breakthrough". In this setting, mineralocorticoid receptor (MR) activation might play an important role in the deterioration of HF when patients are on chronic ACE-I or ARB therapy. It was shown that MR antagonist spironolactone provided additional beneficial effects on LV morphology and function to ARB in experimental myocardial infarction (MI) model rats [15]. In large-scale clinical trials, RALES [16], EPHESUS [17], EMPHASIS-HF [18] and Aldo-DHF study [19], addition of an MR blocker to standard medical therapy including ACE-I or ARB had a beneficial effect on the prognosis of patients with HF or MI.

However, it is still unknown whether the blocking of AT_1 signaling can prevent DM-induced LV dysfunction over the *long-term*. If it couldn't, AT_1-independent MR activation (including aldosterone breakthrough) might have an influence on DM-induced LV dysfunction. Accordingly, the goal of the present study was to determine whether blockade of AT_1 signaling prevented DM-induced LV dysfunction over both the *short-term* and *long-term*. The second goal of this study was to elucidate whether AT_1-independent MR activation has a role in the occurrence of DM-induced LV dysfunction.

Materials and Methods

Experimental Animals

Nine-week-old male mice with knockout of the angiotensin II type 1a receptor ($AT_{1a}R$ KO) were generated (KO mice) [20] and age-matched male mice without $AT_{1a}R$ KO that had the same genetic background (wild-type [WT] mice) were used as controls. The study design is shown in **Figure 1**. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 200 mg/kg; Sigma-Aldrich, Tokyo, Japan), as described previously [21]. Streptozotocin (STZ) was dissolved in sterile sodium citrate buffer. In the control groups, citrate buffer alone was injected. Some KO mice were administered the MR antagonist eplerenone (100 mg/kg BW, Pfizer Inc., NY, USA) for 12 weeks. STZ-treated WT mice (WT-DM), STZ-treated KO mice (KO-DM), vehicle-treated WT mice (WT-control), and vehicle-treated KO mice (KO-control) were raised for 6 or 12 weeks, while STZ-treated KO mice with eplerenone therapy (KO-DM+E) and vehicle-treated KO mice with eplerenone therapy (KO-control+E) were raised for 12 weeks (6w: WT-DM n = 27, WT-control n = 22, KO-DM n = 15, KO-control n = 20. 12w: WT-DM n = 38, WT-control n = 17, KO-DM n = 12, KO-control n = 15, KO-DM+E n = 14, KO-control+E n = 11).

Plasma glucose concentrations were measured with a commercial kit (The Glucose Vision Blood Glucose Monitoring System; Adventure Healthcheck, CA, USA) at 3 days after the injection of STZ or vehicle.

Ethics Statement

This study conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and approval was granted by the ethical review board of Keio University.

Echocardiography

At 6 or 12 weeks after injection of STZ or the vehicle, echocardiography (En Visor C M2540A, Philips, Tokyo, Japan) was performed after anesthesia was induced with a single intraperitoneal injection of ketamine (70 mg/kg) and xylazine (7 mg/kg), as described previously [22]. The heart rate was monitored by electrocardiography in order to evaluate the adequacy of anesthesia. The average of three to four measurements was calculated for each parameter.

Hemodynamic Study

At 6 or 12 weeks after injection of STZ or the vehicle, a 1.4-F micromanometer catheter (Millar Instruments, Inc., TX, USA) was inserted into the LV of each mouse via the right carotid artery under the above-mentioned anesthesia, while the heart rate was monitored by electrocardiography in order to evaluate the adequacy of anesthesia. Then LV pressure curves were recorded and hemodynamic data were obtained, as described previously [23]. Thereafter, the mice were euthanized by an overdose of ketamine and xylazine, their hearts were quickly excised and weighed, and peripheral blood was collected.

Measurement of Aldosterone and Corticosterone

The PAC and plasma corticosterone concentration were determined with a commercially available kit (Aldosterone EIA Kit, Assay Designs, Inc., MI, USA, Corticosterone EIA Kit, Cayman Chemical Company, MI, USA). The corticosterone or aldosterone content of LV tissue was determined by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS), which has sufficient accuracy and precision to measure aldosterone and corticosterone with a high reliability and reproducibility [24].

Real-time PCR

Total cellular RNA was extracted from LV tissue using TRIzol reagent (Invitrogen, CA, USA) as described previously [25]. cDNA was synthesized from total RNA using a High-capacity cDNA archive kit (Applied Biosystems, CA, USA). Real-time PCR was performed with an ABI 7500 Fast real-time PCR system (Applied Biosystems, CA, USA), as described previously [26]. Reaction mixtures containing each set of specific Taqman probes and primers (Applied Biosystems, CA, USA) were added to diluted samples of cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as the internal control.

Western Blotting

After LV myocardial tissues were homogenized in lysis buffer (T-PER Tissue Protein Extraction Reagent, PIERCE, IL, USA), samples were centrifuged and the supernatant was collected as the protein extract. Western blot analysis was performed using a commercially available antibody and proteins were detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Ltd. Buckinghamshire, UK).

Pathological Examination

Excised LV tissues were fixed in 10% formalin and embedded in paraffin, as described previously [27]. Immunohistochemical staining of 4-hydroxy-2-nonenal (4-HNE) was performed with a commercially available kit (Histofine Mouse Stain Kit, Nichirei, Tokyo, Japan). Mouse monoclonal anti-HNE-modified protein antibody (1:50 dilution, NOF Medical Department, Tokyo, Japan) was used as the primary antibody. Samples were examined under a light microscope [28]. Apoptosis was quantified by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method, as described previously [29]. Sections were stained by using a CardioTACS *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Twenty photographs (magnification ×200) were taken of each section. Then the nuclei of cardiomyocytes were manually counted by an observer who was blinded to the experimental conditions.

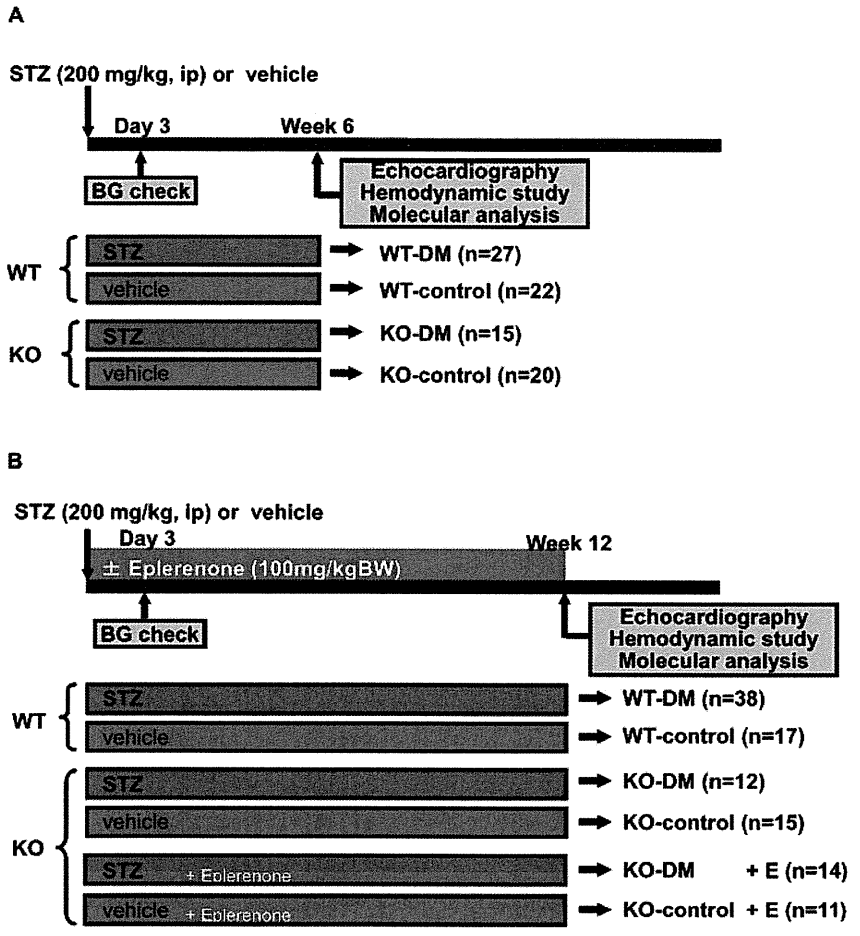

Figure 1. Experimental design. Six-week model (A) and 12-week model (B). STZ, streptozotocin; BG, blood glucose; DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type.
doi:10.1371/journal.pone.0093145.g001

Statistical Analysis

Data are expressed as the mean ± SEM. Multiple comparisons were performed by 1-way analysis of variance, followed by the Tukey-Kramer test. A probability <0.05 was considered to indicate statistical significance.

Results

Blood Glucose and Organ Weights

At 6 and 12 weeks, blood glucose levels were higher in all of the DM groups compared with the respective control groups. There were no differences of blood glucose levels among the DM groups. LV weight corrected by body weight was unchanged in all DM groups compared with the respective control groups (**Table 1**).

Cardiac Function

LV systolic function. At 6 weeks, fractional shortening (FS) showed no significant differences among the 4 groups. With respect to the rate-corrected velocity of circumferential fiber shortening (Vcfc), however, the WT-DM group showed a significant reduction compared with the WT-control group, but the KO-DM group showed no significant change. Hemodynamic assessment revealed that the LV systolic pressure and peak positive dP/dt were similar among the four groups at 6 weeks.

At 12 weeks, both FS and Vcfc were significantly lower in WT-DM mice compared with WT-control mice. However, there were no significant differences of these indices between the KO-DM and KO-control groups (**Figure 2A, 2B**). At 12 weeks, LV systolic pressure was significantly lower in the WT-DM and KO-DM groups compared with the respective control groups. However, there was no significant difference of LV systolic pressure between the KO-DM+E and KO-control+E groups. Peak positive dP/dt was significantly lower in WT-DM mice compared with WT-control mice. However, there were no significant differences of peak positive dP/dt among the KO groups (**Figure 2C, 2D**).

LV diastolic function. At 6 weeks, the E/A ratio calculated from pulsed-wave Doppler measurements of LV inflow and peak negative dP/dt (indices of LV diastolic function) were significantly lower in the WT-DM group compared with the WT-control group. In contrast, there were no significant differences of the E/A ratio or peak negative dP/dt among the KO groups.

At 12 weeks, however, the E/A ratio and peak negative dP/dt were also lower in the KO-DM group compared with the KO-control group. There were no significant differences of the E/A ratio or peak negative dP/dt between the KO-DM+E and KO-control+E groups (**Figure 2E, 2F**). There were no significant differences of heart rate or LV end-diastolic pressure among these groups at any time (data not shown).

LV tissue level of BNP mRNA. Brain natriuretic peptide (BNP) mRNA levels were significantly elevated in WT-DM and

Table 1. Blood glucose level and morphology.

	6w				12w							
	WT		KO		WT		KO					
	control	DM	control	DM	control	DM	control	DM	control+E	DM+E		
	(n=20)	(n=20)	(n=20)	(n=15)	(n=17)	(n=38)	(n=15)	(n=12)	(n=11)	(n=11)		
Blood Glucose(mg/dl)	176±6	521±15*	163±7	477±28*	218±10	540±14**	186±12	510±33 **	221±22	572±17 **		
BW (g)	27.0±0.3	20.0±0.4*	31.0±0.5	25.9±0.8*	29.5±0.5	21.1±0.5**	33.7±0.5	26.8±0.8**	34.8±1.1	24.6±1.2**		
LV weight (mg)	82.7±1.1	62.0±1.6*	86.8±2.0	71.0±2.6*	93.6±2.1	68.2±2.0**	94.2±2.0	75.6±2.8**	89.0±3.1	65.9±4.8**		
LV/BW (mg/g)	3.1±0.0	3.0±0.1	2.8±0.1	2.7±0.1	3.1±0.1	3.2±0.1	2.8±0.1	2.8±0.1	2.6±0.1	2.6±0.1		

Values are expressed as mean±SEM. WT, wild-type mice; KO, AT_{1a} receptor knockout mice; E, eplerenone; BW, body weight; LV, left ventricle. *p<0.05 compared with respective control groups, **p<0.01 compared with respective control groups.

doi:10.1371/journal.pone.0093145.t001

KO-DM mice compared with the respective control groups at both 6 and 12 weeks. However, the BNP mRNA level was similar in KO-DM+E and KO-control+E mice (**Figure 2G**).

Ca^{2+} Handling

At 6 weeks, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) mRNA and protein expression were downregulated in the WT-DM group compared with the WT-control group. (**Figure 3A, 3B**). However, there were no significant differences of SERCA2a mRNA and protein levels between the KO groups. At 12 weeks, SERCA2a mRNA and protein levels were also downregulated in KO-DM mice compared with KO-control mice. Eplerenone treatment prevented these changes (**Figure 3A, 3B**).

Phospholamban mRNA expression was similar among all groups at both 6 and 12 weeks (data not shown). Western blotting revealed that the total phospholamban protein content was similar among all groups (**Figure 3C**). At 6 weeks, the level of phosphorylated phospholamban was significantly reduced in WT-DM mice compared with WT-control mice, although there were no significant differences between the KO groups. At 12 weeks, however, phosphorylated phospholamban levels were also reduced in KO-DM mice compared with KO-control mice. Eplerenone treatment prevented these changes (**Figure 3D**). The ratio of phosphorylated phospholamban to total phospholamban was significantly lower in WT-DM mice than WT-control mice, but the KO groups showed no significant difference at 6 weeks. At 12 weeks, it was also lower in KO-DM mice compared with KO-control mice. Eplerenone treatment prevented these changes (**Figure 3E**).

Apoptosis

At 6 weeks, the number of TUNEL-positive cardiomyocytes was increased in WT-DM mice compared with WT-control mice (3.37 ± 0.40 vs. $1.97\pm0.27/10^3$ nuclei, $p<0.05$), but there was no significant difference between the KO-DM and KO-control groups (2.15 ± 0.48 vs. $1.66\pm0.13/10^3$ nuclei, p=N.S.). At 12 weeks, the number of TUNEL-positive cardiomyocytes was also increased in KO-DM mice compared with KO-control mice (WT-control 2.16 ± 0.35 vs. WT-DM $5.46\pm0.56/10^3$ nuclei, $p<0.01$; KO-control 1.77 ± 0.08 vs. KO-DM 5.27 ± 0.31 nuclei, $p<0.01$). Eplerenone treatment prevented these changes (KO-control+E 1.48 ± 0.16 vs. KO-DM+E 1.38 ± 0.12 nuclei, p=N.S., **Figure 4A**).

At 6 weeks, the Bcl-2 protein level was significantly lower in WT-DM mice, but it showed no significant difference between the KO groups. At 12 weeks, Bcl-2 protein was also downregulated in KO-DM mice compared with KO-control mice. Eplerenone treatment also prevented these changes (**Figure 4B**). Bax protein levels were similar among all groups at both 6 and 12 weeks (**Figure 4C**).

Oxidative Stress

Nicotinamide adenine dinucleotide 3-phosphate (NADPH) oxidase has an important role in the production of reactive oxygen species (ROS) [30] by catalyzing electron transfer from NADPH to molecular oxygen to form O_2^-. Glutathione peroxidase is an enzyme that is upregulated in response to an increase of ROS and catalyzes the reduction of hydrogen peroxide. NADPH oxidase p47phox subunit and glutathione peroxidase (GPx1) mRNA expression were significantly upregulated in WT-DM mice compared with WT-control mice at 6 weeks, whereas there was no significant difference between the KO groups. At 12 weeks, however, p47phox and GPx1 mRNA levels were also upregulated in KO-DM mice compared with KO-

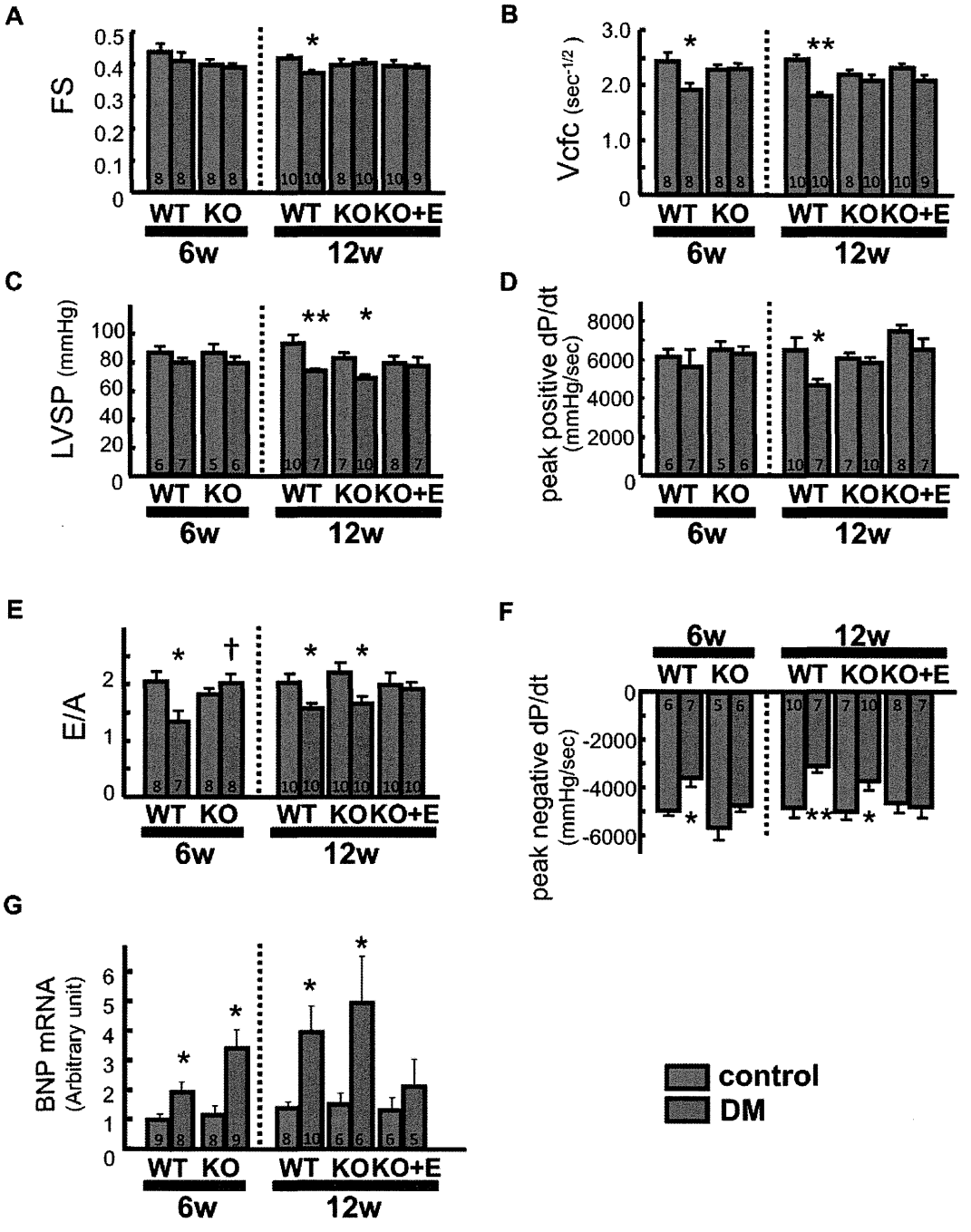

Figure 2. Indices of LV function. Fractional shortening (FS) (A), Vcfc (B), LV systolic pressure (LVSP) (C), peak positive dP/dt (D), E/A ratio (E), peak negative dP/dt (F), and brain natriuretic peptide (BNP) mRNA expression in LV tissue measured by real-time PCR (G). Number of mice per group is indicated in bar graph. Number of mice per group is indicated in the bar graph. Number of mice per group is indicated in the bar graph. Number of mice per group is indicated in the bar graph. Values are the mean±SEM. DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type.*$p<0.05$ vs. the respective control group. **$p<0.01$ vs. the respective control group. †$p<0.05$ vs. WT-DM.
doi:10.1371/journal.pone.0093145.g002

control mice. Eplerenone treatment prevented such changes (**Figure 5A, 5E**). NOX4 mRNA was upregulated in WT-DM and KO-DM mice compared with the respective control groups at both 6 and 12 weeks. Eplerenone prevented such changes in KO-DM mice (**Figure 5B**). Expression of p22phox and NOX2 mRNA was significantly upregulated in WT-DM mice at 6 and 12 weeks, but was similar among KO-groups at both times (**Figure 5C, 5D**).

At 6 weeks, staining for 4-HNE, a byproduct of lipid peroxidation and an index of oxidative stress [10], was stronger in LV tissue obtained from WT-DM mice compared with WT-control mice, whereas there was no significant difference between KO-DM and KO-control mice. At 12 weeks, however, staining for 4-HNE was also stronger in KO-DM mice compared with KO-control mice. Eplerenone treatment prevented these changes (**Figure 6**).

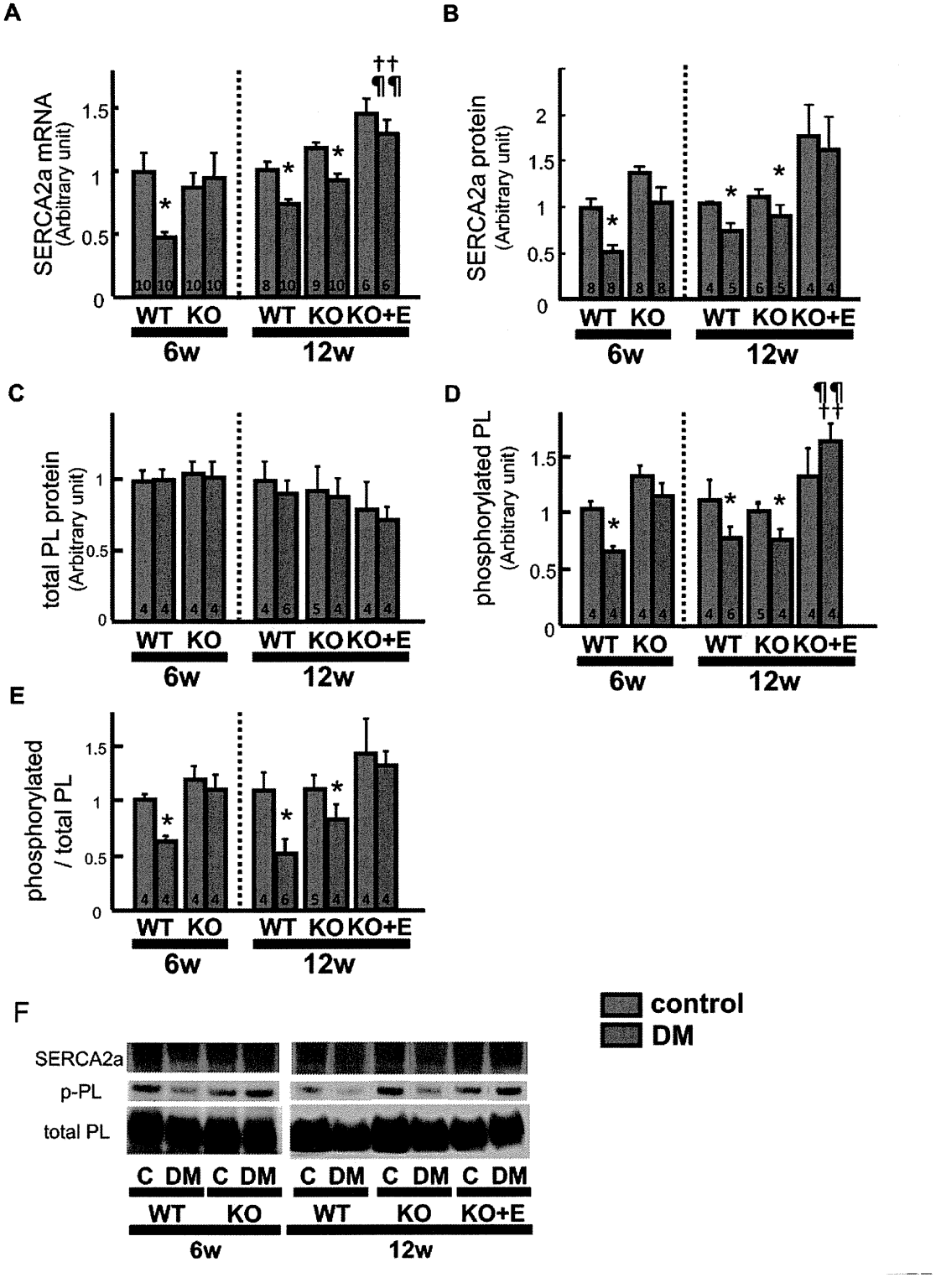

Figure 3. Ca^{2+} handling in the diabetic heart. Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) mRNA expression in LV tissue measured by real-time PCR (A), SERCA2a protein level (B), the contents of total (C) and phosphorylated (D) phospholamban protein in LV tissue determined by Western blotting, the ratio of phosphorylated to total phospholamban (E), and representative images of Western blots (F). Number of mice per group is indicated in bar graph. Values are the mean±SEM. DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type; PL, phospholamban; C, control. *$p<0.05$ vs. the respective control group. ††$p<0.01$ vs. WT-DM, ¶¶$p<0.01$ vs. KO-DM.
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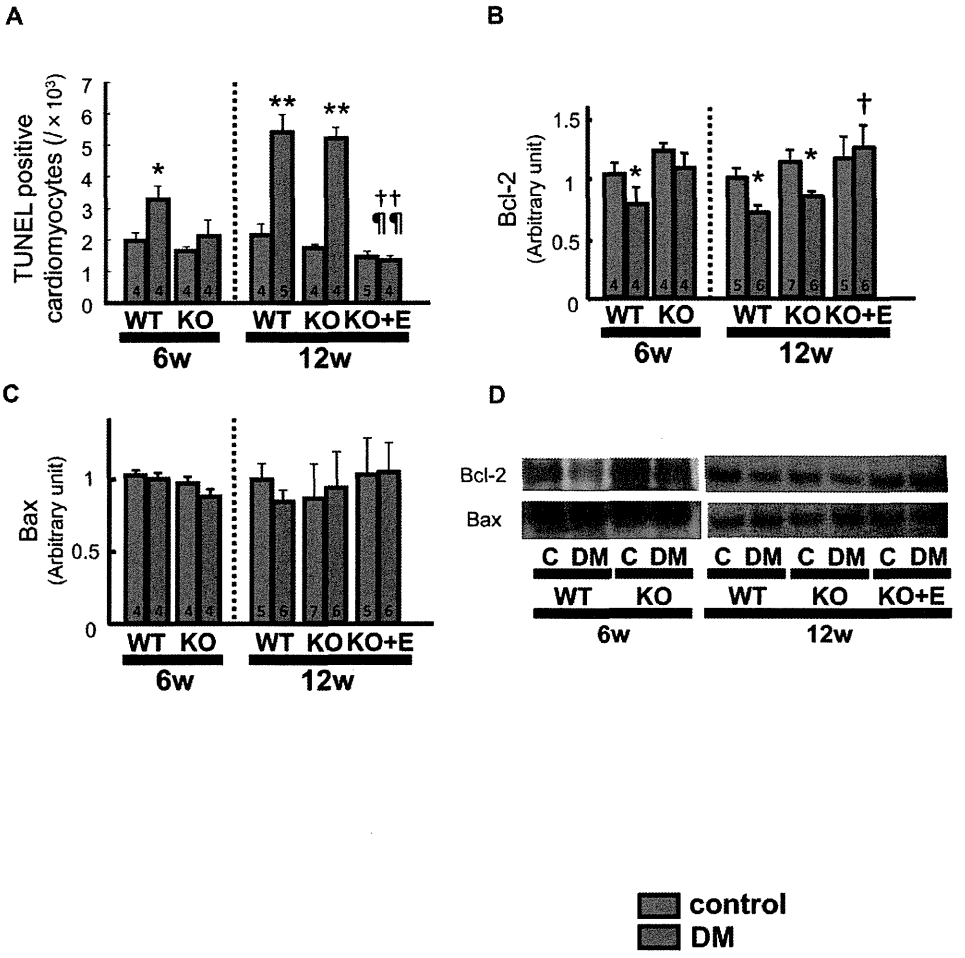

Figure 4. Apoptosis in the diabetic heart. The number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cardiomyocytes in LV tissue (A), expression of Bcl-2 (B) and Bax (C) protein in LV tissue determined by Western blotting, and representative images of the bands for Bcl-2 and Bax protein (D). Number of mice per group is indicated in bar graph. Values are the mean±SEM. DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type; C, control. *p<0.05 vs. the respective control group, **p<0.01 vs. the respective control group, †p<0.05 vs. WT-DM, ††p<0.01 vs. WT-DM, ¶¶p<0.01 vs. KO-DM.
doi:10.1371/journal.pone.0093145.g004

RAAS Activation

At both 6 and 12 weeks, angiotensinogen mRNA expression was upregulated in all of the DM groups compared with the respective control groups (**Figure 7A**). At 6 and 12 weeks, $AT_{1a}R$ expression was upregulated in WT-DM mice compared with WT-control mice (**Figure 7B**). At 6 weeks, the MR mRNA level was 3.9-fold higher in WT-DM mice compared with WT-control mice, but there was no significant difference between the KO-DM and KO-control groups. At 12 weeks, however, the level of MR mRNA was increased by 2.4-fold in WT-DM, and by 3.0-fold even in KO-DM mice. Eplerenone treatment prevented these changes (**Figure 7C**).

PAC was elevated in WT-DM mice at 6 weeks. Although PAC was not elevated in KO-DM mice at 6 weeks, it was significantly elevated at 12 weeks, suggesting that the "aldosterone breakthrough" phenomenon had occurred (**Figure 8A**). PAC was also higher in KO-DM+E mice than KO-control+E mice. However, the aldosterone content of LV tissue was increased in WT-DM mice, but was unchanged in KO-DM mice (**Figure 8C**). That is, "aldosterone breakthrough" was not identified in the local LV tissue RAAS, unlike the systemic RAAS. The plasma corticosterone concentration was elevated in all of the DM groups (**Figure 8B**). In LV tissue from KO-DM mice, however, the corticosterone content was not significantly increased at either 6 or 12 weeks (**Figure 8D**).

Discussion

To the best of our knowledge, this is the first report which clearly showed blockade of AT_1 signaling could prevent, although partially, the chronic deterioration of LV function induced by DM and addition of an MR antagonist had a further cardioprotective effect.

One of the most important findings was the documentation of RAAS activation in the diabetic heart, while genetic ablation of AT_1 signaling prevented LV diastolic dysfunction in diabetic mice at 6 weeks. By 12 weeks, however, ablation of AT_1 signaling could not prevent DM-induced LV diastolic dysfunction. Additional interruption of MR signaling with eplerenone ameliorated these changes.

The second important finding was that aldosterone breakthrough was only demonstrated in the plasma and was not found in local LV tissue.

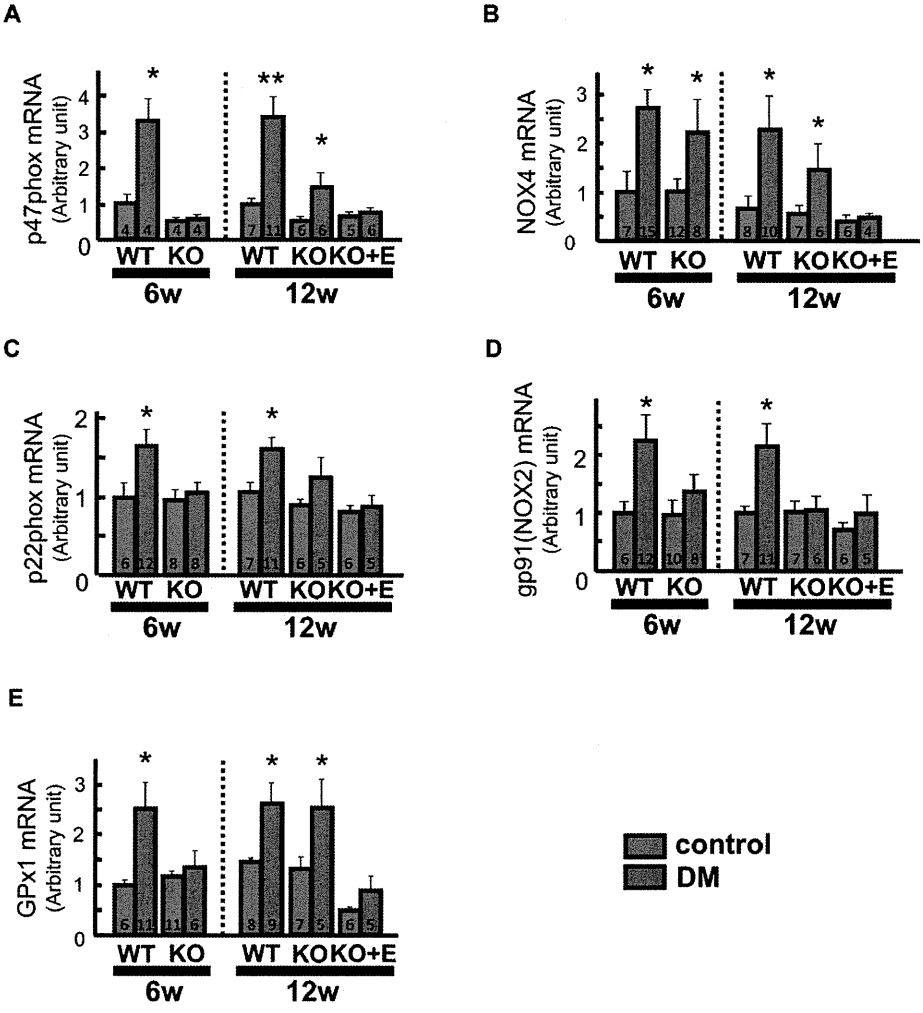

Figure 5. Oxidative stress in the diabetic heart. Expression of mRNAs for nicotinamide adenine dinucleotide 3-phosphate (NADPH) oxidase subunits, p47phox (A), NOX4 (B), p22phox (C), NOX2 (D), and glutathione peroxidase type 1 (GPx1) (E) in LV tissue measured by real-time PCR. Number of mice per group is indicated in bar graph. Values are the mean±SEM. DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type. *$p<0.05$ vs. the respective control group.
doi:10.1371/journal.pone.0093145.g005

The third important finding was that prevention of LV dysfunction by RAAS blockade was accompanied by attenuation of potential mechanisms of LV failure such as abnormal Ca^{2+} handling, oxidative stress, and apoptosis.

RAAS in the Diabetic Heart

Previous reports have shown local renin-angiotensin system activation in the diabetic heart [31]. Blockade of AT_1 signaling with ACE-I or ARB therapy has been shown to reverse DM-induced cardiac dysfunction without affecting the blood glucose level [4,32]. Although such previous studies assessed the effects of AT_1 signaling, the role of MR signaling has not been adequately evaluated so far.

There is a growing body of evidence that the MR mediates a variety of actions in the cardiovascular system and plays an important role in the process of ventricular remodeling independent of blood volume and blood pressure [33,34]. Transgenic mice overexpressing the human MR have a normal blood pressure, but show an increase of LV diameter, as well as systolic dysfunction [35] and a high rate of sudden death linked to severe ventricular arrhythmias, which are prevented by treatment with spironolactone [36]. MR mRNA expression was upregulated in rats with experimental hypertension [37] or MI [38], while spironolactone significantly suppressed the expression of these genes [37,38]. Some *in vitro* studies have also shown that aldosterone significantly increases MR mRNA expression [39]. Since aldosterone production and cardiac sympathetic activity are enhanced by pathological conditions, particularly after MI, it is conceivable that these factors would increase MR expression in the myocardium, although detailed mechanism has not been clarified.

In the present study, we confirmed upregulation of the local RAAS in diabetic hearts. Such upregulation of the local RAAS in the LV of diabetic animal can induce LV dysfunction, as well as cardiomyocyte apoptosis and increased oxidative stress. Genetic RAAS blockade at the AT_1 receptor level ameliorated these changes over the *short-term*. Interestingly, although MR mRNA expression was not upregulated in KO-DM mice at 6 weeks, it was significantly upregulated at 12 weeks (**Figure 7C**). This AT_1-independent MR activation was associated with LV diastolic dysfunction, cardiomyocyte apoptosis, and increased oxidative stress, while the selective MR blocker eplerenone prevented these changes. Therefore, AT_1-independent MR activation might be

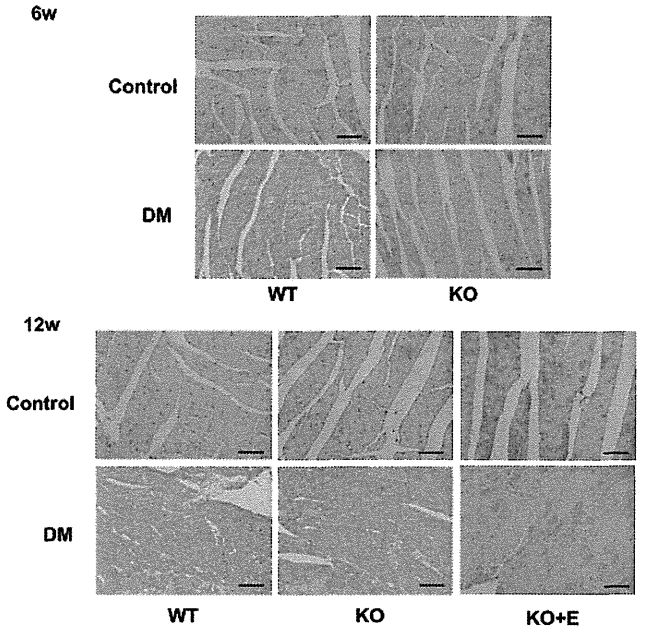

Figure 6. The immunohistochemistry of LV tissue for 4-hydroxy-2-nonenal (4-HNE) in the diabetic heart. Brown staining is positive for 4-HNE, while nuclei are stained blue by hematoxylin. At 6 weeks, staining for 4-HNE was stronger in WT-DM mice compared with WT-control mice, whereas there was no significant difference between KO-DM and KO-control mice. At 12 weeks, however, staining for 4-HNE was also stronger in KO-DM mice compared with KO-control mice. Eplerenone treatment prevented these changes. All pictures are at ×400 magnification. Scale bar = 50 μm.
doi:10.1371/journal.pone.0093145.g006

involved in the mechanism of the development of diabetic cardiac injury.

"Aldosterone Breakthrough" and MR Activation in the Diabetic Heart

In 1981, Staessen *et al.* reported an initial decrease and subsequent increase of PAC in hypertensive patients treated with captopril, an ACE-I [13]. These findings were confirmed by other researchers and MR antagonists were shown to be beneficial in this setting [14,40,41]. The late increase of PAC is related to changes of CYP11B2 (aldosterone synthase) expression in human adrenocortical cells [12].

Several previous studies have revealed that aldosterone is produced in cardiac tissues, particularly in patients with pathological conditions such as MI [22] or HF [42], although its level is much lower than that in the blood. Previous reports have indicated that LV dysfunction occurs in experimental animals with HF in the absence of an increase of PAC, but is reversed by MR blockade [37,43]. In those studies, little aldosterone was detected in the LV tissues. These findings provided evidence that plasma aldosterone independent MR activation in myocardium plays a role in the mechanism of LV dysfunction.

The MR has a high affinity for corticosterone and cortisol, as well as aldosterone [44]. Expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts endogenous glucocorticoids to their receptor-inactive 11-keto analogues, is reported to be extremely low in the heart, indicating that cardiomyocyte MRs are primarily occupied by endogenous glucocorticoids that are present at higher levels than aldosterone [45], although the 11β-HSD2 mRNA expression was found to be upregulated in rats with MI [38]. Although glucocorticoids generally antagonize the MR and act as cardioprotective hormones in nonepithelial cells such as myocytes [46], it has been shown that glucocorticoid effects are modulated by the redox state of cells and that glucocorticoids activate MR signaling in an oxidized state [44].

Although plasma aldosterone independent MR activation in myocardium play a part in the development of MI remodeling and HF, it is not clear whether the same mechanism is involved in the development of diabetic heart injury. Therefore we focused on *plasma* and *local* contents of aldosterone and corticosterone, and MR activation in myocardium with diabetic animals.

The present study demonstrated that expression of MR mRNA in the LV was upregulated in WT-DM mice at 6 weeks and even in KO-DM mice at 12 weeks (**Figure 7C**). In addition, PAC was increased in WT-DM mice at 6 week and also in KO-DM mice at 12 weeks (aldosterone breakthrough, **Figure 8A**). It seems the change of MR mRNA expression was parallel to those of PAC. However, the aldosterone or corticosterone content of the LV was not increased in KO-DM mice (**Figure 8C, 8D**). Collectively, these findings suggest that, in this setting, MR signaling is regulated separately from aldosterone or corticosterone in the myocardium, although *plasma* aldosterone may show a "breakthrough". We could not find any intergroup differences of 11β-HSD2 and CYP11B2 mRNA expression in the LV (data not shown). These findings suggested that the mechanism of MR activation in diabetic hearts did not contain local aldosterone production in the myocardium. The present *in vivo* experiments did not allow us to determine the precise molecular mechanisms by which MR signaling was activated, so further studies are needed to address these issues.

Ca^{2+} Handling Abnormality in the Diabetic Heart as a Potential Mechanism of LV Relaxation Failure

We found that LV relaxation was impaired in diabetic hearts. And the data regarding the components involved in Ca^{2+} handling changed in parallel with the markers of LV relaxation and MR activation. From the present findings, RAAS activation contributed to LV diastolic dysfunction and abnormal Ca^{2+} handling. Previous reports have indicated that the mechanism of impaired LV relaxation involves disturbance of Ca^{2+} handling [7]. In fact, adding SERCA2a to mice with STZ-induced diabetes normalizes both systolic and diastolic function [47]. Also, the Ca^{2+} transient had a slower time to peak and slower decay in LV trabeculae from diabetic rats, along with a slower time course of contraction [48]. Changes of SERCA2a expression and phosphorylation of phospholamban might be responsible for these alterations of Ca^{2+} transients and could play an important role in the occurrence of LV diastolic dysfunction in diabetic hearts, although there are many known factors that influence diastolic function. Collectively, although these findings including previous publications support our suggestion as to potential mechanism of diabetic cardiomyopathy regulated by RAAS, further evaluations are necessary to confirm the detailed mechanism.

Oxidative Stress and Apoptosis in the Diabetic Heart as a Potential Mechanism of LV Failure

In our data, the markers of oxidative stress and apoptosis changed in parallel with the data of LV function and MR activation. The findings of the present study (**Figure 4–6**) suggest that ROS production and cardiomyocyte apoptosis are enhanced in the diabetic heart, at least partly due to activation of the local RAAS, and contribute to the pathogenesis of diabetic cardiomyopathy.

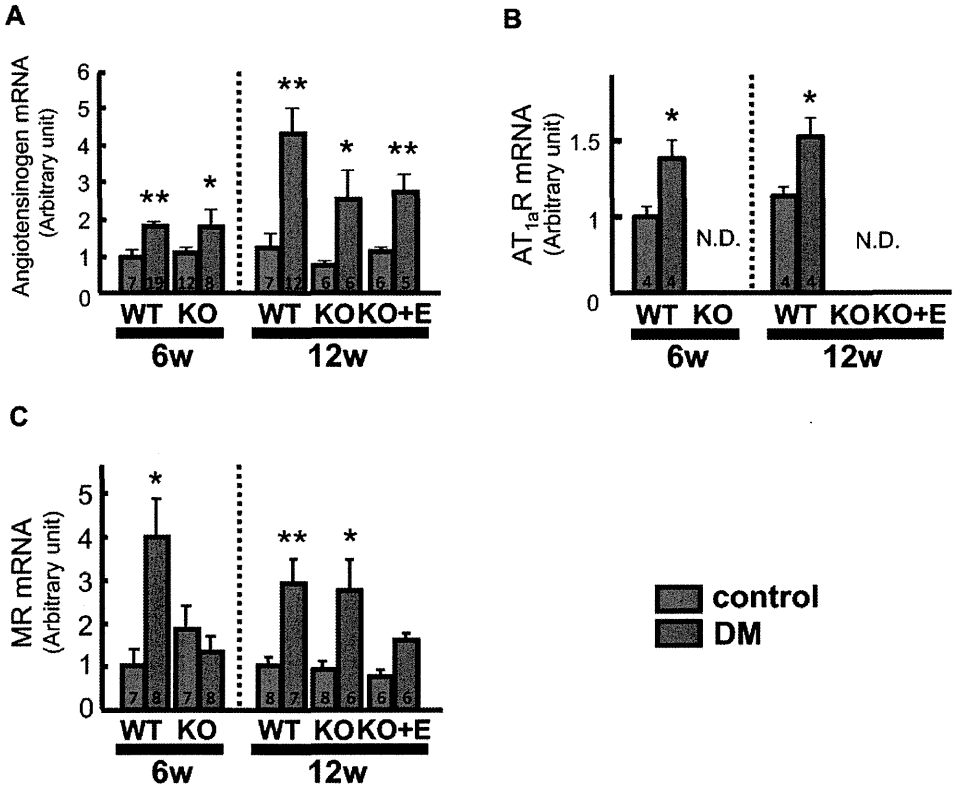

Figure 7. Renin-angiotensin-aldosterone system in the diabetic heart. Expression of mRNAs for angiotensinogen (A), angiotensin II $type_{1a}$ receptor ($AT_{1a}R$) (B), and the mineralocorticoid receptor (MR) (C) in LV tissue measured by real-time PCR. Number of mice per group is indicated in bar graph. Values are the mean±SEM. DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type; N.D., not detected. *p<0.05 vs. the respective control group, **p<0.01 vs. the respective control group.
doi:10.1371/journal.pone.0093145.g007

Previous studies have shown that apoptosis is accelerated in various organs of DM patients including the heart, which is associated with increased oxidative stress [9,10]. Both expression of some RAAS components and apoptosis were enhanced in cardiomyocytes from diabetic mice, while treatment with an ARB inhibited such changes and prevented cardiomyocyte apoptosis [9].

The findings in the present study are consistent with these reports and suggest the possibility that not only AT_1 but MR activation is also involved in these mechanisms, as shown in experimental MI model [15,43].

Clinical Implications

This study showed that AT_1 blockade alone protects the heart from damage caused by DM over the *short-term*, but it is necessary to block both AT_1 and MR signaling over the *long-term*. These findings are consistent with the results of large-scale clinical trials, RALES [16], EPHESUS [17], EMPHASIS-HF [18] and Aldo-DHF study [19], even though the subjects of those studies were patients with HF or MI. In a subanalysis in EPHESUS, the prognosis of diabetic patients with HF following MI was improved by RAAS inhibition through combined therapy with an ACE-I/ARB and MR antagonist [49]. This beneficial effect of RAAS inhibition can be at least partly explained by its protective effect on the heart, and may suggest a strategy for prevention of cardiac complications in diabetic patients.

Limitations

In the present study LV systolic pressure was significantly lower in the WT-DM and KO-DM groups compared with the respective control groups at 12 weeks (**Figure 2C**). One can say the data which represent LV systolic and diastolic function could be influenced by lower blood pressure in DM mice compared with control at 12w. Actually LV systolic pressure was reduced in DM mice at 12 week and it is possible that the deterioration of LV functional indices might reflect it. However, at 6 week Vcfc was already decreased in WT-DM irrespective of no significant difference in blood pressure (**Figure 2B, 2C**). The changes in these indices corresponded to the changes in molecular analysis such as Ca^{2+} handling (SERCA2a expression, phosphorylated phospholamban/total phospholamban ratio) or apoptosis (TUNEL staining). Previous studies showed the disturbance of systolic function in diabetic heart in rodents [5]. Taken these findings together we consider that the decrease of systolic blood pressure might be caused by deterioration of LV contractility. However, it is the limitation that we could not draw the logical conclusion on this issue from the data obtained in the present study since we did not measure the indices that represent LV contractility independent of blood pressure such as end-systolic elastance (Ees) [50].

We used mice with STZ-induced type 1 DM in the present study. However, the prevalence of type 2 DM is much higher than that of type 1 DM, and the present study did not allow us to assess the role of insulin resistance. These are considered to be limitations of the present study.

In conclusion, pathological activation of the RAAS, especially AT_1 independent MR activation, plays a crucial role in the development of DM-induced LV dysfunction. AT_1 blockade alone might be insufficient and additional MR blockade could be

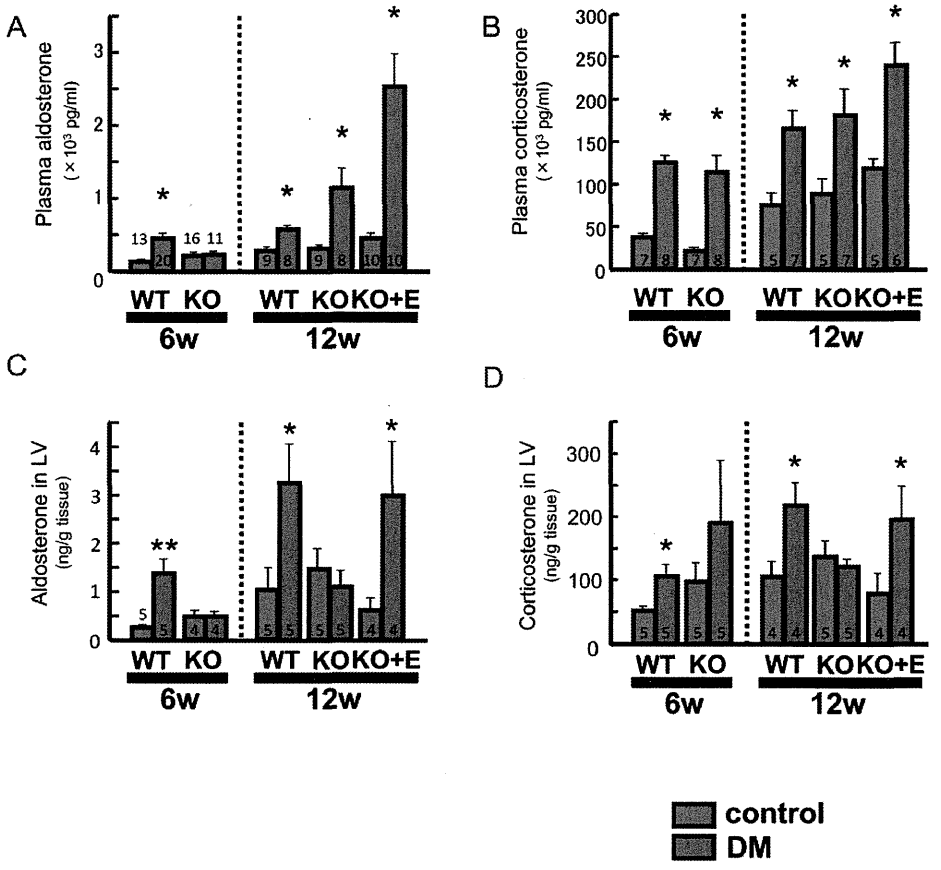

Figure 8. Aldosterone and corticosterone levels. Plasma aldosterone (A) and corticosterone (B) concentrations. Aldosterone content (C) and corticosterone content (D) of LV tissue. Number of mice per group is indicated in bar graph. Values are the mean±SEM. DM, diabetes mellitus; KO, AT_{1a} receptor knockout; WT, wild-type; LV, left ventricle. *p<0.05 vs. the respective control group, **p<0.01 vs. the respective control group.
doi:10.1371/journal.pone.0093145.g008

important for preventing the onset and development of diabetic cardiomyopathy.

Author Contributions

Conceived and designed the experiments: YN TM KF SO TY. Performed the experiments: YN TM HI KK. Analyzed the data: YN TM HI KK TA. Contributed reagents/materials/analysis tools: YN TM HI KK. Wrote the paper: YN TM TY. Checked the integrity of the present study: KF SO TY.

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Presence of Autoantibody Directed Against β_1-Adrenergic Receptors Is Associated With Amelioration of Cardiac Function in Response to Carvedilol: Japanese Chronic Heart Failure (J-CHF) Study

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ABSTRACT

Background: Autoantibody against β_1-adrenergic receptors (β_1-AAb) exerts agonist-like action inducing receptor uncoupling and myocardial damage. We attempted to determine the significance of β_1-AAb in chronic heart failure (CHF) patients who received carvedilol in a substudy of the Japanese Chronic Heart Failure study.

Methods and Results: In this prospective, randomized, multicenter trial, 117 patients were assigned to 2.5 mg, 5 mg, and 20 mg (n = 38, 36, and 43) carvedilol groups according to the target dose. β_1-AAb was positive in 51 patients (44%, P) and negative in 66 (56%, N). The percentage increase of left ventricular ejection fraction over 56 weeks (ΔLVEF) was larger in P than in N ($P = .050$) and in the high-titer group (H) than in the low-titer group (L; $P = .04$). Left ventricular (LV) volume decreased to a greater extent in H than in L over 56 weeks. β_1-AAb titer was significantly correlated with ΔLVEF and the percentage change of LV volume and was an independent predictor of them. No difference was seen in the composite end point (all-cause mortality and hospitalization for cardiovascular diseases or heart failure). However, in patients with dilated cardiomyopathy, it was more common in the 2.5 mg group than in the other groups in N, and it was similar among the 3 groups in P.

Conclusions: Our data suggest that the presence of β_1-AAb is associated with favorable response to carvedilol in CHF. (*J Cardiac Fail 2015;21:198–207*)

Key Words: Autoimmunity, β-blocker, responder, reverse remodeling.

Autoimmune disorder is one of the features characterizing chronic heart failure (CHF) due not only to dilated cardiomyopathy (DCM) but also to other etiologies. Over the past few decades, several autoantibodies have been reported to be present in sera from patients with DCM. Autoantibody directed against β_1-adrenergic receptor (β_1-AR) is found in ~30%–40% of patients with CHF due to DCM.[1–4] Autoantibody directed against the second extracellular loop of β_1-AR (β_1-AAb) shows agonist-like effects[5–8] inducing receptor uncoupling in vitro[9,10] and in vivo,[11] myocyte apoptosis,[12] sustained calcium influx resulting in electric instability of the heart,[13] and persistent myocardial damage.[14] The presence of β_1-AAb is associated with increased mortality in patients with DCM,[15] the occurrence of supraventricular and ventricular arrhythmias,[4,16,17] and sudden death.[4,16] And there are reports that nonspecific and specific removal of β_1-AAb with the use of an immunoadsorption technique could be achieved[18–21] and improved the cardiac function of patients with DCM.[18,19] These results suggest that the presence of β_1-AAb is of pathogenic importance in the induction and progression of cardiomyopathy.

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Manuscript received May 30, 2014; revised manuscript received October 8, 2014; revised manuscript accepted December 10, 2014.

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Funding: Grants from the Ministry of Health, Labor, and Welfare of Japan (14C-2) and the Japan Heart Foundation.

See page 206 for disclosure information.

1071-9164/$ - see front matter

http://dx.doi.org/10.1016/j.cardfail.2014.12.005

Large clinical trials have established that β-blockers improve left ventricular (LV) function and favorably affect survival in patients with CHF.[22,23] Earlier studies, including from our laboratory, have shown that β-blockers might affect biologic actions of β_1-AAb and abolish its adverse effect.[8,11] Our group conducted a proof-of-concept study demonstrating that the presence of β_1-AAb was associated with more favorable response to β-blocker therapy in patients with CHF.[24] Furthermore, in that study, carvedilol was more effective in patients with β_1-AAb than in those without, although there was no difference in patients who were given metoprolol.

Therefore, the purpose of the present study was to determine the significance of the presence of β_1-AAb in patients with CHF who received β-adrenergic receptor blocker, carvedilol, in a substudy of Japanese Chronic Heart Failure (J-CHF) study.[25]

Materials and Methods

Study Populations

This study was prospectively planned and conducted as a substudy of the J-CHF study.[25] Three hundred sixty-four patients were enrolled in the J-CHF study at 131 clinical sites in Japan from July 2003 to January 2008. Among these study subjects, 122 patients who gave informed consent to participate in this substudy were enrolled. The study patients had stable CHF (New York Heart Association [NYHA] functional class II/III, left ventricular ejection fraction [LVEF] ≤40%), were not currently taking carvedilol, were from 20–80 years old, and could be inpatient or outpatient. Key exclusion criteria were cardiogenic shock, systolic blood pressure <80 mm Hg, severe arrhythmia (eg, sustained ventricular tachycardia, ventricular fibrillation), bradycardia (<50 beats/min), 2nd-degree or advanced atrioventricular block, recent myocardial infarction, coronary artery bypass surgery, or percutaneous coronary intervention.

The J-CHF study protocol complies with the principles outlined in the Declaration of Helsinki, and the Institutional Review Board of each participating institution approved the J-CHF study protocol, including the statement of informed consent. Before enrollment, each patient gave informed consent to participate in the J-CHF study and this substudy.

Randomization and Masking

With the use of centralized computer-generated randomization with an algorithm based on the underlying disease, severity, age, and sex, the 364 patients were randomly allocated using a 1:1:1 ratio to 1 of 3 carvedilol groups (2.5 mg, 5 mg, or 20 mg daily). Other β-blockers were prohibited, as were α-blockers, αβ-blockers, and inotropic agents other than digitalis.

Study Design

The study design is outlined in Figure 1. After an 8-week observation period, carvedilol was titrated upward over an 8-week period from 1.25 mg twice daily to the target dose of 10 mg twice daily based on tolerability. Thereafter, patients were seen every 2–8 weeks for the 3-year follow-up. Every 6 months, patients were evaluated for NYHA functional class and specific activity scale (SAS). In addition, an electrocardiogram (ECG), chest X-ray, echocardiography, and laboratory tests, including B-type natriuretic peptide (BNP), were conducted at weeks 0, 24, and 48 of the fixed-dose period. LVEF was measured with the use of the modified Simpson method by means of echocardiography or radionuclide ventriculography. LV end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were calculated from LV end-diastolic diameter and end-systolic diameter, respectively, with the use of the Teichholz method. The presence of β_1-AAb was determined with the use of enzyme-linked immunosorbent assay (ELISA) before carvedilol introduction. In this substudy, 122 patients were enrolled and 117 patients were available for analyses by treatment allocation (Fig. 1).

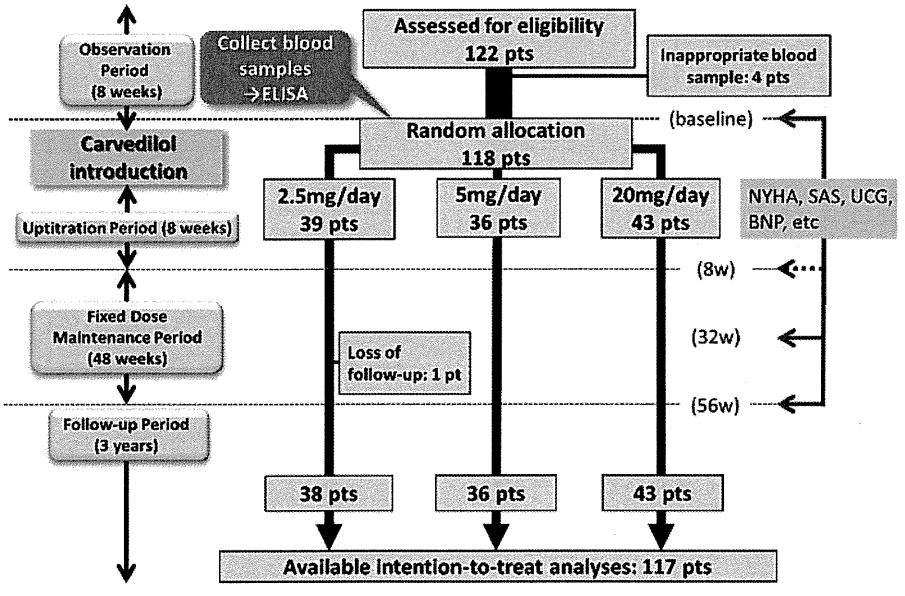

Fig. 1. Substudy protocol and study population. pts, patients; ELISA, enzyme-linked immunosorbent assay; NYHA, New York Heart Association; SAS, specific activity scale; UCG, ultrasonic echocardiography; BNP, B-type natriuretic peptide.

Primary and Secondary End Points

The primary end point was the composite of all-cause mortality and hospitalization for cardiovascular (CV) causes including worsening of heart failure (HF). The secondary end points were all-cause mortality, hospitalization for CV causes, or worsening of symptoms (defined as a decrease of ≥1 Mets in the questionnaire score or an increase of ≥1 NYHA functional class for ≥3 months or a need for modification of HF treatment).

ELISA

The presence of β_1-AAb was determined by means of ELISA with the use of a synthetic peptide corresponding to the putative sequence of the 2nd extracellular loop of human β_1-AR (amino acid sequence number 197–222: H-W-W-R-A-E-S-D-E-A- R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R) as an epitope peptide, as previously described.[4,24,26] Positivity was defined as 2.5 times the background density. We divided the study population into high-titer (≥40-fold) and low-titer (≤20-fold) groups according to β_1-AAb titer.

Statistical Analysis

All values were expressed as mean ± SD. Differences between groups were compared with the use of the nonpaired *t* test or Mann-Whitney *U* rank sum test for unpaired data and with the use of the chi-square test for discrete variables. Two-way repeated-measures analysis of variance (ANOVA) was performed to determine the difference in the time-related changes in LVEF, BNP, LVEDV, and LVESV between β_1-AAb–positive and –negative patients, and between β_1-AAb–high-titer and –low-titer groups. The correlations between β_1-AAb titer and percentage changes in LVEF, LVEDV, and LVESV were analyzed by means of Spearman correlation test. Multiple regression analysis was performed to estimate the independent determinant of the percentage change of LVEF, LVEDV, and LVESV. The variables of age, sex, allocated dose of carvedilol, heart rate at baseline, plasma BNP at baseline, etiology of CHF (coronary artery disease [CAD], DCM, hypertensive heart disease [HHD], or others), and the presence of atrial fibrillation were included in the model. Kaplan-Meier survival curves for primary and secondary end points were calculated according to the presence or absence and high titer or low titer of β_1-AAb, and the differences were analyzed by means of the log-rank test. A *P* value of $<.05$ was considered to be statistically significant.

Results

Patient Characteristics

β_1-AAb was detected in 51 patients (44%) by means of ELISA. Table 1 shows the baseline characteristics of the 2 groups according to the presence or absence of β_1-AAb. The CHF of ~60% of the study subjects was due to DCM. β_1-AAb–positive patients were significantly older than β_1-AAb–negative patients (64 ± 12 y vs 57 ± 14 y; $P = .01$). Fractional shortening tended to be lower in β_1-AAb–positive patients than–negative patients, although it did not reach statistically significant difference (14 ± 6% vs 16 ± 6%; $P = .07$). There were no significant differences in NYHA functional classes, SAS, LVEF, or BNP between the 2 groups. Heart rate and blood pressure also were similar. More patients with β_1-AAb received diuretics than those without β_1-AAb, but there were no differences in the other cardiovascular medications or nonpharmacologic therapy (implantable cardioverter-defibrillator or cardiac resynchronization therapy) between the 2 groups.

Table 1. Baseline Characteristics of the Study Population

	β_1-AAb		
	Negative (n = 66)	Positive (n = 51)	*P* Value
Age (y)	57 ± 14	64 ± 12	.01
Sex (male/female)	53/13	34/17	NS
Etiology			
CAD	19 (29%)	10 (20%)	NS
DCM	38 (57%)	36 (70%)	NS
HHD	4 (6%)	3 (6%)	NS
Others	5 (8%)	2 (4%)	NS
NYHA functional class			
II	56 (85%)	39 (76%)	NS
III	10 (15%)	12 (24%)	NS
SAS	5.0 ± 1.5	5.1 ± 2.0	NS
Atrial fibrillation	12 (18%)	13 (25%)	NS
SBP (mm Hg)	118 ± 16	117 ± 18	NS
HR (beats/min)	79 ± 13	77 ± 13	NS
CTR (%)	53.1 ± 6.3	54.5 ± 6.3	NS
LVEF (%)	30.8 ± 7.5	29.0 ± 7.7	NS
FS (%)	16 ± 6	14 ± 6	.07
LVEDV (mL)	199 ± 55	199 ± 47	NS
LVESV (mL)	136 ± 50	145 ± 43	NS
LAD (cm)	4.1 ± 0.8	4.6 ± 0.7	<.001
BNP (pg/mL)	399 ± 548	447 ± 417	NS
Cr (mg/dL)	0.9 ± 0.3	1.0 ± 0.4	NS
Medication			
ACE-I	25 (38%)	22 (43%)	NS
ARB	35 (53%)	26 (51%)	NS
Diuretics	48 (72%)	45 (88%)	.04
Digitalis	19 (29%)	18 (35%)	NS
Antiarrhythmic agents	3 (5%)	5 (10%)	NS
Calcium blocker	11 (17%)	8 (16%)	NS
Anticoagulants	28 (42%)	24 (47%)	NS
Nonpharmacologic therapy			
ICD	0 (0%)	1 (2%)	NS
CRT	0 (0%)	0 (0%)	NS
Allocation			NS
2.5 mg	23	15	
5 mg	15	21	
20 mg	28	15	
Fixed dose (mg)	9.9 ± 7.9	8.3 ± 7.4	NS
Titration			
2.5 mg	96% (22/23)	100% (15/15)	NS
5 mg	93% (14/15)	90% (19/21)	NS
20 mg	86% (24/28)	93% (14/15)	NS

β_1-AAb, autoantibody against β_1-adrenergic receptor; CAD, coronary artery disease; DCM, dilated cardiomyopathy; HHD, hypertensive heart disease; NYHA, New York Heart Association; SAS, specific activity scale; SBP, systolic blood pressure; HR, heart rate; CTR, cardiothoracic ratio; LVEF, left ventricular ejection fraction; FS, fractional shortening; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LAD, left atrial diameter; BNP, B-type natriuretic peptide; Cr, serum creatinine; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ICD, implantable cardioverter-defibrillator; CRT, cardiac resynchronization therapy.

Values are presented as mean ± SD or n (%).

Dose Assignment and Up-Titration

Dose assignment was similar between β_1-AAb–positive and –negative groups (2.5 mg/5 mg/20 mg: positive, 15/21/

15; negative, 23/15/28; P = NS; Table 1), Final fixed dose and achievement rate of target dose were also similar between the 2 groups (Table 1).

Effect of Carvedilol According to the Presence or Absence of β_1-AAb

During up-titration and fixed-dose maintenance period (total 56 weeks) after carvedilol introduction, there was no adverse effect in this study population. Heart rate significantly decreased in both β_1-AAb–positive and –negative groups 56 weeks after carvedilol introduction (positive: 77 ± 13 to 70 ± 10 beats/min; $P < .001$; negative: 79 ± 13 to 74 ± 12 beats/min; $P < .001$). There was no significant difference in the change of heart rate between these 2 groups (positive: −8 ± 15 beats/min; negative: −5 ± 16 beats/min; P = NS). Analysis of the each allocated group showed heart rate to be significantly reduced in all of the allocated groups (2.5 mg group: 76 ± 11 to 75 ± 13 beats/min; $P < .05$; 5 mg group: 77 ± 14 to 71 ± 9 beats/min; $P < .001$; 20 mg group: 80 ± 14 to 71 ± 12 beats/min; $P < .001$). There was no change in the time course of systolic blood pressure in both β_1-AAb positive and negative patients over the 56 weeks after introduction of carvedilol. There were no significant differences in the time course of LVEF, LVEDV, or LVESV between β_1-AAb–positive and –negative groups (Supplemental Table 1). The percentage increase of LVEF and the percentage decrease of LVEDV over the 56 weeks after introduction of carvedilol tended to be larger in the β_1-AAb–positive group than the –negative group (LVEF +67 ± 79% vs +33 ± 54%, P = .050, Fig. 2A and 2B; LVEDV −24 ± 24% vs −13 ± 25%, P = .050, Supplemental Table 1). The percentage decrease of LVESV was significantly larger in the β_1-AAb–positive group than in the –negative group (−40 ± 32% vs −26 ± 31%; $P < .05$; Supplemental Table 1). When we compared β_1-AAb high-titer (n = 15) and low-titer (n = 103) groups, LVEF tended to be larger in the high-titer group 56 weeks after carvedilol introduction (48.7 ± 12.7% vs 40.6 ± 14.3%; P = .054; Fig. 2C). The percentage increase of LVEF was significantly higher in the high-titer group than in the low-titer group (+85 ± 78% vs +43 ± 65%; P = .04; Fig. 2D). LVEDV decreased to a greater extent in the high-titer group than in the low-titer group (from 182 ± 50 mL to 119 ± 43 mL vs from 202 ± 52 mL to 167 ± 57 mL; $P < .005$ [ANOVA]; Fig. 3A). The percentage decrease of LVEDV was also significantly greater in the high-titer than in the low-titer group (−31 ± 24% vs −16 ± 25%; P = .04; Fig. 3B). Similarly, LVESV decreased to a greater extent in the high-titer group than in the low-titer group (from 136 ± 47 mL to 62 ± 36 mL vs from 141 ± 47 mL to 97 ± 53 mL; P = .048 [ANOVA]; Fig. 3C). The percentage decrease in LVESV was significantly greater in the high-titer than in the low-titer group (−52 ± 22% vs −29 ± 33%; P = .02; Fig. 3D). Plasma BNP decreased in both groups when divided into β_1-AAb–positive and –negative groups (positive: 447 ± 417 to 134 ± 169 pg/mL [$P < .001$]; negative: 399 ± 548 to 193 ± 505 pg/mL [$P < .001$]) as well as high-titer

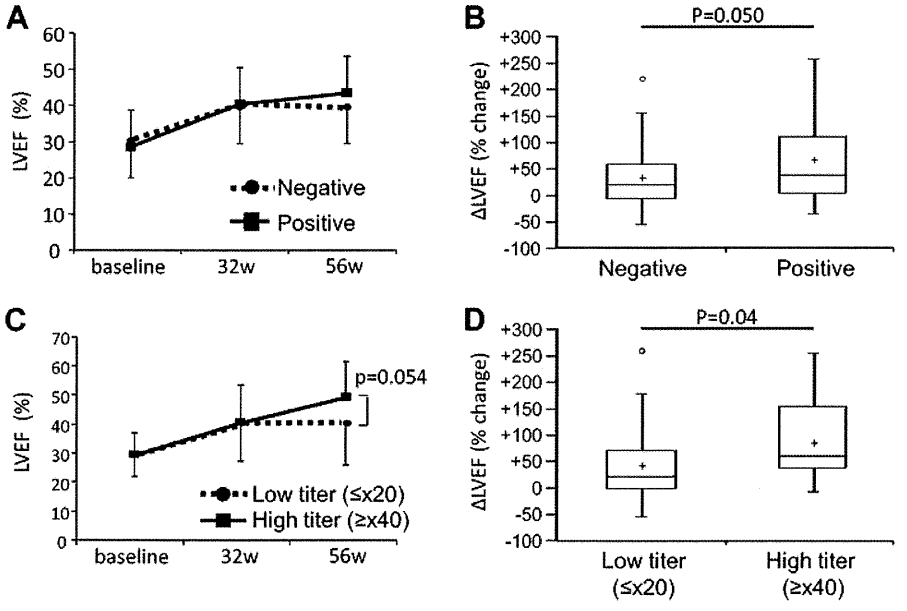

Fig. 2. (A) Serial changes in left ventricular ejection fraction (LVEF) and (B) percentage changes in LVEF (ΔLVEF) over 56 weeks after carvedilol introduction in autoantibody against β_1-adrenergic receptor (β_1-AAb)–positive and –negative patients. The ends of the whiskers represent the lowest data still within 1.5 interquartile range (IQR) of the lower quartile and the highest data still within 1.5 IQR of the upper quartile. The data not included wthin the whiskers are plotted with a small circle; +, mean value. LVEF increased in β_1-AAb–positive and –negative groups but there was no significant difference in the time course of LVEF between these 2 groups (A). (C) Serial changes in LVEF and (D) ΔLVEF in β_1-AAb–high-titer and –low-titer groups. LVEF increased in β_1-AAb–high- and –low-titer groups, but there was no significant difference in time course of LVEF between these 2 groups (C).

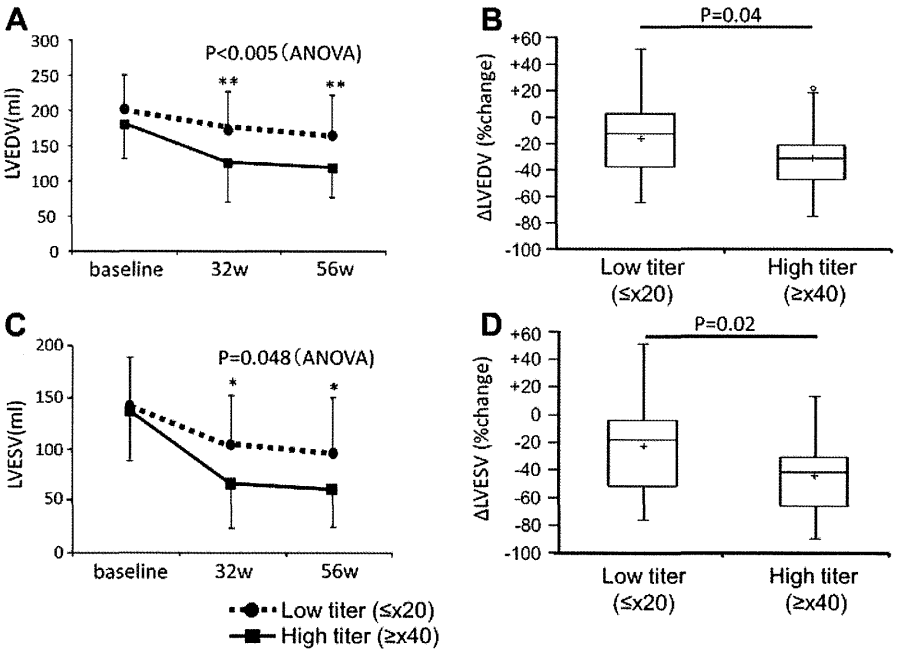

Fig. 3. Serial changes in (A) left ventricular end-diastolic volume (LVEDV) and (C) left ventricular end-systolic volume (LVESV) and percentage changes in (B) LVEDV (ΔLVEDV) and (D) LVESV (ΔLVEDV) over 56 weeks after carvedilol introduction in autoantibody against β_1-adrenergic receptor (β_1-AAb)—high-titer and —low-titer groups. The ends of the whiskers represent the lowest data still within 1.5 interquartile range (IQR) of the lower quartile and the highest data still within 1.5IQR of the upper quartile; +, mean value. $^*P < .05$; $^{**}P < .01$ vs β_1-AAb—high-titer group.

and low-titer groups (high-titer: 498 ± 405 to 82 ± 94 pg/mL [$P = .001$]; low-titer: 408 ± 522 to 180 ± 443 pg/mL [$P < .001$]) over the 56 weeks after carvedilol introduction. There were no significant differences in the change of BNP during the maintenance or follow-up period between β_1-AAb—positive and —negative groups or between high-titer and low-titer groups. The percentage change of LVEF, LVEDV, and LVESV over the 56 weeks after carvedilol introduction showed weak but significant correlation with β_1-AAb titer (LVEF: $r = 0.24$, $P = .02$; LVEDV: $r = -0.26$, $P = .02$; LVESV: $r = -0.27$, $P = .01$; Fig. 4A—C). There was no significant correlation in the change of heart rate or BNP with β_1-AAb titer during the 56 weeks (data not shown). Multiple regression analysis was performed to determine the independent determinants of the percentage change of LVEF, LVEDV, and LVESV.

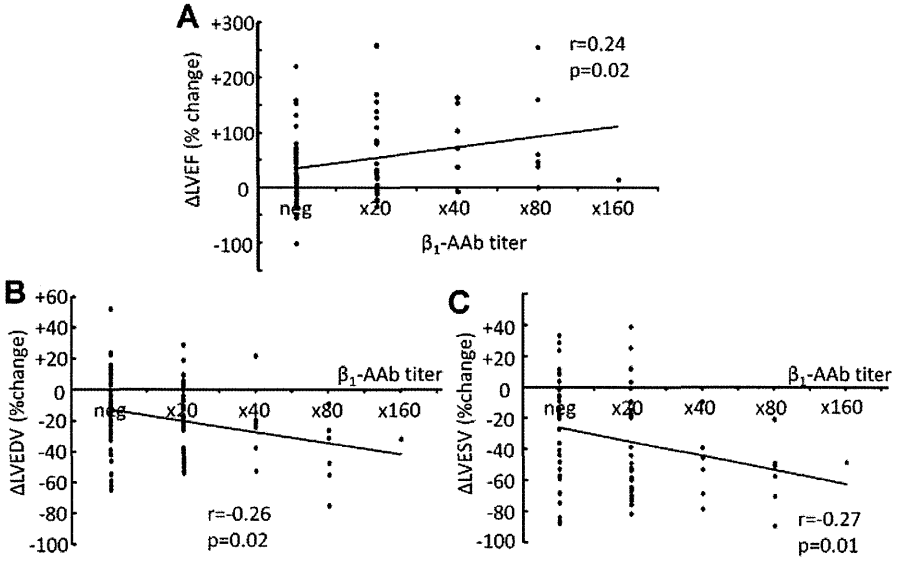

Fig. 4. The correlation between β_1-AAb titer and (A) ΔLVEF, (B) ΔLVEDV and (C) ΔLVESV over 56 weeks after carvedilol introduction. Abbreviations as in Figure 3.

Table 2. Multiple Regression Analysis Predicting the Percentage Changes of LVEF, LVEDV, and LVESV

Variables	ΔLVEF				ΔLVEDV				ΔLVESV			
	β	CI		*P* Value	β	CI		*P* Value	β	CI		*P* Value
Age (y)	−0.119	−0.336	0.097	.28	−0.051	−0.268	0.166	.64	0.026	−0.188	0.240	.81
Sex	0.031	−0.191	0.253	.78	−0.092	−0.306	0.122	.40	−0.090	−0.300	0.121	.40
Allocated dose of carvedilol												
2.5 mg vs 20 mg	0.104	−0.149	0.357	.42	−0.060	−0.304	0.184	.63	−0.063	−0.303	0.178	.61
5 mg vs 20 mg	−0.063	−0.311	0.185	.62	0.068	−0.172	0.308	.58	0.104	−0.132	0.340	.38
HR at baseline (beats/min)	0.046	−0.159	0.252	.65	−0.141	−0.344	0.063	.17	−0.148	−0.349	0.052	.14
BNP at baseline (pg/mL)	0.149	−0.060	0.357	.16	−0.314	−0.520	−0.107	.003	−0.274	−0.478	−0.071	.009
Etiologies of HF												
CAD vs HHD	0.211	−0.107	0.528	.19	0.090	−0.226	0.406	.57	−0.043	−0.354	0.269	.78
CAD vs DCM	−0.151	−0.436	0.133	.29	−0.152	−0.433	0.130	.29	−0.108	−0.385	0.170	.44
CAD vs others	0.105	−0.199	0.410	.49	−0.277	−0.573	0.020	.07	−0.252	−0.544	0.040	.09
Presence of AF	−0.166	−0.370	0.039	.11	0.057	−0.146	0.260	.58	0.003	−0.198	0.203	.98
β_1-AAb titer	0.286	0.067	0.505	.01	−0.227	−0.446	−0.008	0.04	−0.250	−0.466	−0.034	0.02

ΔLVEF, percentage change of left ventricular ejection fraction over 56 weeks; ΔLVEDV, percentage change of left ventricular end-diastolic volume over 56 weeks; ΔLVESV, percentage change of left ventricular end-systolic volume over 56 weeks; CI, confidence interval; AF, atrial fibrillation; other abbreviations as in Table 1.

As presented in Table 2, β_1-AAb titer was significantly associated with percentage change of LVEF, LVEDV, and LVESV.

Clinical Outcomes

During a mean follow-up period of 38 ± 18 months, in Kaplan-Meier analysis there were no significant differences in all-cause mortality (Fig. 5A) or hospitalization for CV cause including HF (Fig. 5B) between the β_1-AAb–positive and –negative groups. There was no significant difference in the occurrence of sudden cardiac death (data not shown). We attempted to determine the difference in the primary end point according to the dose assignment, individually in β_1-AAb–negative and –positive groups. There were no differences in these end points according to the dose assignment in overall patients (data not shown). In DCM patients, however, the incidence of primary end point was significantly higher in the 2.5 mg group than the other groups in the β_1-AAb–negative group (P = .03; Fig. 6A). There was no such difference among the 3 groups in the β_1-AAb–positive group (Fig. 6A). The incidence of worsening of SAS score or change of medication was also significantly higher in the 2.5 mg group than the other groups in the β_1-AAb–negative group (P = .03; Fig. 6B). There was no difference among the 3 groups in β_1-AAb–positive group (Fig. 6B).

Discussion

In the present study, we demonstrated that the presence or titer of β_1-AAb was associated with the response to carvedilol. Increase in LVEF and decrease in LVEDV and LVESV were larger in patients with high titer of β_1-AAb than those without or with low titer of β_1-AAb. Furthermore, in the present study, β_1-AAb titer was significantly correlated with the percentage changes of LVEF, LVEDV, and LVESV. β_1-AAb titer was an independent predictor of them. In DCM patients, the incidence of the primary end point was significantly higher in the 2.5 mg group than the other groups (5 mg or 20 mg) in the β_1-AAb–negative group. However, there

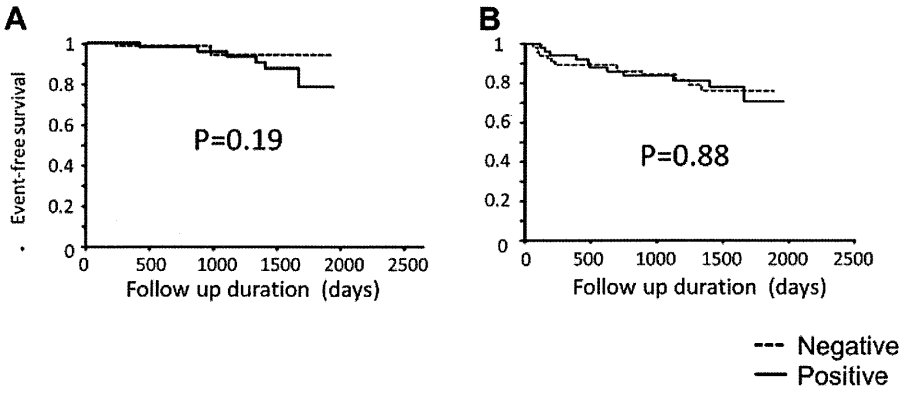

Fig. 5. Kaplan-Meier analysis in the overall population based on the positivity of autoantibody against β_1-adrenergic receptor (β_1-AAb). (A) All-cause mortality. (B) Hospitalization for cardiovascular cause, including the worsening of heart failure.

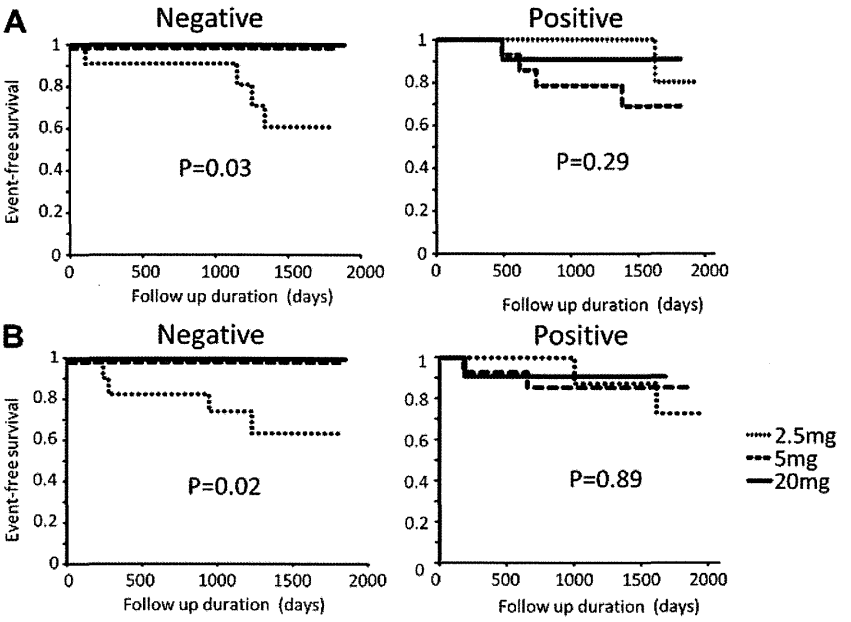

Fig. 6. Kaplan-Meier analysis according to the dose assignment individually in autoantibody against β_1-adrenergic receptor (β_1-AAb)—negative and —positive groups in patients with dilated cardiomyopathy. (A) Primary end point. (B) Worsening of specific activity scale (SAS) score or change of medication. β_1-AAb—negative group: 2.5 mg/5 mg/20 mg group, n = 14/9/14; β_1-AAb—positive group: 2.5 mg/5 mg/20 mg group, n = 10/14/11.

was no such difference among the 3 groups in the β_1-AAb—positive group. From these findings, we conclude that the presence of β_1-AAb is associated with more favorable response to carvedilol in patients with CHF.

Presence of β_1-AAb in β-Blocker Therapy

β-Blocker therapy is well established and the most potent therapy for patients with CHF. However, it is also apparent that clinical responses to this therapy vary substantially by patients. Earlier studies have shown that β_1-AAb has agonist-like action inducing receptor uncoupling.[9–11] The presence of β_1-AAb was associated with worse cardiac function in patients with advanced CHF.[3] In addition, β-blockers might affect biologic actions of β_1-AAb.[8,11,27] We therefore hypothesized that β-blockers might work especially well in the presence of β_1-AAb.

Determinants of Reverse Remodeling During Carvedilol Therapy

In the present study, β_1-AAb titer was significantly correlated with the percentage change of LVEF, LVEDV, and LVESV after carvedilol introduction, and it was a significant determinant of percentage change of LVEF, LVEDV, and LVESV according to multiple regression analysis. The inconsistency in the response to β-blocker for CHF patients has been recognized as a serious issue in clinical practice. Most earlier investigations that have addressed this issue focused on the genetic polymorphism of the molecules related to β-blocker therapy, such as β_1-AR,[28] G-protein—coupled receptor kinase 5,[29] and CYP2D6 (which is involved in drug degradation),[30] and so on. The findings in the present study showed the presence of β_1-AAb is also one of the factors associated with reverse remodeling represented as the changes in LVEF, LVEDV, and LVESV during carvedilol therapy for patients with CHF.

Clinical Evidence Regarding the Efficacy of β-Blockers in the Presence of β_1-AAb

Previously we conducted a proof-of-concept study that demonstrated the presence of β_1-AAb to be associated with more favorable response to β-blocker therapy in patients with CHF.[24] Furthermore, in that study, carvedilol was more effective in patients with β_1-AAb than in those without, although there was no difference between the 2 groups in patients who were given metoprolol. On the other hand, an earlier report showed that patients with β_1-AAb showed greater improvement in cardiac function than those without β_1-AAb with the use of metoprolol. However, in that study up-titration was feasible in a shorter period and maximal dose was higher in patients who had β_1-AAb compared with those who did not have β_1-AAb.[31] From the findings, we could not come to a conclusion on whether the presence of β_1-AAb plays a role in mediating the efficacy of metoprolol. Therefore, we sought to determine the significance of the presence of β_1-AAb in patients with CHF who received carvedilol in this substudy of the J-CHF study. To our knowledge, this is the 1st report that prospectively examined these issues, including the prognosis of CHF patients and the change of cardiac function, in a randomized multicenter trial.

The results of the present study are consistent with those of our proof-of-concept study regarding the more favorable

response to β-blocker therapy in patients with β_1-AAb. Namely, the increase of LVEF and the decrease of LV volume were larger in β_1-AAb—positive or —high-titer than in —negative or —low-titer patients. Whereas the proof-of-concept study was a single-center study that enrolled a relatively small number of patients and cardiac function was assessed 16 weeks after β-blocker introduction, the present study was a multicenter study and cardiac function was assessed as long as 56 weeks after carvedilol introduction. The present study demonstrated that β_1-AAb titer was a significant determinant of the percentage change of LVEF and LV volume. Furthermore, the clinical end points were examined prospectively and precisely by an End Point Classification Committee. Therefore, the results in the present study provide a higher level of evidence regarding the efficacy of carvedilol in the presence of β_1-AAb.

Proposed Mechanisms Underlying the Beneficial Effects of β-Blockers in the Presence of β_1-AAb

Although it is conceivable that different kinds of β-blockers could show a range of inhibitory effect against β_1-AAb, only a few published reports have validated this issue. Nikolaev et al examined the effects of different kinds of β-blockers on β_1-AAb in vitro, which showed the superior blocking effect of carvedilol compared with metoprolol or bisoprolol.[27] However, the detailed mechanism of the difference among β-blocker agents remains unknown. Carvedilol preferentially binds higher-affinity receptors which couple G-protein and have ability to activate intracellular downstream signaling.[32] Receptor activation was more profoundly affected by carvedilol in cells expressing the Arg^{389} variant of β_1-AR, which is associated with greater stimulation of downstream signaling compared with those expressing the Gly^{389} variant.[33,34] These properties were not observed with the use of metoprolol. From these findings, carvedilol potentially could more profoundly affect high-affinity adrenergic receptors activated by the presence of β_1-AAb than those in the absence of β_1-AAb.

On the other hand β_1-AAb enhanced the proliferation of T lymphocytes and inhibited the secretion of interferon-γ while promoting an increase in interleukin-4 levels in vitro.[35] From these findings it is also possible that β_1-AAb may enhance humoral immunity and that β-blocker may even affect favorably the autoimmunity of certain populations through T lymphocytes. Miao et al showed β_1-AAb titer decreased 1 year after initiation of β-blocker therapy.[31] We did not follow β_1-AAb titer after the treatment was initiated. Further investigation will be necessary to figure out this issue.

Long-Term Outcomes

We previously showed that the presence of β_1-AAb was associated with sudden cardiac death in patients with DCM. In addition, we found that use of β-blockers was a negative predictor of high-risk ventricular tachycardia in patients who had such autoantibodies.[4] Other researchers also showed the presence of β_1-AAb was associated with increased all-cause and CV mortality risk in DCM but not in ischemic cardiomyopathy patients.[15] In the present study, we could not find any differences in sudden death or admission due to CV causes including HF between β_1-AAb—positive and —negative groups. However, in DCM patients, the incidence of the primary end point was significantly higher in the 2.5 mg group than in the other groups (5 mg and 20 mg) in the β_1-AAb—negative group. On the other hand, there was no such difference among the 3 groups in the β_1-AAb—positive group. It might mean that a lower dose, 2.5 mg, of carvedilol might be sufficient to prevent CV events for β_1-AAb—positive patients, whereas it dose-dependently decreased cardiac events in β_1-AAb—negative patients.

Also, of note, there was no such difference in primary end point according to the dose assignment in analysis including all patients. Theoretically, in DCM, the manifestation of autoantibody should be a primary phenomenon as a consequence of autoimmune disorder. On the other hand, in coronary artery disease or hypertensive heart disease (HHD), it might be an epiphenomenon secondary to immunologic response to myocardial damage such as ischemia or ventricular overloading. Actually, 1 earlier study has shown that β_1-AAb from valvular heart disease or HHD did not have ability to activate β_1-AR expressed in cardiomyocytes in vitro.[36] Therefore, the presence of β_1-AAb has more significant impact on the pathophysiology of CHF and the prognosis, especially in patients with DCM.

Study Limitations

There are some limitations of the present study. First, we used ELISA for the detection of AAb against the 2nd extracellular loop of β_1-AR. We did not assess the functional aspect of β_1-AAb. As mentioned above, in earlier studies all β_1-AAbs detected by means of ELISA were not necessarily functionally active.[3,36] In addition, AAb against the 1st extracellular loop domain of β_1-AR was shown to have stimulatory effect on β_1-AR, although cyclic adenosine monophosphate signals induced by these AAbs were significantly lower than AAbs against the 2nd extracellular loop.[27] Second, because this study was conducted as a substudy, we were not allowed to plan it with a factorial design to avoid the imbalance of β_1-AAb positivity or titer among the carvedilol dosing groups. Third, we could not find any differences in the change of plasma BNP between β_1-AAb—positive and —negative groups or between high-titer and low-titer groups, even though the differences in the change of LVEF and LV volume were seen. The mechanism of this discrepancy is unknown. It has been shown that age, anemia, and renal dysfunction are independently associated with plasma BNP levels in patients with HF.[37,38] In the present study, age was older, hemoglobin concentration was lower, and serum creatinine concentration was higher in the β_1-AAb—positive group, although it did not reach statistically significant difference (data not shown), and these findings might mean that the

cardiorenal syndrome was more evident in the β_1-AAb–positive group. Fourth, prescription rate of diuretics was higher in the β_1-AAb–positive group than in the –negative group. Earlier studies have shown that β_1-AAb–positive patients have poorer cardiac function.[3] The difference of the prescription rate might reflect such findings, although in the present study there was no difference in cardiac function between β_1-AAb–positive and –negative groups at baseline. Fifth, as mentioned above, we did not follow β_1-AAb titer after the treatment was started.

Conclusion

Carvedilol is more effective in improving cardiac dysfunction and reversing remodeling in patients who have β_1-AAb. Carvedilol may affect biologic actions of β_1-AAb on receptor signaling in patients with CHF who have β_1-AAb. Further investigation will be needed to elucidate the detailed mechanism.

Disclosures

None.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cardfail.2014.12.005.

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