Agonist-Independent Constitutive Activity of Angiotensin II Receptor Promotes Cardiac Remodeling in Mice


See Editorial Commentary, pp 542–544

Abstract—The angiotensin II (Ang II) type 1 (AT1) receptor mainly mediates the physiological and pathological actions of Ang II, but recent studies have suggested that AT1 receptor inherently shows spontaneous constitutive activity even in the absence of Ang II in culture cells. To elucidate the role of Ang II–independent AT1 receptor activation in the pathogenesis of cardiac remodeling, we generated transgenic mice overexpressing AT1 receptor under the control of α-myosin heavy chain promoter in angiotensinogen-knockout background (AT1tg-AgtKO mice). In AT1tg-AgtKO hearts, redistributions of the Gαq11 subunit into cytosol and phosphorylation of extracellular signal-regulated kinases were significantly increased, compared with angiotensinogen-knockout mouse hearts, suggesting that the AT1 receptor is constitutively activated independent of Ang II. As a consequence, AT1tg-AgtKO mice showed spontaneous systolic dysfunction and chamber dilatation accompanied by severe interstitial fibrosis. Progression of cardiac remodeling in AT1tg-AgtKO mice was prevented by treatment with candesartan, an inverse agonist for the AT1 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 AT1 receptor under basal conditions contributes to the cardiac remodeling even in the absence of Ang II, when the AT1 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 (AT1) receptor is a 7 transmembrane spanning G protein-coupled receptor (GPCR), and the activation of AT1 receptor is involved in regulating pathophysiological processes of the cardiovascular system. In principle, the AT1 receptor is activated on binding to Ang II, which is produced systemically or locally after pathogenesis of cardiac remodeling, we generated transgenic mice overexpressing AT1 receptor under the control of α-myosin heavy chain promoter in angiotensinogen-knockout background (AT1tg-AgtKO mice). In AT1tg-AgtKO hearts, redistributions of the Gαq11 subunit into cytosol and phosphorylation of extracellular signal-regulated kinases were significantly increased, compared with angiotensinogen-knockout mouse hearts, suggesting that the AT1 receptor is constitutively activated independent of Ang II. As a consequence, AT1tg-AgtKO mice showed spontaneous systolic dysfunction and chamber dilatation accompanied by severe interstitial fibrosis. Progression of cardiac remodeling in AT1tg-AgtKO mice was prevented by treatment with candesartan, an inverse agonist for the AT1 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 AT1 receptor under basal conditions contributes to the cardiac remodeling even in the absence of Ang II, when the AT1 receptor is upregulated in the heart. (Hypertension. 2012;59:627-633.) ● Online Data Supplement

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coding region of the AT₁ receptor gene have not been identified in hypertension or primary hyperaldosteronism.9,10 Although knock-in mice with a constitutively activating mutation (substitution of Asn¹¹ 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 AT₁ receptor leads to some phenotypic abnormalities even under circumstances where the production of Ang II is genetically inhibited.

Therefore, we generated transgenic mice overexpressing AT₁ receptor under the control of α-myosin heavy chain promoter in the angiotensinogen (Agt) knockout background. Here, we show that constitutive activity of the AT₁ receptor indeed contributes to cardiac remodeling independent of Ang II even in vivo, when the AT₁ 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’s 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
AT₁ Receptor Is Constitutively Activated Without the Involvement of Ang II in AT₁ Transgenic-Angiotensinogen Knockout Mice Hearts
To elucidate the pathogenic role of Ang II–independent AT₁ receptor activation in the hearts, we crossed transgenic mice overexpressing human AT₁ receptor under the control of cardiac-specific α-myosin heavy chain promoter (AT₁Tg) with angiotensinogen knockout mice (AgtKO) to generate AT₁Tg-AgtKO mice. First, we examined the expression levels of renin-angiotensin system components. Although the mRNA level of the AT₂ receptor (Agr2) was significantly higher in AT₁Tg-AgtKO hearts than in AgtKO hearts, there was no significant difference in protein levels of the AT₂ receptor between AT₁Tg-AgtKO and AgtKO hearts (Figure S1 in the online-only Data Supplement). Furthermore, the mRNA levels of the AT₁b receptor (Agtr1b), angiotensin-converting enzyme (Ace), and renin (Ren1 and Ren2) did not differ significantly between AT₁Tg-AgtKO and AgtKO hearts (Figure S1A).

We next determined the density of the AT₁ receptor (Bₘₐₓ values of receptor binding) in membranes isolated from the ventricles of AgtKO and AT₁Tg-AgtKO mice by radioligand binding assays using [¹²⁵I]-[Sar¹, Ile⁸] Ang II as ligand. Consistent with the previous report,12 the Bₘₐₓ of AT₁ receptor was increased by >200-fold in AT₁Tg-AgtKO hearts compared with AgtKO hearts (AT₁Tg-AgtKO: 5.41±1.79 pmol/mg of protein; AgtKO: 24.0±13.9 pmol/mg of protein; n=4 per group; P<0.01). Next, to evaluate whether the AT₁ receptor is constitutively activated in the AT₁Tg-AgtKO hearts, we examined redistribution of Goq₄₁ into the cytosolic fraction and phosphorylation of extracellular signal–regulated kinases (ERKs) in AgtKO and AT₁Tg-AgtKO hearts. On activation of the AT₁ receptor, the heterotrimeric Gq subunits dissociates into α and βγ subunits, and the GTP-bound Goq subunit stimulates diverse intracellular signaling pathways, including the ERK pathway.15,16 Redistribution of Goq₄₁ subunits from the particulate to the cytosolic fraction was significantly increased in AT₁Tg-AgtKO hearts compared with AgtKO hearts (Figure 1A). In addition, the levels of phosphorylated ERKs in AT₁Tg-AgtKO hearts was significantly increased compared with AgtKO hearts (Figure 1B). These results suggest that the AT₁ receptor is upregulated and constitutively activated without the involvement of Ang II in the AT₁Tg-AgtKO hearts.

AT₁Tg-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 AT₁Tg-AgtKO mice at 20 weeks of age (Table). However, morphological and physiological analysis revealed progressive chamber dilatation, contractile dysfunction, and interstitial fibrosis in AT₁Tg-AgtKO mice, whereas cardiac structure and function were normal in AgtKO mice. At 20 weeks of age, AT₁Tg-AgtKO mice displayed ~1.5-fold increase in heart/body ratio (BPs) and pulse rates revealed that these parameters did not differ significantly between AgtKO and AT₁Tg-AgtKO mice at 20 weeks of age (Table). However, morphological and physiological analysis revealed progressive chamber dilatation, contractile dysfunction, and interstitial fibrosis in AT₁Tg-AgtKO mice, whereas cardiac structure and function were normal in AgtKO mice. At 20 weeks of age, AT₁Tg-AgtKO mice displayed ~1.5-fold increase in heart/body ratio (BPs) and pulse rates revealed that these parameters did not differ significantly between AgtKO and AT₁Tg-AgtKO mice at 20 weeks of age (Table). However, morphological and physiological analysis revealed progressive chamber dilatation, contractile dysfunction, and interstitial fibrosis in AT₁Tg-AgtKO mice, whereas cardiac structure and function were normal in AgtKO mice. At 20 weeks of age, AT₁Tg-AgtKO mice displayed ~1.5-fold increase in heart/body ratio.
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 AT1Tg-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 AT1Tg-AgtKO hearts compared with AgtKO hearts (Figure 2C). These results indicate that upregulation of the AT1 receptor induced spontaneous and progressive cardiac remodeling in AT1Tg-AgtKO mice in spite of systemic deficiency of Ang II.

Cardiac Remodeling in AT1Tg-AgtKO Mice Is Prevented by Treatment With an Inverse Agonist for the AT1 Receptor

We examined whether an AT1 receptor blocker candesartan could prevent the progression of cardiac remodeling in AT1Tg-AgtKO mice. In cultured cells, candesartan reduces the basal activity of both the wild-type AT1 receptor and constitutively active AT1 mutant receptors, suggesting that candesartan is an inverse agonist for the AT1 receptor.1 Candesartan also suppresses mechanical stretch-induced hemo-

Tab. Measurement of Heart Weight, Heart Rate, and BP in AgtKO and AT1Tg-AgtKO Mice at 20 wk of Age

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AgtKO</th>
<th>AT1Tg-AgtKO</th>
</tr>
</thead>
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<tr>
<td>BW, g</td>
<td>31.0±3.4</td>
<td>30.2±3.5</td>
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<td>HW/BW, mg/g</td>
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<td>57.3±6.0</td>
<td>55.7±7.4</td>
</tr>
<tr>
<td>Mean BP, mm Hg</td>
<td>65.7±5.3</td>
<td>66.0±5.0</td>
</tr>
</tbody>
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BW indicates body weight; HR, heart rate; HW/BW, heart/body weight ratio; BP, blood pressure; AgtKO, angiotensinogen-knockout; AT1Tg, angiotensin II type 1 transgenic.

*P<0.01 vs sham.

Figure 1. Constitutive activation of angiotensin II type 1 (AT1) receptor in AT1 transgenic (AT1Tg)-angiotensinogen-knockout (AgtKO) hearts. A, Immunoblot analysis of Gaq11 in cytosolic fraction (CF) and particulate fraction (PF) extracted from AgtKO (n=6) and AT1Tg-AgtKO (n=6) hearts. The quantitation of the Gaq11 in CF/PF is shown as a bar graph. Data are presented as mean±SEM. **P<0.01 vs AT1Tg-AgtKO mice. B, Immunoblot analysis of phosphorylated extracellular signal-regulated kinases (ERK; p-ERKs) and total ERKs in AgtKO (n=8) and AT1Tg-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.
because Ang II is not produced in AT\textsubscript{1}Tg-AgtKO mice. Collectively, these results suggest that cardiac remodeling in AT\textsubscript{1}Tg-AgtKO mice was prevented by candesartan, an inverse agonist for the AT\textsubscript{1} receptor, but not by candesartan-7H, which cannot inhibit Ang II–independent AT\textsubscript{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\textsubscript{3} receptor controls histaminergic neuron activity in rodents.\textsuperscript{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.\textsuperscript{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.\textsuperscript{21,22}

In the present work, we provide experimental evidence that transgenic myocardial overexpression of the wild-type AT\textsubscript{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 (AT1) transgenic (AT1Tg-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 = 100 in each group). *P < 0.05, **P < 0.01 vs vehicle-treated group.

B, Heart:body weight ratios and gross hearts in AgtKO and AT1Tg-AgtKO 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 AT1Tg-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 AT1Tg-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 AT1Tg-AgtKO 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 AT1Tg-AgtKO 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; x, 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 AT1 receptor in native tissues, we used AgtKO mice, deficient in the production of Ang II.13 Furthermore, AT1Tg-AgtKO mice developed cardiac remodeling regardless of whether they were the offspring of Agr+/− females or Agr−/− females (Figure S3), suggesting that maternal or placental angiotensinogen had little influence on the postnatal development of cardiac remodeling in AT1Tg-AgtKO mice. Among the renin-angiotensin system components, the mRNA level of the AT2 receptor was significantly upregulated in AT1Tg-AgtKO hearts compared with AgtKO hearts (Figure S1A), but the protein level of the AT1 receptor was comparable between AT1Tg-AgtKO and AgtKO hearts. Therefore, we believe that constitutive activity of the AT1 receptor is sufficient for inducing structural and functional cardiac remodeling, when the AT1 receptor is upregulated in the hearts.

Redistribution of Gαq11 into the cytosolic fraction in AT1Tg-AgtKO hearts (Figure 1A) indicates that constitutive activity of the AT1 receptor is mediated through the Gαq11-dependent signaling pathway. On binding to Ang II, the AT1 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 AT1 receptors are constitutively internalized and recycled when overexpressed in HEK293 cells.24 In contrast, we showed previously, by immunofluorescence analysis, that the wild-type AT1 receptor was predominantly localized in the plasma membrane of HEK293 cells expressing the AT1 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 AT1Tg-AgtKO and AgtKO hearts (Figure S4). Therefore, we suppose that, in the absence of Ang II, wild-type AT1 receptor stochastically undergoes subtle and transient conformational changes, leading to partial activation of Gαq11-dependent signaling without inducing detectable receptor internalization. The AT1 receptor can also stimulate G protein–independent diverse signaling pathways involving β-arrestins, tyrosine kinases, reactive oxygen species, and AT1 receptor–associated proteins.19 Further structure-function analysis will be needed to elucidate the full breadth of the molecular mechanisms and signal transduction network that mediate agonist-independent AT1 receptor activation in the hearts.

It has been reported that the AT1 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 AT1 receptor was increased ~8-fold in pressure-overloaded mice after transverse aortic constriction (Bmax: 142.9±36.5 fmol/mg; n=3) compared with sham-operated mice (Bmax: 16.4±4.9 fmol/mg; n=3). In addition, it has been reported that the AT1 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 AT1 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 AT1 receptor in the pathogenesis of cardiovascular and metabolic disorders.

We also demonstrate that treatment with candesartan, inverse agonist for the AT1 receptor, effectively prevents cardiac remodeling in AT1Tg-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 We also demonstrate that treatment with candesartan, inverse agonist for the AT1 receptor, effectively prevents cardiac remodeling in AT1Tg-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 AT1 receptor was significantly higher than that of losartan.2 According to a recent article,34 the use of candesartan is 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 AT1 receptor contributes to the progression of cardiac remodeling even in the absence of Ang II, when the AT1 receptor is upregulated in the heart. Inverse agonism of ARBs provides therapeutic effects in the prevention of cardiac remodeling induced by constitutive activity of AT1 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 ofGPCRs.

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Disclosures

None.

References


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/content/59/5/e51.full.pdf

Data Supplement (unedited) at:
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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.

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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.
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. 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* +/+ (AT1Tg), *AGTR1a* Tg/o/*Agt* -/- (AT1Tg-AgtKO), and *AGTR1a* o/o/*Agt* -/- (AgtKO) mice. We also generated *AGTR1a* Tg/o/*Agt* +/- (AT1Tg-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 Committee 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 sphygmanometer (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’-cgccagcacatgtgta-3’ and 5’-ggaggggtgaattaaca-3’, No. 32; *Agtr2*, 5’-ggagctcggaactgaaagc-3’ and 5’-ctgcagcaactccaaattctt-3’, No. 41; *Ace*, 5’-tatgcccctggaacctgat-3’ and 5’-gatggctctccccacctt-3’, No. 78; *Ren1*, 5’-ggagagagttgctctgtctcttata-3’ and 5’-tgctacactctgggcc-3’, No. 3; *Ren2*,

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5’-catggagaatggagacgactt-3’ and 5’-cacagtgattccacccacag-3’, No. 102; Nppa,
5’-cacagatctgatggatttcaaga-3’ and 5’-cctcatcttctaccggcatc-3’, No. 25; Nppb,
5’-gtcagtgattccacccacag-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’-
tgcctgtggatctgc-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-\( \alpha \)\( \text{G} \)/11 antibody, goat polyclonal
anti-GAPDH antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal
anti-phospho-ERK1/2 antibody (Cell Signaling Technology), rabbit polyclonal
anti-ERK1/2 antibody (Invitrogen), rabbit polyclonal anti-\( \beta \)2 receptor antibody
(Alomone Labs), mouse monoclonal anti-GRK2 antibody (Santa Cruz), and mouse
monoclonal anti-\( \beta \)-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 analysis
Hearts were excised, fixed immediately in 10% neutralized
formalin, and embedded in paraffin. Serial sections at 5 \( \mu \)m were stained with
Masson’s trichrome for evaluation of fibrosis.

Radioligand receptor binding assay
Radioligand-binding assays were performed as
described previously 3-5. The protein in membrane fraction was incubated with 100
pM \(^{125}\text{I}-[\text{Sar}^1, \text{Ile}^8]\) Ang II (Perkin Elmer) for 1 hr at 22°C. 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.
References


Figure S1. Expression levels of the renin-angiotensin system components in AT1Tg-AgtKO and AgtKO hearts. 
(A) The mRNA expressions of the renin-angiotensin system components in AT1Tg-AgtKO (n = 6) and AgtKO hearts (n = 6) at 20 weeks of age. Data are presented as mean ± SEM. * P < 0.05 versus AgtKO mice. 
(B) Immunoblot analysis of AT2 receptor in AgtKO (n = 4) and AT1Tg-AgtKO (n = 4) hearts at 20 weeks of age. GAPDH was used as an internal control for loading. The quantitation of the AT2 receptor/GAPDH is shown as a bar graph. Data are presented as mean ± SEM. NS, not significant (P > 0.05).
Figure S2. Systolic BP in AT$_1$Tg mice treated with vehicle ($n = 9$), AgtKO mice treated with vehicle ($n = 6$), AT$_1$Tg-AgtKO mice treated with vehicle ($n = 6$), candesartan cilexetil (Can) (1 mg/kg/day, $n = 8$) or candesartan-7H (Can-7H) (1 mg/kg/day, $n = 5$ or 20 mg/kg/day, $n = 5$). BP was measured in 20-week-old mice after the treatment for 14 weeks. Data are presented as mean ± SEM. **$P < 0.01$ versus AgtKO mice.
Figure S3. AT1Tg-AgtKO mice developed cardiac remodeling independently of the effects of maternal or placental angiotensinogen during the fetal period. Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AT1Tg-AgtKO offspring of Agt +/- females ($n = 4$) or Agt +/- females ($n = 4$), measured by echocardiogram at 6 and 10 weeks of age. Data are presented as mean ± SEM.
Figure S4. Immunoblot analysis of GRK2 and β-arrestin 1/2 in particulate fraction (PF) and cytosolic fraction (CF) extracted from AgtKO (n = 4) and AT1Tg-Agt KO (n = 4) hearts. The quantitation of GRK2 in PF/CF and β-arrestin 1/2 in PF/CF is shown as bar graphs. Data are presented as mean ± SEM. NS, not significant (P > 0.05).