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

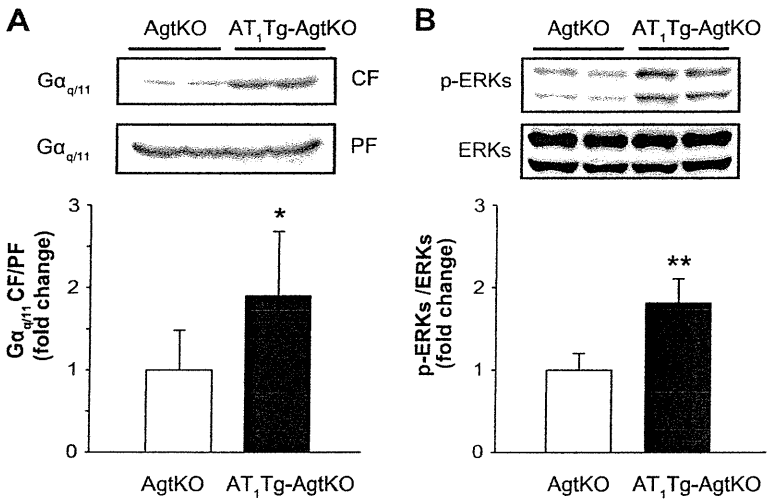

Figure 1. Constitutive activation of angiotensin II type 1 (AT_1) receptor in AT_1 transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) hearts. **A**, Immunoblot analysis of $G\alpha_{q/11}$ in cytosolic fraction (CF) and particulate fraction (PF) extracted from AgtKO (n=6) and AT_1Tg-AgtKO (n=6) hearts. 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 hetical 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)

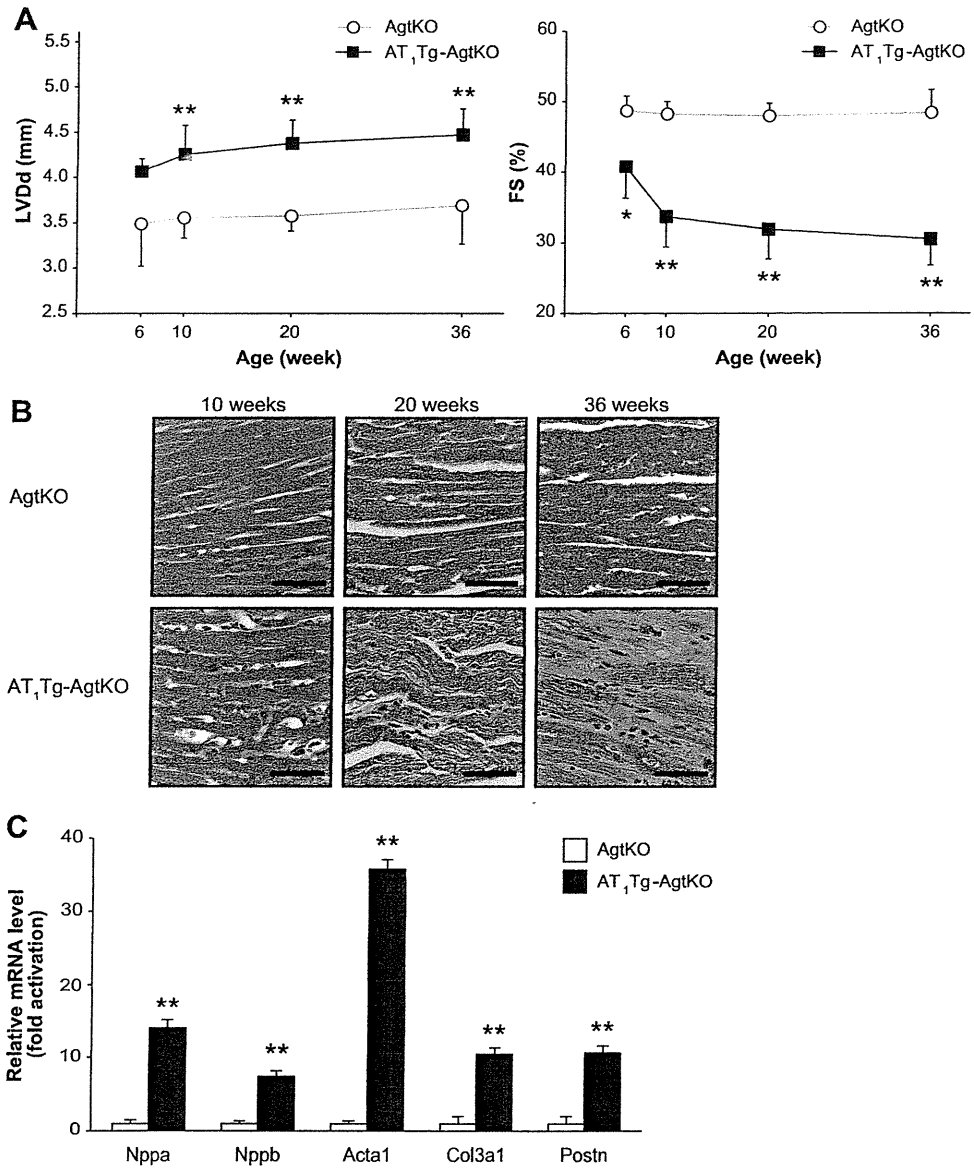

Figure 2. Spontaneous development of cardiac remodeling in angiotensin II type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice. **A**, Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AgtKO (n=7–9) and AT_1Tg-AgtKO (n=9–11) mice measured by echocardiogram 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

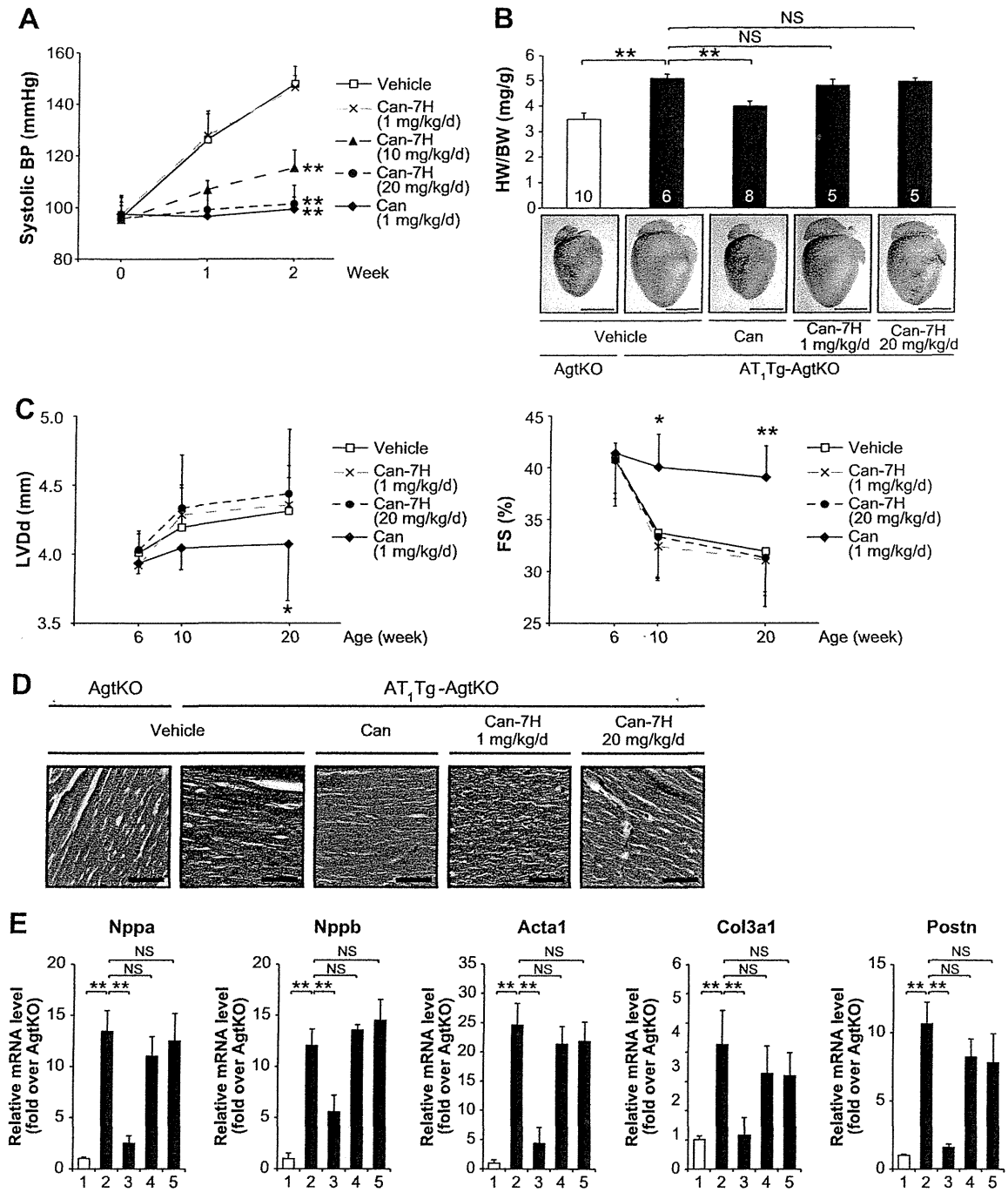

Figure 3. Prevention of cardiac remodeling in angiotensin II (Ang II) type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice by candesartan but not by candesartan-7H. **A**, Blood pressure–lowering effects of candesartan cilexetil (Can) and candesartan-7H (Can-7H) in Ang II–infused mice. 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

From the Department of Cardiovascular Science and Medicine (N.Y., K.I., Ip.S., R.Y., Y.O., T.M.), Chiba University Graduate School of Medicine, Chiba, Japan; Departments of Cardiovascular Medicine (H.A., Y.K.-S., C.Y., M.Y., T.O., I.K.) and Cardiovascular Regenerative Medicine (A.T.N., Ic.S.), Osaka University Graduate School of Medicine, Suita, Japan; Department of Medical Science and Cardiorenal Medicine (K.T., S.U.), Yokohama City University Graduate School of Medicine, Yokohama, Japan; Lady Davis Institute for Medical Research (P.P.), Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal, Quebec, Canada; Laboratory of Cardiac Growth and Differentiation (M.N.), Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada.

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.

(*Hypertension.* 2012;59:e51.)

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYP.0b013e31825416f4

ONLINE SUPPLEMENT

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

Noritaka Yasuda[1], Hiroshi Akazawa[2], Kaoru Ito[1], Ippei Shimizu[1], Yoko Kudo-Sakamoto[2], Chizuru Yabumoto[2], Masamichi Yano[2], Rie Yamamoto[1], Yukako Ozasa[1], Tohru Minamino[1], Atsuhiko T. Naito[3], Toru Oka[2], Ichiro Shiojima[3], Kouichi Tamura[4], Satoshi Umemura[4], Mona Nemer[5], Issei Komuro[2]

1. Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
2. Department of Cardiovascular Medicine,
3. Department of Cardiovascular Regenerative Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
4. Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
5. Laboratory of Cardiac Growth and Differentiation, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 550 Cumberland, Ottawa, Ontario K1N 6N5, Canada

Correspondence to:

Issei Komuro, M.D., Ph.D.

Department of Cardiovascular Medicine,
Osaka University Graduate School of Medicine,
2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
TEL: +81-6-6879-3631
FAX: +81-6-6879-3639
E-mail: komuro-tky@umin.ac.jp

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'-gatggctctccccacctt-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.

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Figure S1

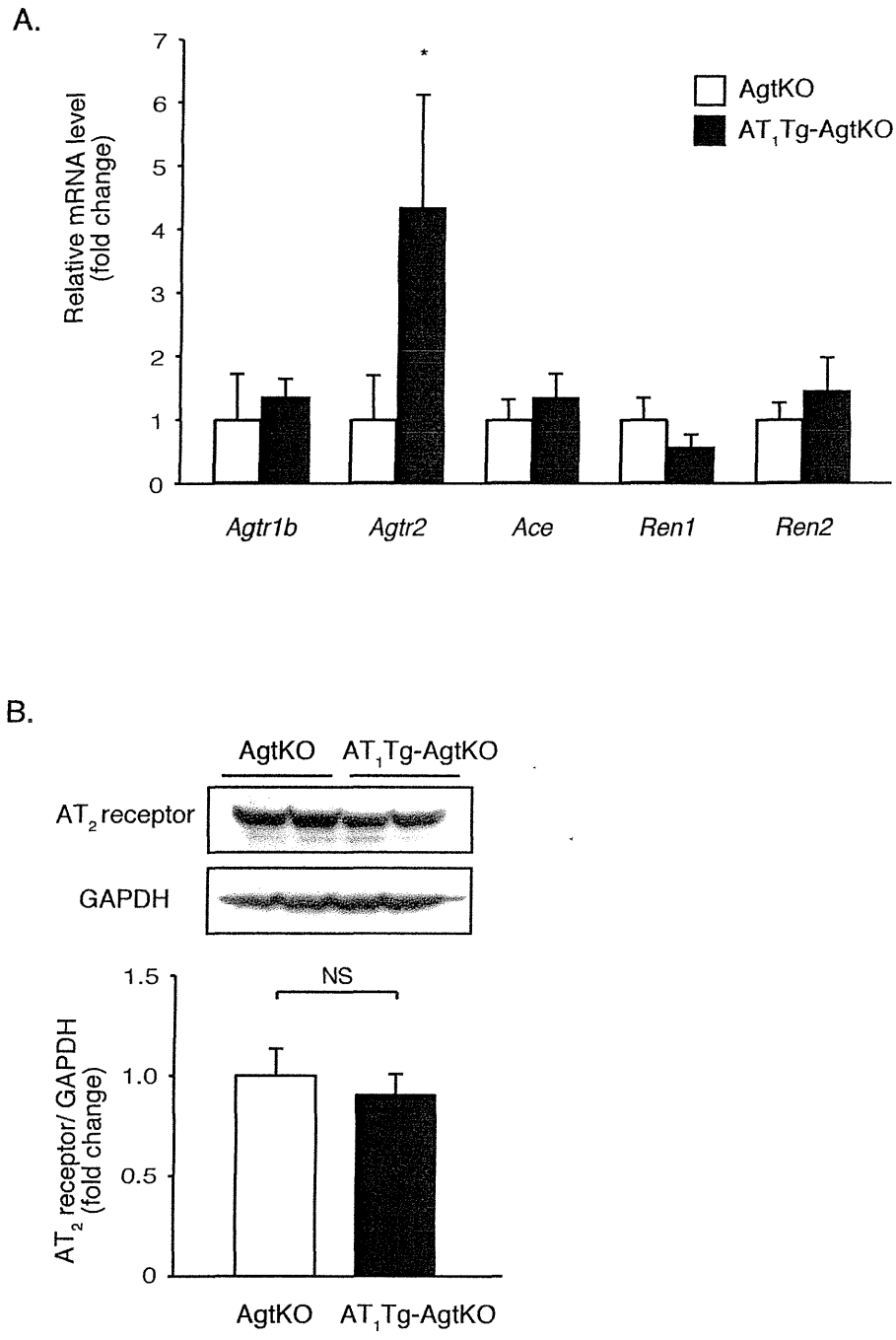

Figure S1. Expression levels of the renin-angiotensin system components in AT_1Tg-Agt KO and AgtKO hearts. (A) The mRNA expressions of the renin-angiotensin system components in AT_1Tg-Agt KO ($n = 6$) and AgtKO hearts ($n = 6$) at 20 weeks of age. Data are presented as mean ± SEM. * $P < 0.05$ versus AgtKO mice. (B) Immunoblot analysis of AT_2 receptor in AgtKO ($n = 4$) and AT_1Tg-AgtKO ($n = 4$) hearts at 20 weeks of age. GAPDH was used as an internal control for loading. The quantitation of the AT_2 receptor /GAPDH is shown as a bar graph. Data are presented as mean ± SEM. NS, not significant ($P > 0.05$).

Figure S2

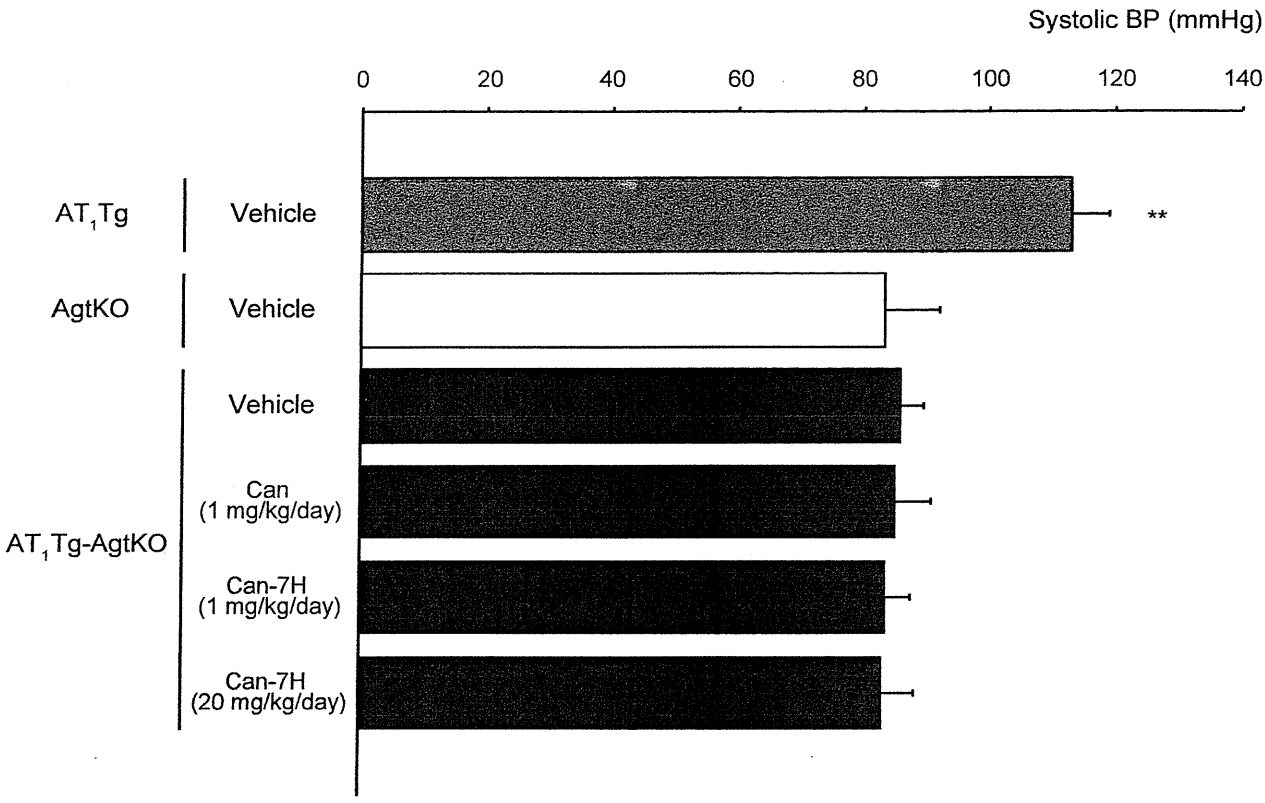

Figure S2. Systolic BP in AT_1Tg mice treated with vehicle ($n = 9$), AgtKO mice treated with vehicle ($n = 6$), AT_1Tg-AgtKO mice treated with vehicle ($n = 6$), candesartan cilexetil (Can) (1 mg/kg/day, $n = 8$) or candesartan-7H (Can-7H) (1 mg/kg/day, $n = 5$ or 20 mg/kg/day, $n = 5$). BP was measured 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

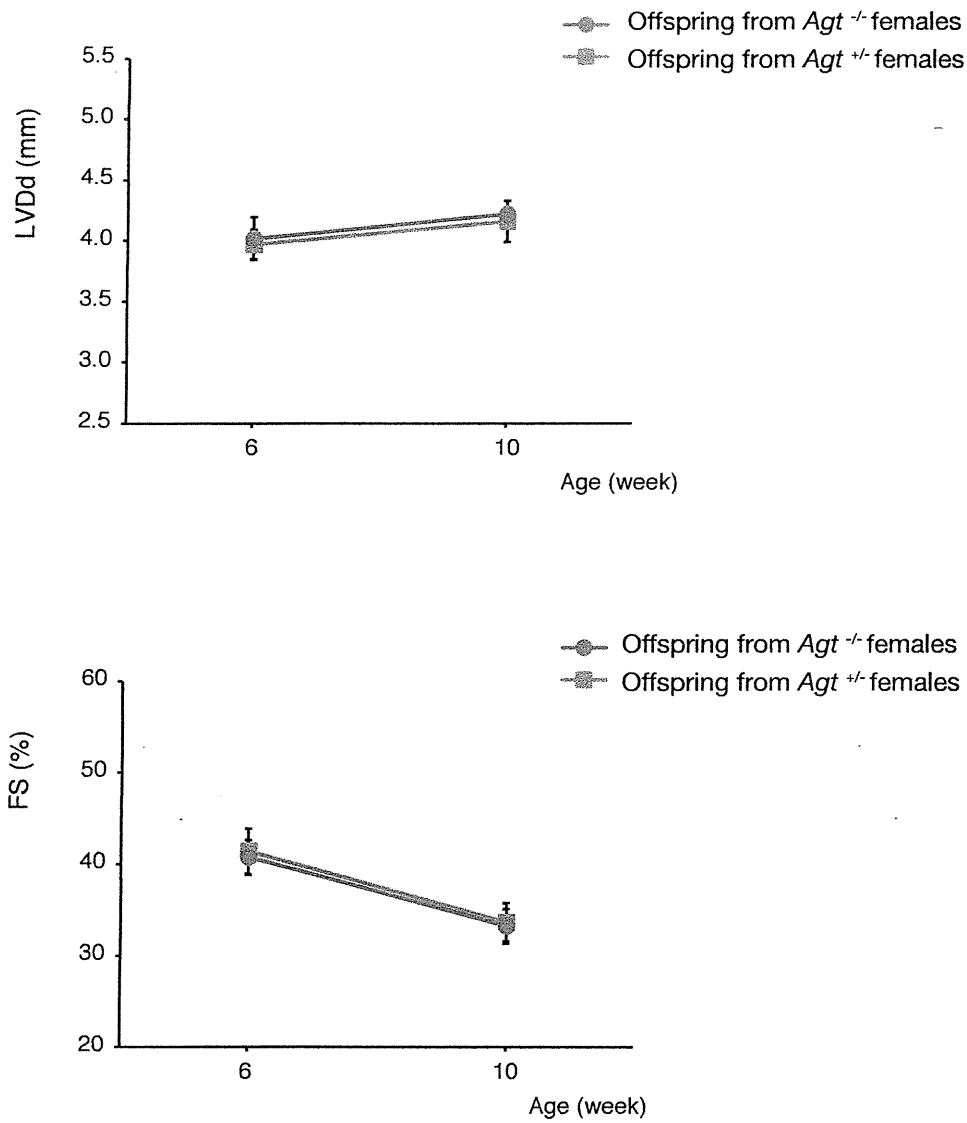

Figure S3. AT_1Tg-AgtKO mice developed cardiac remodeling independently of the effects of maternal or placental angiotensinogen during the fetal period. Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AT_1Tg-AgtKO offspring of *Agt* $^{+/-}$ females ($n = 4$) or *Agt* $^{-/-}$ females ($n = 4$), measured by echocardiogram at 6 and 10 weeks of age. Data are presented as mean ± SEM.

Figure S4

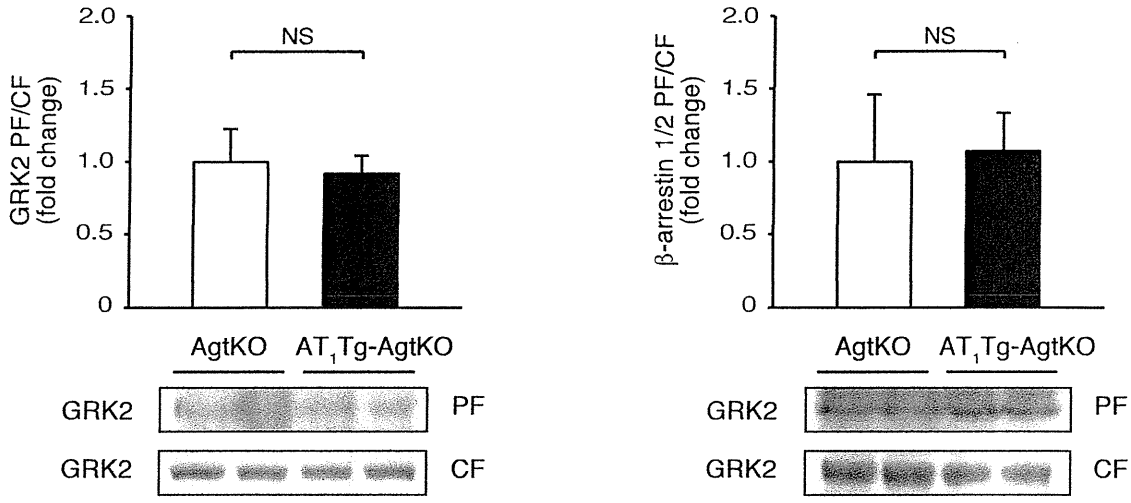

Figure S4. Immunoblot analysis of GRK2 and β-arrestin 1/2 in particulate fraction (PF) and cytosolic fraction (CF) extracted from AgtKO ($n = 4$) and AT_1Tg-Agt KO ($n = 4$) hearts. The quantitation of GRK2 in PF/CF and β-arrestin 1/2 in PF/CF is shown as bar graphs. Data are presented as mean ± SEM. NS, not significant ($P > 0.05$).

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Review Article

Emerging concept of anti-hypertensive therapy based on ambulatory blood pressure profile in chronic kidney disease

Kouichi Tamura, Tomohiko Kanaoka, Masato Ohsawa, Sona Haku, Kengo Azushima, Akinobu Maeda, Toru Dejima, Hiromichi Wakui, Motoko Ozawa, Atsu-ichiro Shigenaga, Yoshiyuki Toya, Satoshi Umemura

Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Japan.

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Abstract: Presently hypertensive patients with chronic kidney disease (CKD) particularly diabetic nephropathy are increasing in number, and cardiovascular and renal complications are the most common cause of death in these patients. The control of blood pressure (BP) is an important issue in cardiovascular and renal protection in hypertensive patients with CKD. Although hypertension is usually diagnosed based on measurements of BP recorded during a visit to a physician, that is, office BP, several studies have shown that target organ damage and prognosis are more closely associated with ambulatory BP than with office BP. It should be important to achieve the target absolute BP levels in hypertensive patients obtained either by office or home measurements or by ambulatory recordings for the cardiovascular and renal protection. Noninvasive techniques for measuring ambulatory BP have allowed BP to be monitored during both day and night. Additionally, ambulatory BP monitoring can provide information on circadian BP variation and short-term BP variability, which is suggested to be associated with cardiovascular and renal morbidity and mortality. This review will briefly summarize the emerging concept of anti-hypertensive therapy based on ambulatory BP profile in hypertensive patients with CKD.

Keywords: Blood pressure variability, diabetic nephropathy, hypertension, chronic kidney disease, renin-angiotensin system.

Introduction

Presently hypertensive patients with CKD and diabetes are increasing in number, and cardiovascular complications are the most common cause of death in these hypertensive patients. Thus, it would be of considerable value to identify the mechanisms involved in the cardiovascular events associated with hypertension complicated by CKD and diabetes. Ambulatory blood pressure (BP) monitoring has allowed an easier and more accurate determination of the circadian rhythm of BP under different pathophysiological conditions. The circadian pattern of BP in hypertensive patients with CKD and diabetes has been found to exhibit a blunted nocturnal decrease in BP, which is associated with autonomic neuropathy and nephropathy in these hypertensive patients [1]. The loss of nocturnal BP dipping has been considered to be a risk factor for the progression of nephropathy and to be of prognostic value with respect to target organ damage and cardiovascular morbidity in these CKD patients [2-4].

Estimation of ambulatory short-term BP variability

Ambulatory BP monitoring allows the acquisition of valuable information on not only the average 24-h BP, but also the variations in the BP values that happen during the course of daily life. Among the information obtained by ambulatory BP monitoring, previous studies have shown that BP variability is a complex phenomenon that involves both short- and long-lasting changes [5]. Thus the 24-h BP varies not only because of a reduction in BP during nighttime sleep and increase in the morning, but also because of sudden, quick, and short-lasting

changes that occur both during the daytime and, to a lesser extent, at nighttime. This phenomenon, short-term BP variability, has been shown to depend on sympathetic vascular modulation and on atherosclerotic vascular changes [6,7]. Several previous animal studies showed that exaggerated short-term BP variability without significant changes in mean BP induced chronic cardiovascular inflammation and remodeling [8,9]. Short-term BP variability is also suggested to be clinically relevant by the fact that hypertensive patients with similar 24-h mean BP values exhibit more severe organ damage when the short-term BP variability is greater [7,10-16].

Home-measured BP variability and CKD

On the other hand, several clinical studies have provided epidemiological basis for supporting the greater accuracy of home BP monitoring compared with clinic pressures for prognosis of fatal and nonfatal cardiovascular disease in long-term follow-up surveys and in cross-sectional studies. There is a general consensus that home BP monitoring is more convenient, available, and less costly than ambulatory BP monitoring, but the superiority of ambulatory BP monitoring for special clinical problems (i.e., 1) detection of non-dippers or need for sleep pressures in chronic renal disease, autonomic neuropathies, and sleep apnea; 2) estimation of short-term BP variability) is also clearly recognized [17]. Surveys of both physicians and patients suggest that home BP monitoring is both appreciated and recognized as a valuable strategy. Several experts in the field of hypertension research and care have published appeals to expand the use of home BP monitoring for routine care and to have it supported by health care systems.

Concerning home-measured BP variability, a previous study showed that high day-by-day BP variability is associated with increases in total, cardiovascular, and stroke mortality, independently of BP value and other cardiovascular risk factors in the general population of Ohasama study [18]. In the state of type 2 diabetes, while high short-term BP variability on ambulatory BP monitoring is reported to be associated with atherosclerosis and proteinuria in hypertensive patients with type 2 diabetes [11,19,20], the recent study by Ushigome et al. adds further information on the clinical relevance of home-measured BP variability in the pathophysiology of diabetic nephropathy [21]. Although the hypothesis that home-measured BP variability favors the development of nephropathy in type 2 diabetes is appealing, the cross-sectional nature of this study makes it impossible to evaluate the causal relationships between day-by-day BP variability and diabetic nephropathy. Further studies, such as outcome studies focusing on whether a therapeutic intervention reducing day-by-day BP variability also carries additional prognostic benefit by a concomitant suppression of the development of diabetic nephropathy, are warranted to confirm the prognostic value of home-measured BP variability.

Effects of Ang II type 1 receptor-specific blockers (ARB) on ambulatory short-term BP variability in diabetic nephropathy patients

Presently, inhibitors of renin-angiotensin system (RAS), such as ARB and angiotensin-converting enzyme inhibitors (ACEI) are recommended as the first-line anti-hypertensive medication to treat hypertensive patients with CKD, particularly those with albuminuria. Inhibitors of RAS exerts the BP lowering effects through the suppression of circulating and tissue RAS and the additional anti-proteinuric effect through the inhibition of intra-glomerular hypertension. With respect to effects of RAS inhibitors on ambulatory BP profile in hypertensive patients with CKD, we performed a series of clinical studies by administrating ARB to hypertensive CKD patients including those on dialysis therapy.

We examined whether ARB would improve ambulatory short-term BP variability in hypertensive patients with diabetic nephropathy. A total of 30 patients with type 2 diabetes along with hypertension and overt nephropathy were enrolled in this randomized, two-period, crossover trial of 12 weeks of treatment with losartan and telmisartan [11]. After 12 weeks of treatment, 24-h, daytime, and nighttime short-term BP variability, assessed on the basis of the coefficient of variation of ambulatory BP, was significantly decreased by telmisartan. Both of losartan and telmisartan reduced urinary protein excretion and baPWV. However, compared with losartan, telmisartan significantly decreased urinary protein excretion, baPWV, and low frequency (LF)-to-high frequency (HF) ratio, an index of sympathovagal balance. Multiple regression analysis showed significant correlations between

urinary protein excretion and baPWV, 24-h LF-to-HF ratio, nighttime systolic BP, and 24-h short-term systolic BP variability. Although the results of AMADEO study showed that telmisartan was more effective than losartan in reducing proteinuria in hypertensive patients with diabetic nephropathy at levels of office BP that were not different between the telmisartan and losartan treatment groups, the possible mechanisms involved in this difference in antiproteinuric effect were not elucidated [22]. The results of this study suggest that ARB, particularly telmisartan, is effective in reducing proteinuria in hypertensive patients with overt diabetic nephropathy, partly through inhibitory effects on ambulatory short-term BP variability and sympathetic nerve activity, in addition to its longer duration of action on nighttime BP reduction.

Accumulating evidence has shown that CKD patients with diabetes are increasing in number, and renal and cardiovascular complications are the most common cause of death in these patients. Thus, it is important to identify the mechanisms involved in the progression of renal impairment and cardiovascular injury associated with diabetic nephropathy. Recent evidence also indicated that multifactorial intervention is able to reduce the risk of cardiovascular disease and death among patients with diabetes and microalbuninuria [23]. Thus, in another study we examined the effects of intensified multifactorial intervention, with tight glucose regulation and the use of valsartan and fluvastatin, on ambulatory BP profile, estimated glomerular filtration rate (eGFR), and urinary albumin to creatinine ratio (UACR), in hypertensive patients with type 2 diabetes mellitus and overt nephropathy [20]. In this study we showed that the intensified multifactorial intervention including the use of valsartan and fluvastatin is able to improve ambulatory BP profile, preserve renal function, and reduce urinary albumin excretion in type 2 diabetic hypertensive patients with overt nephropathy.

Effects of ARB on ambulatory short-term BP variability in hemodialysis patients

Although cardiovascular disease is the leading cause of mortality in CKD patients on dialysis therapy, ARB is reported be effective in reducing cardiovascular events in patients undergoing hemodialysis [24,25]. Thus, we examined whether ARB would improve ambulatory short-term BP variability in hypertensive patients on hemodialysis [12]. In this study hypertensive patients on hemodialysis therapy were randomly assigned to the losartan treatment group or the control treatment group. After 6- and 12-months of treatment, nighttime short-term BP variability, assessed on the basis of the coefficient of variation of ambulatory BP, was significantly decreased in the losartan group, but remained unchanged in the control group. Compared with the control group, losartan significantly decreased left ventricular mass index (LVMI), baPWV, and the plasma levels of brain natriuretic peptide and advanced glycation end products (AGE). Furthermore, multiple regression analysis showed significant correlations between changes in LVMI and changes in nighttime short-term BP variability, as well as between changes in LVMI and changes in the plasma levels of AGE. These results suggest that ARB is beneficial for the suppression of pathological cardiovascular remodeling though its inhibitory effect on ambulatory short-term BP variability during nighttime. A recent study also shows that a direct renin inhibitor aliskiren was effective for BP control and may have cardiovascular protective effects in hypertensive CKD patients on hemodialysis [26].

Effects of ARB on ambulatory short-term BP variability in peritoneal dialysis patients

Among CKD patients on peritoneal dialysis, we examined whether addition of ARB, including high-dose ARB, to conventional antihypertensive treatment could improve BP variability in hypertensive patients [15]. Hypertensive patients on chronic peritoneal dialysis therapy were randomly assigned to the ARB treatment groups either by candesartan or valsartan, or the control group. After the 6-months treatment, 24-h ambulatory BP values were similarly decreased in both the control group and ARB groups. However, short-term BP variability assessed on the basis of the standard deviation of 24-h ambulatory BP was significantly decreased in the ARB groups, but remained unchanged in the control group. Furthermore, parameters of cardiovascular remodeling assessed by natriuretic peptides, echocardiography, and baPWV were significantly improved in the ARB groups but not in the control group. These results indicate that ARB treatment is beneficial for the suppression of pathological cardiovascular remodeling with a decrease in BP variability in

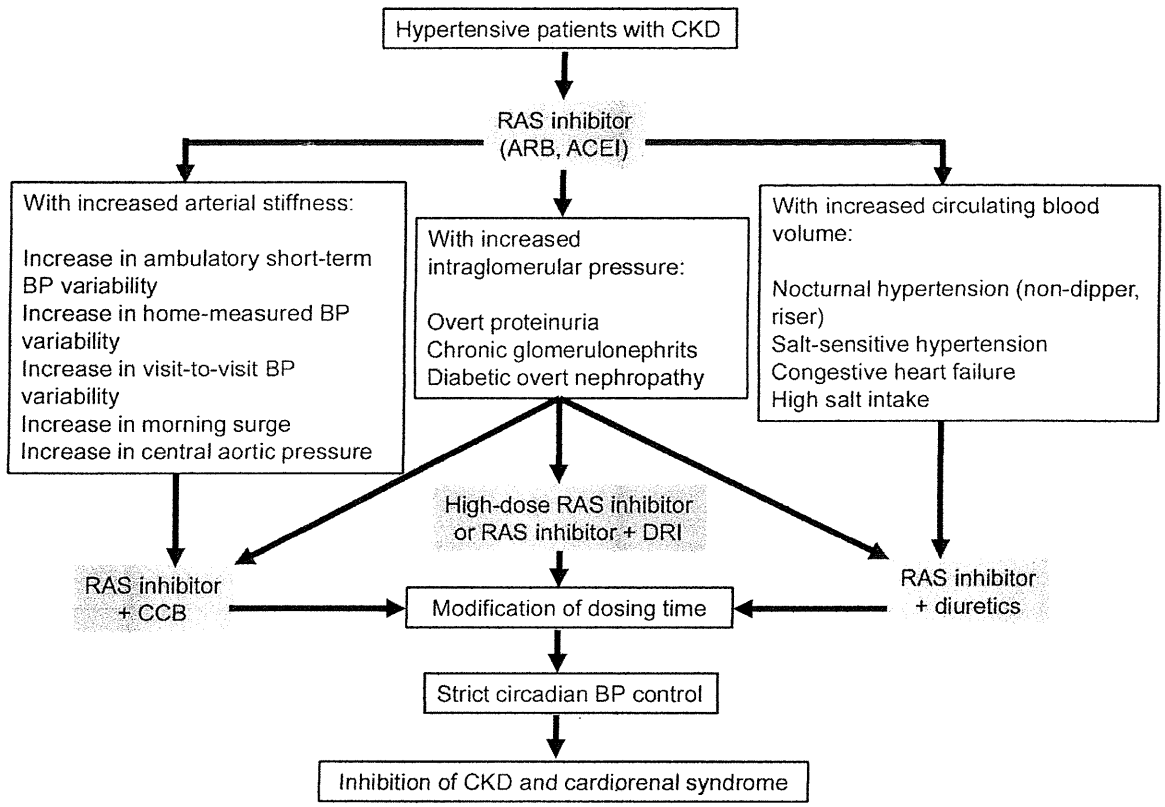

Figure 1. Schema showing the proposed strategy of RAS inhibitor-based combination therapy for hypertensive patients with CKD. ACEI, angiotensin-converting enzyme inhibitor; ARB, Ang II type 1 receptor-specific blocker; BP, blood pressure; CKD, chronic kidney disease; CCB, calcium channel blocker; DRI, direct renin inhibitor; RAS, renin-angiotensin system.

hypertensive patients on peritoneal dialysis

RAS inhibitor-based combination therapy in CKD

Although clinical guidelines specify that inhibitors of RAS are the drugs of choice for the treatment of hypertension in patients with CKD, the results of previous meta-analysis indicate that the benefits of RAS inhibitors on renal outcomes in clinical trials mainly result from a BP-lowering effect [27]. Thus, present guidelines also recommend RAS inhibitors-based combination therapy to achieve the target office BP level. The results of GUARD study showed that combination therapy with a RAS inhibitor and thiazide diuretic resulted in a greater reduction in albuminuria compared to that with a RAS inhibitor and calcium channel blocker (CCB) [28]. Previous studies showed that in CKD patients who have a sodium-sensitive type of hypertension, BP failed to fall during the night, thereby exhibiting non-dipper or riser types of ambulatory BP profile which correspond to abnormality of circadian BP rhythm. Although the sodium sensitivity of BP with non-dipper or riser types of ambulatory BP profile contributes as an independent risk factor for cardiovascular morbidity, both sodium restriction and thiazide diuretics are able to shift circadian BP rhythm from riser or non-dipper to dipper. Thus, RAS inhibitors-based combination therapy with thiazide diuretics may have an additional therapeutic advantage to relieve the renal and cardiovascular risks by different ways: systemic BP reduction and normalization of circadian BP rhythm.

On the other hand, the results of ACCOMPLISH study showed that combination therapy with a RAS inhibitor and CCB slows progression of nephropathy and inhibits cardiovascular death to a greater extent with a better preservation of eGFR, compared to combination therapy with a

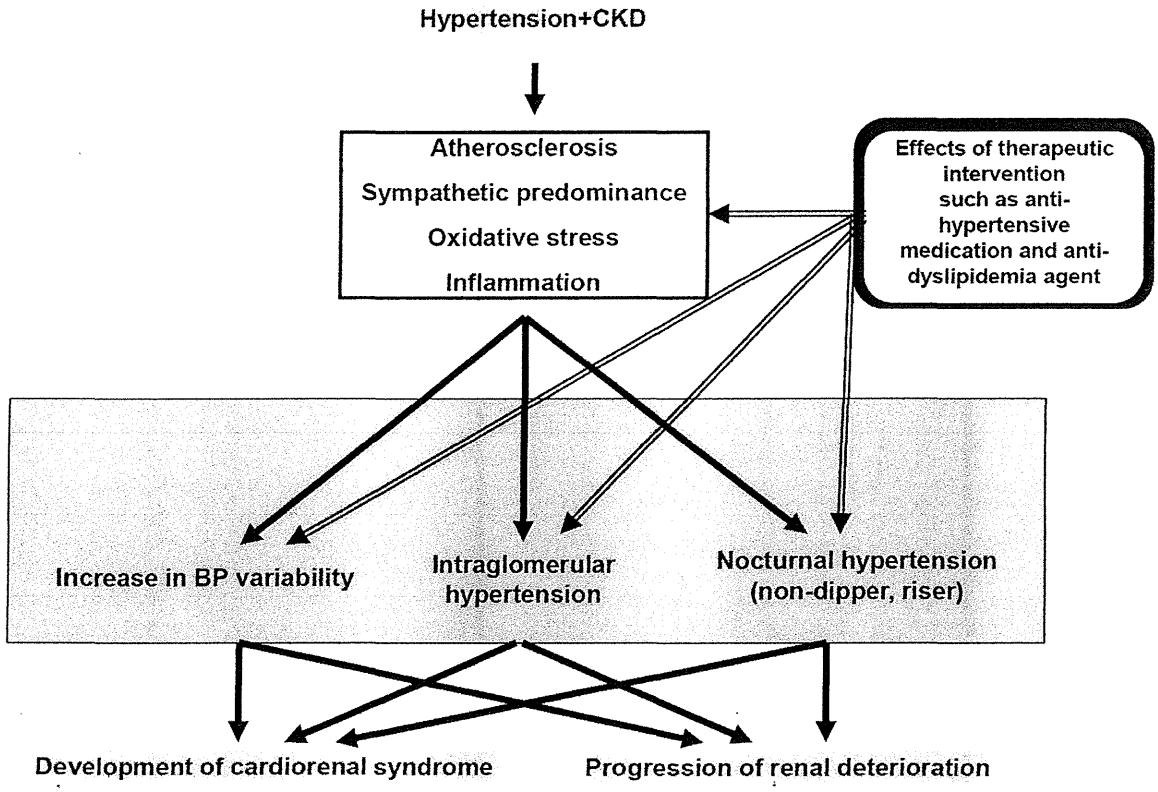

Figure 2. Increasing importance of clinical studies examining effects of various therapeutic intervention such as anti-hypertensive medication and anti-dyslipidemia agent on altered ambulatory BP profile in hypertensive patients with CKD. BP, blood pressure; CKD, chronic kidney disease.

RAS inhibitor and thiazide diuretic in high-risk hypertensive patients with CKD [29]. Although the detailed mechanistic basis for this difference in cardiorenal protection, in spite of similar mean 24-hour systolic and diastolic BP patterns by combination therapy [30], should be resolved by future studies, combination therapy with a RAS inhibitor and CCB is reported to effectively decrease central aortic pressure and ambulatory short-term BP variability with a preventive effect on the progression of arterial stiffness [31,32].

The direct renin inhibitor aliskiren is available as alternative or complementary approaches to pharmacological RAS blockade. Direct renin inhibitors constitute a novel class of RAS antagonists that block the conversion of angiotensinogen to angiotensin I. Aliskiren, the first approved compound of this class, reduces BP levels with similar potency as ACE inhibitor and ARB. Aliskiren as add-on treatment to standard therapy including the optimal dose of losartan, in the AVOID study, reduced albuminuria and slowed development of renal dysfunction more than placebo across different levels of eGFR in patients with type 2 diabetes, hypertension, and nephropathy [33-35]. The long-term nephroprotective potential of aliskiren-based therapy and its superiority over existing therapies as a possible first-line regimen remains to be elucidated.

Chronotherapy as a possible another therapeutic option in CKD

Finally, given that nocturnal BP non-dipping is a potential independent risk factor for CKD progression and development of cardiorenal syndrome, the timing of administration of antihypertensive drugs may be of relevance. Even compounds with recommended once-daily administration based on their pharmacokinetic properties may reduce nocturnal BP level more efficiently when applied in the evening, thereby