then recycling to the plasma membrane.[23] It has been reported that constitutively active mutant AT_1 receptors are constitutively internalized and recycled when overexpressed in HEK293 cells.[24] In contrast, we showed previously, by immunofluorescence analysis, that the wild-type AT_1 receptor was predominantly localized in the plasma membrane of HEK293 cells expressing the AT_1 receptor.[1] In addition, the expression levels of GPCR kinase 2 and β-arrestins in the particulate fraction relative to the cytosolic fraction were comparable between AT_1Tg-AgtKO and AgtKO hearts (Figure S4). Therefore, we suppose that, in the absence of Ang II, wild-type AT_1 receptor stochastically undergoes subtle and transient conformational changes, leading to partial activation of $G\alpha_{q11}$-depentent signaling without inducing detectable receptor internalization. The AT_1 receptor can also stimulate G protein–independent diverse signaling pathways involving β-arrestins, tyrosine kinases, reactive oxygen species, and AT_1 receptor–associated proteins.[15] Further structure-function analysis will be needed to elucidate the full breadth of the molecular mechanisms and signal transduction network that mediate agonist-independent AT_1 receptor activation in the hearts.

It has been reported that the AT_1 receptor is upregulated in stressed hearts of spontaneously hypertensive rats,[25] 2-kidney 1-clip renovascular hypertensive rats,[25] Tsukuba hypertensive mice,[26] and rats with myocardial infarction.[27] Furthermore, we observed that cardiac expression of the AT_1 receptor was increased $\approx$8-fold in pressure-overloaded mice after transverse aortic constriction (B_{max}: 142.9$\pm$36.5 fmol/mg; n=3) compared with sham-operated mice (B_{max}: 16.4$\pm$4.9 fmol/mg; n=3). In addition, it has been reported that the AT_1 receptor is upregulated in response to low-density lipoprotein cholesterol,[28] insulin,[29] glucose,[30] progesterone,[31] and inflammatory cytokines, such as interleukin 1α or interleukin 6,[32,33] in vascular cells. Therefore, it seems quite reasonable to assume that enhancement of constitutive activity of the AT_1 receptor through upregulation of receptor expression may accelerate the progression of atherosclerosis in patients with hypercholesterolemia or diabetes mellitus, especially after menopause. Further studies in animal models will be required to clarify the roles of constitutive activity of the AT_1 receptor in the pathogenesis of cardiovascular and metabolic disorders.

We also demonstrate that treatment with candesartan, inverse agonist for the AT_1 receptor, effectively prevents cardiac remodeling in AT_1Tg-AgtKO mice. The inverse agonist activity of ARBs may provide clinical advantage of inhibiting both Ang II–dependent and -independent receptor activation and, thus, be an important pharmacological parameter defining the beneficial effects on organ protection.[3] Several ARBs are currently available for the treatment of hypertension and heart failure with reduced left ventricular ejection fraction, and their potency of inverse agonist activity differs according to the distinct chemical structure of the drug.[3] For example, the inhibitory effect of olmesartan on both constitutive activity and stretch-induced activation of the AT_1 receptor was significantly higher than that of losartan.[2] According to a recent article,[34] the use of candesartan was associated with lower all-cause mortality than the use with losartan in a Swedish registry of patients with heart failure. Although EXP3174, an active metabolite of losartan, can act as an inverse agonist,[8] it is tempting to speculate that the potent inverse agonist activity of candesartan may explain some of its association with lower mortality in patients with heart failure.

Perspectives

Blockade of the renin-angiotensin system has been shown to be beneficial in patients with hypertension, especially those with cardiovascular and metabolic complications. Our findings show that constitutive activity of the AT_1 receptor contributes to the progression of cardiac remodeling even in the absence of Ang II, when the AT_1 receptor is upregulated in the heart. Inverse agonism of ARBs provides therapeutic effects in the prevention of cardiac remodeling induced by constitutive activity of AT_1 receptor and, thus, has potential impact on long-term outcomes in patients with hypertension. Our work is the first proof-of-principle experiment, to our knowledge, on the in vivo importance of constitutive activity of a native GPCR in the pathogenesis of diseases. Beyond in vitro pharmacological tools, inverse agonists emerge as promising pharmacological candidates in treating diseases caused by enhancement of constitutive activity through upregulation of GPCRs.

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Disclosures

None.

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Correction

In the *Hypertension* article by Yasuda et al (Yasuda N, Akazawa H, Ito K, Shimizu I, Kudo-Sakamoto Y, Yabumoto C, Yano M, Yamamoto R, Ozasa Y, Minamino T, Naito AT, Oka T, Shiojima I, Tamura K, Umemura S, Nemer M, Komuro I. Agonist-Independent Constitutive Activity of Angiotensin II Receptor Promotes Cardiac Remodeling in Mice. *Hypertension.* 2012;59:627–633), corrections have been made.

Pierre Paradis's name was erroneously omitted from the author line. He has made the transgenic mice AGTR1, which are very important for this study.

The corrected author line and affiliations are as follows: Noritaka Yasuda, Hiroshi Akazawa, Kaoru Ito, Ippei Shimizu, Yoko Kudo-Sakamoto, Chizuru Yabumoto, Masamichi Yano, Rie Yamamoto, Yukako Ozasa, Tohru Minamino, Atsuhiko T. Naito, Toru Oka, Ichiro Shiojima, Kouichi Tamura, Satoshi Umemura, Pierre Paradis, Mona Nemer, Issei Komuro

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On page 628, first paragraph of the Methods section, the first sentence following the subheading, the name of a gene is not correct: it should be "*AGTR1*", not "*AGTR1a*". This change affects none of the observations or conclusions made in the article.

The authors regret these errors.

These corrections have been made to the current online version of the article, which is available at http://hyper.ahajournals.org/content/59/3/627.full.

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ONLINE SUPPLEMENT

AGONIST-INDEPENDENT CONSTITUTIVE ACTIVITY OF ANGIOTENSIN II RECEPTOR PROMOTES CARDIAC REMODELING IN MICE

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Supplemental Materials and Methods

Mice, TAC operation, and transthoracic echocardiography

Mice expressing the human *AGTR1a* gene under the control of *α-myosin heavy chain* (*MHC*) promoter and mice deficient for *Agt* gene were previously described [1, 2]. We crossed *AGTR1a* $^{Tg/o}$ mice (on the C57BL/6 background) with *Agt* $^{-/-}$ mice (on the ICR background), and then bred the resulting *AGTR1a* $^{Tg/o}$/*Agt* $^{+/-}$ offspring with *Agt* $^{+/-}$ mice to generate *AGTR1a* $^{Tg/o}$/*Agt* $^{+/+}$ (AT_1Tg), *AGTR1a* $^{Tg/o}$/*Agt* $^{-/-}$ (AT_1Tg-AgtKO), and *AGTR1a* $^{o/o}$/*Agt* $^{-/-}$ (AgtKO) mice. We also generated *AGTR1a* $^{Tg/o}$/*Agt* $^{-/-}$ (AT_1Tg-AgtKO) by crossing *AGTR1a* $^{Tg/o}$/*Agt* $^{+/-}$ with *Agt* $^{-/-}$ mice. C57BL/6 mice were purchased from Japan SLC. Candesartan and candesartan-7H were synthesized in Takeda Pharmaceutical Co., Ltd., and administered via drinking water. For TAC operation, 10-week-old male mice were anesthetized by i.p. injection of pentobarbital (50 mg/kg), and respiration was artificially controlled with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths/min. The transverse aorta was constricted with 7-0 nylon strings by ligating the aorta with splinting a blunted 27 gauge needle, which was removed after the ligation. After aortic constriction, the chest was closed and mice were allowed to recover from anesthesia. We confirmed that the magnitude of initial pressure elevation after aortic banding was identical in all groups of mice. The surgeon had no information about the mice used in this study. For evaluation of cardiac dimensions and contractility, transthoracic echocardiography was performed on conscious mice with Vevo 770 Imaging System using a 25 MHz linear probe (Visual Sonics). All protocols were approved by the Institutional Animal Care and Use Commmittee of Chiba University.

Ang II infusion and BP measurement

Ang II (Sigma-Aldrich) was dissolved in 0.9% saline. Eight-week-old C57BL/6J male mice were treated with Ang II (0.6 mg/kg/day) or vehicle for 2 weeks using an osmotic mini-pump (ALZET model 2002; Durent Corp.). The systolic and diastolic BP and pulse rates were measured in conscious mice noninvasively by a programmable sphygmomanometer (BP-98A, Softron) using the tail-cuff method.

Real-time RT-PCR analysis

Total RNA was extracted by using RNeasy Kit (Qiagen), and single-stranded cDNA was transcribed by using QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol. We conducted quantitative real-time PCR analysis with the Universal ProbeLibrary Assays (Roche Applied Science), according to the manufacturer's instructions. Amplification conditions were initial denaturation for 10 min at 95°C followed by 45 cycles of 10 s at 95°C and 25 s at 60°C. Individual PCR products were analyzed by melting-point analysis. The expression level of a gene was normalized relative to that of *Gapdh* by using a comparative Ct method. The primer sequences and Universal Probe numbers were designed with the ProbeFinder software as following: *Agtr1b*, 5'-cgccagcagcactgtaga-3' and 5'-ggaggggtgaattcaaaa-3', No. 32; *Agtr2*, 5'-ggagctcggaactgaaagc-3' and 5'-ctgcagcaactccaaattctt-3', No. 41; *Ace*, 5'-tatgcccctggaacctgat-3' and 5'-gatggctctccccaccttt-3', No. 78; *Ren1*, 5'-ggaggaagtgttctctgtctactaca-3' and 5'-tcgctacctcctagcaccac-3', No. 3; *Ren2*,

5'-catggagaatggagacgactt-3' and 5'-cacagtgattccacccacag-3', No. 102; *Nppa*, 5'-cacagatctgatggatttcaaga-3' and 5'-cctcatcttctaccggcatc-3', No. 25; *Nppb*, 5'-gtcagtcgtttgggctgtaac-3' and 5'-agacccaggcagagtcagaa-3', No. 71; *Acta1*, 5'-agctatgagctgcctgacg-3' and 5'-atccccgcagactccatac-3', No. 9; *Col3a1*, 5'-tcccctggaatctgtgaatc-3' and 5'-tgagtcgaattggggagaat-3', No. 49; *Postn*, 5'-cgggaagaacgaatcattaca-3' and 5'- accttggagacctctttttgc-3', No. 10; *Gapdh*, 5'-tgtccgtcgtggatctgac-3' and 5'-cctgcttcaccaccttcttg-3', No. 80.

Western blot analysis and subcellular fractionation

Protein samples were fractionated with SDS–PAGE, transferred to PVDF membranes (GE Healthcare Biosciences). The blotted membranes were incubated with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Immunoreactive signals were visualized using ECL Plus Western Blotting Detection System (GE Healthcare Biosciences). Following antibodies were used: rabbit polyclonal anti-$G\alpha_{q/11}$ antibody, goat polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-phospho-ERK1/2 antibody (Cell Signaling Techonlogy), rabbit polycloncal anti-ERK1/2 antibody (Invitrogen), rabbit polyclonal anti-AT_2 receptor antibody (Alomone Labs), mouse monoclonal anti-GRK2 antibody (Santa Cruz), and mouse monoclonal anti-β-arrestin 1/2 antibody (Santa Cruz).

For subcellular fractionation, heart samples were homogenized in lysis buffer (25 mM Tris HCl pH 7.4, 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 5 mM DTT) plus protease inhibitors (Complete mini; Roche Applied Science). The lysates were centrifuged at 500 *g* for 20 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 100,000 *g* for 60 min, and the supernatant was designated as the cytosolic fraction. The pellets were then resuspended as the membrane-particulate fraction in lysis buffer with 1% Triton X-100.

Histological analysisHearts were excised, fixed immediately in 10% neutralized formalin, and embedded in paraffin. Serial sections at 5 μm were stained with Masson's trichrome for evaluation of fibrosis.

Radioligand receptor binding assayRadioligand-binding assays were performed as described previously [3-5]. The protein in membrane fraction was incubated with 100 pM ^{125}I-[Sar^1, Ile^8] Ang II (Perkin Elmer) for 1 hr at 22℃. Binding reaction was terminated by filtering the incubation mixture through Whatman GF/C glass filters (GE healthcare Biosciences), and the residues were extensively washed further with binding buffer. The bound ligand fraction was determined from the counts per minute (cpm) remaining on the membrane. Binding kinetics values were determined with the LIGAND computer program (Elsevier-Biosoft), as previously described [3-5].

Statistics

All data are presented as means ± SEM. Two-group comparison was analyzed by unpaired 2-tailed Student's *t* test, and multiple-group comparison was performed by one-way ANOVA followed by the Fisher`s PLSD test for comparison of means. A probability value of $P < 0.05$ was considered to be statistically significant.