Heart failure is one of the major public health problems in an aging society,1,2 and hypertension is the most prevalent risk factor of heart failure.3–5 Left ventricular (LV) hypertrophy is an initial adaptive response against the increased LV afterload; however, long-term myocardial wall stress may affect the LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Mechanical load and several neurohumoral factors are suggested to progress the LV hypertrophy and LV structural integrity. Functional decompensation occurs with preserved systolic contractile function. Intraperitoneal administration of human recombinant osteoprotegerin, but not subcutaneous injection of anti-receptor activator of nuclear factor-κb ligand antibody, to the angiotensin II–infused osteoprotegerin−/− mice for 28 days ameliorated the progression of heart failure without affecting systolic blood pressure. These results underscore the biological activity of osteoprotegerin in preserving myocardial structure and function during the angiotensin II–induced cardiac hypertrophy, independent of receptor activator of nuclear factor-κb ligand activity. In addition, the antiapoptotic and profibrotic actions of osteoprotegerin that emerged from our data might be involved in the mechanisms. (Hypertension. 2016;67:848-856. DOI: 10.1161/HYPERTENSIONAHA.115.06689.)

Key Words: angiotensin II • apoptosis • fibrosis • hypertension • hypertrophy
of RANK ligand (RANKL). RANKL is produced primarily from bone and thymus, whereas osteoprotegerin mRNA is widely distributed in various organs associated with bone metabolism, such as thyroid, kidney, and intestines; however, it is also highly expressed in the heart and vasculature. Plasma concentration of osteoprotegerin not only increases with age in a healthy aged population but also elevated in patients with cardiac hypertrophy, heart failure, and myocardial infarction and its level may determine the prognosis. We reported a possible association of osteoprotegerin production and RAS activation in human heart. Whether the relationship between increased serum osteoprotegerin and heart disease is compensatory, causal, or merely an association is an important unanswered question. This study aimed at addressing whether endogenous RANKL/RANK/osteoprotegerin axis may contribute to the pathogenesis of cardiac hypertrophy and heart failure interacted with RAS in parallel with osteoporosis, using mice lacking the osteoprotegerin gene and overexpressing the RANKL gene.

Figure 1. A–D, Effects of angiotensin II (Ang II) infusion (250 ng/kg per minute, SC) on systolic blood pressure (SBP) (A), ratio of heart weight (HW) to body weight (BW) (B), and type 1 collagen (C) and osteoprotegerin (OPG) mRNA (D) in the left ventricle (LV) of 8-week-old Wistar rats with or without Ang II type 1 receptor antagonist RNH-6270 (0.5 mg/kg, orally) at days 7 and 14. Data are shown as the mean±SD (sham [n=10], Ang II infusion with [n=15] or without RNH-6270 [n=13] at day 7, and sham [n=7] Ang II infusion with [n=6] or without RNH-6270 [n=9] at day 14), and analyzed by multiple comparisons with Tukey 1-way ANOVA test at days 7 and 14, respectively. *P<0.05, **P<0.01, ***P<0.001. Open bar, sham; filled bar, Ang II; crosshatched bar, Ang II+RNH-6270. The gene expression was normalized to 18S ribosomal RNA (rRNA) and the oligonucleotide sequences and probes are shown in Table S1. E, Localization of OPG, Mallory-Azan, vimentin, S100A4, or CD68 in serial cross sections of LV in Ang II-infused Wistar rat at day 7. Scale bar, 50 μm.
Methods

The experimental protocols are detailed under in the online-only Data Supplement.

Experimental Procedure

We administered synthetic Ang II subcutaneously using the implanted miniosmotic pump to 8-week-old male Wistar rats, osteoprotegerin−/−, RANKL-transgenic (Tg), and wild-type (WT) mice. In addition, we gave human recombinant (hr) osteoprotegerin/OCIF or anti-mouse RANKL rat monoclonal antibody to the Ang II–infused osteoprotegerin−/− mice. We measured systolic blood pressure (SBP) by tail-cuff plethysmography and performed transthoracic echocardiography and then collected blood samples and organs.

Cell Culture

We isolated cardiac myocytes and fibroblasts from the ventricles of 1-day-old Wistar rats and incubated them with synthetic Ang II with or without its receptor antagonists.

Real-Time Quantitative polymerase Chain Reaction

cDNA was amplified with oligonucleotide primers and TaqMan probes labeled with 6-carboxy-fluorescein as reporter fluorescence and 6-carboxy-tetramethyl-rhodamine as quencher fluorescence by real-time quantitative reverse transcriptase polymerase chain reaction. We used relative standard curve method using a serial dilution of cDNA (mouse heart) for both the target and the housekeeping gene. The standard curve is a straight line composed of the fractional polymerase chain reaction cycle numbers, defined as threshold cycle (Ct) at which signal of the reporter fluorescence is greater than the minimal detection level (detection limit). The amount of gene for unknown samples was accomplished by measuring Ct using the standard curve. We listed the oligonucleotide sequences and probes in Table S1 in the online-only Data Supplement.

Histology

We stained the slides of rat LV cross sections with primary antibodies such as osteoprotegerin, vimentin, S100A4, or CD68. In addition, we stained the LV cross-sections with Mallory-Azan (rats and mice) and Sirius red (mice).

Apoptotic Cells

We assessed to detect the apoptotic cells in the slides of LV cross sections by in situ detection of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling staining.

RANKL Concentration

We measured plasma concentration of mouse RANKL in duplicate by Quantikine ELISA kit.

Micro–Computed Tomography

We performed micro–computed tomographic scan using right lower extremity of mice. We set the measurement area at the proximal metaphysis of femur and calculated the trabecular separation using a 3-dimensional image analyzing system.

Statistical Analysis

Data analyses were performed using GraphPad Prism 5 (La Jolla, CA). We assessed comparison of the 2 variables with Student t test and multiple comparisons with 1-way ANOVA and 2-way ANOVA (genotype × Ang II). The respective method for statistical
test is described in the figure legends. We presented the data as the means±SD, and *P*<0.05 was considered significant.

**Results**

**Ang II Induces Osteoprotegerin mRNA Through AT1 Receptor in Rat Heart**

Subcutaneous infusion of Ang II (250 ng/kg per minute) to 8-week-old male rats for 14 days raised SBP (Figure 1A), increased the ratio of heart weight/body weight (Figure 1B), accompanied by the increase of type 1 collagen mRNA (Figure 1C) in the LV. Ang II type 1 receptor blocker RNH-6270 (0.5 mg/kg per day) had a greater effect on attenuating the type 1 collagen synthesis than on ameliorating cardiac hypertrophy at day 14. Furthermore, Ang II significantly increased osteoprotegerin mRNA in the LV by 3.9-fold at day 7 and by 2.6-fold at day 14, compared with the controls, whereas RNH-6270 treatment decreased the expression (Figure 1D).

Immunoreactivity of osteoprotegerin was more intensely stained in the interstitial area than cardiocytes of rats with Ang II infusion and was in accordance with the staining for vimentin, S100A4, or CD68 (Figure 1E).

**Osteoprotegerin Synthesis of Cardiac Fibroblast and Cardiomyocyte Stimulation With Ang II In Vitro**

Both cardiomyocytes and fibroblasts expressed osteoprotegerin mRNA similarly at the basal condition (Figure 2A). Ang II (10^{-7} mol/L) led to a 2-fold induction of osteoprotegerin mRNA in the first 1 hour, compared with controls, and it decreased for 24 hours in cardiac fibroblasts, but it was to a lesser extent in the cardiomyocytes (Figure 2B). The effect of Ang II on osteoprotegerin mRNA stimulation was observed maximally at 10^{-7} mol/L between 10^{-8} and 10^{-6} mol/L in cardiac fibroblasts (Figure 2C). On the contrary, pretreatment with RNH-6270 (10^{-6} and 10^{-7} mol/L) blunted the Ang II (10^{-7} mol/L)–induced osteoprotegerin mRNA, but not with the Ang II type 2 receptor antagonist, PD-123319 (10^{-6} and 10^{-7} mol/L) in these cells (Figure 2D).

**Myocardial Osteoprotegerin/RANK/RANKL mRNA and Soluble RANKL With or Without Ang II Stimulation**

Raw Ct of osteoprotegerin, RANK, and RANKL expression is summarized (Figure S1A–S1C). Ang II infusion for 28 days stimulated osteoprotegerin mRNA in LV but not in thymus, lung, liver, kidney, or spleen of WT mice (Figure 3A), and osteoprotegerin mRNA was not detected in osteoprotegerin−/− mice (Figure 3B). LV RANK expressed in a similar manner in WT and osteoprotegerin−/− mice with or without Ang II stimulation (Figure 3C). LV RANKL mRNA was too low to detect in most controls of WT and osteoprotegerin−/− mice, and Ang II tended to stimulate it in both types of mice (Figure 3D). On the contrary, osteoprotegerin−/− mice displayed ≈30-fold higher concentration of RANKL in the circulation, compared with WT mice regardless of Ang II infusion (Figure 3E).

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**Figure 3.** Comparisons of osteoprotegerin (OPG) mRNA in various organs in wild-type (WT) mice (A), and OPG (B), receptor activator of nuclear factor-κB (RANK; C) and RANK ligand (RANKL; D) mRNA in the left ventricular, and soluble RANKL concentration (E) in WT and OPG−/− mice with or without angiotensin II (Ang II) infusion (1000 ng/kg per minute) subcutaneously for 28 d. Data are shown as the means±SD (A, WT mice with [n=4] without [n=6] Ang II stimulation; B–D, WT mice with [n=18] or without [n=21] Ang II stimulation; OPG−/− mice with [n=16] or without [n=12] Ang II stimulation; E, WT mice with [n=3] or without [n=4] Ang II stimulation; OPG−/− mice with [n=4] or without [n=4] Ang II stimulation), and analyzed by 2-way ANOVA followed by Bonferroni post hoc test. *P*<0.05, **P*<0.01, ***P*<0.001. We used thymus and bone marrow (BM) as positive control (n=2). The gene expression was normalized to 18S ribosomal RNA (rRNA), and the oligonucleotide sequences and probes are shown in Table S1.
Ang II Stimulation Enhances Cardiac Hypertrophy and Functional Decompensation in Osteoprotegerin−/− Mice

Ang II infusion achieved an equivalent level of SBP at day 28 in both types of mice (Figure 4A), but increased heart weight (HW) to body weight (BW) (B), atrial natriuretic peptide (ANP) mRNA (C), % interstitial fibrosis area (D), numbers of transferase-mediated dUTP nick end-labeling–positive apoptotic cells (E) and trabecular separation (Tb.Sp) at proximal metaphysis of femur (F) in the left ventricle (LV) of 8-week-old wild-type (WT) and osteoprotegerin (OPG)−/− mice. ANP mRNA was normalized by 18S ribosomal RNA (rRNA), and expressed as % of control WT mice. The oligonucleotide sequences and probes are shown in Table S1. Right, Representative macroscopic pictures, longitudinal and cross-sections of hearts stained with Mallory-Azan in WT and OPG−/− mice with or without Ang II infusion. Data are shown as the means±SD (WT mice with [A and B, n=16; C, n=14; D, n=9; E, n=8; F, n=19] or without [A and B, n=14; C, n=17; D, n=12; E, n=10; F, n=16] Ang II stimulation; OPG−/− mice with [A and B, n=18; C, n=12; D, n=10; E, n=12; F, n=19] or without [A and B, n=13; C, n=12; D, n=13; E, n=7; F, n=13] Ang II stimulation), and analyzed by 2-way ANOVA followed by Bonferroni post hoc test. **P<0.01, ***P<0.001.

Ang II Impairs the Syntheses of Procollagen α1 and Syndecan-1 in Osteoprotegerin−/− Mice

Raw Ct of procollagen α1 and syndecan-1 expression is summarized (Figure S1D–S1E). Ang II infusion stimulated lesser extent of syntheses for procollagen α1 (Figure 6A) and syndecan-1 (Figure 6B) mRNA in osteoprotegerin−/− mice than in WT mice.

Effects of Recombinant Osteoprotegerin/OCIF and Anti-RANKL Antibody on the Exacerbation of Heart Failure Elicited by Ang II Infusion

hrOCIF (10 mg/kg, IP) every 2 days for 28 days or anti-RANKL antibody (5 mg/kg, IC) on days 1, 14, and 28 decreased the trabecular separation at femur in Ang II–infused osteoprotegerin−/− mice (Figure 7A). The respective treatment did not affect SBP (Figure 7B), but hrOCIF significantly reduced the heart weight/body weight ratio (Figure 7C) and increased LV %fractional shortening (Figure 7D), but no such changes were observed in mice treated with anti-RANKL.
antibody. Microscopic observation displayed that hrOCIF significantly decreased the number of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling–positive apoptotic cells in the LV myocardium, whereas anti-RANKL antibody had little effect on it (Figure 7E). Neither hrOCIF nor anti-RANKL antibody affected the extent of fibrotic area in Ang II–infused osteoprotegerin−/− mice (Figure 7F).

**Discussion**

Our data suggest that important cross talk exists between osteoprotegerin synthesis and RAS in rodent heart. In addition, our data highlight that osteoprotegerin plays a role for preserving structure and function against Ang II–induced cardiac hypertrophy.

Ang II stimulates the osteoprotegerin synthesis in vascular smooth muscle cells.29 Accordingly, we demonstrated that Ang II increased the osteoprotegerin mRNA in rat LV tissues. Immunoreactivity of osteoprotegerin was more intensely stained in CD68-macrophages than in S100A4-fibroblasts or cardiocytes in hypertrophied LV. Nondepressor dose of AT1-receptor antagonism decreased the osteoprotegerin mRNA expression, suggesting a direct regulation of osteoprotegerin synthesis mediated through AT1-receptor. In cell culture experiment, osteoprotegerin was synthesized in both cardiomyocytes and fibroblasts, and fibroblasts are susceptible to activation by Ang II than cardiomyocytes. However, the dose of Ang II to cardiac tissue was different from cell culture, and the magnitude of osteoprotegerin synthesis in response to Ang II was greater in cardiac tissues than in cultured cells, suggesting that there are other agonists and infiltrating inflammatory cells that crosstalk with Ang II might engage osteoprotegerin upregulation in vivo.

We previously reported that myocardial secretion of osteoprotegerin correlated with concentric hypertrophy, whereas no such observation was made for eccentric hypertrophy in...
Thus, we hypothesized that Ang II stimulation of osteoprotegerin might exert a protective mechanism for LV dilation. To clarify the pathophysiological roles of endogenous osteoprotegerin in Ang II–induced cardiac hypertrophy, we conducted experiments using mice lacking the osteoprotegerin gene. It is interesting to note that Ang II infusion to the 8-week-old osteoprotegerin−/− mice for 28 days developed LV dilation with reduced contractile function, and this was comparable with the WT mice, despite equivalent elevation of SBP. One of the most striking histological features associated with LV dilation was the reduction of interstitial fibrosis accompanied by procollagen α1 synthesis in response to Ang II in osteoprotegerin−/− mice. These data suggest that it is likely that osteoprotegerin mediates the fibrotic action of Ang II. In supporting our study, Toffoli et al30 reported that recombinant osteoprotegerin promotes the fibrosis in vascular wall. Myocyte apoptosis is another pathological feature associated with the progression of heart failure.31 Osteoprotegerin exerts antiapoptosis/survival through RANKL-dependent or RANKL-independent mechanism,32–34 and our data correspond to the increase in number of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling–positive apoptotic cells in the LV of Ang II–infused osteoprotegerin−/− mice. It is reasonable to speculate that Ang II–mediated cardiac damage was accentuated in osteoprotegerin−/− mice because of their inability to mount an appropriate fibrotic response and

Figure 6. Gene expressions of procollagen 1α (A) and syndecan-1 (B) with or without angiotensin II (Ang II) stimulation in wild-type (WT) and osteoprotegerin (OPG)−/− mice. Data are shown as the mean±SD (A, WT mice with [n=9] or without [n=8] Ang II stimulation; OPG−/− mice with [n=17] or without [n=18] Ang II stimulation; B, WT mice with [n=14] or without [n=9] Ang II stimulation; OPG−/− mice with [n=16] or without [n=12] Ang II stimulation) and analyzed by 2-way ANOVA followed by Bonferroni post hoc test. **P<0.01, ***P<0.001. Respective gene expression was normalized by 18S ribosomal RNA (rRNA), and the oligonucleotide sequences and probes are shown in Table S1.

Figure 7. Effects of human recombinant (hr) osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) or anti–receptor activator of nuclear factor-κB ligand (RANKL) antibody to the angiotensin II (Ang II)–infused OPG−/− mice for 28 d on trabecular separation (Tb.Sp) at proximal metaphysis of femur (A), systolic blood pressure (SBP) (B), ratio of heart weight (HW) to body weight (BW) (C), %fractional shortening (D), the number of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)–positive cells (E), and %fibrosis area assessed by Sirius red staining (F). We administered hrOCIF (10 mg/kg IP) every 2 d for 28 d, or anti-mouse RANKL rat monoclonal antibody (5 mg/kg SC) on days 1, 14, and 28. Data are shown as the mean±SD (Ang II–infused OPG−/− mice without any treatment [n=18], with hrOPG/OCIF [n=20] or anti-RANKL antibody [n=9] administration), and analyzed by multiple comparisons with Tukey 1-way ANOVA test. *P<0.05, **P<0.01, ***P<0.001.
stimulation of myocyte apoptosis against the increased BP. We further addressed that recombinant osteoprotegerin/OCIF administration ameliorated the development of heart failure accompanied by decreasing the number of apoptotic cells without affecting SBP in these mice.

This study also refers to the pathological roles of RANKL in the development of heart failure. Soluble form of RANKL increases in peripheral blood and in bone marrow in accordance with the severity of human heart failure. Consistently, osteoprotegerin−/− mice revealed a marked elevation of RANKL in the circulation. Because osteoprotegerin exerts biological activity through neutralizing the RANKL activity as a competitive receptor, we initially hypothesized a functional relationship between osteoprotegerin and RANKL in the development of systolic heart failure. Consistently to the previous report, our data revealed that RANKL expression was too low to detect in the LV. In addition, we demonstrated that RANKL−transgenic mice exhibited the concentric hypertrophy, as in the case with WT mice (Figure S2A–S2H). More importantly, the inhibition of RANKL activity with RANKL-transgenic mice had little effect on LV remodeling, further supporting that the increased osteoprotegerin may represent a compensatory response that minimizes cardiac damage independently of RANKL. Osteoprotegerin binds not only to RANKL but also to various ligands such as tumor necrosis factor–related apoptosis ligand–induced apoptosis in myeloma cells. Because osteoprotegerin binds to syndecan-1 by its heparin-binding domain, we speculate that syndecan-1–mediated interaction with osteoprotegerin may protect from cardiomyocyte death and organize appropriate matrix network.

Perspectives

RANKL/RANK/osteoprotegerin axis has been identified to play crucial roles to regenerating and remodeling the bone. Osteoprotegerin is also expressed in the heart, suggesting its biological actions beyond the bone metabolism. This study provides new insight into osteoprotegerin in interaction with RAS in the evolution of systolic heart failure. Osteoprotegerin−/− mice exhibited progression of LV dilation with impaired systolic contractile function in Ang II–induced hypertension, and the weak mounting of collagen and increase of myocyte apoptosis are supposed to explain the LV dilation. This article also describes a biological action of osteoprotegerin independent of hemodynamics and RANKL. Taken together, our data underscore that osteoprotegerin exclusively at local sites is important in maintaining the LV structural integrity and function against high BP.

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Disclosures

None.

References

Novelty and Significance

What Is New?

- Angiotensin II stimulates the osteoprotegerin synthesis in rodent heart.
- Mice lacking the osteoprotegerin gene develop systolic heart failure during hypertension.

What Is Relevant?

- Osteoprotegerin has recognized to work as a competitive receptor for receptor activator of nuclear factor (NF)-κB to inhibit the binding of receptor activator of nuclear factor (NF)-κB ligand on bone metabolism. We report here that osteoprotegerin preserves the myocardial structure against angiotensin II stimulation.

Summary

Osteoprotegerin plays a critical role in angiotensin II–induced cardiac remodeling, and lack of this gene leads to dilated left ventricular chamber, resulting in functional left ventricular decompensation.
Angiotensin II Stimulation of Cardiac Hypertrophy and Functional Decompensation in Osteoprotegerin-Deficient Mice

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Angiotensin II stimulation of cardiac hypertrophy and functional decompensation in osteoprotegerin-deficient mice

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Materials and Methods
All protocols for the present experiments were reviewed and approved by the University of Miyazaki Institutional Animal Care and Use Committee (approval number 2012-506-3) and Genetic Modification Safety Committee of Miyazaki University (approval number 403). All studies were conducted in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (Revised 2015).

Experimental procedure
Rats and mice were housed in a temperature and light-controlled room (25±1 °C; 12/12-h light/dark cycle) with free access to normal chow and water.

Rats: We used 8-week-old male Wistar rats (Charles River, Japan) weighing 200 to 250 g randomly divided to three groups; controls (sham), and those received synthetic angiotensin II (Ang II) (Sigma) with or without antagonism of the Ang II type 1 receptor (AT1-R). Under anesthesia with intraperitoneal administration of 50 mg/kg pentobarbital sodium, hypertension was induced by 250 ng/kg/min Ang II (Sigma) infusion subcutaneously for 14 days by mini-osmotic pumps (Alzet, model 2002; DURECT Co.). AT1-R antagonist RNH-6270 (0.5 mg/kg, provided by Daiichi-Sankyo Co., Ltd, Tokyo, Japan) dissolved in 2.5% NaHCO3 was started to give to rats orally one day before implanting the mini-osmotic pump, and continued up to the end of study.

Mice: OPG−/− mice (genetic background of C57BL/6) were generated by targeted disruption of the gene, and OPG−/− mice were bred with OPG−/− mice. RANKL-transgenic (Tg) mice (genetic background of C57BL/6) were generated to express the murine RANKL in hepatocytes by using the human serum amyloid P component promoter, and bred with wild-type (WT) mice (C57BL/6J) purchased from Japan Clea Co. (Tokyo, Japan). Phenotype of each mouse was verified at every mating by PCR analysis of the tail DNA. We used 8-week-old male OPG−/− mice and age-matched WT mice (Japan Clea Co.), and 8-week-old male RANKL-Tg mice and age-matched WT littermates in this study. We anesthetized OPG−/−, RANKL-Tg and age-matched WT mice by injecting 2,2,2-tribromoethanol (Avertin) at 200 mg/kg intraperitoneally. We infused either Ang II (1000 ng/kg/min dissolved in 0.9% saline) or vehicle (0.9% saline) subcutaneously for 28 days using the implanted mini-osmotic pump (Alzet, Model 1004; DURECT Co.). In another setting of experiments, we administered human recombinant (hr) OPG/ osteoclastogenesis inhibitory factor (OCIF) (10 mg/kg, intraperitoneally, provided by Daiichi-Sankyo Co., Ltd, Tokyo, Japan) every 2 days from day 1 to day 28, or anti-mouse RANKL rat monoclonal antibody (5 mg/kg,
subcutaneously, Oriental Yeast Co., Ltd, Tokyo, Japan) on days 1, 14 and 28 to the Ang II-infused OPG<sup>−/−</sup> mice.

**Blood pressure**

We measured systolic blood pressure of rats and mice 3 times by tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan) in a conscious situation, and averaged them.

**Echocardiography**

We carried out transthoracic echocardiography to mice with a 15-MHz high-frequency linear transducer connected to SONOS 5500 system (Philips) in a conscious state<sup>4</sup>. Wall thickness and chamber dimension were determined from M-mode tracings; left ventricular (LV) wall thickness was evaluated in LV posterior wall (LVPWd) at diastole. End-diastolic dimension was obtained at the point of maximal LV diastolic internal dimension (LVIDd), and LV systolic internal dimension (LVIDs) was obtained at the time point of most anterior systolic excursion with minimal chamber dimension. All LV dimensions are presented as the average of 5 consecutive measurements by 3 experienced readers. The % fractional shortening, a measure of LV systolic performance, was calculated from M-mode-derived LV dimensions using the following formula: (LVIDd-LVIDs)/LVIDd x100%.

**Sample collection**

We collected blood samples from right ventricle under anesthesia with intraperitoneal administration of 50 mg/kg pentobarbital sodium, and mixed them in 10 µl of 10 mg/mL 2-[[2-[bis(carboxymethyl)amino]ethyl](carboxymethyl)amino]acetic acid and 0.7 mg/mL aprotinin. They were centrifuged at 3,000 rpm, 4 °C for 10 minutes, and the plasma was stored at −80 °C until use. After weighing the whole heart, we removed atria and right ventricle, and the middle part of LV was fixed in 4% paraformaldehyde, and the rest of them were frozen in liquid nitrogen. We also had taken thymus and bone marrow from the femur from WT and OPG<sup>−/−</sup> mice.

**Cell culture**

We isolated cardiac myocytes and fibroblasts from the ventricles of 1-day-old Wistar rats<sup>5,6</sup>. In the first protocol, either cardiac myocytes or fibroblasts were cultured in serum-free Dulbecco’s modified Eagle’s medium supplemented with 10 µg/mL insulin, 5 µg/mL transferrin and 7 ng/mL sodium selenite for 48 hours, and were then treated
with $10^{-7}$ mol/L Ang II for the indicated time periods. In the second protocol, the cardiac fibroblasts were exposed to the indicated concentrations ($10^{-6}$ to $10^{-9}$ mol/L) of Ang II for 1 hour. In the third protocol, we pretreated cultured cardiac fibroblasts with either Ang II type 1 receptor antagonist, RNH-6270 (provided by Sankyo Co., Ltd) or Ang II type 2 receptor antagonist, PD-123,319 (Sigma) for 30 minutes, and then added Ang II ($10^{-7}$ mol/L) to the culture medium for 1 hour. We collected the cells at the indicated times followed by washing with phosphate-buffered saline 3 times, and stored them at -80 °C until use.

**RNA isolation and real-time quantitative PCR**

We extracted total RNA with TRIzol Reagent (GIBCO BRL), and used 1 µg for synthesizing complementary DNA (cDNA) by means of SuperScript reverse transcriptase (Life Technologies Inc.). cDNA was then amplified with oligonucleotide primers and TaqMan probes labeled with 6-carboxy-fluorescein as reporter fluorescence and 6-carboxy-tetramethyl-rhodamine as quencher fluorescence by real-time quantitative RT-PCR (ABI Prism 7300 Sequence Detector, Applied Biosystems). We used relative standard curve method using a serial dilution of cDNA (mouse heart) for both the target and housekeeping gene. The standard curve is a straight line composed of the fractional PCR cycle numbers, defined as threshold cycle (Ct) at which signal of the reporter fluorescence is greater than the minimal detection level (detection limit). The amount of gene for “unknown samples” was accomplished by measuring Ct, and determined the initial copy number using the standard curve. We listed the oligonucleotide sequences and probes in Table S1 in the online-only Data Supplement. The PCR products were of the expected molecular size and the gene expression levels were normalized relative to the level of 18S ribosomal RNA (rRNA).

**Immunohistochemistry**

Ventricular tissues, fixed in 4% paraformaldehyde and embedded in paraffin wax, were sectioned at 3 µm thickness. After deparaffinization with xylene and graded alcohol, we immersed the slides in 3% hydrogen peroxide to block the endogenous peroxidase activity, and thereafter incubated with Protein Block (DakoCytomation) for 10 minutes at room temperature to reduce the nonspecific background. Then, we incubated the slide sections at 4 °C overnight with the following primary antibodies; OPG (10 µg/mL, clone 98A1071; IMGENIX), vimentin (clone L1843, DakoCytomation), S100A4 (1 µg/mL, ab27957; abcam) or CD68 (0.8 µg/mL, clone ED1, Millipore). After overnight reaction with antibodies, we incubated the slide sections with EnVision+.
(Dakocytomation) for 30 minutes at room temperature, visualized them with 0.05% 3, 3’-diaminobenzidine containing hydrogen peroxide and counterstained with hematoxylin. We dehydrated the slide sections in xylene, and coverslipped. To retrieve the antigens, we autoclaved the slide sections at 121 °C for 15 minutes in 10 mmol/L citrate buffer (pH 6.0) for S100A4, or incubated with 0.05% pronase at 37 °C for 10 minutes for CD68 before reacting with the primary antibodies. We scanned the slides at x200 magnification under the Olympus BX53F microscope (Olympus, Tokyo, Japan). Negative control staining was carried out by omitting the first antibody or by using a non-immune isotype-matched IgG.

**Fibrosis**

We visualized the distribution of myocardial fibrosis by Mallory-Azan in rats. We also stained the slide tissue sections of mouse heart with 0.1% picrosirius red (Direct Red 80, Sigma, St Louis, MO, USA) dissolved in saturated picric acid for 10 minutes. We scanned 3 separate parts in the cross-sectional area of LV at the level of papillary muscle under the polarized light at x100 magnification (Olympus BX53F microscope, Olympus, Tokyo, Japan), and analyzed the images (WinROOF v.6.5, Mitani Co., Japan). We determined collagen volume fraction in the interstitial space between the myocardial fibers by calculating the ratio of collagen area to the selected myocardial tissue area, while we omitted fibrosis of the perivascular, epi- and endo-cardial areas from the analysis.

**Apoptotic cells**

Apoptotic cells in the LV cross-sections were assessed by in situ detection of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining (MEBSTAIN Apoptosis Kit II, Medical & Biological Laboratories, Co., Ltd., Nagoya, Japan). The TUNEL index was calculated as the ratio of TUNEL-positive nuclei to total nuclei counterstained with 0.5 µg/mL Propidium Iodide. We used rat thymus for positive control.

**RANKL concentration**

We measured plasma concentration of mouse RANKL in duplicate by Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

**Micro-computed tomography (µCT)**

We took the right lower extremity from mice body after sacrificing, and removed soft
tissues surrounding the bone. Then, we performed μCT scan (ScanXmate-L090H; Comscantecno, Kanagawa, Japan) at 70 kV and 114 μA, and acquired a total of 496 micro-tomographic slices with a slice thickness of 17.0 μm in 496 x 496 matrices (voxel size; 17.0 x 17.0 x 17.0 μm). To analyze the trabecular bone microstructure, we used a 3D image analyzing system (TRI/3D-BON; Ratoc System Engineering Co. Ltd., Tokyo, Japan). The measurement area was set to the secondary trabecular area at the proximal metaphysis of femur (2.0 mm trimming), and we calculated the trabecular separation (Tb.Sp) based on direct measures by a distance transformation method

References


10. Hildebrand T, Rüegsegger P. Quantification of bone microarchitecture with the
### Table S1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe sequences</th>
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S, sense strand; AS, antisense strand

OPG, osteoprotegerin; RANK, receptor activator of nuclear factor-κb; RANKL, receptor activator of nuclear factor-κb ligand; ANP, atrial natriuretic peptide
Detection limit (dot line) based on the threshold cycle (Ct) by real-time quantitative PCR in OPG (A), RANK (B), RANKL (C), procollagen α1 (D) and syndecan-1 (E) in WT and OPG⁻/⁻ mice with or without stimulation of angiotensin II (Ang II) for 28 days.
Effects of Ang II infusion (1000 ng/kg/min) subcutaneously for 28 days on systolic blood pressure (SBP) (A), ratio of heart weight (HW) to body weight (BW) (B), LVPWd (C), LVIDd (D), LVIDs (E), %fractional shortening (F), trabecular separation (Tb.Sp) at proximal metaphysis of femur (G) and soluble RANKL concentration (H) in 8-week-old wild-type (WT) and RANKL-transgenic (Tg) mice. LVPWd, LV posterior wall dimension; LVIDd, LV diastolic internal dimension; LVIDs, LV systolic internal dimension. Data are shown as the means ± standard deviation [WT mice with (A, n=10; B, n=7; C-F, n=8; G, n=6; H, n=4) Ang II stimulation; RANKL-Tg mice with (A, n=10; B, n=10; C-F, n=9; G, n=10; H, n=10) or without (A, n=10; B, n=6; C-F, n=9; G, n=9; H, n=6) Ang II stimulation], and analyzed by 2-way ANOVA followed by Bonferroni post hoc test. ***p<0.001.