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

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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 coding region of the AT_1 receptor gene have not been identified in hypertension or primary hyperaldosteronism.[9,10]

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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 *AGTR1a* 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 βγ 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

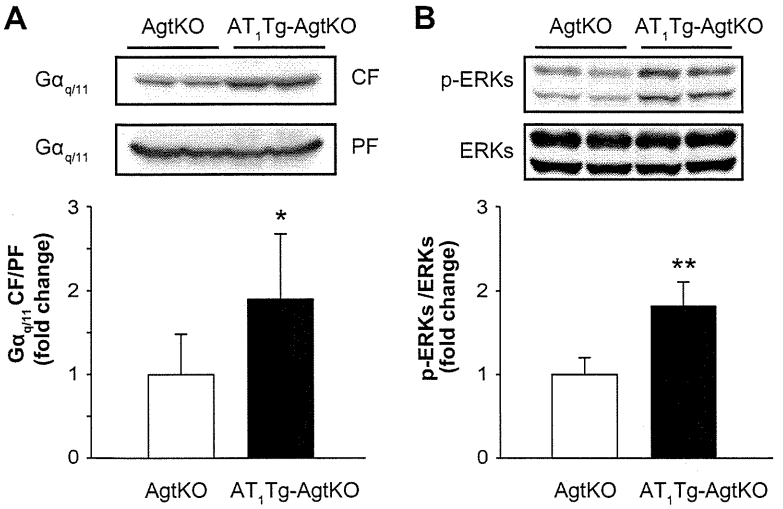

Figure 1. Constitutive activation of angiotensin II type 1 (AT_1) receptor in AT_1 transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) hearts. **A**, Immunoblot analysis of $G\alpha_{q/11}$ in cytosolic fraction (CF) and particulate fraction (PF) extracted from AgtKO (n=6) and AT_1Tg-AgtKO (n=6) hearts.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

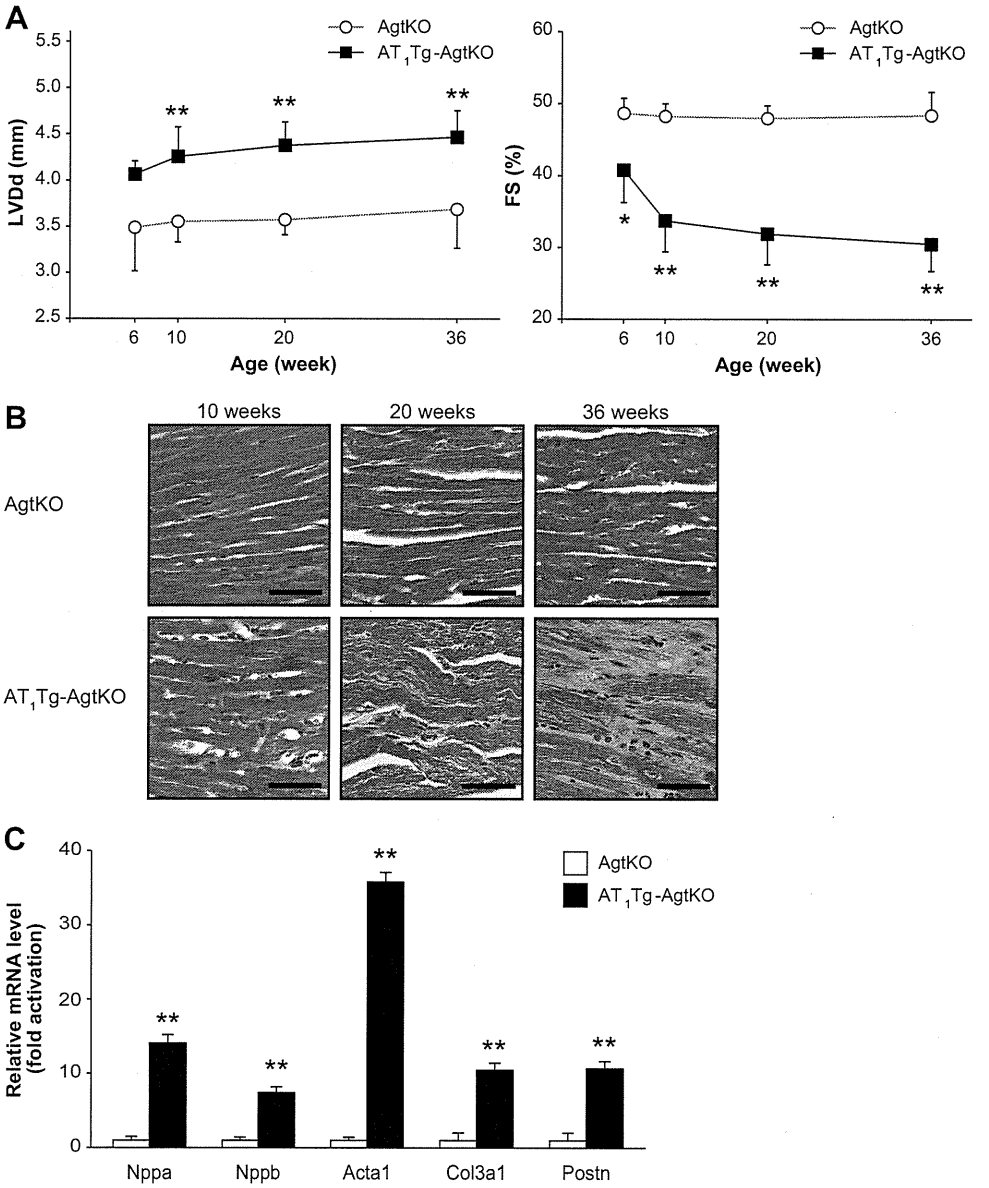

Figure 2. Spontaneous development of cardiac remodeling in angiotensin II type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice. **A**, Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AgtKO (n=7–9) and AT_1Tg-AgtKO (n=9–11) mice measured by echocardiogram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

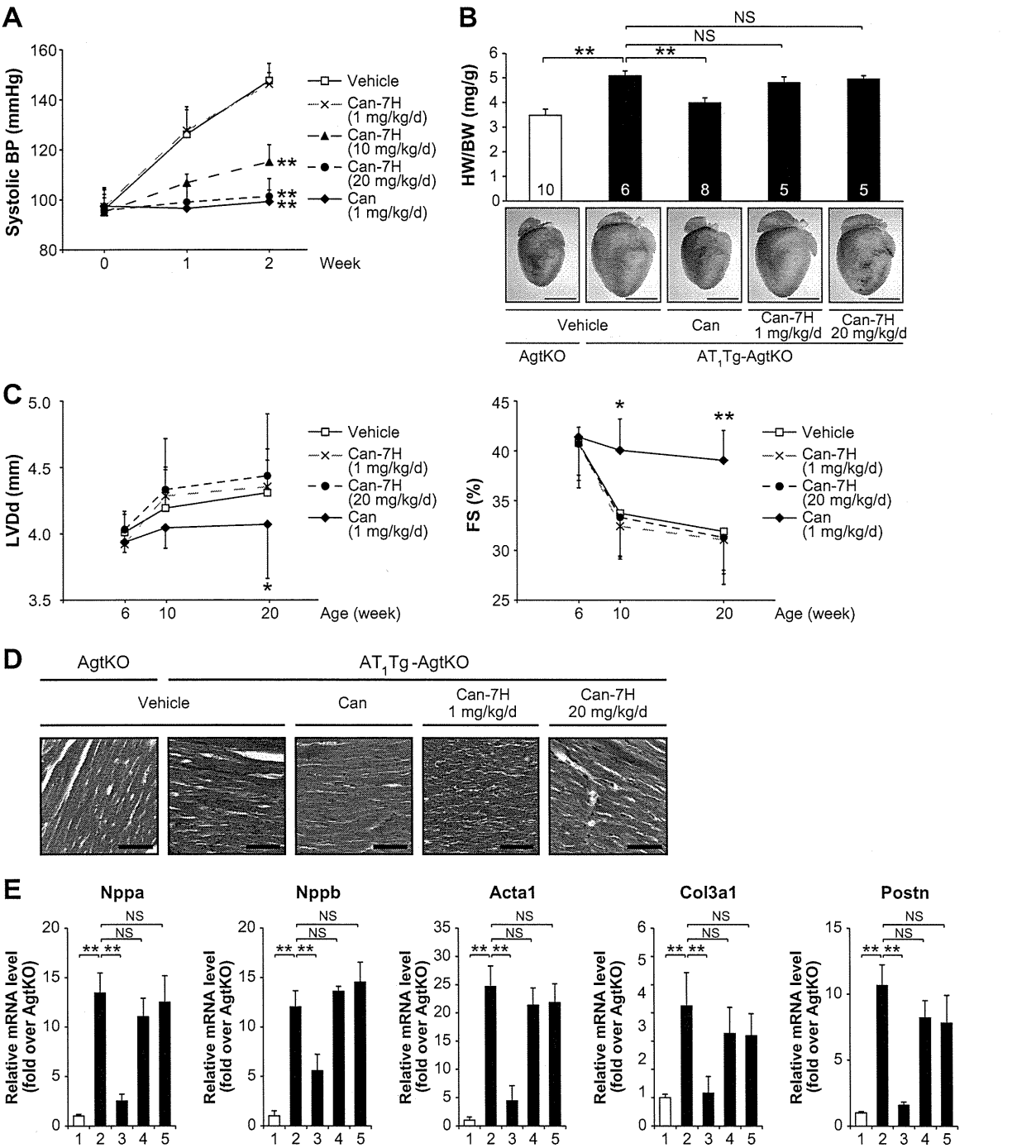

Figure 3. Prevention of cardiac remodeling in angiotensin II (Ang II) type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice by candesartan but not by candesartan-7H. **A**, Blood pressure–lowering effects of candesartan cilexetil (Can) and candesartan-7H (Can-7H) in Ang II–infused mice. 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 ≈8-fold in pressure-overloaded mice after transverse aortic constriction (B_{max}: 142.9±36.5 fmol/mg; n=3) compared with sham-operated mice (B_{max}: 16.4±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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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'-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

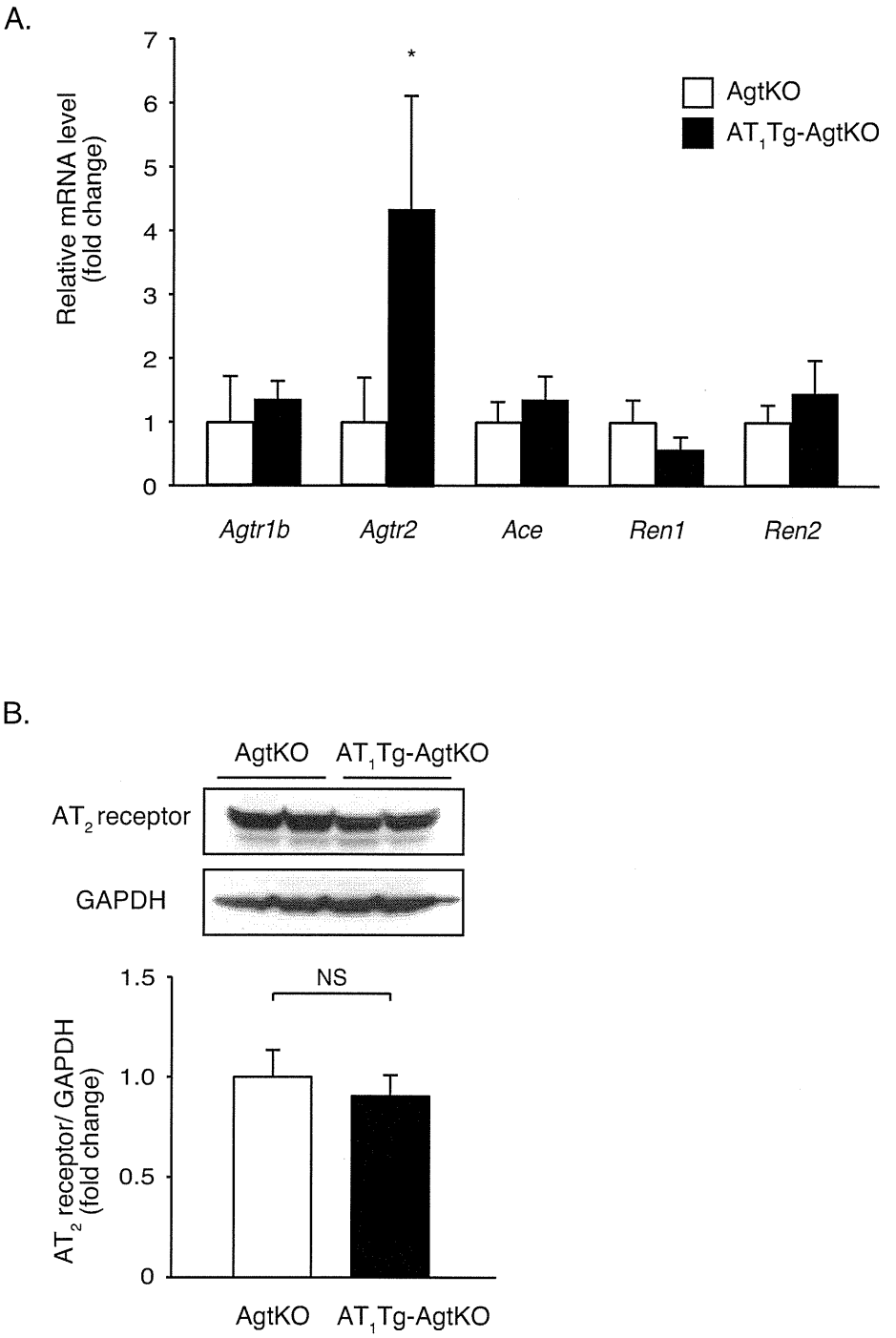

Figure S1. Expression levels of the renin-angiotensin system components in AT_1Tg-Agt KO and AgtKO hearts. (A) The mRNA expressions of the renin-angiotensin system components in AT_1Tg-Agt KO ($n = 6$) and AgtKO hearts ($n = 6$) at 20 weeks of age. Data are presented as mean ± SEM. * $P < 0.05$ versus 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

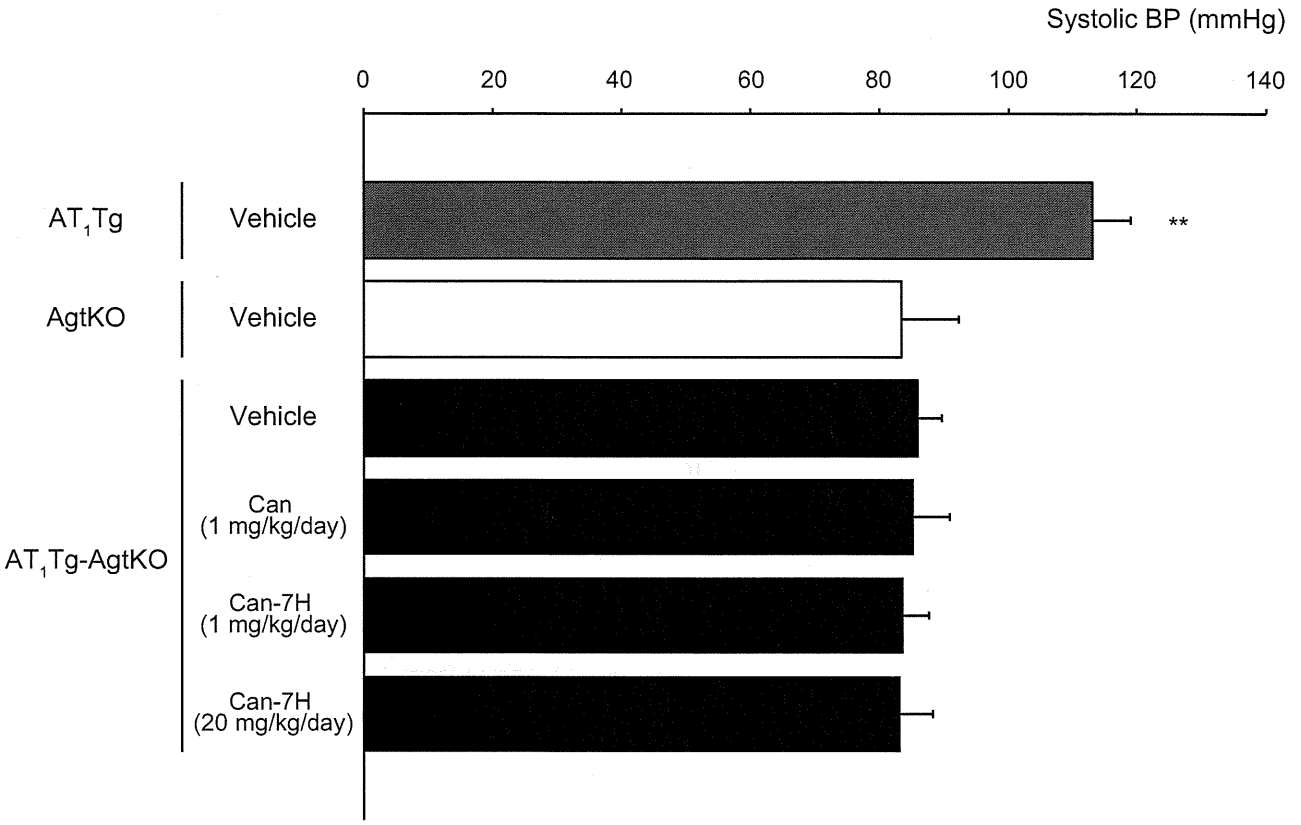

Figure S2. Systolic BP in AT_1Tg mice treated with vehicle ($n = 9$), AgtKO mice treated with vehicle ($n = 6$), AT_1Tg-AgtKO mice treated with vehicle ($n = 6$), candesartan cilexetil (Can) (1 mg/kg/day, $n = 8$) or candesartan-7H (Can-7H) (1 mg/kg/day, $n = 5$ or 20 mg/kg/day, $n = 5$). BP was measured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

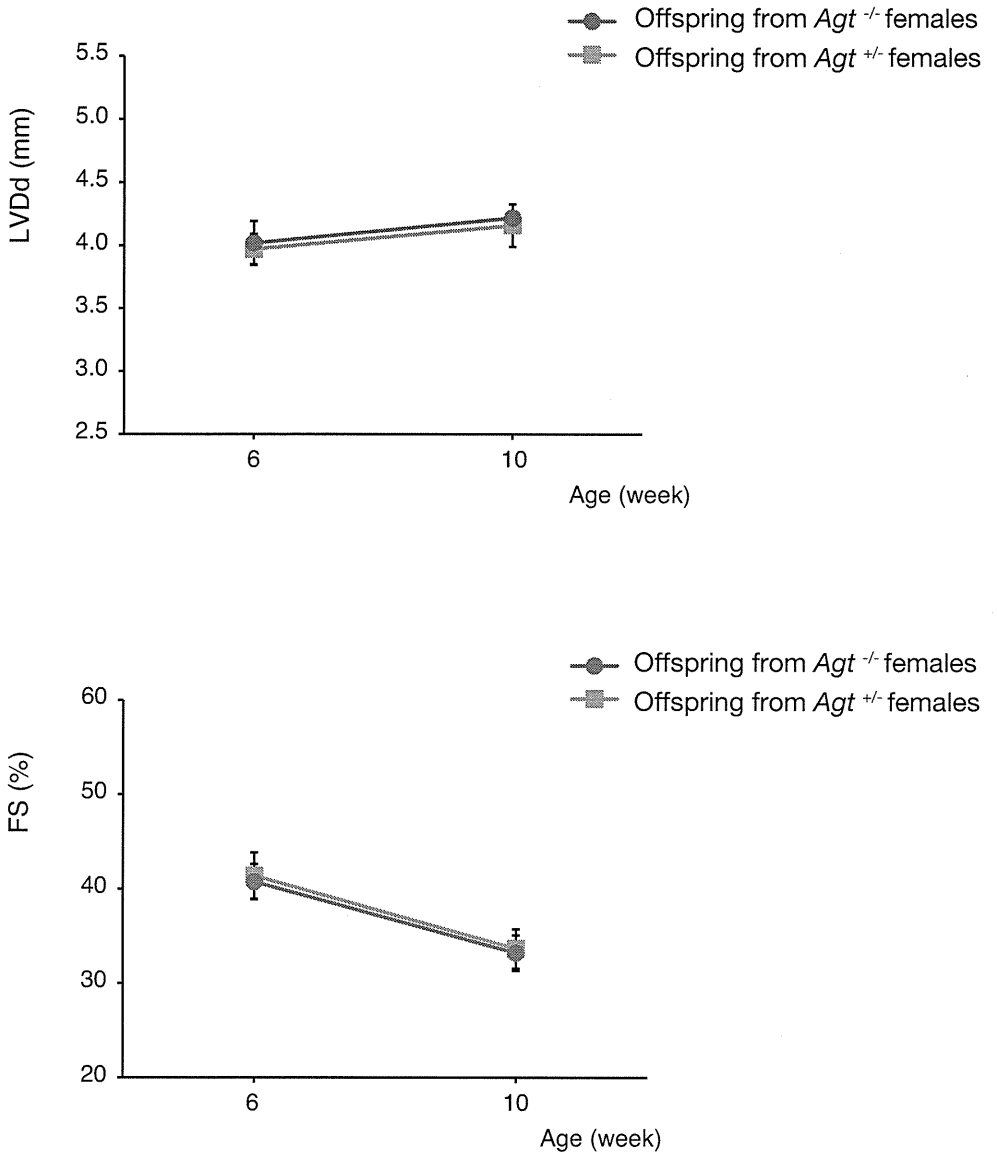

Figure S3. AT_1Tg-AgtKO mice developed cardiac remodeling independently of the effects of maternal or placental angiotensinogen during the fetal period. Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AT_1Tg-AgtKO offspring of *Agt* $^{+/-}$ females ($n = 4$) or *Agt*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

Figure S4

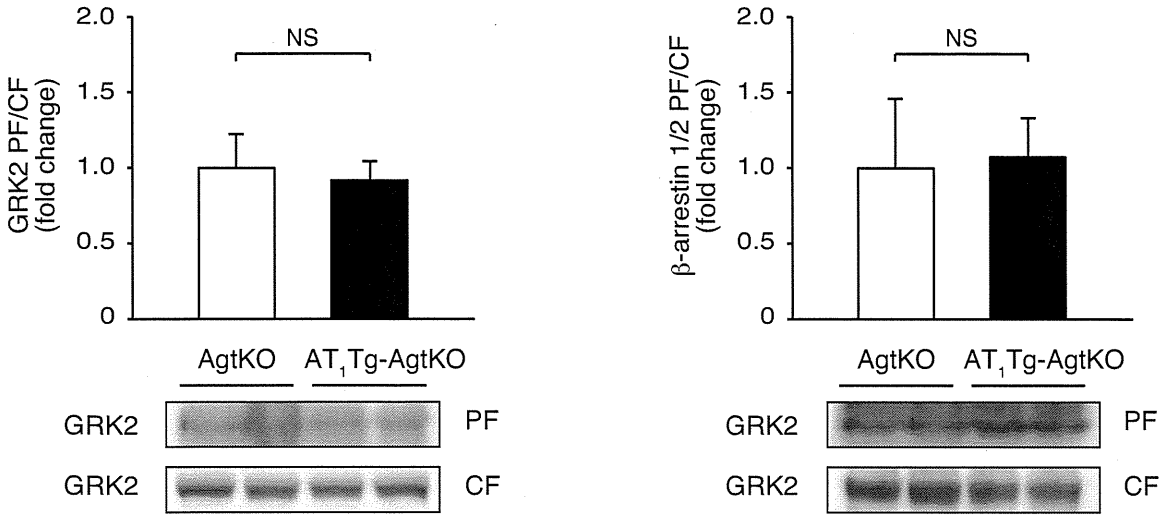

Figure S4. Immunoblot analysis of GRK2 and β-arrestin 1/2 in particulate fraction (PF) and cytosolic fraction (CF) extracted from AgtKO ($n = 4$) and AT_1Tg-Agt KO ($n = 4$) hearts. The quantitation of GRK2 in PF/CF and β-arrestin 1/2 in PF/CF is shown as bar graphs. Data are presented as mean ± SEM.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

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Original article

Role of regulatory T cells in atheroprotective effects of granulocyte colony-stimulating factor

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ABSTRACT

We and others have previously reported that granulocyte colony-stimulating factor (G-CSF) prevents left ventricular remodeling and dysfunction after myocardial infarction in animal models and human. We have also reported that G-CSF inhibits the progression of atherosclerosis in animal models, but its precise mechanism is still elusive. So, we examined the effects of G-CSF on atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) mice. Twelve-week-old male $ApoE^{-/-}$ mice were subcutaneously administrated with 200 μg/kg of G-CSF or saline once a day for 5 consecutive days per a week for 4 weeks. Atherosclerotic lesion of aortic sinus was significantly reduced in the G-CSF-treated mice compared with the saline-treated mice (35% reduction, $P<0.05$). G-CSF significantly reduced the expression level of interferon-γ by 31% and increased the expression level of interleukin-10 by 20% in atherosclerotic lesions of aortic sinus. G-CSF increased the number of $CD4^+CD25^+$ regulatory T cells in lymph nodes and spleen, and enhanced the suppressive function of regulatory T cells *in vitro*. G-CSF markedly increased the number of Foxp3-positive regulatory T cells in atherosclerotic lesions of aortic sinus. Administration of anti-CD25 antibody (PC61) that depletes regulatory T cells abrogated these atheroprotective effects of G-CSF. Moreover, in $ApoE^{-/-}/CD28^{-/-}$ mice, that lack regulatory T cells, the protective effects of G-CSF on atherosclerosis were not recognized. These findings suggest that regulatory T cells play an important role in the atheroprotective effects of G-CSF.

1. Introduction

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in arterial walls. Over the past two decades, an understanding of the importance of inflammation in the initiation and progression of atherosclerosis has greatly increased. Under normal conditions, the endothelial cells of the arterial wall resist adhesion and aggregation of leukocytes and promote fibrinolysis. When activated by stimuli such as hypertension, smoking, insulin resistance or inflammation, the endothelial cells express a series of adhesion molecules that selectively recruit various classes of leukocytes. Blood monocytes, which are the most numerous inflammatory cells within the atherosclerotic lesions, adhere to the dysfunctional endothelial surface by binding to leukocyte adhesion molecules [1]. Helper T cells and the associated cytokines have been reported to play a crucial role in the pathophysiology of atherosclerosis. $CD4^+CD25^+$ regulatory T cells (Tregs) subset constitutes 5–10% of all peripheral $CD4^+$ T cell population and are specialized for the suppressions of both type 1 helper T (Th1) and type 2 helper T (Th2) immune responses [2]. Tregs, as dedicated suppressors of diverse immune responses and important gatekeepers of immune homeostasis, contribute to the maintenance of the peripheral tolerance. Recently, it was reported that Tregs prevent the progression of atherosclerosis and a defect in Tregs favors atherogenesis [3–5].

Granulocyte colony-stimulating factor (G-CSF) is a member of a group of glycoproteins called hematopoietic cytokines. G-CSF induces the release of hematopoietic stem cells and endothelial progenitor cells from bone marrow into the peripheral blood circulation [6]. Moreover, G-CSF has been reported to modulate immune system and ameliorate immune-mediated diseases of animals [7]. We and others have previously reported that G-CSF prevents left ventricular remodeling and dysfunction after myocardial infarction (MI) in animal models and human [8–12]. G-CSF activates multiple signaling pathways such as Akt and Janus family kinase-2 and signal transducer and activation of transcription-3 (Jak2-STAT3) pathway in cardiac myocytes. G-CSF decreases cardiomyocyte death and increases the number of blood vessels, suggesting the importance of direct actions of G-CSF on the myocardium rather than through mobilization and differentiation of

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stem cells. We and others also reported that G-CSF prevents the progression of atherosclerosis [13–16]. G-CSF significantly reduced the stenosis score of coronary artery and lipid plaque area of thoracic aorta in the myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHL-MI) rabbits and prevented an increase in neointima/media ratio in the vascular injury model of rabbit [13]. However, its precise mechanism of G-CSF on atherosclerosis has been still elusive. In human and animal studies, G-CSF stimulation alters the T cell function and modulates the balance between Th1 and Th2 immune responses by affecting cytokine production [17]. G-CSF stimulation reduces cytotoxic activity and proliferative response of human and murine T cells [18]. Recently, G-CSF was reported to prevent autoimmune type 1 diabetes, graft-versus-host disease and transplanted heart allograft acceptability through enhancement of $CD4^+CD25^+$ Tregs subset [19,20]. Therefore, the aim of this study was to elucidate whether Tregs subset is involved in the atheroprotective mechanism of G-CSF.

2. Materials and methods

2.1. Animal model of atherosclerosis

Apolipoprotein E-deficient ($ApoE^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Twelve-week-old male $ApoE^{-/-}$ mice were fed with a proatherogenic diet (1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, 0.5% sodium cholate) (Oriental Yeast Co., Tokyo, Japan) ad libitum for 28 days [21]. These animals were then divided into 2 groups: saline group that received saline and G-CSF group that received G-CSF (rhG-CSF, Kirin Brewery Co., Ltd., Tokyo, Japan). Mice were subcutaneously administrated with 200 μg/kg of G-CSF or same volume (100 μl) of saline once a day for 5 consecutive days per a week for 4 weeks. After 28 days of G-CSF or saline treatment, mice were sacrificed and analyzed.

To deplete Tregs, twelve-week-old male $ApoE^{-/-}$ mice were intraperitoneally injected 100 μg of purified anti-mouse CD25 antibody (clone PC61; Biolegend, San Diego, CA) at day 0 and day 14. These Tregs-depleted mice started proatherogenic diet and each treatment from day 0. At 28 days after first injection, mice were sacrificed and analyzed. To determine the role of Tregs in the mechanisms of G-CSF-induced atheroprotective effect, we used $CD28^{-/-}$ mice. The construction and characterization of $CD28^{-/-}$ mice has been described previously [22]. $ApoE^{-/-}$ mice were crossed with $CD28^{-/-}$ mice and the heterozygous progeny were intercrossed to generate $ApoE^{-/-}/CD28^{-/-}$ double knockout (DKO) mice. Animal genotype was identified by a polymerase chain reaction (PCR)-based assay. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

2.2. Atherosclerotic lesion assessment at aortic sinus

After overnight fasting, blood was collected by the cardiac puncture under anesthetic condition using pentobarbital sodium (60 mg/kg intraperitoneal injection). Serum total cholesterol and high-density lipoprotein cholesterol levels were determined by high-performance liquid chromatography at SRL (Tokyo, Japan).

The aorta was perfused *in situ* with phosphate buffered saline (PBS), and the heart was removed and the proximal aorta containing the aortic sinus was embedded in OCT compounds (Tissue-Tek, CA). Five sections (10 μm thickness) of the aortic sinus were collected from each mouse and stained with Oil Red-O (Sigma-Aldrich, St Louis, MO) as described previously [21]. For the quantitative analysis of the area of atherosclerosis, section images were captured digitally (Axio Vision, Zeiss, Germany) and the average lesion areas of five separate sections from each mouse were obtained with Image J 1.38 (National Institutes of Health, MD).

2.3. Immunohistochemistry

Immunohistochemical staining with CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA), MOMA-2 (BMA Biomedicals AG, Switzerland), IFN-γ (Biosource International, Camarillo, CA), interleukin-10 (IL-10) (Santa Cruz Biotechnology) and Foxp3 (FJK-16 s, eBioscience, San Diego, CA) of atherosclerotic lesions at the aortic sinus were performed. Quantitative analysis of MOMA-2, IFN-γ and IL-10-immunostaining were evaluated as a ratio of the positive-stained area to total plaque area in the atherosclerotic lesion. At least three sections per mouse were examined for each immunostaining and appropriate negative controls were used. To evaluate the extent of nonspecific binding in the immunohistochemical experiments, control sections were incubated in the absence of primary antibody. As negative controls of the stainings of IFN-γ and IL-10, aorta of wild type mice were used. To define the subtype of infiltrated macrophages in the atherosclerotic lesions, we analyzed the expression level of CD11c, an M1 macrophage marker [23].

2.4. Quantitative real-time PCR at aortic sinus and whole aorta

At 28 days after first injection, mice were sacrificed under anesthetic condition using pentobarbital sodium (60 mg/kg intraperitoneal injection). The aorta was perfused *in situ* with phosphate buffered saline (PBS), and the whole aorta with aortic sinus was removed. These samples were frozen with liquid nitrogen and stored at −80 °C until each assay. Quantitative real-time PCR (qRT-PCR) analysis for transforming growth factor-β (TGF-β) was performed as described previously [24]. Total RNA was extracted from sample using the RNeasy kit (QIAGEN, Valencia, CA). We used 0.5 μg of total RNA to generate cDNA using the Super Script VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). qRT-PCR was carried out on a LightCycler system (Roche, Mannheim, Germany) using probes from Universal Probe Library and the TaqMan Master Mix. Sequence of primers and the respective Universal Probe Library probes were as follows: Tgf-beta: forward; CACCATCCATGACATGAACC, reverse; CCGCACACAGCAGTTCTTC; IFN-γ: forward; ATCTGGAGGAACTGGCAAA, reverse; CAAGACTTCAAAGAGTCTGAGGTA; IL-10: forward; CAGAGCCACATGCTCCTAGA, reverse; TGTCCAGCTGGTCCTTTGTT; Gapdh: forward; TGTCCGTCGTGGATCTGAC, reverse; CCTGCTTCACCACCTTCTTG. Relative expression of target genes was calculated with the comparative CT method. Each

Table 1
Body weight and cholesterol levels.

	$ApoE^{-/-}$				DKO	
			PC61			
	saline	G-CSF	saline	G-CSF	saline	G-CSF
Body weight, g	22.7 ± 1.2	22.4 ± 0.8	22.4 ± 0.9	23.6 ± 1.1	24.2 ± 0.6	23.1 ± 0.7
Total cholesterol, mg/dl	3306 ± 171	3527 ± 207	3321 ± 359	3259 ± 245	3767 ± 514	3965 ± 507
HDL cholesterol, mg/dl	29.3 ± 7.3	24.5 ± 4.8	30.8 ± 7.4	28.8 ± 7.5	23.2 ± 6.2	26.2 ± 7.9

HDL indicates high-density lipoprotein. DKO indicates $ApoE^{-/-}/CD28^{-/-}$ double knockout mice. $ApoE^{-/-}$ mice and DKO mice were treated by saline or G-CSF for 28 days. The means ± SEM of 10 animals are shown.

sample was run in duplicate, and the results were systematically normalized using Gapdh.

2.5. Mobilization procedure of Tregs and flow cytometry analysis

Twelve-week-old male C57BL/6 mice received G-CSF at a dose of 200 μg of G-CSF or saline once a day for 5 consecutive days per a week for 4 weeks. At day 28, the total cells of spleen and inguinal lymph nodes were taken and the cell surface phenotype of splenocytes and lymphocytes were analyzed by flow cytometry. All cells were incubated in ice cold PBS supplemented with 3% fetal calf serum and 0.1% azide. One million cells per sample were incubated for 15 minutes at ice cold temperature with the following mAbs: Phycoerythrin (PE)-conjugated anti-mouse CD4 (clone L3T4, BD Biosciences, Le Pont de Claix, France), Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD25 (clone 7D4, BD Biosciences, Le Pont de Claix, France).

At day 28, the total cells of spleen were taken and the cell phenotypes of lymphocytes were analyzed by flow cytometry. After magnetic activated cell sorter (MACS) with $CD4^+$ antibody and the stimulation with phorbol myristate acetate (PMA) and ionomycin, the cells were incubated with permeabilization buffer and stained with FITC-labeled anti-IFN-γ antibody and PE-labeled anti-IL-4 antibody for analysis with a flow cytometer [19]. $CD4^+$IFN-γ^+IL-4^- cells and $CD4^+$IFN-γ^- IL-4^+ cells were defined as Th1 cells and Th2 cells, respectively, and the Th1/Th2 ratio was calculated.

Thirty thousand events were acquired using EPICS ALTRA flow cytometric analysis (Beckman Coulter, Fullerton, CA). Analysis of the acquired data was performed using Expo32 MultiCOMP software (Beckman Coulter, Fullerton, CA).

2.6. Isolation and functional assays of Tregs

Tregs were isolated from spleen cell suspensions by the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec, Tokyo, Japan), which depletes samples of non-$CD4^+$ T cells followed by positive selection of $CD4^+CD25^+$ cells. The purity of the isolated $CD4^+CD25^+$ cells was

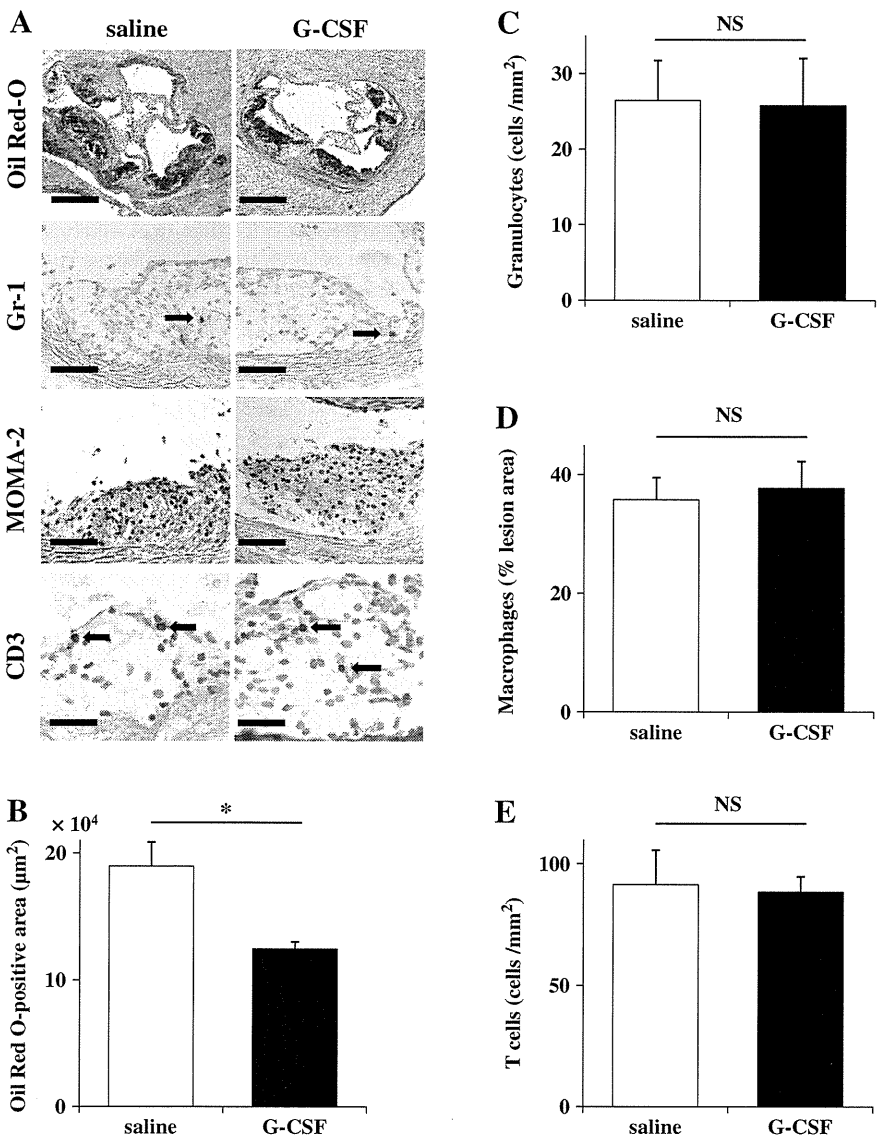
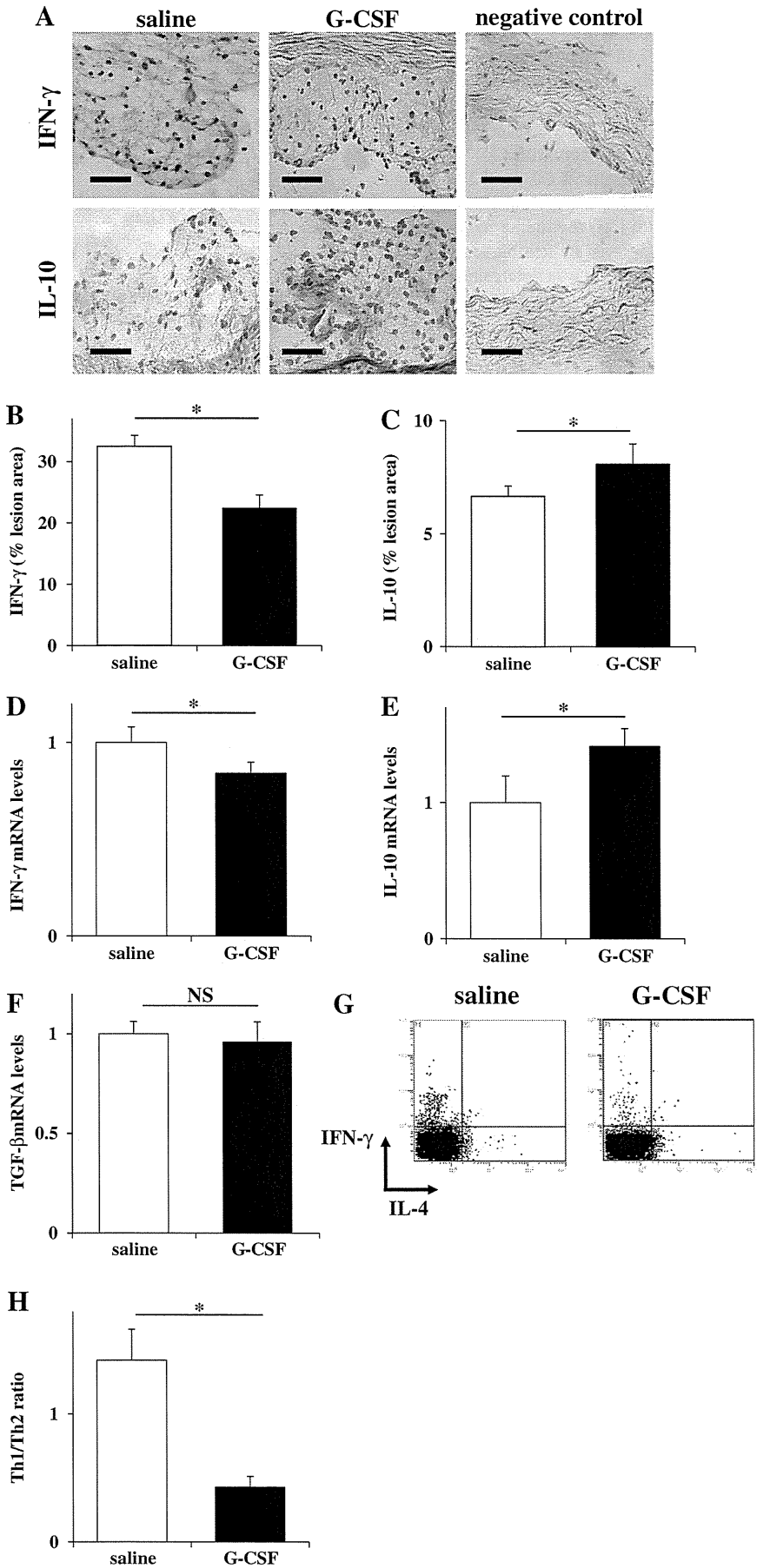


Fig. 1. Effects of G-CSF on atherosclerotic lesions at the aortic sinus in $ApoE^{-/-}$ mice. (A) Representative photographs of atherosclerotic lesion formation (Oil Red-O staining) (Scale bars; 400 μm) and immunostaining of Gr-1 (Scale bars; 100 μm), MOMA-2 (Scale bars; 100 μm) and CD3 (Scale bars; 50 μm) at the aortic sinus of each mouse fed atherogenic diet in the saline group and the G-CSF group. (B) Quantitative analysis of the degree of atherosclerosis at the aortic sinus in both groups. The average lesion area of five sections at the aortic sinus from each mouse was quantified morphometrically as described in Materials and methods. (C) The number of infiltrated granulocytes in the atherosclerotic lesion calculated by immunostaining with Gr-1. (D) The lesion area of infiltrated macrophages in the atherosclerotic lesion calculated by immunostaining with MOMA-2. (E) The number of infiltrated T lymphocyte in the atherosclerotic lesion calculated by immunostaining with CD3. *$P<0.05$. NS indicates that there is no significant difference between the groups. The means ± SEM of 10 animals are shown.


A
saline
G-CSF
negative control
IFN-γ
IL-10
B
IFN-γ (% lesion area)
30
20
10
0
*
saline
G-CSF
C
IL-10 (% lesion area)
10
5
0
*
saline
G-CSF
D
IFN-γ mRNA levels
1
0
*
saline
G-CSF
E
IL-10 mRNA levels
1
0
*
saline
G-CSF
F
TGF-βmRNA levels
1
0.5
0
NS
saline
G-CSF
G
saline
G-CSF
IFN-γ
IL-4
H
Th1/Th2 ratio
1
0
*
saline
G-CSF

>85% by fluorescence-activated cell sorter analysis. All suppression assays were performed in 96-well round-bottom plates in a final volume of 200 μl/well of RPMI 1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% FCS, 10 mmol/l Hepes, 50 μmol/l 2β-mercaptoethanol and antibiotics. Before the assay, 96-well plates were coated with 50 μl of a final concentration of 10 μg/ml anti-CD3 (Cedar-Lane Laboratories, Burlington, NC). The $CD4^+CD25^-$ responder cells were plated at 5×10^4/well alone or in combination with $CD4^+CD25^+$ Tregs at 5×10^4/well and incubated at 37 °C with 5% CO_2 for 72 h. Then 1×10^5 irradiated (3000 rads) splenocytes were added as antigen-presenting cells. Cultures were pulsed with [^{3}H] thymidine for the last 16 h of culture [25]. Cell proliferation was assayed by scintillation counting (β counter). Percent inhibition of proliferation was determined from the following formula: 1 - (median [^{3}H] thymidine uptake of 1:1 $CD4^+CD25^+$:$CD4^+CD25^-$ coculture/median [^{3}H] thymidine uptake of $CD4^+CD25^+$ cells).

2.7. Statistical analysis

Data are presented as mean ± SEM. An unpaired Student *t*-test was used to detect significant differences when two groups were compared. One-way ANOVA was used to compare the differences among four groups with Fisher's PLSD test for post hoc analysis. $P<0.05$ was considered statistically significant.

3. Results

3.1. Atherosclerosis at aortic sinus

There were no significant differences between the saline- and the G-CSF-treatment groups in body weight, serum total cholesterol and high-density lipoprotein cholesterol levels (Table 1). The degree of atherosclerotic legion assessed by Oil Red-O staining at the aortic sinus was significantly reduced in the G-CSF-treated mice compared with the saline-treated mice (G-CSF group: $12.4\pm0.6\times10^4$ μm^2 vs. saline group: $19.0\pm1.9\times10^4$ μm^2, $P<0.05$) (Fig. 1A, B). There was no significant change in the number of infiltrated granulocytes in atherosclerotic lesions between the two groups (G-CSF group: 22.1 ± 18 cells/mm^2 vs. saline group: 23.5 ± 14 cells/mm^2, $P=0.87$) (Fig. 1A, C). Immunohistochemical staining on atherosclerotic lesions of aortic sinus revealed that the percentage of cross-sectional area occupied by macrophages (MOMA-2-positive staining) was not significantly different between the two groups (G-CSF group: 37.7 ± 13% vs. saline group: 35.8 ± 9.9%, $P=0.75$) (Fig. 1A, D). The ratio of CD11c-positive M1 macrophages to total infiltrated cells was not changed in the atherosclerotic lesions of the G-CSF-treated mice compared to the saline-treated mice (G-CSF group: 8.2 ± 1.1% vs. saline group: 9.0 ± 0.5%, $P=0.31$). The number of CD3-positive T cells was not also significantly different between the two groups (G-CSF group: 88.4 ± 19 cells/mm^2 vs. saline group: 91.5 ± 37 cells/mm^2, $P=0.87$) (Fig. 1A, E).

3.2. Effect of G-CSF on cytokine expression

To elucidate the mechanisms by which G-CSF reduced the degree of atherosclerosis, we examined the local cytokine expression in the saline- and the G-CSF-treated mice. Level of proinflammatory cytokine IFN-γ in the atherosclerotic legions was lower in the G-CSF-treated mice compared with the saline-treated mice (G-CSF group: 22.4 ± 2.7% vs. saline group: 32.5 ± 2.2%, $P<0.05$) (Fig. 2A, B). Meanwhile, G-CSF treatment increased the level of antiinflammatory cytokine IL-10 in the atherosclerotic legions (G-CSF group: 8.1 ± 0.9% vs. saline group: 6.7 ± 0.5%, $P<0.05$) (Fig. 2A, C). The expression levels of IFN-γ and IL-10 mRNA were also significantly different between the two groups (IFN-γ; G-CSF group: 0.8 ± 0.1% vs. saline group: 1.0 ± 0.1%, $P<0.05$, IL-10; G-CSF group: 1.4 ± 0.1% vs. saline group: 1.0 ± 0.2%, $P<0.05$) (Fig. 2D, E). Since we could not perform TGF-β immunostaining due to technical problems, we examined the expression level of TGF-β mRNA by using qRT-PCR. The expression level of TGF-β mRNA was not significantly different between the two groups (Fig. 2F).

We examined the effects of G-CSF on Th1/Th2 balance. The Th1/Th2 ratio was significantly reduced in the G-CSF-treated mice (G-CSF group: 0.4 ± 0.1 vs. saline group: 1.4 ± 0.2, $P<0.01$) (Fig. 2G, H).

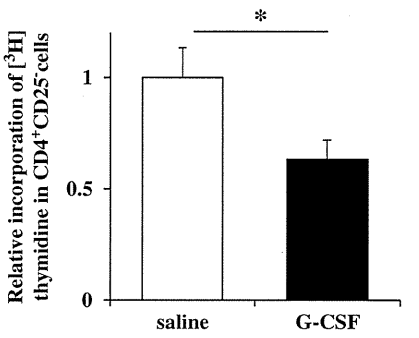


Fig. 3. Effects of G-CSF on the inhibitory function of Tregs. $CD4^+CD25^-$ effector T cells were co-cultured with Tregs from the saline- or the G-CSF-treated mice at the ratio of 1:1 and the incorporation of [^{3}H] thymidine in $CD4^+CD25^-$ cells was counted. *$P<0.05$. The means ± SEM of 6 animals are shown.

3.3. Effect of G-CSF on function of Tregs

To evaluate whether the function of Tregs is influenced by G-CSF treatment, we compared the suppressive function of the Tregs isolated from the saline- and the G-CSF-treated mice. $CD4^+CD25^+$ Tregs from each mouse were co-cultured with $CD4^+CD25^-$ effector cells from wild type mice at the ratio of 1:1 in cell number. Tregs from the G-CSF-treated mice significantly inhibited the incorporation of [^{3}H] thymidine in $CD4^+CD25^-$ effector cells compared with Tregs from the saline-treated mice (Fig. 3).

3.4. Effect of G-CSF on the number of Tregs

To elucidate the mechanism of antiinflammatory effects of G-CSF, we investigated whether G-CSF increases the number of Tregs or not. First of all, we examined the systemic effects of G-CSF on the number of Tregs by flow cytometry analysis of cells from spleen and inguinal lymph nodes of C57BL/6 mice. G-CSF increased the number of $CD4^+CD25^+$ Tregs in spleen (G-CSF group: $249.0\pm18.0\times10^4$ cells vs. saline group: $88.2\pm4.2\times10^4$ cells, $P<0.01$) (Fig. 4A, B) and inguinal lymph nodes (G-CSF group: $54.0\pm5.2\times10^3$ cells vs. saline group: $31.0\pm2.8\times10^3$ cells, $P<0.01$) (Fig. 4A, C).

We next examined whether G-CSF enhances the number of Tregs in atherosclerotic lesions. G-CSF significantly increased the number of Foxp3-positive Tregs in the atherosclerotic legions of aortic sinus (Fig. 5A, B). The number of Foxp3-positive regulatory T cells was 3.1-fold increased at the atherosclerotic lesions of the G-CSF-treated

Fig. 2. Effects of G-CSF on cytokine levels in atherosclerotic lesion at the aortic sinus. (A) Panels are representative photographs of immunostaining with IFN-γ and IL-10 in the atherosclerotic lesion of the G-CSF-treated mice and the saline-treated mice and the aorta of wild type mice (negative control). (B) Levels of IFN-γ calculated in the atherosclerotic legion of the G-CSF-treated mice and the saline-treated mice. (C) Levels of IL-10 calculated in the atherosclerotic legion of the G-CSF-treated mice and the saline-treated mice. (D) Expression levels of IFN-γ mRNA detected by real-time PCR analysis. (E) Expression levels of IL-10 mRNA detected by real-time PCR analysis. (F) Expression levels of TGF-β mRNA detected by real-time PCR analysis. (G) The proportions of $CD4^+$IFN-γ^+IL-4^- cells and $CD4^+$IFN-γ^-IL-4^+ cells from spleen of the G-CSF- and the saline-treated mice assessed by flow cytometric analysis. (H) The Th1/Th2 ratio in spleen of the G-CSF- and the saline-treated mice assessed by flow cytometric analysis. *$P<0.05$. NS indicates that there is no significant difference between the two groups. Scale bars indicates 50 μm. The means ± SEM of 10 animals are shown.