right atrial pressure and useful for monitoring volume status in patients with heart failure.[10]

Cardiac Catheterization

Cardiac catheterization was performed before and 1 month after surgery. Before left ventriculography, LV systolic pressure, end-diastolic pressure, pulmonary capillary wedge pressure, systolic and mean PAP, and right atrial pressure were measured. Using biplane cine-ventriculography, LV end-diastolic volume, LV end-systolic volume, and LVEF were determined. Cardiac output was measured by the thermodilution method. Volumes and cardiac output were indexed by body surface area.

The purposes of cardiac catheterization and its invasive nature were explained in detail to all patients, and only those who gave informed consent underwent catheterizations. The indications for postoperative catheterization were not selective and the procedure was performed by a cardiologist under appropriate hydration conditions. Preoperative coronary arteriography was performed in all 208 patients, whereas serial left ventriculography and hemodynamic measurements were performed in 154 (74%) and 109 (52%), respectively.

Surgical Procedures

All operations were performed with bicaval cannulation, mild hypothermic cardiopulmonary bypass, and meticulous myocardial preservation with intermittent cold blood cardioplegia. Coronary artery bypass grafting was generally performed first. The approach to the mitral valve was generally through a superior transseptal method. The mitral valve was repaired using a restrictive mitral annuloplasty technique with an undersized ring.[11] Ring size was determined after careful intraoperative measurements of the height of the anterior leaflet and intertrigonal distance and then downsizing by 2 to 3 sizes. No other adjunct procedures were performed on the valve itself. Surgical ventricular reconstruction was performed when a broad anteroapical or anteroseptal asynergy (akinesis or dyskinesis) was demonstrated by left ventriculography and a postoperative LV end-diastolic volume index >90 mL/m^2 was anticipated. Our criteria for adding surgical ventricular reconstruction to restrictive mitral annuloplasty were mainly based on 2 previous investigations.[12,13]

Perioperative Management for Patients With Hemodialysis

Hemodialysis was performed in all patients with ESRD 12 to 24 hours before surgical intervention. During surgery, an extracorporeal ultrafiltration method was used to appropriately maintain volume status. Hemodialysis was routinely resumed on the second postoperative day or earlier if volume overload or hyperkalemia was present.

Clinical Follow-Up

Every 6 months to 1 year, each patient was assessed in the department as well as by their primary cardiologist. Functional status was assessed according to New York Heart Association criteria and plasma brain natriuretic peptide (BNP) level. The primary study end point was mortality during follow-up, and the second was defined as the composite of mortality and readmission for heart failure. Diagnosis of postoperative recurrent heart failure was based on clinical symptoms, physical signs, or radiological evidence of pulmonary congestion.

Clinical follow-up examinations were completed for all patients (100%) with a mean duration of 49±25 months (range, 4–126 months) for survivors. The cumulative follow-up period was 728 patient-years.

Statistical Analysis

Continuous variables are summarized as means±SDs or SEs when describing data presented in a figure and categorical variables as frequencies and proportions. For the continuous variables, comparisons between 2 groups were made using an unpaired *t* test. Comparisons among 3 groups were made using one-way analysis of variance. For categorical variables, 3 groups were compared using Fisher exact or a Kruskal-Wallis test. Correlations between continuous variables were tested with Pearson correlation coefficient (*r*). Hemodynamic data (LV end-diastolic volume index, LV end-systolic volume index, LVEF, LV end-diastolic pressure, mean PAP, cardiac index) were analyzed using an analysis of covariance model, including factors for the corresponding baseline value as the covariate, group, and interaction between them. Functional (plasma BNP) and echocardiographic (LV end-diastolic dimension, LV end-systolic dimension, LVEF, systolic PAP) data over time after surgical intervention were analyzed using a mixed-effects model for repeated measures, including factors for the corresponding baseline value, group, time, and interaction between group and time. The following covariance structures were considered: unstructured, compound symmetrical, first-order autoregressive, and Toeplitz. The covariance structure that provided the best fit according to Akaike information criterion was used in the analysis. Assessment time points were treated as categorical factors. The analysis of covariance and mixed-effects models included adjustments for age, etiology of cardiomyopathy, with or without diuretics, and laboratory examination findings, which showed significant differences among the groups (Table 1).

The linearized mortality rate was computed by dividing the number of patients experiencing an event by patient-years at risk. Survival and adverse event-free curves were estimated using the Kaplan-Meier method, and compared using an overall log-rank test, followed by a post hoc pairwise log-rank test. The association of group with adverse events was examined using Cox proportional-hazards models with adjustments for all other covariates presented in baseline demographics, echocardiographic, and surgical data (see the online-only Data Supplement Appendix). Factors obtaining a probability value <0.05 in the univariate Cox proportional hazards analysis were then entered appropriately into the multivariate fashion. Results are summarized as hazard ratios and 95% CIs. Multiplicity in pairwise comparisons was corrected by the Bonferroni procedure. All probability values are 2-sided and values of $P<0.05$ were considered to indicate statistical significance. Statistical analyses were performed using JMP 7.0 (SAS Institute, Cary, NC), SAS statistical software (Version 9.2; SAS Institute), and SPSS (Version 17.0; SPSS Inc).

Results

Clinical Outcomes

Overall hospital mortality was 6.3% for all patients and 6.3%, 6.7%, and 5.3% for the control, late CKD, and ESRD groups, respectively, with no intergroup differences.

Among 195 operative survivors, 49 late deaths occurred during the follow-up period with a linearized mortality rate of 6.8% per patient-year. Patients with ESRD tended to die from infectious or bleeding disorders rather than cardiac causes, whereas those with late CKD died more often from cardiac-related causes such as heart failure or sudden death than the ESRD group (Table 2). Actuarial survival at 2 and 5 years was 66%±7% and 45%±8% in late CKD and 78%±10% and 70%±12% in ESRD compared with 87%±3% and 77%±4%, respectively, in the control group (median survival, 40, 67, and 118 months, respectively; Figure 1). Patients with late CKD had a worse postoperative survival ($P<0.0001$ versus control group), whereas patients with ESRD had nearly comparable overall survival as compared with the control ($P=0.27$ versus control). Similarly, freedom from mortality and readmission for heart failure at 2 and 5 years was 50%±8% and 18%±7% in late CKD ($P<0.0001$ versus control, $P=0.01$ versus ESRD) and 72%±11% and 64%±12% in ESRD ($P=1$ versus control) as compared with 77%±4% and 52%±5%, respectively, in the control group (median event-free survival, 26, 67, and 63 months, respectively; Figure 2).

The Cox proportional hazards model with adjustments for baseline demographics, echocardiographic, and surgical data showed that late CKD (hazard ratio, 2.6; 95% CI, 1.6–4.2;

Table 2. Late Mortality and Morbidity

	Control (n=135)	Late CKD (n=42)	ESRD on HD (n=18)
Late death	22 (16%)	20 (48%)	7 (39%)
Heart failure (LOS)	13	11	0
Sudden death	1	4	0
Infectious disorder	2	2	3
Cerebrovascular accident	1	2	1
Gastrointestinal bleeding	0	0	2
Malignancy	5	0	0
Unknown	0	1	1
Major complications			
Readmission for heart failure	32	12	2
Recurrent MR	7	4	1
Introduction of dialysis	2	5	...
Myocardial infarction	2	1	1
Cerebrovascular accident	2	0	0
CRT-D implantation	30	10	2

CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis; LOS, low output syndrome; MR, mitral regurgitation; CRT-D, cardiac resynchronization therapy defibrillator.

$P<0.0001$) but not ESRD on hemodialysis (hazard ratio, 1.0; 95% CI, 0.5–2.3; $P=0.91$) was associated with postoperative adverse events defined as mortality and readmission for heart failure.

Pre- and Postoperative Hemodynamic Data

From baseline to 1 month after surgery, LV volumes were substantially decreased in all groups with no intergroup differences in regard to the postoperative values (interaction effects $P>0.05$, group effects $P>0.05$; Figure 3). LVEF changed from 26%±8% to 32%±12%, 25%±7% to 26%±10% and 24%±7± to 33%±8% in the control, late CKD, and ESRD groups, respectively, providing evidence that patients with late CKD showed less improvement in LVEF than the other 2 groups (interaction effect $P=0.027$). LV systolic pressure did not change, whereas LV end-diastolic pressure was decreased in all groups. Systolic and mean PAP were decreased in all groups, whereas right atrial pressure did not change. Cardiac index increased from 2.5±0.7 to 2.8±0.6, 2.4±0.5 to 2.6±0.6, and 2.9±0.7 to 3.1±0.8 L/min/m^2 in the control, late CKD, and ESRD groups, respectively. These findings suggested that improvements in LV function and hemodynamics could be obtained at 1 month after surgery, irrespective of preoperative renal function status, with no substantial intergroup differences for those postoperative values.

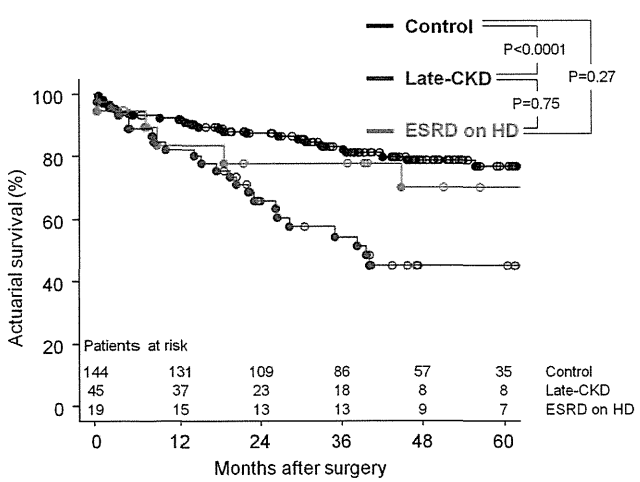

Figure 1. Actuarial survival. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis.

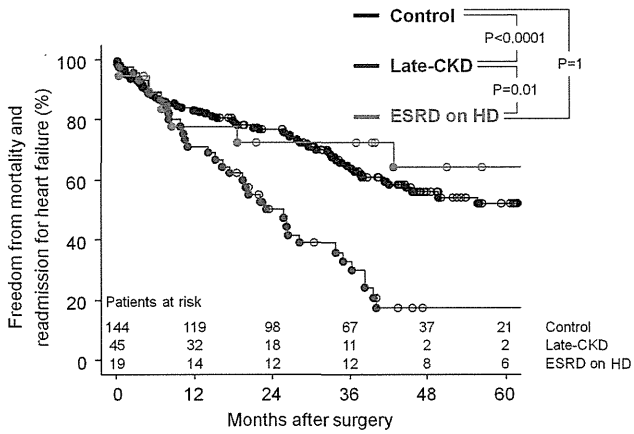

Figure 2. Freedom from mortality and readmission for heart failure. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis.

Serial Echocardiographic Data

LV dimensions were decreased and LVEF was increased at 1 month after surgery in all groups (Figure 4), with subsequent changes in each group apparently distinctive. In the control, these improvements (LV reverse remodeling) persisted during the 2-year follow-up period. As compared with the control group, patients with ESRD showed further decreases in LV dimensions and improvement in LVEF during the follow-up period. In contrast, patients with late CKD showed a gradual reincrease in LV dimensions and less improvement in LVEF than the other groups (interaction effects $P<0.05$ for all).

From baseline to 1 month after surgery, systolic PAP was decreased from 46±13 to 33±11, 49±18 to 35±12, and 49±14 to 34±8 mm Hg in the control, late CKD, and ESRD groups, respectively, showing nearly identical systolic PAP values at 1 month for the 3 groups. The systolic PAP value at 2 years after surgery was 36±14 mm Hg in the control and 33±11 mm Hg in the ESRD group, suggesting that improvement in systolic PAP was nearly completely sustained over time in those 2 groups. In contrast, patients with late CKD showed a gradual reincrease in systolic PAP and had a high PAP value (44±18 mm Hg) at the 2-year follow-up examination.

IVC Dimension

Mean IVC dimension at the latest examination was significantly greater in the late CKD group as compared with the others (Figure 5).

Symptoms and Serial BNP Level

Among patients in 3 study groups, the proportion with New York Heart Association Class I heart failure (no symptoms)

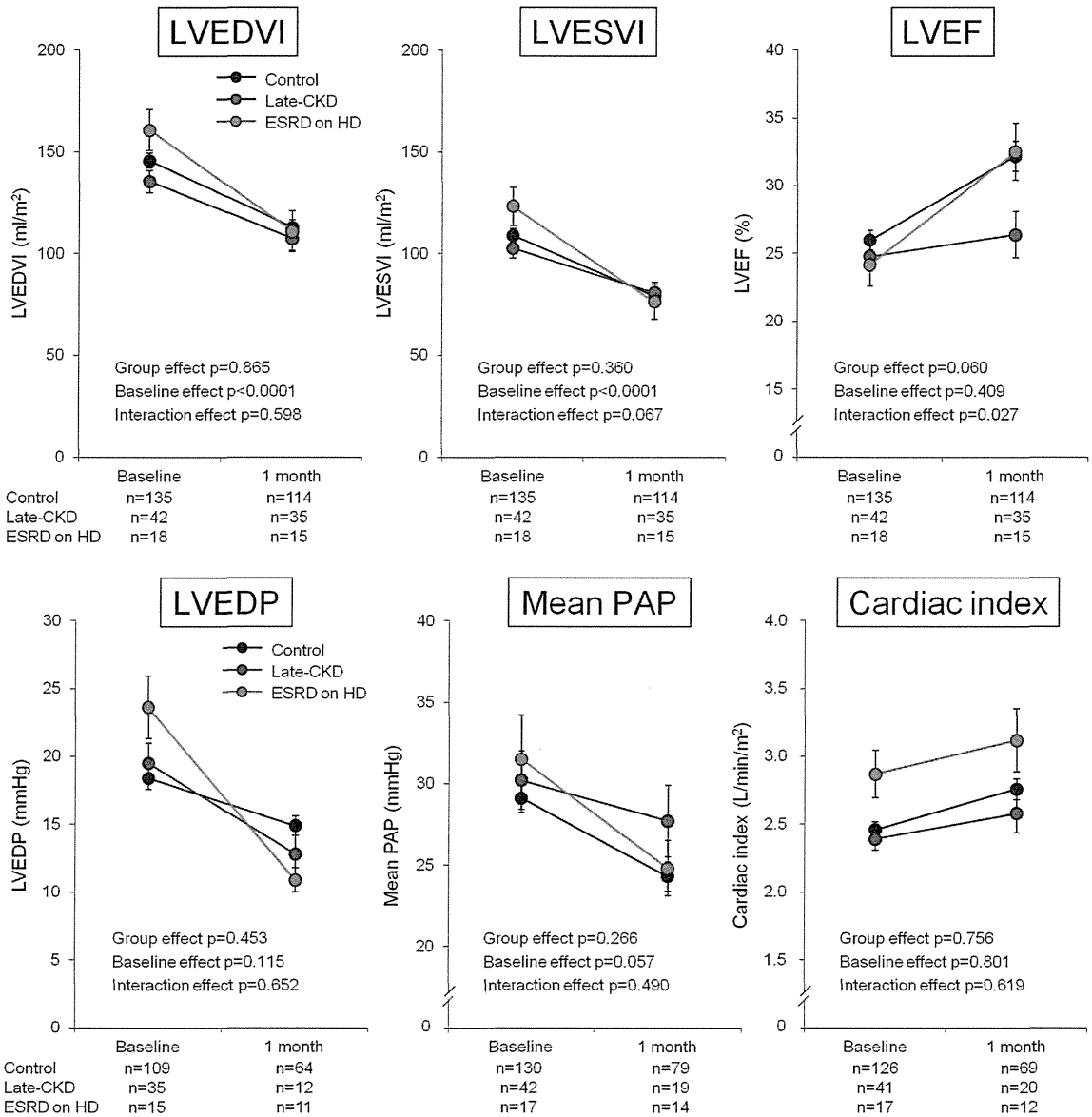

Figure 3. Pre- and postoperative hemodynamic changes. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis; LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; LVEF, left ventricular ejection fraction; LVEDP, left ventricular end-diastolic pressure; PAP, pulmonary artery pressure. Data are presented as mean±SE.

increased and the proportion with Class III or IV heart failure decreased from baseline to the last follow-up visit (91%–15% for the control, 91%–33% for the late CKD, and 95%–17% for the ESRD group, respectively; Figure 6). The symptoms improved by an average of 1.1, 0.7, and 1.2 New York Heart Association classes in the control, late CKD, and ESRD groups, respectively ($P=0.054$ for the difference among the 3 groups in the change from baseline).

Plasma BNP concentration in the control group was decreased at 1 month after surgery and this improvement was sustained during the 2-year follow-up period. As compared with the control, patients with late CKD showed less improvement in plasma BNP value over time, whereas those with ESRD showed remarkable improvement in that value at 1 month and a gradual improvement thereafter (interaction effect $P=0.001$; Figure 7).

Relationship Between IVC Dimension and Plasma BNP Concentration

There was a significant positive correlation between BNP level and IVC dimension at 1 ($r=0.47$, $P<0.0001$) and 2 ($r=0.44$, $P<0.0001$) years after surgery (Figure 8).

Discussion

The major findings can be summarized as follows: (1) restrictive mitral annuloplasty for advanced cardiomyopathy could be performed for high-risk patients with late CKD or ESRD with an acceptable early mortality comparable to that seen in the control; (2) postoperative (1-month) cardiac catheterization resulted in a substantial decrease in LV volume and improvement of hemodynamic status with no intergroup differences for those postoperative values; (3) patients with ESRD had nearly comparable

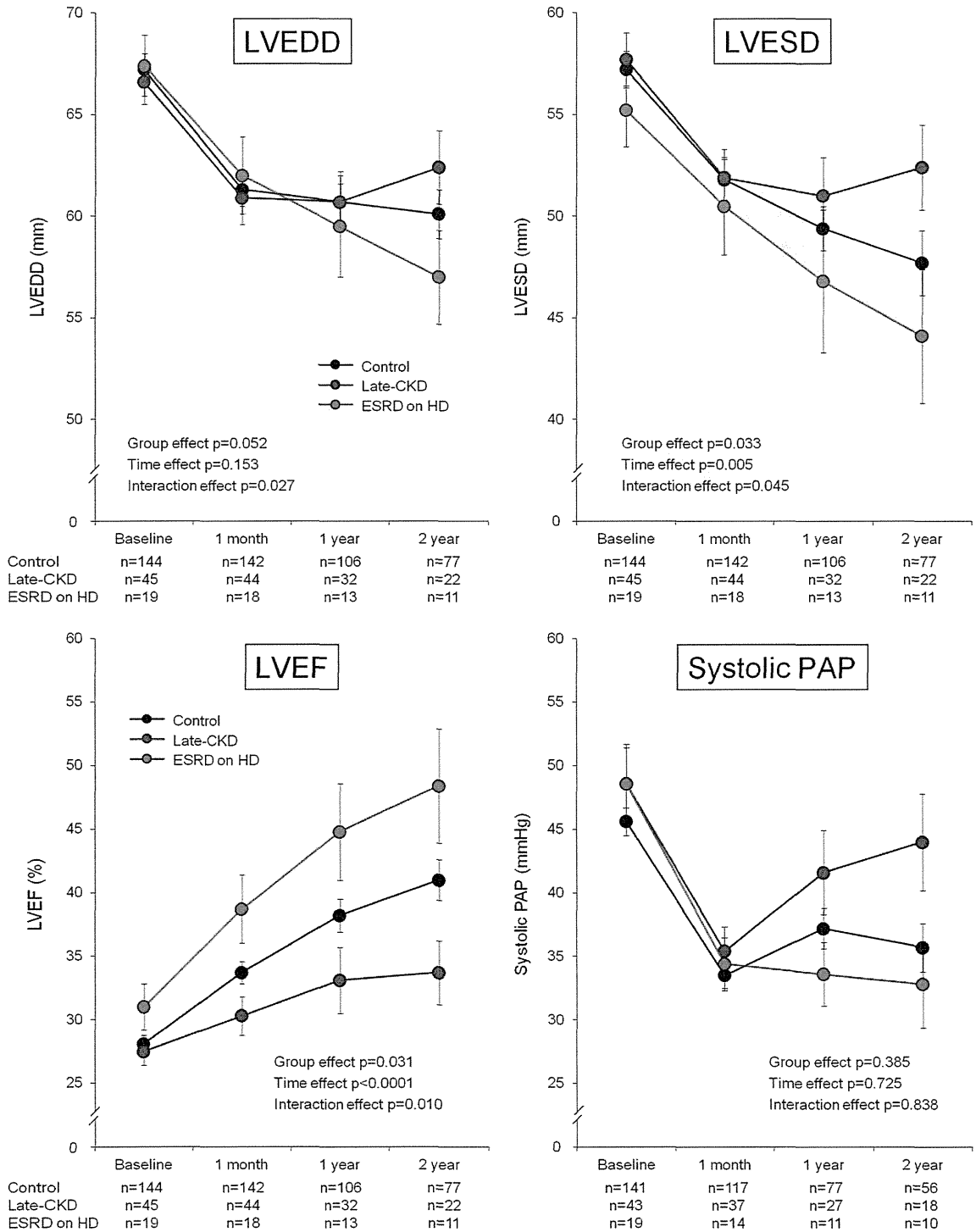

Figure 4. Serial echocardiographic findings. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic dimension; PAP, pulmonary artery pressure. Data are presented as mean±SE.

postoperative outcomes as compared with the control and significantly better outcomes than the late CKD group in terms of freedom from mortality and readmission for heart failure; and (4) patients in the control and ESRD groups showed further improvements in plasma BNP level during the 2-year follow-up period as compared with the late CKD group.

The 5.3% rate of early mortality seen in the patients with ESRD is favorable as compared with that in previous reports, including 20 studies with a total of 863 patients on chronic hemodialysis who underwent various cardiac procedures.[14] Furthermore, the overall survival rate seen in our patients with ESRD is satisfactory given that outcome in patients with heart failure requiring regular hemodialysis is extremely poor with a 3-year survival rate <15% after hospitalization for chronic heart failure.[15]

There are limited data available regarding acute hemodynamic changes after a restrictive annuloplasty in patients with

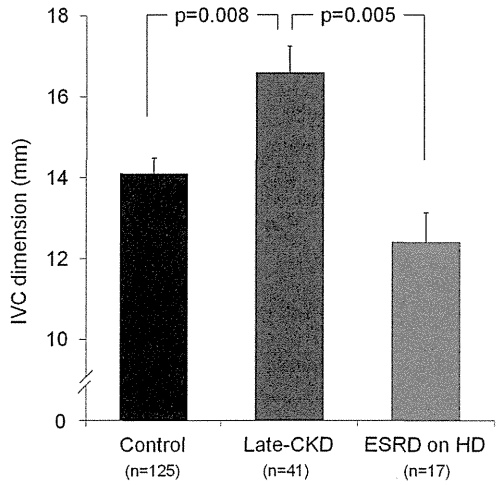

Figure 5. IVC dimension at latest follow-up examination. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis; IVC, inferior vena cava. Comparisons among 3 groups were made using one-way analysis of variance followed by the post hoc pairwise unpaired *t* test. Data are presented as mean±SE.

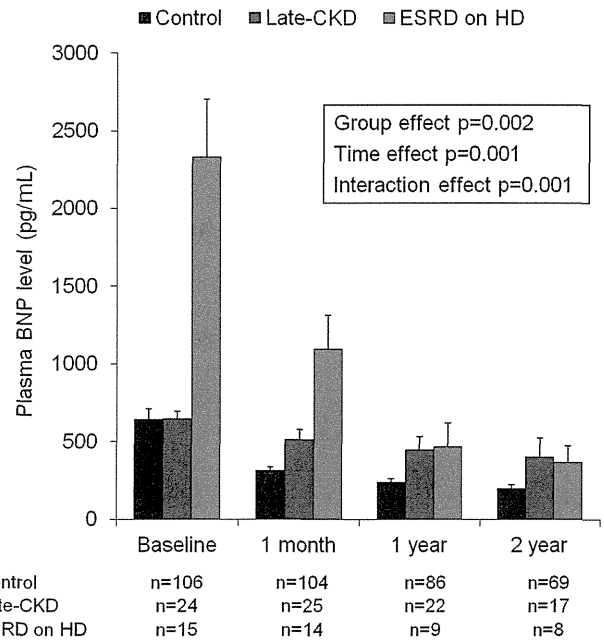

Figure 7. Serial changes in plasma BNP levels. BNP indicates brain natriuretic peptide.

ESRD. Chang et al[16] reported favorable short-term results of 5 patients with chronic hemodialysis undergoing mitral valve repair for "uremic cardiomyopathy" that mimicked the pathophysiological disorder in patients with dilated cardiomyopathy resulting from chronic volume overload. Their echocardiographic findings showed that LV end-diastolic volume decreased from 168±20 at baseline to 113±36 mL, whereas LV end-systolic volume decreased from 91±38 to 52±26 mL after mitral valve repair for uremic cardiomyopathy, similar to that seen in our patients with ESRD. Importantly, cardiac catheterization in the present study allowed confirmation of improvements in LV volume and function as well as other hemodynamic parameters including LV end-diastolic pressure, mean PAP, and cardiac index, irrespective of preoperative renal function status. Furthermore, our serial echocardiographic examinations extending over 2 years revealed that the sustained improvements in LV function (reverse LV remodeling) and hemodynamics seen in patients with ESRD were comparable to those in the control group, indicating the efficacy of surgical intervention in patients with ESRD.

Plasma BNP concentration may be a useful biomarker of heart failure and provide prognostic information.[17] Sustained improvements in BNP level in the control and ESRD groups may indicate that relief of heart failure obtained immediately

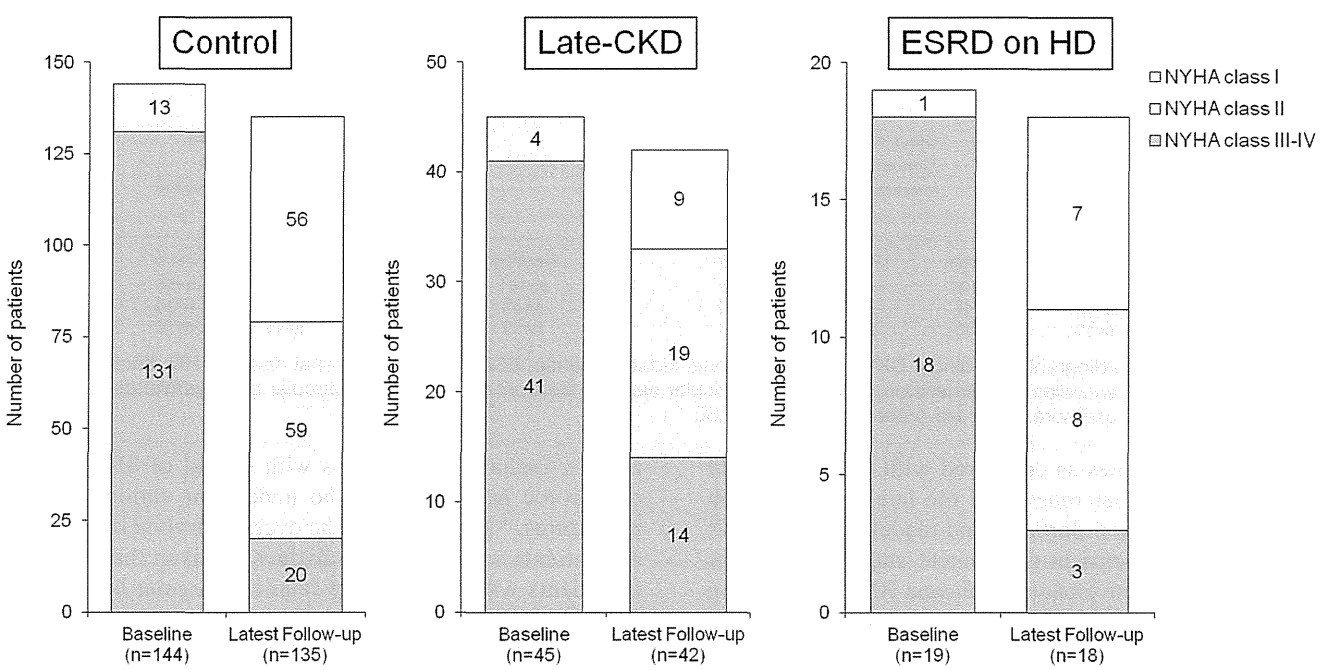

Figure 6. Heart failure symptoms at baseline and at the last follow-up visit. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis.

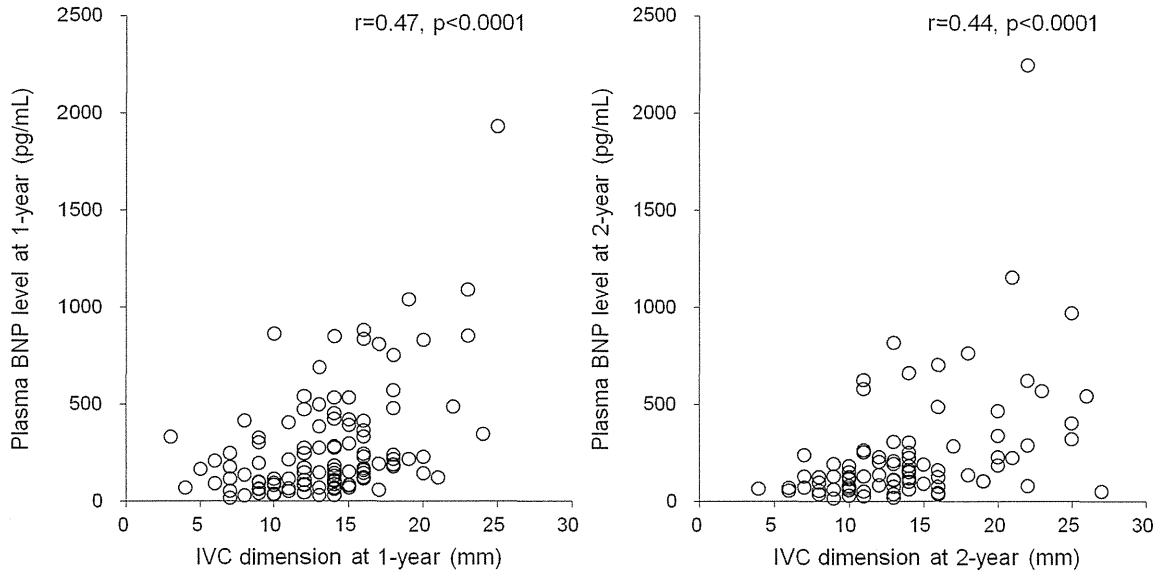

Figure 8. Relationship between IVC dimension and plasma BNP level. CKD indicates chronic kidney disease; ESRD, end-stage renal disease; HD, hemodialysis; IVC, inferior vena cava; BNP, brain natriuretic peptide.

after surgery was sustained over time, possibly accounting for the better outcome than seen in the late CKD group. In contrast, plasma BNP in patients with late CKD was decreased at 1 month after surgery along with a substantial decrease in LV volume confirmed by left ventriculography; however, that value remained substantially high at all follow-up time points, suggesting abnormal hemodynamics and unfavorable functional status despite significant MR improvement in the patients with late CKD.

Finally, we found a significant difference in the late outcome between the late CKD and ESRD groups despite no intergroup differences in postoperative LV and hemodynamic function, except for LVEF. Patients with ESRD exhibited a smaller IVC dimension than those with late CKD at the latest follow-up examination and also had higher rates of freedom from mortality and readmission for heart failure as compared with those with late CKD. Because IVC dimension is an indicator of volume status in patients with heart failure,[10] our findings regarding IVC dimension led us to speculate that volume management for the ESRD group was performed more appropriately than for the late CKD group during the follow-up period. Indeed, it is more difficult to control body fluid volume balance in patients with renal dysfunction not receiving hemodialysis than in those with ESRD on hemodialysis.[18] Our speculation may also be supported by the positive correlation found between BNP level and IVC dimension after surgery, indicating that the increased plasma BNP levels might have been partially caused by LV volume overload. Furthermore, the lower rate of mortality due to heart failure seen in patients with ESRD may be accounted for by adequate fluid removal and volume management. Our data suggested that the favorable late outcome seen in patients with ESRD, as compared with those with late CKD, was not mainly attributed to the surgical intervention itself, but rather adequate postoperative volume management. Therefore, we consider that postoperative volume management is important for better outcome in patients with functional MR and advanced cardiomyopathy; thus careful and meticulous monitoring of volume status is mandatory, especially for patients with late CKD.

Study Limitations

This study was retrospective in nature and investigated a small number of subjects; thus, any conclusions are limited. In particular, the sample size for ESRD on hemodialysis is very small and may have involved biased sampling, which might have led to the result showing no difference in survival between the patients with ESRD and control subjects. Inclusion of patients with different etiologies for heart failure and patients who had undergone concomitant surgical intervention such as coronary artery bypass grafting or surgical ventricular reconstruction might have influenced the results. However, such concomitant procedures are usually required for sick patients with a similar clinical and pathophysiologic status despite the etiology of LV dysfunction. We only analyzed patients with functional MR secondary to advanced cardiomyopathy considered suitable by referring cardiologists to undergo restrictive annuloplasty. No information is available regarding the number of patients not referred for surgical intervention during the same time period, because of the extremely high risk considered by their primary care physician.

It remains controversial whether patients with end-stage heart failure and functional MR can benefit from mitral valve repair.[19] In our study, patients with normal or mildly impaired renal function (control group) and those with ESRD on hemodialysis showed improvements in LV volume and function, decreases in plasma BNP level, and a satisfactory overall survival rates. Our results from meticulous follow-up examinations, including invasive cardiac catheterization at 1 month after surgery as well as sequential BNP levels, show that mitral valve repair improved hemodynamics and symptoms in those patients. However, the lack of an untreated control group did not allow us to investigate the survival

benefit conferred by mitral valve repair in patients with ESRD on hemodialysis. Additional randomized studies with higher numbers of patients and longer follow-up periods are necessary to confirm our results.

Conclusion

Mitral valve repair for medically refractory functional MR and Stage C/D heart failure yielded improvement in LV function and hemodynamics with an acceptably low hospital mortality, irrespective of preoperative renal function status. Patients with ESRD showed favorable late outcome in terms of freedom from mortality and readmission for heart failure as compared with those with late CKD. Further studies are needed to assess the survival benefit of mitral valve repair in patients with end-stage renal disease and advanced heart failure.

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Disclosures

None.

References

1. Kazory A, Ross EA. Contemporary trends in the pharmacological and extracorporeal management of heart failure: a nephrologic perspective. *Circulation*. 2008;117:975–983.
2. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med*. 2004;351:1296–1305.
3. Kottgen A, Russell SD, Loehr LR, Crainiceanu CM, Rosamond WD, Chang PP, Chambless LE, Coresh J. Reduced kidney function as a risk factor for incident heart failure: the Atherosclerosis Risk In Communities (ARIC) study. *J Am Soc Nephrol*. 2007;18:1307–1315.
4. Heywood JT. The cardiorenal syndrome: lessons from the ADHERE database and treatment options. *Heart Fail Rev*. 2004;9:195–201.
5. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF III, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J; CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009;150:604–613.
6. Rahmanian PB, Adams DH, Castillo JG, Vassalotti J, Filsoufi F. Early and late outcome of cardiac surgery in dialysis-dependent patients: single-center experience with 245 consecutive patients. *J Thorac Cardiovasc Surg*. 2008;135:915–922.
7. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. National Kidney Foundation. *Am J Kidney Dis*. 2002;39:1–266.
8. Pepi M, Tamborini G, Galli C, Barbier P, Doria E, Berti M, Guazzi M, Fiorentini C. A new formula for echo-Doppler estimation of right ventricular systolic pressure. *J Am Soc Echocardiogr*. 1994;7:20–26.
9. Kircher BJ, Himelman RB, Schiller NB. Noninvasive estimation of right atrial pressure from the inspiratory collapse of the inferior vena cava. *Am J Cardiol*. 1990;66:493–496.
10. Goonewardena SN, Gemignani A, Ronan A, Vasaiwala S, Blair J, Brennan JM, Shah DP, Spencer KT. Comparison of hand-carried ultrasound assessment of the inferior vena cava and N-terminal pro-brain natriuretic peptide for predicting readmission after hospitalization for acute decompensated heart failure. *J Am Coll Cardiol Cardiovasc Imaging*. 2008;1:595–601.
11. Bolling SF, Pagani FD, Deeb GM, Bach DS. Intermediate-term outcome of mitral reconstruction in cardiomyopathy. *J Thorac Cardiovasc Surg*. 1998;115:381–388.
12. Kitamura S. Excision of left ventricular aneurysm and large scarred myocardium following myocardial infarct: Postoperative and follow-up studies based on hemodynamics and left ventricular function. *Nippon Kyobu Geka Gakkai Zasshi*. 1976;24:1343–1364.
13. Ohara K. Clinical studies concerning surgical indication for excision of left ventricular aneurysm or asynergy following myocardial infarction. *Nippon Kyobu Geka Gakkai Zasshi*. 1977;25:907–927.
14. Horst M, Mehlhorn U, Hoerstrup SP, Suedkamp M, de Vivie ER. Cardiac surgery in patients with end-stage renal disease: 10-year experience. *Ann Thorac Surg*. 2000;69:96–101.
15. Trespalacios FC, Taylor AJ, Agodoa LY, Bakris GL, Abbott KC. Heart failure as a cause for hospitalization in chronic dialysis patients. *Am J Kidney Dis*. 2003;41:1267–1277.
16. Chang JP, Kao CL. Mitral valve repair in uremic congestive cardiomyopathy. *Ann Thorac Surg*. 2003;76:694–697.
17. Zoccali C, Mallamaci F, Benedetto FA, Tripepi G, Parlongo S, Cataliotti A, Cutrupi S, Giacone G, Bellanuova I, Cottini E, Malatino LS; Creed Investigators. Cardiac natriuretic peptides are related to left ventricular mass and function and predict mortality in dialysis patients. *J Am Soc Nephrol*. 2001;12:1508–1515.
18. Takami Y, Horio T, Iwashima Y, Takiuchi S, Kamide K, Yoshihara F, Nakamura S, Nakahama H, Inenaga T, Kangawa K, Kawano Y. Diagnostic and prognostic value of plasma brain natriuretic peptide in non-dialysis-dependent CRF. *Am J Kidney Dis*. 2004;44:420–428.
19. Wu AH, Aaronson KD, Bolling SE, Pagani FD, Welch K, Koelling TM. Impact of mitral valve annuloplasty on mortality risk in patients with mitral regurgitation and left ventricular systolic dysfunction. *J Am Coll Cardiol*. 2005;45:381–387.

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

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See Editorial Commentary, pp 542–544

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

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

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

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

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

Methods

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

Mice, Transverse Aortic Constriction Operation, and Transthoracic Echocardiography

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

Ang II Infusion and BP Measurement

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

Real-Time RT-PCR Analysis

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

Western Blot Analysis and Histological Analysis

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

Radioligand Receptor Binding Assay

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

Statistics

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

Results

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

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

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

AT_1Tg-AgtKO Mice Display Progressive Cardiac Remodeling

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

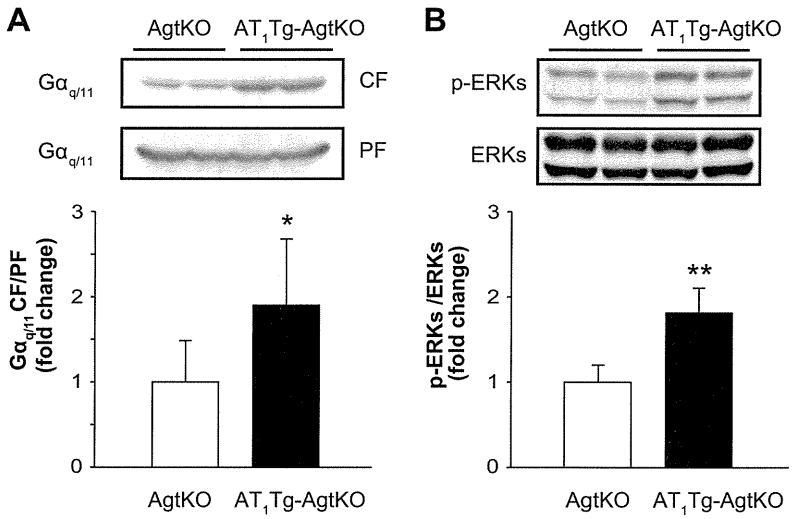

Figure 1. Constitutive activation of angiotensin II type 1 (AT_1) receptor in AT_1 transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) hearts. **A**, Immunoblot analysis of $G\alpha_{q/11}$ in cytosolic fraction (CF) and particulate fraction (PF) extracted from AgtKO (n=6) and AT_1Tg-AgtKO (n=6) hearts.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

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

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

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

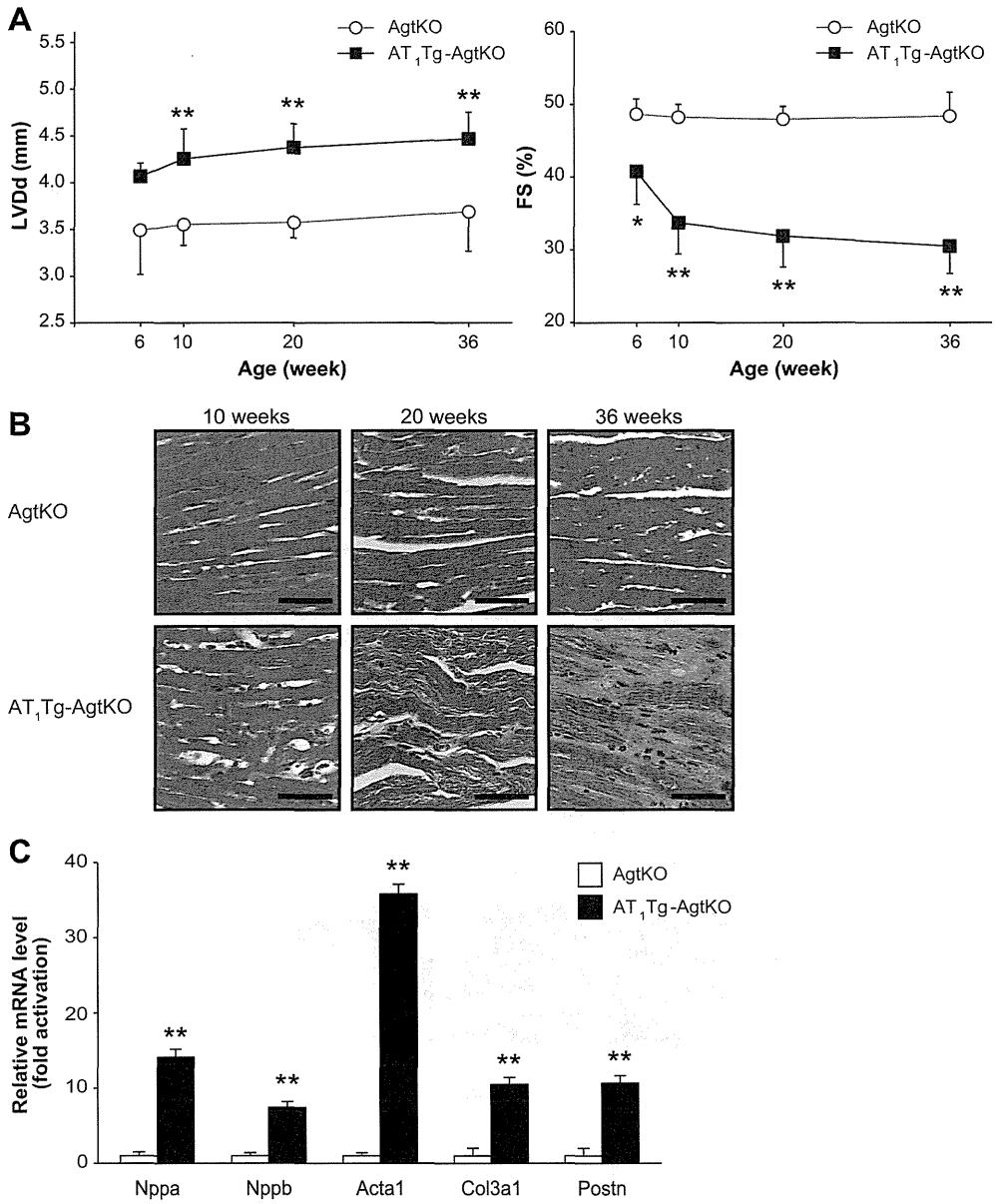

Figure 2. Spontaneous development of cardiac remodeling in angiotensin II type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice. **A**, Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AgtKO (n=7–9) and AT_1Tg-AgtKO (n=9–11) mice measured by echocardiogram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

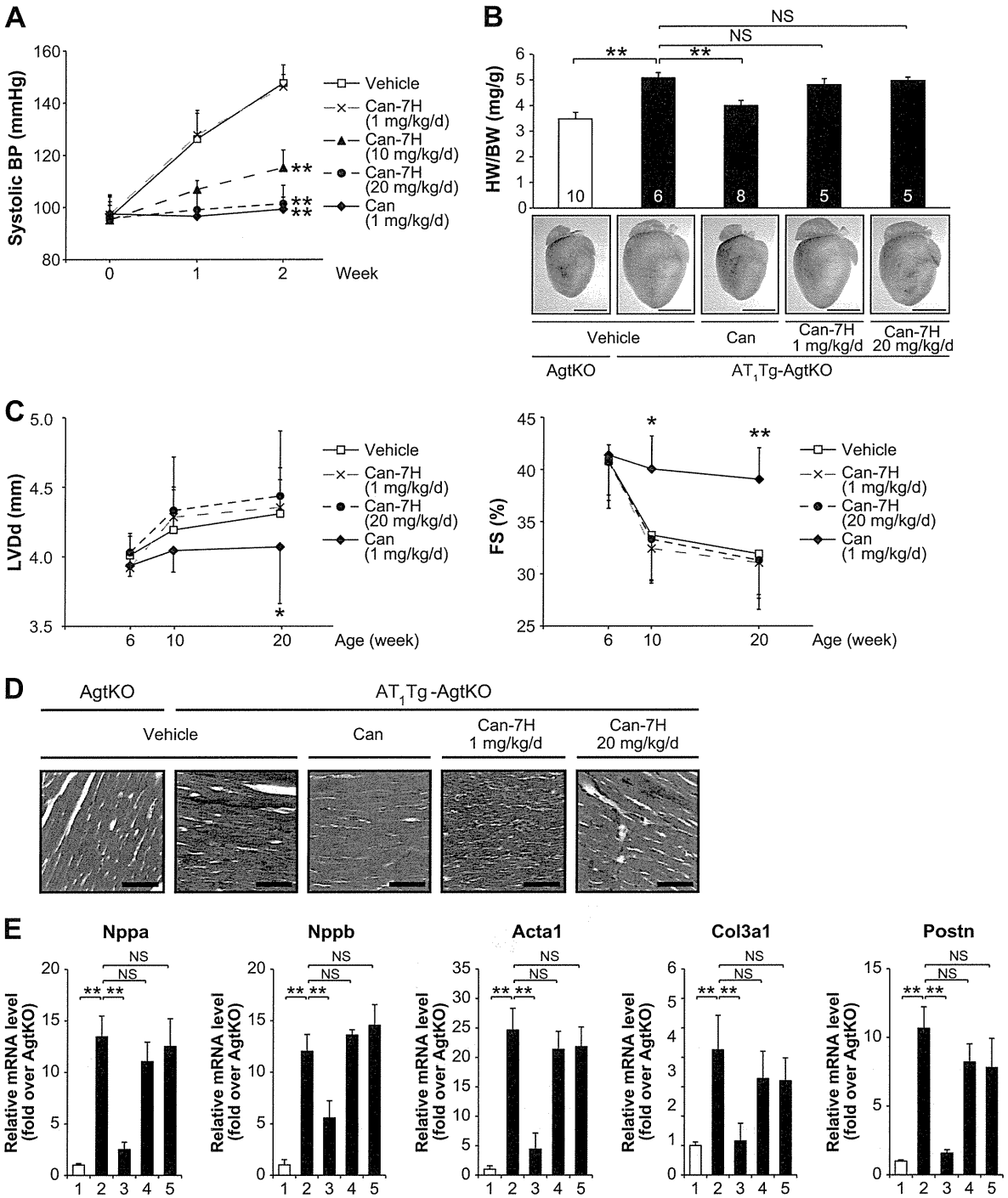

Figure 3. Prevention of cardiac remodeling in angiotensin II (Ang II) type 1 (AT_1) transgenic (AT_1Tg)-angiotensinogen-knockout (AgtKO) mice by candesartan but not by candesartan-7H. **A**, Blood pressure–lowering effects of candesartan cilexetil (Can) and candesartan-7H (Can-7H) in Ang II–infused mice. 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 $\approx$8-fold in pressure-overloaded mice after transverse aortic constriction (B_{max}: 142.9$\pm$36.5 fmol/mg; n=3) compared with sham-operated mice (B_{max}: 16.4$\pm$4.9 fmol/mg; n=3). In addition, it has been reported that the AT_1 receptor is upregulated in response to low-density lipoprotein cholesterol,[28] insulin,[29] glucose,[30] progesterone,[31] and inflammatory cytokines, such as interleukin 1α or interleukin 6,[32,33] in vascular cells. Therefore, it seems quite reasonable to assume that enhancement of constitutive activity of the AT_1 receptor through upregulation of receptor expression may accelerate the progression of atherosclerosis in patients with hypercholesterolemia or diabetes mellitus, especially after menopause. Further studies in animal models will be required to clarify the roles of constitutive activity of the AT_1 receptor in the pathogenesis of cardiovascular and metabolic disorders.

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

Perspectives

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

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Disclosures

None.

References

1. Yasuda N, Miura S, Akazawa H, Tanaka T, Qin Y, Kiya Y, Imaizumi S, Fujino M, Ito K, Zou Y, Fukuhara S, Kunimoto S, Fukuzaki K, Sato T, Ge J, Mochizuki N, Nakaya H, Saku K, Komuro I. Conformational switch of angiotensin II type 1 receptor underlying mechanical stress-induced activation. *EMBO Rep.* 2008;9:179–186.
2. Qin Y, Yasuda N, Akazawa H, Ito K, Kudo Y, Liao CH, Yamamoto R, Miura S, Saku K, Komuro I. Multivalent ligand-receptor interactions elicit inverse agonist activity of AT(1) receptor blockers against stretch-induced AT(1) receptor activation. *Hypertens Res.* 2009;32:875–883.
3. Akazawa H, Yasuda N, Komuro I. Mechanisms and functions of agonist-independent activation in the angiotensin II type 1 receptor. *Mol Cell Endocrinol.* 2009;302:140–147.
4. Milligan G. Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol.* 2003;64: 1271–1276.
5. Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, Makita N, Iwanaga K, Zhu W, Kudoh S, Toko H, Tamura K, Kihara M, Nagai T, Fukamizu A, Umemura S, Iiri T, Fujita T, Komuro I. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. *Nat Cell Biol.* 2004;6:499–506.
6. Mederos y Schnitzler M, Storch U, Meibers S, Nurwakagari P, Breit A, Essin K, Gollasch M, Gudermann T. Gq-coupled receptors as mechano-sensors mediating myogenic vasoconstriction. *EMBO J.* 2008;27: 3092–3103.
7. Rakesh K, Yoo B, Kim IM, Salazar N, Kim KS, Rockman HA. β-Arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. *Sci Signal.* 2010;3:ra46.
8. Miura S, Fujino M, Hanzawa H, Kiya Y, Imaizumi S, Matsuo Y, Tomita S, Uehara Y, Karnik SS, Yanagisawa H, Koike H, Komuro I, Saku K. Molecular mechanism underlying inverse agonist of angiotensin II type 1 receptor. *J Biol Chem.* 2006;281:19288–19295.
9. Sachse R, Shao XJ, Rico A, Finckh U, Rolfs A, Reincke M, Hensen J. Absence of angiotensin II type 1 receptor gene mutations in human adrenal tumors. *Eur J Endocrinol.* 1997;137:262–266.
10. Davies E, Bonnardeaux A, Plouin PF, Corvol P, Clauser E. Somatic mutations of the angiotensin II (AT1) receptor gene are not present in aldosterone-producing adenoma. *J Clin Endocrinol Metab.* 1997;82: 611–615.
11. Billet S, Bardin S, Verp S, Baudrie V, Michaud A, Conchon S, Muffat-Joly M, Escoubet B, Souil E, Hamard G, Bernstein KE, Gasc JM, Elghozi JL, Corvol P, Clauser E. Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice. *J Clin Invest.* 2007;117:1914–1925.
12. Paradis P, Dali-Youcef N, Paradis FW, Thibault G, Nemer M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proc Natl Acad Sci U S A.* 2000; 97:931–936.
13. Tanimoto K, Sugiyama F, Goto Y, Ishida J, Takimoto E, Yagami K, Fukamizu A, Murakami K. Angiotensinogen-deficient mice with hypotension. *J Biol Chem.* 1994;269:31334–31337.
14. Miura S, Feng YH, Husain A, Karnik SS. Role of aromaticity of agonist switches of angiotensin II in the activation of the AT1 receptor. *J Biol Chem.* 1999;274:7103–7110.
15. Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol.* 2006;20:953–970.
16. Zhai P, Galeotti J, Liu J, Holle E, Yu X, Wagner T, Sadoshima J. An angiotensin II type 1 receptor mutant lacking epidermal growth factor receptor transactivation does not induce angiotensin II-mediated cardiac hypertrophy. *Circ Res.* 2006;99:528–536.
17. Shibouta Y, Inada Y, Ojima M, Wada T, Noda M, Sanada T, Kubo K, Kohara Y, Naka T, Nishikawa K. Pharmacological profile of a highly potent and long-acting angiotensin II receptor antagonist, 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl)biphenyl-4- yl]methyl]-1H-benzimidazole-7-carboxylic acid (CV-11974), and its prodrug, (+/−)-1-(cyclohexyloxycarbonyloxy)-ethyl 2-ethoxy-1-[[2′-(1H-tetrazol-5- yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate (TCV-116). *J Pharmacol Exp Ther.* 1993;266:114–120.
18. Morisset S, Rouleau A, Ligneau X, Gbahou F, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC, Arrang JM. High constitutive activity of native H3 receptors regulates histamine neurons in brain. *Nature.* 2000;408:860–864.
19. Srinivasan S, Lubrano-Berthelier C, Govaerts C, Picard F, Santiago P, Conklin BR, Vaisse C. Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. *J Clin Invest.* 2004;114:1158–1164.
20. Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J Clin Invest.* 2006;116: 760–768.
21. Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G. Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature.* 1993;365:649–651.
22. Shenker A, Laue L, Kosugi S, Merendino JJ Jr, Minegishi T, Cutler GB Jr. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature.* 1993;365:652–654.
23. Shenoy SK, Lefkowitz RJ. Angiotensin II-stimulated signaling through G proteins and β-arrestin. *Sci STKE.* 2005;2005:cm14.
24. Miserey-Lenkei S, Parnot C, Bardin S, Corvol P, Clauser E. Constitutive internalization of constitutively active agiotensin II AT(1A) receptor mutants is blocked by inverse agonists. *J Biol Chem.* 2002;277: 5891–5901.
25. Suzuki J, Matsubara H, Urakami M, Inada M. Rat angiotensin II (type 1A) receptor mRNA regulation and subtype expression in myocardial growth and hypertrophy. *Circ Res.* 1993;73:439–447.
26. Fujii N, Tanaka M, Ohnishi J, Yukawa K, Takimoto E, Shimada S, Naruse M, Sugiyama F, Yagami K, Murakami K, Miyazaki H. Alterations of angiotensin II receptor contents in hypertrophied hearts. *Biochem Biophys Res Commun.* 1995;212:326–333.
27. Nio Y, Matsubara H, Murasawa S, Kanasaki M, Inada M. Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction. *J Clin Invest.* 1995;95:46–54.
28. Nickenig G, Jung O, Strehlow K, Zolk O, Linz W, Scholkens BA, Bohm M. Hypercholesterolemia is associated with enhanced angiotensin AT1-receptor expression. *Am J Physiol.* 1997;272:H2701–H2707.
29. Nickenig G, Roling J, Strehlow K, Schnabel P, Bohm M. Insulin induces upregulation of vascular AT1 receptor gene expression by posttranscriptional mechanisms. *Circulation.* 1998;98:2453–2460.
30. Sodhi CP, Kanwar YS, Sahai A. Hypoxia and high glucose upregulate AT1 receptor expression and potentiate ANG II-induced proliferation in VSM cells. *Am J Physiol Heart Circ Physiol.* 2003;284:H846–H852.
31. Nickenig G, Strehlow K, Wassmann S, Baumer AT, Albory K, Sauer H, Bohm M. Differential effects of estrogen and progesterone on AT(1) receptor gene expression in vascular smooth muscle cells. *Circulation.* 2000;102:1828–1833.
32. Wassmann S, Stumpf M, Strehlow K, Schmid A, Schieffer B, Bohm M, Nickenig G. Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin II type 1 receptor. *Circ Res.* 2004;94:534–541.
33. Sasamura H, Nakazato Y, Hayashida T, Kitamura Y, Hayashi M, Saruta T. Regulation of vascular type 1 angiotensin receptors by cytokines. *Hypertension.* 1997;30:35–41.
34. Eklind-Cervenka M, Benson L, Dahlstrom U, Edner M, Rosenqvist M, Lund LH. Association of candesartan vs losartan with all-cause mortality in patients with heart failure. *JAMA.* 2011;305:175–182.

Correction

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

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

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

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

The authors regret these errors.

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

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ONLINE SUPPLEMENT

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

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Supplemental Materials and Methods

Mice, TAC operation, and transthoracic echocardiography

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

Ang II infusion and BP measurement

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

Real-time RT-PCR analysis

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

References

1. Paradis P, Dali-Youcef N, Paradis FW, Thibault G, Nemer M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proc Natl Acad Sci U S A.* 2000;97:931-936.
2. Tanimoto K, Sugiyama F, Goto Y, Ishida J, Takimoto E, Yagami K, Fukamizu A, Murakami K. Angiotensinogen-deficient mice with hypotension. *J Biol Chem.* 1994;269:31334-31337.
3. Miura S, Feng YH, Husain A, Karnik SS. Role of aromaticity of agonist switches of angiotensin II in the activation of the AT1 receptor. *J Biol Chem.* 1999;274:7103-7110.
4. Yasuda N, Miura S, Akazawa H, Tanaka T, Qin Y, Kiya Y, Imaizumi S, Fujino M, Ito K, Zou Y, Fukuhara S, Kunimoto S, Fukuzaki K, Sato T, Ge J, Mochizuki N, Nakaya H, Saku K, Komuro I. Conformational switch of angiotensin II type 1 receptor underlying mechanical stress-induced activation. *EMBO Rep.* 2008;9:179-186.
5. Akazawa H, Yasuda N, Miura S, Komuro I. Assessment of inverse agonism for the angiotensin II type 1 receptor. *Methods Enzymol.* 2010;485:25-35.