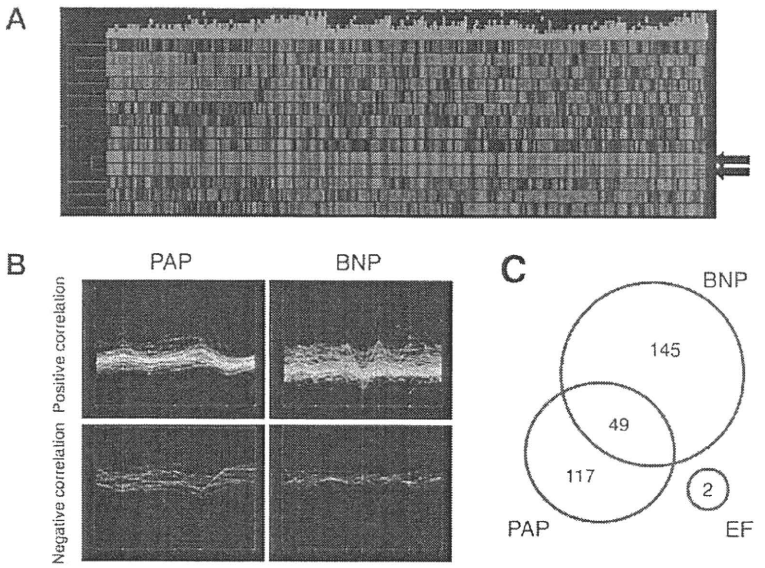

Fig. 1. The gene expression profile of human failing or non-failing myocardium. Gene expression levels of myocardial samples from 12 patients with severe heart failure and from two normals were analyzed using microarray. (A) Heat maps showing the genes with differential expression between the 12 failing myocardial samples and the two non-failing myocardial samples. Red color indicates upregulated gene expression. Green color indicates downregulated gene expression. Arrows indicate non-failing samples. (B) Expression profile of positively or negatively correlated genes to pulmonary artery pressure (PAP) or brain natriuretic peptide (BNP) mRNA level ($r > 0.7$). (C) Venn diagram of genes correlated with PAP, BNP, and ejection fraction.

Table 1
Datasets of genes whose expressions were correlated to clinical parameters.

	PAP	EF	BNP mRNA level
Positive correlation			
Number	124	1	175
Function	Cardiovascular system development and function Cell death	–	Cardiovascular system development and function Cell cycle
Representative genes	*ARNT, MYOCD, SMARCA4* *BGN, CFLAR, EEF2, MTPN*	*LMAN1L*	*BTG1, NPPA, NPPB, SERPNF1* *CKS1B, DDR2, FCGR2B, FN1*
Negative correlation			
Number	42	1	19
Function	Skeletal and muscular system development and function Cellular assembly and organization	–	Skeletal and muscular system development and function Cellular assembly and organization
Representative genes	*PIK3R1, PRKAR1A, SLMAP* *C19ORF20, RAB9A, SYNGAP1, TTN*	*FMO2*	*ACTC1, RBBP4, TTN*

The function of gene sets was analyzed by Ingenuity Pathway Analysis.
PAP, pulmonary artery pressure; EF, ejection fraction; BNP, brain natriuretic peptide.

representative genes are shown in Table 1. Interestingly, both gene sets correlated positively with PAP and BNP mRNA level were most associated with the same functional category of "cardiovascular system development and function", although the included genes were different. Similarly, the gene sets correlated negatively with both PAP and BNP mRNA level had most association with common functional categories of "skeletal and muscular system development and function" and "cellular assembly and organization".

Selection of 12 genes for in vitro screening

Among the genes selected using clinical parameters, we selected those genes that showed high expression levels in the heart by performing microarray analysis. On the basis of their novelty determined by a literature-based search, we selected four genes for further investigation (Table 2). Concurrently, to identify possible drug targets, we included four orphan GPCRs and four additional genes (three enzyme-encoding genes and one ion-channel protein-encoding gene) in the further analysis. The *RHOQ* and *STK38* genes were selected based on their correlation with BNP mRNA level and PAP, respectively. *GPR161* and *NBC1* were selected owing to their high expression level in the heart. *GPR37L1, GPR35, F2RL2,* and *MMP23B* were selected because of their high expression level in the heart, and their association with the cardiac diseases-related genes listed in the database was determined by *in silico* analysis.

Functional analysis of genes on the basis of adenovirus-mediated overexpression of proteins in neonatal rat cardiomyocytes

To determine which of the selected genes were associated with the physiological functions of the heart, we first generated adenovirus vectors for each gene listed in Table 2 and transfected these vectors into neonatal rat cardiomyocytes. Next, we evaluated the hypertrophic reaction, viability, and morphology of the transfected cardiomyocytes. Among the 12 selected genes, three adenovirus-mediated genes decreased the incorporation of [^{3}H]phenylalanine in neonatal rat cardiomyocytes (Table 2); the expression of one

Table 2
In vitro functional screening of the 12 candidate genes.

Probe set ID	Gene symbol	Gene name	Criteria for selection	p value	[^{3}H]PA intake	Fluorescence of Alamar blue	Cellular morphology
Genes relevant to clinical parameters							
75678_at	*MYLK3*	Myosin light chain kinase 3	Correlation with PAP ($r = 0.792$)	0.00262	No change	No change	Spiking
49333_at	*XPR1*	Xenotropic and polytropic retrovirus receptor	Correlation with PAP ($r = 0.765$), GPCR, change in CHF	0.00045	No change	No change	No change
38435_at	*PRDX4*	Peroxiredoxin 4	Correlation with BNP ($r = 0.863$)	0.00024	Increased	Decreased	No change
45314_at	*SMOC2*	SPARC related modular calcium binding 2	Correlation with both PAP and BNP ($r = 0.715$ and 0.758, respectively)	0.00444	No change	No change	No change
Genes encoding orphan GPCRs							
35544_at	*GPR37L1*	G-protein-coupled receptor 37 like 1	Orphan GPCR, downregulated in CVD	>0.005	Decreased	Decreased	Apoptosis
31700_at	*GPR35*	G-protein-coupled receptor 35	Orphan GPCR, upregulated in MI	0.00216	Decreased	Decreased	Hypertrophy
45204_at	*F2RL2*	Coagulation factor II (thrombin) receptor-like 2	GPCR, change in CVD	>0.005	Increased	No change	No change
40299_at	*GPR161*	G-protein-coupled receptor 161	GPCR, expression in heart	>0.005	Decreased	Decreased	No change
Genes encoding interesting enzymes or ion-channels							
38950_at	*MMP23B*	Matrix metallopeptidase 23B	Family of MMP, change in CHF	>0.005	No change	Decreased	No change
35285_at	*NBC1*	Na^+–HCO^{3-} cotransporter 1	Expression in heart	>0.005	No change	Decreased	No change
87788_at	*RHOQ*	Ras homolog gene family, member Q	Expression in DCM, correlation with BNP ($r = 0.711$)	>0.005	No change	No change	No change
78801_at	*STK38*	Serine/threonine kinase 38	Kinase activity, correlation with PAP ($r = 0.736$)	>0.005	No change	No change	No change

PAP, pulmonary artery pressure; GPCR, G-protein-coupled receptor; CHF, congestive heart failure; BNP, brain natriuretic peptide; CVD, cardiovascular disease; MI, myocardial infarction; DCM, dilated cardiomyopathy; PA, phenylalanine. *p* value indicates the significance of the difference between the gene expression level of failing and non-failing myocardium.

gene promoted [^{3}H]phenylalanine incorporation; and the overexpression of six genes lowered the viability of cardiomyocytes, which was evaluated by Alamar blue assay. We also evaluated the phenotype of transfected cardiomyocytes. Unlike control cells, *MYLK3*-adenovirus-transfected cardiomyocytes were spike shaped. The overexpression of *GPR37L1* induced apoptosis of cardiomyocytes. The transfection of *NBC1*-adenoviral vectors modified the beating rate of cardiomyocytes (data not shown). Then, we analyzed each gene that encoded a distinct cardiomyocyte phenotype by developing gene-targeted mouse models.

In vivo analysis using transgenic and knockout mice

To study the *in vivo* role of the selected genes, we developed genetically modified mice: three transgenic (Tg) mice for *Mylk3*, *Gpr37l1*, or *Nbc1* and three knockout (KO) mice for *Gpr37l1*, *Gpr35*, or *Mmp23*. We estimated hemodynamic parameters using Miller catheter and the heart weight (HW)/body weight (BW). As shown in Fig. 2A, we found that the blood pressure of *Gpr37l1*-KO mice was significantly higher than that of *Gpr37l1*-Tg mice by 61.7 mmHg ($p < 0.01$). Further, the blood pressure of *Gpr35*-KO mice was higher than that of wild type (WT) littermate by 37.5 mmHg ($p < 0.01$). Overexpression with or knockout of *Mylk3*, *Mmp23*, or *Nbc1* did not result in a significant change in the systolic blood pressure. The HW/BW of *Mylk3*-Tg mice was lower than that of *Mylk3*-WT mice (Fig. 2B). The HW/BW was higher in *Gpr37l1*-KO mice than in *Gpr37l1*-Tg mice. The HW/BW in mice with *Nbc1*, *Gpr35*, or *Mmp23* manipulations did not show any difference. These data showed that modification of *Gpr37l1*, *Gpr35*, or *Mylk3* can produce a distinct cardiovascular phenotype *in vivo*.

Discussion

The present study identified heart failure-related genes using a novel strategy that was different from the conventional microarray analysis approach. Firstly, we constructed global gene expression profiles to analyze the gene expression in 12 human samples of failing myocardium and two samples of non-failing myocardium. Secondly, we prepared datasets of heart failure-related genes associated with the severity of heart failure; this approach is unique to our study and has not been published before. Thirdly, we selected four genes from these datasets by microarray analysis and a literature-based search. We also included four orphan GPCR genes and four other genes with high expression in the heart as possible drug targets for heart failure treatment. Fourthly, we screened the *in vitro* functions of these 12 genes by achieving adenovirus-mediated overexpression of these genes in rat cardiomyocytes. Finally, we generated gene-targeted mouse models of the five selected genes and screened the *in vivo* functions of these genes. Our novel strategy using a microarray analysis revealed three potential targets, namely, *MYLK3*, *GPR37L1*, and *GPR35* for diagnosing and managing heart failure.

End-stage heart failure caused by a variety of cardiovascular diseases including hypertension, cardiomyopathy, and ischemic heart disease features a common phenotype of reduced cardiac function and dilated cardiac chamber. This result strongly suggested the existence of common genes during the development of heart failure, including the genes encoding natriuretic peptides. To identify novel diagnostic or therapeutic targets for heart failure, such as natriuretic peptides, several microarray analyses of genes expressed in failing myocardium have been performed in the last decade by comparing the gene expression levels between different pairs of samples, such as non-failing versus failing hearts [4–6], failing hearts before versus after placement of left-ventricular assisting device [7,8], hypertrophic versus failing hearts [9], ischemic versus non-ischemic hearts [10]. However, the severity of heart failure is not fixed, but varies from mild to severe heart failure in these studies. To identify the therapeutic targets for heart failure effectively, we believe that it is important to consider the severity of heart failure with microarray data analysis. In this study, we prepared new datasets of heart failure-associated genes that were selected from gene expression profiles of 12 human failing myocardial samples using clinical parameters. A number of genes were associated with PAP, which is an index for the severity of heart failure, whereas only two genes correlated with EF, which is an index for cardiac contractility. This result implies that the stress caused to the heart, and not the ability of cardiac contraction, regulates gene expression in heart failure. We also selected heart failure-related genes whose expression correlated to

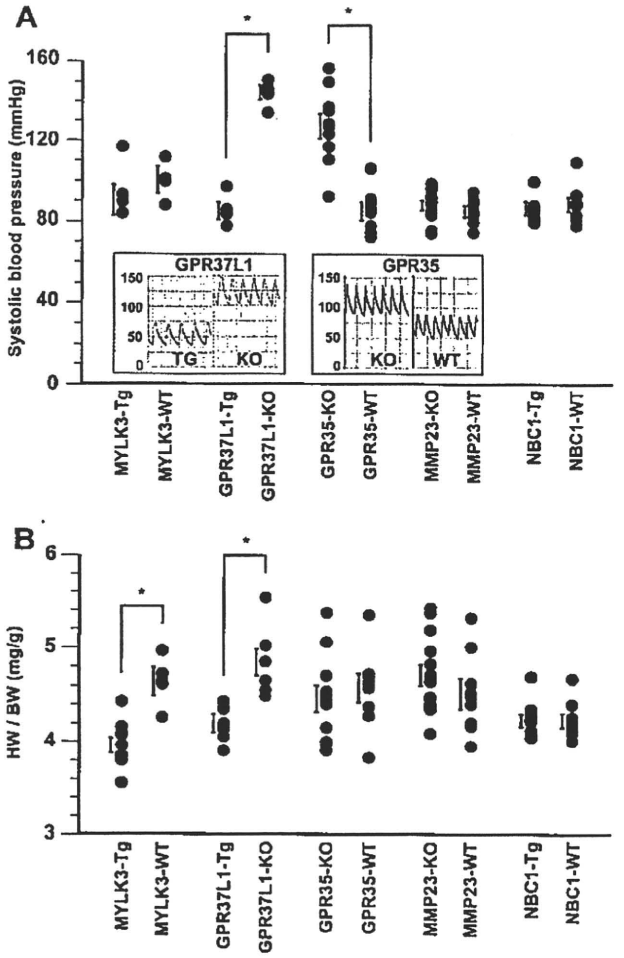

Fig. 2. *In vivo* functional analysis using gene-targeting mice of the *Mylk3*, *Gpr37l1*, *Gpr35*, *Mmp23*, and *Nbc1* genes. Blood pressure and heart weight (HW)/body weight (BW) of transgenic (Tg), knockout (KO) and their wild type (WT) littermate mice of each gene were investigated. Values are means ± SEM. $^{*}p < 0.01$. (A) Systolic blood pressure measured using Millar catheter inserted via right carotid artery. The monitoring chart shows representative data of *Gpr37l1*- and *Gpr35*-manipulated mice. (B) HW/BW ratio of each gene-targeting mouse.

the BNP mRNA level, which is the best known indicator of heart failure. The approach used in our study can help in efficient identification of the diagnostic or therapeutic targets for heart failure rather than only comparing two types of samples such as failing versus non-failing myocardium. Among the genes from these new datasets, we focused on the genes exhibiting high expression in heart tissues and finally selected four genes for performing the screening of functional analysis *in vitro*. The expression level of *MYLK3* gene was highly correlated to PAP, and this gene was detected only in the heart tissue. Recently, we reported that *MYLK3* plays a crucial role in sarcomere assembly via phosphorylation of myosin regulatory light chain 2V (MLC2v) [13]. We also showed that the knockdown of *MYLK3* by using a morpholino oligo caused immature sarcomere formation leading to ventricular dilation in zebrafish. These results indicate that *MYLK3* is strongly associated with the pathophysiology of heart failure. Chan et al. also reported that MYLK3 phosphorylates MLC2v and regulates sarcomere organization [15]. These reports affirm the reliability of our original strategy that involves the microarray analysis of failing myocardium. Among these genes, most genes including *XPR1*, *PRDX4*, and *SMOC2* have not been reported to link with cardiovascular phenotypes and were not included in many gene expression profiles published previously.

Next, we performed *in vivo* functional analysis of five selected genes, and we found that gene-targeted mouse models of *Mylk3*, *Gpr37l1*, or *Gpr35* showed the cardiovascular phenotype. As described above, *Mylk3* plays a crucial role in failing heart. In this study, we identified two GPCRs, namely, *Gpr37l1* and *Gpr35*, whose modification affects systolic blood pressure or HW/BW. To our knowledge, this is the first report about the role of these genes in cardiovascular system.

GPCRs constitute one of the largest protein families, but many GPCRs remain to be orphaned. GPR35 is now known to have some ligands such as kynurenic acid (KYNA) [16], zaprinast [17], and 5-nitro-2-(3-phenylpropylamino) benzoic acid [18]. These agonists mobilize intracellular calcium concentration. Therefore, lowering systolic blood pressure in *Gpr35*-KO mice can be induced by modulating calcium release from calcium-storing organelles. Among the three agonists, only KYNA is produced endogenously as a metabolite of tryptophan. Although GPR35 gene expression is supposed to be specific to immune cells and gastrointestinal tract, we found that GPR35 gene expression increased in failing myocardium. In an inflammatory state, interferon γ induces idolemine 2,3-dioxygenase, a rate-limiting enzyme involved in tryptophan degradation, resulting in a substantial increase in KYNA. Inflammation is thought to be involved in the pathogenesis of dilated cardiomyopathy as well as myocardial infarction. Hence there is a possibility that a KYNA-GPR35 signaling plays a role in the pathogenesis of cardiovascular diseases.

Unlike GPR35, GPR37L1 is still orphaned. However, we found that *Gpr37l1*-KO mice showed significant high blood pressure and high HW/BW as compared to Tg mice, which implies the existence of cardiovascular-related function of *Gpr37l1*. Identification of the ligand and the function of this orphan receptor are awaited.

Although no significant phenotype was observed in *Mmp23* and *Nbc1*-Tg mice, we have been investigating their cardiac function in pathological condition such as myocardial infarction or hypertension and determined their detrimental effect on heart failure (data not shown).

In the present study, we determined 12 novel heart failure-related genes by integrating an original method with parameters that indicated disease severity. Further, we assessed these possible targets of drug discovery. *MYLK3*, *GPR37L1*, and *GPR35* were the newly identified targets that play an interesting role in the cardiovascular system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.076.

References

[1] Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. The SOLVD Investigators, N. Engl. J. Med. 325 (1991) 293–302.
[2] Effect of metoprolol CR/XL in chronic heart failure: metoprolol CR/XL randomised intervention trial in congestive heart failure (MERIT-HF), Lancet 353 (1999) 2001–2007.

[3] M. Packer, M.R. Bristow, J.N. Cohn, W.S. Colucci, M.B. Fowler, E.M. Gilbert, N.H. Shusterman, The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group, N. Engl. J. Med. 334 (1996) 1349–1355.
[4] J. Yang, C.S. Moravec, M.A. Sussman, N.R. DiPaola, D. Fu, L. Hawthorn, C.A. Mitchell, J.B. Young, G.S. Francis, P.M. McCarthy, M. Bond, Decreased SLIM1 expression and increased gelsolin expression in failing human hearts measured by high-density oligonucleotide arrays, Circulation 102 (2000) 3046–3052.
[5] J.D. Barrans, P.D. Allen, D. Stamatiou, V.J. Dzau, C.C. Liew, Global gene expression profiling of end-stage dilated cardiomyopathy using a human cardiovascular-based cDNA microarray, Am. J. Pathol. 160 (2002) 2035–2043.
[6] F.L. Tan, C.S. Moravec, J. Li, C. Apperson-Hansen, P.M. McCarthy, J.B. Young, M. Bond, The gene expression fingerprint of human heart failure, Proc. Natl. Acad. Sci. USA 99 (2002) 11387–11392.
[7] B.C. Blaxall, B.M. Tschannen-Moran, C.A. Milano, W.J. Koch, Differential gene expression and genomic patient stratification following left ventricular assist device support, J. Am. Coll. Cardiol. 41 (2003) 1096–1106.
[8] J.L. Hall, E.J. Birks, S. Grindle, M.E. Cullen, P.J. Barton, J.E. Rider, S. Lee, S. Harwalker, A. Mariash, N. Adhikari, N.J. Charles, L.E. Felkin, S. Polster, R.S. George, L.W. Miller, M.H. Yacoub, Molecular signature of recovery following combination left ventricular assist device (LVAD) support and pharmacologic therapy, Eur. Heart J. 28 (2007) 613–627.
[9] J. Rysa, H. Leskinen, M. Ilves, H. Ruskoaho, Distinct upregulation of extracellular matrix genes in transition from hypertrophy to hypertensive heart failure, Hypertension 45 (2005) 927–933.
[10] M.M. Kittleson, S.Q. Ye, R.A. Irizarry, K.M. Minhas, G. Edness, J.V. Conte, G. Parmigiani, L.W. Miller, Y. Chen, J.L. Hall, J.G. Garcia, J.M. Hare, Identification of a gene expression profile that differentiates between ischemic and nonischemic cardiomyopathy, Circulation 110 (2004) 3444–3451.
[11] A.S. Barth, R. Kuner, A. Buness, M. Ruschhaupt, S. Merk, L. Zwermann, S. Kaab, E. Kreuzer, G. Steinbeck, U. Mansmann, A. Poustka, M. Nabauer, H. Sultmann, Identification of a common gene expression signature in dilated cardiomyopathy across independent microarray studies, J. Am. Coll. Cardiol. 48 (2006) 1610–1617.
[12] M. Asakura, M. Kitakaze, Global gene expression profiling in the failing myocardium, Circ. J. 73 (2009) 1568–1576.
[13] O. Seguchi, S. Takashima, S. Yamazaki, M. Asakura, Y. Asano, Y. Shintani, M. Wakeno, T. Minamino, H. Kondo, H. Furukawa, K. Nakamaru, A. Naito, T. Takahashi, T. Ohtsuka, K. Kawakami, T. Isomura, S. Kitamura, H. Tomoike, N. Mochizuki, M. Kitakaze, A cardiac myosin light chain kinase regulates sarcomere assembly in the vertebrate heart, J. Clin. Invest. 117 (2007) 2812–2824.
[14] M. Asakura, M. Kitakaze, S. Takashima, Y. Liao, F. Ishikura, T. Yoshinaka, H. Ohmoto, K. Node, K. Yoshino, H. Ishiguro, H. Asanuma, S. Sanada, Y. Matsumura, H. Takeda, S. Beppu, M. Tada, M. Hori, S. Higashiyama, Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy, Nat. Med. 8 (2002) 35–40.
[15] J.Y. Chan, M. Takeda, L.E. Briggs, M.L. Graham, J.T. Lu, N. Horikoshi, E.O. Weinberg, H. Aoki, N. Sato, K.R. Chien, H. Kasahara, Identification of cardiac-specific myosin light chain kinase, Circ. Res. 102 (2008) 571–580.
[16] J. Wang, N. Simonavicius, X. Wu, G. Swaminath, J. Reagan, H. Tian, L. Ling, Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35, J. Biol. Chem. 281 (2006) 22021–22028.
[17] Y. Taniguchi, H. Tonai-Kachi, K. Shinjo, Zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, is an agonist for GPR35, FEBS Lett. 580 (2006) 5003–5008.
[18] Y. Taniguchi, H. Tonai-Kachi, K. Shinjo, 5-Nitro-2-(3-phenylpropylamino)-benzoic acid is a GPR35 agonist, Pharmacology 82 (2008) 245–249.

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ORIGINAL CONTRIBUTION

A histamine H_2 receptor blocker ameliorates development of heart failure in dogs independently of β-adrenergic receptor blockade

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Abstract Histamine has a positive inotropic effect on ventricular myocardium and stimulation of histamine H_2 receptors increases the intracellular cAMP level via Gs protein, as dose stimulation of β-adrenergic receptors, and worsens heart failure. To test whether a histamine H_2 receptor blocker had a beneficial effect in addition to β-adrenergic receptor blockade, we investigated the cardioprotective effect of famotidine, a histamine H_2 receptor blocker, in dogs receiving a β-blocker. We induced heart failure in dogs by rapid ventricular pacing (230 beats/min). Animals received no drugs (control group), famotidine (1 mg/kg daily), carvedilol (0.1 mg/kg daily), or carvedilol plus famotidine. Both cardiac catheterization and echocardiography were performed before and 4 weeks after the initiation of pacing. Immunohistochemical studies showed the appearance of mast cells and histamine in the myocardium after 4 weeks of pacing. In the control group, the left ventricular ejection fraction (LVEF) was decreased after 4 weeks compared with before pacing (71 ± 2 vs. $27 \pm 2\%$, $p < 0.05$) and mean pulmonary capillary wedge pressure (PCWP) was increased (8 ± 1 vs. 19 ± 3 mmHg). Famotidine ameliorated the decrease of LVEF and increase of PCWP, while the combination of carvedilol plus famotidine further improved both parameters compared with the carvedilol groups. These beneficial effects of famotidine were associated with a decrease of the myocardial cAMP level. Histamine H_2 receptor blockade preserves cardiac systolic function in dogs with pacing-induced heart failure, even in the presence of β-adrenergic receptor blockade. This finding strengthens the rationale for using histamine H_2 blockers in the treatment of heart failure.

Keywords Heart failure · Histamine · Histamine H_2 receptor blocker · β-Adrenergic receptor blocker

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Introduction

Chronic heart failure (CHF) is one of the major causes of morbidity and mortality worldwide, and is characterized by neurohormonal imbalances that include activation of the sympathetic nervous system [9, 15]. β-Adrenergic receptor blockade is a established treatment of CHF because it protects the heart from the harmful effects of the sympathetic nervous system that are partly mediated via cyclic adenosine monophosphate (cAMP)-dependent pathways [2, 34]. Interestingly, histamine H_2 receptors are linked to Gs proteins that facilitate the production of cAMP (as are β-adrenergic receptors) and are expressed in the heart [18, 29, 33]. Histamine has a positive inotropic effect on human ventricular myocardium and chronotropic effects [3, 12], and also autonomic control of the heart [21]. Indeed, we previously reported that famotidine, a histamine H_2

receptor blocker, protected the heart against ischemia-reperfusion injury in dogs [1] and also improved both symptoms of CHF and ventricular remodeling in the clinical setting [16]. Although the maximum inotropic effects of substances acting through cAMP were decreased in diseased myocardium [6], famotidine, a histamine H_2 receptor blocker, exerts negative effects on cardiac performance [13], the roles of the histamine would have remained unclear in the state of heart failure.

In addition, it is still unclear whether histamine H_2 receptor blockers have a protective effect against CHF by reducing the myocardial accumulation of cAMP and whether there is an additive effect of histamine H_2 receptor blockade in the presence of β-adrenergic receptor blockade.

Therefore, we investigated the effect of a histamine H_2 receptor blocker on cardiac performance and myocardial cAMP accumulation in dogs with pacing-induced heart failure, and also investigated whether there was an additive effect of combined histamine H_2 receptor blocker and β-blocker therapy on cardiac performance.

Methods

Materials

The histamine H_2 receptor blocker famotidine was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Carvedilol, a β-adrenergic receptor blocker, was obtained from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-histamine antibody was obtained from Progen (Queensland, Australia).

Animal preparation

Beagle dogs (Oriental Yeast Co. Ltd., Tokyo, Japan) weighing 8–10 kg were sedated with intravenous sodium pentobarbital at a dose of 25 mg/kg. After intubation with a cuffed endotracheal tube, anesthesia was maintained with 0.5–1% isoflurane and an equal mixture of air and oxygen. Ventilation was provided with a tidal volume of 22 mL/kg at a rate of 15 times per minute. A bipolar pacing lead (Model BT-45P, Star Medical Inc., Tokyo, Japan) was advanced under fluoroscopic guidance through the right jugular vein to the right ventricular (RV) apex and was connected to an external programmable pacemaker (VOO mode; Model SIP-501, Star Medical Inc., Tokyo, Japan) that was implanted in a subcutaneous pocket in the neck. The success of this procedure was confirmed by electrocardiography. Antibiotics were given after surgery, and the dogs were allowed to recover fully. Then heart failure was induced by rapid right ventricular pacing at a rate of 230 beats/min for 4 weeks as the model mimicking heart failure in human, as reported previously [22, 23, 27].

All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, 1996 revision) and were approved by the ethical committee for laboratory animal use of the National Cardiovascular Center in Japan.

Echocardiography

Transthoracic echocardiography was performed by using an echocardiographic system equipped with a 2–4 MHz phased-array transducer (SONOS 5500, Hewlett Packard, Massachusetts, USA) in conscious dogs before pacemaker implantation and 30 min after the cessation of RV pacing at 4 weeks. Good two-dimensional short-axis views of the left ventricle were obtained at the level of the papillary muscles for guided M-mode measurement of the left ventricular (LV) end-diastolic dimension (LVDd), LV end-systolic dimension (LVDs), LV fractional shortening (LVFS), and LV ejection fraction (LVEF). All measurements were made by two observers, who were blinded to the source of the tracings.

Hemodynamic studies

LV pressure and mean aortic pressure were measured by pressure amplifiers connected to a pig tail catheter (5F, Terumo Co. Ltd., Tokyo, Japan) that was inserted into the left ventricle from the left femoral artery. Pulmonary capillary wedge pressure (PCWP) was measured with a 7 Fr Swan-Ganz catheter (American Edwards Laboratories, California, USA). LV dP/dt was analyzed using software (Data viewer, Yokogawa Electric Corporation, Tokyo, Japan). These studies were performed both before and after 4 weeks of RV pacing or 4 weeks after pacemaker implantation in the sham group.

Measurement of the myocardial cAMP level

The myocardial cyclic AMP (cAMP) level was measured as described previously [8]. Briefly, a sample of frozen cardiac muscle was homogenized mechanically in 500 mL of frozen hydrochloric acid (0.1 N) with a mechanical homogenizer. The homogenate was thawed and centrifuged at $5{,}000 \times g$ at room temperature for 15 min and then a 100 mL aliquot of the supernatant was employed to measure cAMP with a sensitive radioimmunoassay (cyclic AMP kit; Yamasa Shoyu Co., Choshi, Japan).

Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously [24]. Briefly, myocardial tissue samples were fixed in 10% formalin and embedded in paraffin. Then

5-μm-thick sections were cut and preincubated with 3% hydrogen peroxide. Rabbit polyclonal anti-histamine antibody (1:1,000 dilution) was added, and incubation was done at room temperature overnight. Next, the sections were incubated with biotinylated anti-rabbit immunoglobulin for 30 min and subsequently with horseradish peroxidase-labeled streptavidin solution for 30 min. The slides were rinsed in tris-buffered saline after each incubation step. Peroxidase activity was visualized by incubation with diaminobenzidine hydrochloride solution.

Experimental protocols

Protocol 1: effects of famotidine on cardiac performance and myocardial cAMP accumulation in dogs with pacing-induced heart failure

After pacemaker implantation, the dogs were randomly assigned to a sham group ($n = 6$) without pacing, a control group ($n = 7$) with pacing only, and a famotidine group ($n = 5$) with pacing plus the daily oral administration of famotidine (1 mg/kg per day). The dose of famotidine was chosen on the basis of previous reports [30, 36]. Echocardiography and measurement of hemodynamic parameters were performed before and 4 weeks after pacemaker implantation. After the measurement of hemodynamic parameters, myocardial tissue samples were obtained and quickly placed into liquid nitrogen for storage at −80°C until measurement of cAMP levels.

Protocol 2: effects of famotidine on cardiac performance in dogs with pacing-induced heart failure under β-adrenergic receptor blockade

Next, we examined the additive effect of histamine H_2 receptor blockade on the development of CHF. After pacemaker implantation, the dogs were randomly assigned to a carvedilol group ($n = 6$) that received daily oral administration of carvedilol (0.1 mg/kg per day) or a carvedilol + famotidine group ($n = 6$) that received daily oral administration of both carvedilol (0.1 mg/kg per day) and famotidine (1 mg/kg per day).

Statistical analysis

Results are expressed as the mean ± SEM. Comparison of time-course changes between the groups was performed by two-way repeated measures analysis of variance (ANOVA). For comparison of mast cell counts and cAMP levels between the groups, the Mann–Whitney U test was performed. A p value < 0.05 was considered to represent statistical significance.

Results

Mast cells and histamine expression

Mast cells were detected in the myocardium by toluidine blue staining. Consistent with previous reports [8, 26], we observed an increase of mast cells in the failing hearts compared with the number of cells in the sham group (Fig. 1a). Immunohistochemical analysis showed an increase of histamine expression indicating increased degranulation of mast cells in failing hearts compared with the level in the sham group (Fig. 1b).

Effect of famotidine on cardiac performance and myocardial cAMP in dogs with pacing-induced heart failure

Both mean aortic pressure and heart rate before pacing were similar in the control group (104 ± 5 mmHg and

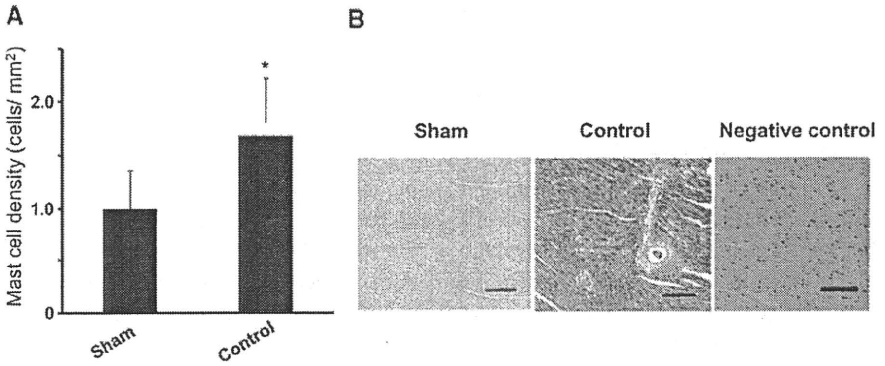

Fig. 1 Mast cell density and histamine expression in the failing heart. **a** Mast cell density in the heart. Values are the mean ± SEM. *$p < 0.05$ versus the sham group. **b** Immunostaining with an anti-histamine antibody. **a** Representative staining of a heart from the sham group. **b** Representative staining of a heart from the control group. **c** Negative control section incubated without the primary antibody. The *scale bars* indicate 50 μm

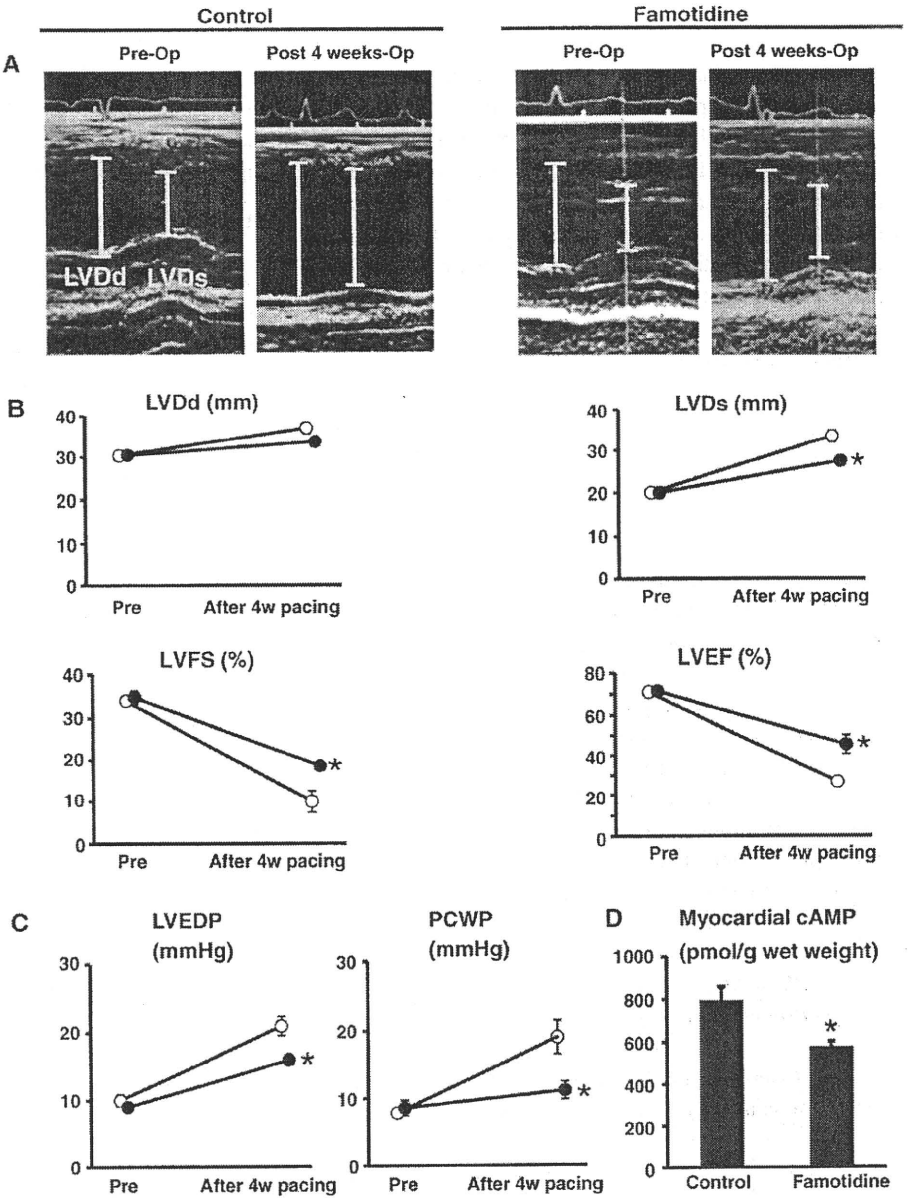

Fig. 2 Effects of famotidine on echocardiographic and hemodynamic parameters and myocardial cAMP levels. **a** Representative 2D echocardiograms. **b** Quantitative analysis of echocardiographic parameters in the control and famotidine groups. *Open* and *closed circles* indicate the control group and the famotidine group, respectively. **c** LVEDP and mean PCWP in the control and famotidine groups. **d** Myocardial cAMP levels in each group. All values are the mean ± SEM. $^*p < 0.05$ versus the sham group

117 ± 7 bpm, respectively), and the famotidine group (101 ± 3 mmHg and 115 ± 8 bpm, respectively). These parameters did not significantly differ among the groups. LV d*P*/d*t* was 3,592 ± 512 mmHg/s in the control group and 3,981 ± 528 mmHg/s in the famotidine group. Four weeks after surgery, neither hemodynamic nor echocardiographic data showed any changes compared with the preoperative values in the sham group (data not shown). Four weeks after rapid RV pacing, an administration of famotidine significantly limited the increase of both LVDd and LVDs, as well as the decrease of both LVFS and LVEF (33.4 ± 0.8 mm, 27.4 ± 1.2 mm, 18.5 ± 2.6% and 45.4 ± 4.8%, respectively), compared with the findings in the control group (37.0 ± 1.4 mm, 33.4 ± 1.4 mm, 9.9 ± 1.0% and 26.7 ± 2.4%, respectively) (Fig. 2a, b). Four weeks after RV pacing, LV end-diastolic pressure (LVEDP) and PCWP of the famotidine group (16 ± 2 and 11 ± 1 mmHg, respectively), were both significantly lower compared with the values in the control group (21 ± 2 and 19 ± 2 mmHg and, respectively) (Fig. 2c). LV d*P*/d*t* after RV pacing was significantly preserved higher in the famotidine group (2,601 ± 216 mmHg/s) compared with that in control group (2,077 ± 124 mmHg/s) ($p < 0.05$).

The myocardial cAMP level was significantly higher in the control group (796 ± 111 pmol/g wet weight) compared with that in the sham group (597 ± 77 pmol/g wet

weight), while it was significantly lower in the famotidine group (577 ± 28 pmol/g wet weight) compared with the control group ($p < 0.05$) (Fig. 2d).

Additive effects of famotidine and a β-blocker on cardiac performance in dogs with pacing-induced heart failure

Before pacing, mean aortic pressure and heart rate were both similar in the carvedilol group (101 ± 7 mmHg and 111 ± 8 bpm, respectively), and the famotidine + carvedilol group (93 ± 2 mmHg, 106 ± 7 bpm, respectively), and these parameters did not significantly differ among the groups. LV d*P*/d*t* was 3,672 ± 417 mmHg/s in the carvedilol group and 3,941 ± 284 mmHg/s in the famotidine + carvedilol group. After rapid RV pacing for 4 weeks, both LVDd and LVDs were decreased and both LVFS and LVEF were increased (33 ± 0.4 mm, 25 ± 0.7 mm, 28 ± 2%, and 54 ± 3%, respectively), in the famotidine + carvedilol group compared with the respective values in the carvedilol group (34 ± 1 mm, 28 ± 1 mm, 23 ± 1%, and 38 ± 5%, respectively) (Fig. 3a).

Four weeks after RV pacing, LVEDP and PCWP of the carvedilol + famotidine group (12 ± 3 and 10 ± 4 mmHg, respectively), were both significantly reduced compared with the respective values in the carvedilol group (16 ± 2 and 15 ± 1 mmHg, respectively) (Fig. 3b). LV d*P*/d*t* after RV pacing was preserved higher in the famotidine + carvedilol group (3,382 ± 252 mmHg/s) compared with that in carvedilol group (2,740 ± 321 mmHg/s) ($p < 0.05$).

Furthermore, the myocardial cAMP level was significantly lower in the carvedilol + famotidine group (488 ± 45 pmol/g wet weight) compared with that in the carvedilol group (615 ± 28 pmol/g wet weight) (Fig. 3c).

Discussion

In the present study, we demonstrated that (1) myocardial histamine expression was increased by pacing-induced heart failure, (2) the histamine H_2 receptor blocker famotidine improved cardiac performance (gauged by echocardiographic and hemodynamic parameters) along with a reduction of myocardial cAMP accumulation, and (3) there was an additive effect of combined histamine H_2 receptor and β-adrenergic receptor blockade on cardiac performance in dogs with pacing-induced heart failure.

Impact of histamine blockade on cardiac failure

Histamine is one of the autacoids, and is stored and released by mast cells in the human heart, as well as in other organs or

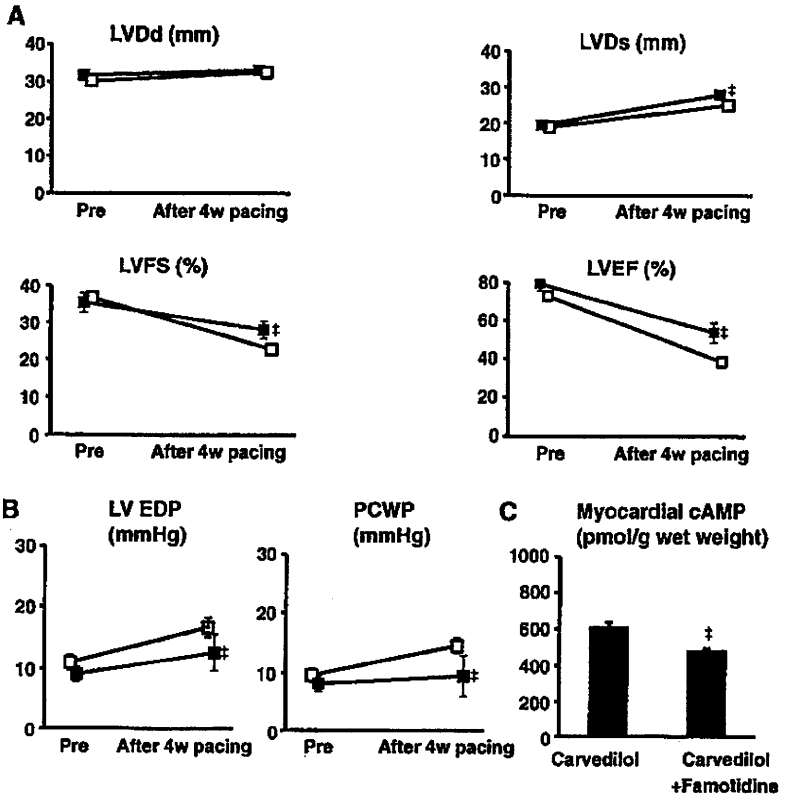

Fig. 3 Additive effect of famotidine and carvedilol on cardiac performance. **a** Quantitative analysis of echocardiographic parameters in the carvedilol and carvedilol + famotidine groups. *Open* and *closed squares* indicate the carvedilol group and the carvedilol + famotidine group, respectively. **b** LVEDP and mean PCWP in the carvedilol group and carvedilol + famotidine group. *Open* and *closed squares* indicate the carvedilol group and the carvedilol + famotidine group, respectively. **c** Myocardial cAMP levels in each group. All values are the mean ± SEM. *$p < 0.05$ versus the carvedilol group

tissues [8]. Recently, the number of mast cells and the myocardial histamine level were found to increase in the hearts of patients with idiopathic dilated cardiomyopathy or ischemic cardiomyopathy [26]. Histamine modulates various cellular functions via the activation of four different G-protein-coupled receptors (H_{1-4} receptors) [29]. As is well known, histamine H_2 receptors located on gastric cells promote the production of gastric acid [10] and histamine H_2 receptor blockers have been widely used for the treatment of peptic ulcer. Interestingly, histamine H_2 receptors are also expressed in canine and human ventricular myocardium [14, 18], although the expression levels were different among species [18]. Consisted with the previous studies, we confirmed the presence of histamine H_2 receptor in the canine heart using quantitative reverse-transcriptase PCR (data not shown).

In the present study, the precise locations of histamine receptors in myocytes or vessels in the canine hearts remained unclear. However, since famotidine did not decrease blood pressure in this study, the accumulating lines of evidence would suggest that histamine H_2 blockers did not have the potent effect on the vessels compared with cardiomyocytes. On the other hand, since histamine H_1 receptors are abundantly expressed in the vessels in most animal species [29], histamine H_1 receptor blocker has the effects on vessels. In addition, stimulation of H_2 receptors transduces the intracellular signals via Gs protein, as does the stimulation of β-adrenergic receptors. Moreover, histamine has a positive inotropic effect on human ventricular myocardium and has been suggested to have a role in cardiovascular diseases [3, 11]. Although it was reported that the maximum inotropic effects of histamine receptor stimulation were less than those mediated by beta-adrenergic receptors [5, 35], the roles of histamine receptor blockade have remained unclear compared with those of beta-adrenergic receptor.

Based on these backgrounds, we previously proposed that histamine H_2 receptor blockers could provide a novel therapeutic strategy for heart failure, and we have reported that histamine H_2 blocker treatment may have a cardioprotective effect in patients with chronic heart failure [16]. In the present study, we found that myocardial histamine expression was increased in dogs with pacing-induced heart failure compared with that in sham dogs on immunohistochemical analysis. Also, the histamine H_2 receptor blocker, famotide, prevented the development of heart failure induced by rapid RV pacing and lessened the myocardial accumulation of cAMP. These findings suggest that histamine H_2 receptor blockade exerts a cardioprotective effect along with the amelioration of myocardial cAMP accumulation. Recently it was reported that increased cardiac adenylyl cyclase expression is associated with mortality after myocardial infarction in rats [31]. It has been controversial for the role of myocardial cAMP in the heart failure [17]. Although increased cAMP acutely induced the improvement of ventricular function, several trials with either beta-adrenergic agonists or PDE inhibitors have revealed an increase in mortality [17, 20]. In the present study, in the dogs with the drugs that decreases myocardial cAMP levels, the development of heart failure was substantially attenuated, however further investigation will be needed to solve the roles of cAMP in the onset and progression of heart failure.

Additive effects of histamine H_2 blocker and β-blocker therapy on cardiac performance

β-Blockers have long been established as useful agents for chronic heart failure [2, 7, 25, 32]. These drugs act by preventing intracellular Ca overload, because β-adrenergic stimulation promotes Ca overload via Gs protein [34]. Histamine H_2 receptor blockade also prevents Ca overload [28], so we hypothesized that the combination of a histamine H_2 receptor blocker and a β-blocker would exert a stronger cardioprotective effect than either agent alone. Almost all of the patients in our earlier study, which showed that histamine H_2 receptor blockers were effective for the treatment of CHF, were also on β-blocker therapy [16], suggesting that there was an additive effect of histamine H_2 receptor and β-adrenergic receptor blockade in patients with CHF. In the present study, we demonstrated that the combination of a histamine H_2 receptor blocker and a β-blocker prevented the development of heart failure compared with β-blocker monotherapy. Thus, histamine H_2 receptor blockers have a potential clinical role in the treatment of CHF.

Rationale of the present study

The present study provided strong experimental evidence that histamine H_2 receptor blockade improves the pathophysiology of CHF. We have already reported about the beneficial effects of famotidine in patients with heart failure [16]. However, clinical research may be confounded by unexpected errors due to (1) the influence of other drugs being used by patients with CHF, (2) variation in the severity of CHF between patients, and (3) variation in the duration of CHF. Therefore, it was important to prove that histamine H_2 receptor blockade improves CHF in a controlled experimental setting (canine cardiomyopathy model) to support the clinical use of famotidine for CHF. Furthermore, to determine the merit of famotidine in heart failure patients, the present study would be a basis to design a prospective randomized double-blinded study.

Limitations

There are several limitations in this study. First, carvedilol blocks alpha$_1$-, beta$_1$-, beta$_2$- receptors, decreased the cardiac effects of norepinephrine, and has additional antioxidant and antiproliferative effects [4, 19]. In the present study, we did not address that carvedilol has the pleiotropic effects and is not just a beta-blocker.

Second, we did not measure cardiac output as a cardiac contractive index in the present study. However, we have previously reported that our tachycardia-induced heart failure model in dogs using the same procedure of the present study, revealed the low output state that mimics heart failure in human [27]. Our untreated dogs in the heart failure group were strongly suggested in the low output state because of decreased the level of ejection fraction as much as our previous study. In the present study, we analyzed the values of dP/dt as the index of contractility by measuring LV pressure using a pig tail catheter.

In summary, despite these limitations, we demonstrated that the histamine H_2 receptor blockade preserves cardiac systolic function in dogs with pacing-induced heart failure, even in the presence of β-adrenergic receptor blockade. This finding strengthens the rationale for the beneficial effects of histamine H_2 blockers in the treatment of heart failure.

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Conflict of Interest None.

References

1. Asanuma H, Minamino T, Ogai A, Kim J, Asakura M, Komamura K, Sanada S, Fujita M, Hirata A, Wakeno M, Tsukamoto O, Shinozaki Y, Myoishi M, Takashima S, Tomoike H, Kitakaze M (2006) Blockade of histamine H_2 receptors protects the heart against ischemia and reperfusion injury in dogs. J Mol Cell Cardiol 40:666–674
2. Bristow MR, Gilbert EM, Abraham WT, Adams KF, Fowler MB, Hershberger RE, Kubo SH, Narahara KA, Ingersoll H, Krueger S, Krueger S, Young S, Shusterman N (1996) Carvedilol produces dose-related improvements in left ventricular function and survival in subjects with chronic heart failure. Circulation 94:2807–2816
3. Bristow MR, Ginsburg R, Harrison DC (1982) Histamine and the human heart: the other receptor system. Am J Cardiol 49:249–251
4. Bristow MR (1997) Mechanism of action of beta-blocking agents in heart failure. Am J Cardiol 80:26L–40L
5. Brodde OE, Hillemann S, Kunde K, Vogelsang M, Zerkoski HR (1992) Receptor systems affecting force of contraction in the human heart and their alterations in chronic heart failure. J Heart Lung Transplant 11:S164–S174
6. Brown L, Lorenz B, Erdmann E (1986) Reduced positive inotropic effects in diseased human ventricular myocardium. Cardiovasc Res 20:516–520
7. CIBIS II Investigators and Committees (1999) The cardiac insufficiency bisoprolol study II (CIBIS II): a randomized trial. Lancet 353:9–13
8. Dvorak AM (1986) Mast-cell degranulation in human hearts. N Engl J Med 315:969–970
9. Eichhorn EJ (1998) Restoring function in failing hearts: the effects of beta blockers. Am J Med 104:163–169
10. Gantz I, Schaffer M, DelValle J, Logsdon C, Campbell V, Uhler M, Yamada T (1991) Molecular cloning of a gene encoding the histamine H_2 receptor. Proc Natl Acad Sci USA 88:5937
11. Hara M, Ono K, Hwang MW, Iwasaki A, Okada M, Nakatani K, Sasayama S, Matsumori A (2002) Evidence for a role of mast cells in the evolution to congestive heart failure. J Exp Med 195:375–381
12. Hattori Y (1999) Cardiac histamine receptors: their pharmacological consequences and signal transduction pathways. Methods Find Exp Clin Pharmacol 21:123–131
13. Hinrichsen H, Halabi A, Kirsch W (1990) Hemodynamic effects of different H_2-receptor antagonists. Clin Pharmacol Ther 48:302–308
14. Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R, Haas HL (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. Pharmacol Rev 49:253–278
15. Jessup M, Brozena S (2003) Heart failure. N Engl J Med 348:2007–2018
16. Kim J, Ogai A, Nakatani S, Hashimura K, Kanzaki H, Komamura K, Asakura M, Asanuma H, Kitamura S, Tomoike H, Kitakaze M (2006) Impact of blockade of histamine H_2 receptors on chronic heart failure revealed by retrospective and prospective randomized studies. J Am Coll Cardiol 48:1378–1384
17. Leineweber K, Bohm M, Heusch G (2006) Cyclic adenosine monophosphate in acute myocardial infarction with heart failure Slayer or savior? Circulation 114:365–367
18. Matsuda N, Jesmin S, Takahashi Y, Hatta E, Kobayashi M, Matsuyama K, Kawakami N, Sakuma I, Gando S, Fukui H, Hattori Y, Levi R (2004) Histamine H_1 and H_2 receptor gene and protein levels are differentially expressed in the hearts of rodents and humans. J Pharmacol Exp Ther 309:786–795
19. Metra M, Giubbini R, Nodari S, Boldi E, Modena MG, Cas LD (2000) Differential effects of beta-blockers in patients with heart failure. Circulation 102:546–551
20. Movsesian MA (1999) Beta-adrenergic receptor agonists and cyclic nucleotide phosphodiesterase inhibitors: shifting the focus from inotropy to cyclic adenosine monophosphatase. J Am Coll Cardiol 34:318–324
21. Nault MA, Milne B, Parlow JP (2002) Effects of the selective H_1 and H_2 histamine receptor antagonists loratadine and ranitidine on autonomic control of the heart. Anesthesiology 96:336–341
22. Neumann T, Heusch G (1997) Myocardial, skeletal muscle, and renal blood flow during exercise in conscious dogs with heart failure. Am J Physiol 273:H2452–H2457
23. Neumann T, Vollmer A, Schaffner TH, Hess OM, Heusch G (1999) Diastolic dysfunction and collagen structure in canine pacing-induced heart failure. J Mol Cell Cardiol 31:179–192
24. Okada K, Minamino T, Tsukamoto Y, Liao Y, Tsukamoto O, Takashima S, Hirata A, Fujita M, Nagamachi Y, Nakatani T,

Yutani C, Ozawa K, Ogawa S, Tomoike H, Hori M, Kitakaze M (2004) Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. Circulation 110:705–712

25. Packer M, Coats AJ, Fowler MB, Fowler MB, Katus HA, Krum H, Mohacsi P, Rouleau JL, Tendera M, Castaigne A, Roecker EB, Schultz MK, DeMets DL, Carvedilol Prospective Randomized Cumulative Survival Study group (2001) Effect of carvedilol on survival in severe chronic heart failure. N Engl J Med 344:1651–1658
26. Patella V, Marino I, Arbustini E, Lamparter-Schummert B, Verga L, Adt M, Marone G (1998) Stem cell factor in mast cells and increased mast cell density in idiopathic and ischemic cardiomyopathy. Circulation 97:971–978
27. Sasaki H, Asanuma H, Fujita M, Takahama H, Wakeno M, Ito S, Ogai A, Asakura M, Kim J, Minamino T, Takashima S, Sanada S, Sugimachi M, Komamura K, Mochizuki N, Kitakaze M (2009) Metformin prevents progression of heart failure in dogs: role of AMP-activated protein kinase. Circulation 119:2568–2577
28. Schultz G, Rosenthal W, Hescheler J (1990) Role of G proteins in calcium channel modulation. Annu Rev Physiol 52:275–292
29. Simons FE (2004) Advances in H_1-antihistamines. N Engl J Med 351:2203–2217
30. Sugiyama A, Satoh Y, Takahara A, Nakamura Y, Shimizu-Sasamata M, Sato S, Miyata K, Hashimoto K (2003) Famotidine does not induce long QT syndrome: experimental evidence from in vitro and in vivo test systems. Eur J Pharmacol 466:137–146
31. Takahashi T, Tang T, Lai C, Roth DM, Rebolledo B, Saito M, Lew WYW, Clopton P, Hammond K (2006) Increased cardiac adenylyl cyclase expression is associated with increased survival after myocardial infarction. Circulation 114:388–396
32. The MERIT-HF Study Group (1999) Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure (MERIT-HF). Lancet 353:2001–2007
33. Trautwein W, Hescheler J (1990) Regulation of cardiac L-type calcium current by phosphorylation and G proteins. Annu Rev Physiol 52:257–274
34. Xiao RP, Cheng H, Zhou YY, Kuschel M, Lakatta EG (1999) Recent advances in cardiac beta(2)-adrenergic signal transduction. Circ Res 85:1092–1100
35. Zeekowski HR, Broede A, Kunde K, Hillemann S, Schafer E, Vogelsang M, Michel MC, Brodde OE (1993) Comparison of the positive inotropic effects of serotonin, histamine, angiotensin II, endothelin and isoprenaline in the isolated human right atrium. Naunyn-Schemiedeberg's Arch Phamacol 347:347–352
36. Zhou R, Moench P, Heran C, Lu X, Mathias N, Faria TN, Wall DA, Hussain MA, Smith RL, Sun D (2005) pH-dependent dissolution in vitro and absorption in vivo of weakly basic drugs: development of canine model. Pharm Res 22:188–192

Spironolactone use at discharge was associated with improved survival in hospitalized patients with systolic heart failure

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Background The RALES trial demonstrated that spironolactone improved the prognosis of patients with heart failure (HF). However, it is unknown whether the discharge use of spironolactone is associated with better long-term outcomes among hospitalized systolic HF patients in routine clinical practice. We examined the effects of spironolactone use at discharge on mortality and rehospitalization by comparing with outcomes in patients who did not receive spironolactone.

Methods The JCARE-CARD studied prospectively the characteristics and treatments in a broad sample of patients hospitalized with worsening HF and the outcomes were followed with an average of 2.2 years of follow-up.

Results A total of 946 patients had HF with reduced left ventricular ejection fraction (LVEF) (<40%), among whom spironolactone was prescribed at discharge in 435 patients (46%), but not in 511 patients (54%). The mean age was 66.3 years and 72.2% were male. Etiology was ischemic in 39.7% and mean LVEF was 27.1%. After adjustment for covariates, discharge use of spironolactone was associated with a significant reduction in all-cause death (adjusted hazard ratio 0.612, $P = .020$) and cardiac death (adjusted hazard ratio 0.524, $P = .013$).

Conclusions Among patients with HF hospitalized for systolic dysfunction, spironolactone use at the time of discharge was associated with long-term survival benefit. These findings provide further support for the idea that spironolactone may be useful in patients hospitalized with HF and reduced LVEF. (Am Heart J 2010;0:1-7.)

Aldosterone plays an important role in the development and progression of chronic heart failure (HF). It induces vascular damage,[1,2] cardiac hypertrophy,[3-5] and fibrosis.[6-9] Higher level of serum aldosterone has been shown to be an independent predictor of increased mortality risk in patients with HF.[10] The RALES demonstrated that spironolactone reduces the risk of mortality and morbidity in patients with HF and systolic dysfunction.[11] Current guidelines from American College of Cardiology/American Heart Association/AHA and European Society of Cardiology recommend the use of spironolactone in HF patients with reduced left ventricular ejection fraction (LVEF) who were symptomatic under the use of angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) and diuretics.[12,13] However, RALES was performed among carefully selected severe HF patients with current or recent HF of New York Heart Association (NYHA) functional class IV. In addition, it excluded the patients with a serum creatinine concentration of >2.5 mg/dL. Moreover, the use of β-blockers was as low as 10% among the patients enrolled in RALES. Therefore, the patients in the RALES were clearly different from those in the "real world" under current standard practice for HF who are more elderly and have more comorbidities including hypertension, diabetes, and renal dysfunction. However, many patients who received new prescriptions for spironolactone after the publication of RALES have been reported not to have severe HF and about one third had renal dysfunction.[14] These findings indicated that the effect of spironolactone on outcomes needed to be assessed in an unselected population of patients with HF.

The JCARE-CARD studied prospectively the characteristics and treatments in a broad sample of patients hospitalized with HF in Japan from January 2004 to June

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2005, and the outcomes including death and rehospitalization were followed until 2008.[15-20] The JCARE-CARD enrolled 2,675 patients admitted with HF in a Web-based registry at 164 participating hospitals with an average of follow-up of 2.2 years.

The aim of the present study was to analyze the prognostic value of spironolactone on the mortality and rehospitalization by evaluating the relationship between discharge use of spironolactone and clinical outcomes among patients hospitalized with systolic HF registered in the JCARE-CARD database.

Methods

Patients

The details of the JCARE-CARD have been described previously.[15-20] Briefly, eligible patients were those hospitalized because of worsening HF as the primary cause of admission. The study hospitals were encouraged to register the patients as consecutively as possible. For each patient, baseline data included (1) age, sex, and body mass index (BMI); (2) causes of HF; (3) medical history; (4) prior procedures; (5) vital signs at discharge; (6) laboratory data at discharge; (7) echocardiographic data; and (8) medication use at discharge. The data were entered using a Web-based electronic data capture system licensed by the JCARE-CARD (www.jcare-card.jp).

From the database of a total cohort of 2,675 patients registered in JCARE-CARD, the present analysis used the data of 946 patients who had systolic dysfunction defined as LVEF <40% and did not have valvular heart disease as a cause of HF. They were divided into 2 groups according to the spironolactone use (n = 435; 46%) or no spironolactone use (n = 511; 54%) at the time of discharge from the index hospitalization.

Outcomes

The status of all patients was surveyed by June 2008 and the following information of the outcomes was obtained from the participating cardiologists by using a Web-based electronic data capture system: (1) all-cause death; (2) cardiac death, defined as death due to HF, myocardial infarction, and other causes such as pulmonary embolism; (3) rehospitalization due to an exacerbation of HF that required more than continuation of their usual therapy on prior admission; and (4) the composite end point of all-cause death and rehospitalization due to HF. The end points were adjudicated by the cardiologists in each participating hospital. Of 946 patients, 99 patients (10.5%) missed during the follow-up were excluded from the follow-up analysis. Follow-up data were obtained in 847 (89.5%) of 946 patients. Of 847 patients, 396 patients were in the group of spironolactone use and 451 patients were in that of no spironolactone use. Mean postdischarge follow-up was 801 ± 300 days (2.2 ± 0.8 years).

The hypothesis being tested was whether spironolactone use at hospital discharge would be associated with lower mortality and rehospitalization rates during the follow-up compared with no spironolactone use.

Statistical analysis

Patient characteristics and treatments were compared using Pearson χ^2 test for categorical variables, Student t test for normally distributed continuous variables, and Mann-Whitney U test for continuous variables not normally distributed. Only patients who survived the index hospitalization were included in the follow-up analysis. Cumulative event-free rates during the follow-up were derived using the method of Kaplan and Meier. The relationship between the spironolactone use at discharge and outcomes was evaluated among patients with multivariable adjustment. The covariates, age, BMI, serum creatinine at discharge, systolic blood pressure at discharge, LVEF, and medication use (calcium channel blocker, antiarrhythmic, warfarin), were used in developing the postdischarge Cox proportional hazard models.

The results were reported as hazard ratio (HR), 95% CI, and P value. Hazard ratio for outcomes when spironolactone was used was compared with not used. A P value of <.05 was used for criteria for variables to stay in the model. SPSS version 16.0 J for Windows (SPSS, Chicago, IL) was used for all statistical analyses.

The JCARE-CARD was funded by the Health Sciences Research Grants from the Japanese Ministry of Health, Labor and Welfare (Comprehensive Research on Cardiovascular Diseases), the Japan Heart Foundation, and Japan Arteriosclerosis Prevention Fund. The authors are solely responsible for the design and conduct of this study, all study analyses, the drafting and editing of the paper, and its final contents.

Results

Patient characteristics

The present study included 946 patients with the mean age of 66.3 ± 13.7 years and 72.2% men (Table I). The causes of HF were ischemic heart disease in 39.6%, dilated cardiomyopathy in 36.3%, and hypertensive heart disease in 21.6%. The mean LVEF was 27.1% ± 7.3%.

Characteristics of patients prescribed spironolactone at discharge and those not receiving it prescription were compared in Table I. Patients discharged with spironolactone had significantly higher BMI. Cause of HF, medical history such as hypertension and diabetes, and treatment procedures such as coronary revascularization did not differ between groups. Systolic blood pressure at discharge was significantly lower in patients with spironolactone use. However, diastolic blood pressure was not different. Estimated glomerular filtration rate was significantly lower and the prevalence of renal dysfunction defined as serum creatinine ≥2.5 mg/dL was greater in patients without spironolactone use. Left ventricular end-diastolic and end-systolic diameters were significantly greater in patients with spironolactone use and LVEF tended to be lower, which, however, did not reach statistical significance.

Use of other medications at hospital discharge was compared between groups in Table II. The use of ACE inhibitor or ARB was as high as 90% in both groups, and that of β-blocker was 65%. Importantly, the use of these guideline-based standard medications was similar between spironolactone use and no spironolactone use groups. However, diuretics, antiarrhythmics, and warfa-

Table I. Patient characteristics

Characteristics	Total (n = 946)	Spironolactone use (n = 435)	No spironolactone use (n = 511)	P
Age (y [mean ± SD])	66.3 ± 13.7	65.2 ± 14.4	67.3 ± 13.1	.052
Male (%)	72.2	73.3	71.2	.472
BMI (kg/m^2)	22.7 ± 4.2	23.0 ± 4.4	22.4 ± 4.0	.043
Causes of HF (%)				
Ischemic	39.6	38.6	40.5	.554
Dilated cardiomyopathy	36.3	39.3	33.7	.072
Hypertensive	21.6	21.1	21.9	.775
Medical history (%)				
Hypertension	50.7	49.1	52.2	.346
Diabetes mellitus	33.1	33.6	32.7	.790
Hyperlipidemia	28.9	28.5	29.2	.826
Hyperuricemia	51.3	49.2	53.0	.245
Prior stroke	13.8	12.9	14.5	.484
COPD	6.0	6.3	5.8	.734
Smoking	46.6	45.1	47.9	.403
Prior myocardial infarction	34.9	35.3	34.5	.797
Atrial fibrillation	24.3	21.7	26.6	.077
Sustained VT/VF	9.2	11.0	7.6	.068
Procedures (%)				
PCI	20.7	20.8	20.6	.950
CABG	11.9	13.3	10.7	.217
ICD	3.8	3.9	3.7	.879
CRT	2.4	3.4	1.6	.061
Vital signs at discharge				
NYHA functional class	1.7 ± 0.6	1.7 ± 0.6	1.7 ± 0.6	.416
NYHA functional class 1or 2 (%)	93.9	94.5	93.3	.468
Heart rate (beat/min)	70.6 ± 12.0	70.6 ± 12.0	70.6 ± 12.0	.988
SBP (mmHg)	113.5 ± 17.1	111.8 ± 17.3	114.9 ± 16.7	.008
DBP (mmHg)	66.2 ± 11.6	65.8 ± 12.1	66.6 ± 11.2	.596
Laboratory data at discharge				
eGFR (mL min^{-1} 1.73 m^{-2})	53.8 ± 24.2	55.9 ± 20.3	52.1 ± 26.9	.017
Serum creatinine ≥2.5 mg/dL	7.9	3.7	11.5	<.001
Hemoglobin (g/dL)	12.9 ± 2.3	13.0 ± 2.1	12.8 ± 2.5	.657
Plasma BNP (pg/mL)	383 ± 534	376 ± 534	388 ± 535	.554
Echocardiographic data				
LV EDD (mm)	61.5 ± 9.4	62.6 ± 9.3	60.6 ± 9.3	.003
LV ESD (mm)	53.0 ± 9.4	54.0 ± 9.4	52.2 ± 9.3	.007
LVEF (%)	27.1 ± 7.3	26.7 ± 7.4	27.5 ± 7.3	.100

Data are shown as percentage or means ± SD.
COPD, Chronic obstructive pulmonary disease; *VT/VF*, ventricular tachycardia/fibrillation; *PCI*, percutaneous coronary intervention; *CABG*, coronary artery bypass grafting; *ICD*, implantable cardioverter defibrillator; *CRT*, cardiac resynchronization therapy; *SBP*, systolic blood pressure; *DBP*, diastolic blood pressure; *eGFR*, estimated glomerular filtration rate; *BNP*, B-type natriuretic peptide; *LV*, left ventricular; *EDD*, end-diastolic diameter; *ESD*, end-systolic diameter; *EF*, ejection fraction.

rin were prescribed more in patients with spironolactone use. On the other hand, calcium channel blocker was used more in patients without spironolactone use.

Postdischarge clinical outcomes according to spironolactone use

During the follow-up of 2.2 years after hospital discharge, the rates of adverse outcomes were as follows: all-cause death 17.8%, cardiac death 11.8%, sudden cardiac death 2.2%, rehospitalization due to the worsening HF 33.4%, and all-cause death or rehospitalization 40.0%. The unadjusted rates of cardiac death were significantly lower in patients with spironolactone use (Table III).

After adjustment for covariates in multivariable Cox proportional hazard models, discharge use of spironolactone, which compared to no spironolactone use, was associated with a reduced risk of all-cause death (HR 0.619, 95% CI 0.413-0.928, P = .020) and cardiac death (HR 0.524, 95% CI 0.315-0.873, P = .013) (Table III and Figure 1). However, spironolactone use was not associated with the risk of rehospitalization due to worsening HF and the combined end point of all-cause death or rehospitalization.

Furthermore, in the subgroup of patients with NYHA functional class I or II, discharge use of spironolactone was associated with a reduced risk of all-cause death (adjusted HR 0.605, 95% CI 0.389-0.940, P = .026) and cardiac death (adjusted HR 0.492, 95% CI 0.276-0.876,

Table II. Medication use at hospital discharge

	Total (N = 946)	Spironolactone use (n = 435)	No spironolactone use (n = 511)	P
ACE inhibitor (%)	44.3	44.6	44.0	.861
ARB (%)	45.6	47.4	44.0	.306
β-Blocker (%)	65.9	66.7	65.2	.628
Diuretics (%)	88.1	100	77.9	<.001
Digitalis (%)	28.8	30.3	27.4	.318
Calcium channel blocker (%)	17.1	11.7	21.7	<.001
Nitrates (%)	22.6	22.3	22.9	.827
Antiarrhythmics (%)	20.9	26.9	15.9	<.001
Aspirin (%)	49.2	48.5	49.7	.713
Warfarin (%)	42.9	46.7	39.7	.032
Statin (%)	23.1	23.7	22.7	.722

Table III. Unadjusted and adjusted HRs for outcomes according to spironolactone use

	Number (%)				
Outcomes	Spironolactone use (n = 396)	No spironolactone use (n = 451)	HR	95% CI	P
All-cause death	59 (14.9%)	92 (20.4%)			
Unadjusted			0.746	0.537-1.035	.078
Adjusted for covariates			0.619	0.413-0.928	.020
Cardiac death	36 (9.1%)	64 (14.2%)			
Unadjusted			0.655	0.435-0.986	.041
Adjusted for covariates			0.524	0.315-0.873	.013
Rehospitalization	125 (31.6%)	158 (35.0%)			
Unadjusted			0.902	0.713-1.141	.389
Adjusted for covariates			0.788	0.592-1.048	.101
All-cause death or rehospitalization	150 (37.9%)	189 (41.9%)			
Unadjusted			0.912	0.735-1.130	.398
Adjusted for covariates			0.820	0.632-1.064	.136

The Cox regression model was used in the analysis adjusted for the following covariates; age, BMI, serum creatinine at discharge, systolic blood pressure at discharge, LVEF, and medication use (calcium channel blocker, antiarrhythmic, warfarin). Patients with no spironolactone use were a reference group.

P = .016) compared to no spironolactone use after adjustment for covariates.

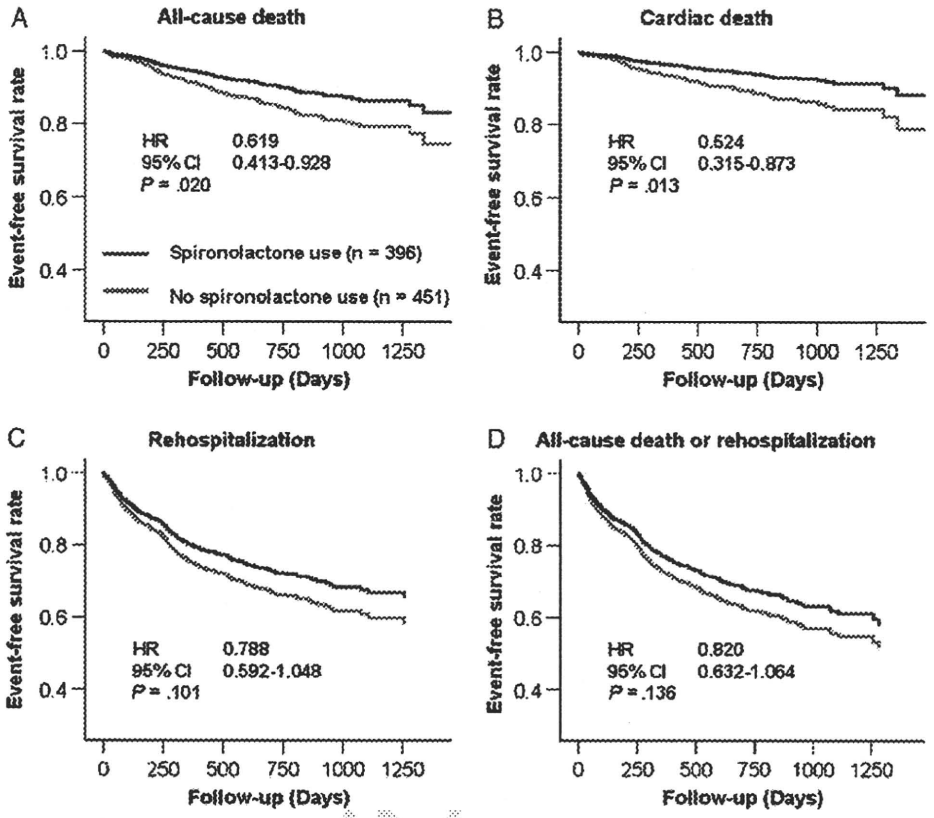
However, in the subgroup patients with serum creatinine ≥2.5 mg/dL (10 patients with spironolactone use and 39 patients with no use), spironolactone use was not significantly associated with the outcomes.

Discussion

The present study suggested that, among patients hospitalized with HF and reduced EF, spironolactone use at discharge was associated with a significant reduction in the risk of cardiac death during the long-term follow-up up to 2.2 years. These findings extended the results of RALES conducted in selected chronic severe HF patients to heterogeneous HF patients with significant survival benefit.

Results from the randomized clinical trial RALES demonstrated that spironolactone significantly improved outcomes in patients with severe HF.[11] RALES enrolled 1,663 patients who had severe HF (NYHA functional class III or IV) and LVEF of no more than 35%. The findings from RALES were further supported by another randomized clinical trial, the EPHESUS, which enrolled 6,632 patients after acute myocardial infarction with LVEF ≤40% and HF.[21] In the EPHESUS, eplerenone, a selective aldosterone antagonist with less adverse effects than spironolactone, reduced the relative risk of death during a mean follow-up of 16 months when added to conventional treatment including ACE inhibitor or ARB and β-blocker. Recent systemic review of 19 randomized clinical trials comprising 10,807 patients demonstrated a 20% reduction in all-cause mortality with the use of aldosterone blockade in clinically heterogeneous groups of patients with LV dysfunction.[22] These studies demonstrated that the addition of aldosterone antagonists in patients with systolic HF and ongoing symptoms despite optimal treatment with ACE inhibition and β-blockers could substantially reduce overall mortality.[23] On the other hand, they found a paucity of evidence on the effects of aldosterone antagonists in patients with diastolic HF or in patients with systolic HF but less severe symptoms.[22]

Figure 1



Kaplan-Meier survival curves free from all-cause death (**A**), cardiac death (**B**), rehospitalization due to worsening HF (**C**), and all-cause death or rehospitalization (**D**) in hospitalized patients with spironolactone use (black lines, n = 396) versus no spironolactone use (red lines, n = 451) at discharge.

More importantly, the patients enrolled in RALES and EPHESUS were recognized as unrepresentative of the general HF population in routine clinical practice. In fact, after the publication of RALES, there was a rapid increase in the rate of prescriptions for spironolactone and in hyperkalemia-associated mortality and morbidity in older patients with HF in Ontario, Canada.[24] This might be explained by the clear difference between the patients in the RALES and those in the "real world" because of the strict inclusion and exclusion criteria that are common to all clinical trials.[25] Furthermore, it may be also due to the recent and rapidly increasing use of β-blockers, which inhibit the release of renin, in patients with HF compared to those enrolled in RALES.[25] Therefore, uncertainty pertaining to the applicability of these findings to the population of patients with HF persists, and it is of critical importance to analyze the registry data of HF patients. The present results extended the previous findings to the "real world" by showing that spironolactone could improve the long-term outcomes in heterogeneous HF patients.

In the present study, >90% of patients had less severe symptom (NYHA functional class I or II) (Table I). The patients with spironolactone use had better renal function and more dilated LV than those with no spironolactone use. According to the European Society of Cardiology and American College of Cardiology/American Heart Association guidelines, the addition of a low-dose aldosterone antagonist should be considered in all patients with a LVEF ≤35% and severe symptomatic HF (NYHA functional class III or IV) unless contraindicated or not tolerated.[12,13] Therefore, in hospitalized patients with severe HF, treatment with an aldosterone antagonist has been recommended to be initiated before discharge.[13] However, published data have suggested that spironolactone was widely used with HF without consideration of their functional class or LVEF and optimization of background treatment with ACE inhibitor and β-blockers.[14] Many patients treated with spironolactone are distinctly dissimilar from those in RALES and the effects of therapy in these patients remain unknown. Therefore, the efficacy of aldosterone

antagonist in patients with reduced LVEF but less severe symptoms needs to be tested by an ongoing large-scale clinical trial, the EMPHASIS-HF trial (ClinicalTrials.gov Identifier NCT00133003), which will enroll 2,584 patients with NYHA functional class II symptoms. The present results suggested that spironolactone use could improve the long-term outcomes in patients with systolic HF and even less severe symptoms (NYHA functional class I or II). These findings should reassure clinicians that the use of spironolactone at discharge can provide an opportunity to improve outcomes for HF patients with severe as well as milder symptoms. Several explanations have been postulated for the beneficial effects of spironolactone in HF. First, spironolactone could induce reverse LV remodeling.[26-28] Spironolactone was demonstrated to improve LV function and decrease plasma BNP levels in patients with chronic HF.[26] In addition, it could also improve exercise tolerance in these patients.[27] Second, spironolactone could decrease cardiac fibrosis.[29] The data from RALES demonstrated that serum procollagen type III amino-terminal peptide (PIIINP) levels, markers of cardiac fibrosis, were significantly higher in HF patients and decreased by the treatment of these patients with spironolactone. Third, spironolactone could improve endothelial function in asymptomatic or mild HF patients when added to optimal treatment including β-blocker.[30]

Study limitations

Several limitations inherent in the design of the registry should be considered. First, the documentation of spironolactone use at hospital discharge might not accurately reflect continuation over time or start after discharge. Moreover, we did not collect the information regarding the dose of spironolactone and whether spironolactone was initiated during or before hospitalization. Second, the information regarding the serum potassium concentration was not obtained in our database. Therefore, we could not assess the impact of hyperkalemia in the outcomes in this study. Third, the present study was not a prospective randomized trial and, despite covariate adjustment, other measured and unmeasured factors may have influenced outcomes. Specifically, severer renal dysfunction, inadequate antiarrhythmic therapy including the use of ICD and antiarrhythmics, and disproportionate use of medications such as calcium channel blockers might affect the outcomes in patients with no spironolactone use, although these confounders were corrected in this study. Fourth, we could not evaluate whether the advantage of spironolactone would persist in the subgroup of renal dysfunction (serum creatinine ≥2.5 mg/dL) because the number of patients was so small for this type of analysis. It thus remained to be assessed exclusively in HF patients associated with renal dysfunction. Finally, data were dependent on the accuracy of documentation and abstraction by individual medical centers that participated in this study. Especially, the end points were adjudicated by the participating cardiologists. Moreover, the present study excluded 10.5% of the overall cohort of patients from the follow-up analysis because end points could not be determined. The patients lost to follow-up might influence the overall outcomes. However, the patient characteristics and medication use at discharge were similar between patients with follow-up and those lost to follow-up except for only 2 variables including history of diabetes (32.8% vs 35.4%, $P = .012$) and diastolic blood pressure (65.9 ± 11.4 vs 68.8 ± 12.8 mmHg, $P = .031$).

Conclusions

Among patients hospitalized for HF and reduced LVEF, treatment with spironolactone at discharge was associated with significantly reduced risk of cardiac death. Widespread use of spironolactone could substantially improve the outcomes in the larger numbers of HF patients in routine clinical practice.

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References

1. Rocha R, Chander PN, Zuckerman A, et al. Role of aldosterone in renal vascular injury in stroke-prone hypertensive rats. Hypertension 1999;33:232-7.
2. Rocha R, Rudolph AE, Frierdich GE, et al. Aldosterone induces a vascular inflammatory phenotype in the rat heart. Am J Physiol Heart Circ Physiol 2002;283:H1802-10.
3. Duprez DA, Bauwens FR, De Buyzere ML, et al. Influence of arterial blood pressure and aldosterone on left ventricular hypertrophy in moderate essential hypertension. Am J Cardiol 1993;71:17A-20A.
4. Navarro-Lopez F, Coca A, Pare JC, et al. Left ventricular hypertrophy in asymptomatic essential hypertension: its relationship with aldosterone and the increase in sodium-proton exchanger activity. Eur Heart J 1993;14(Suppl J):38-41.
5. Schunkert H, Hense HW, Muscholl M, et al. Associations between circulating components of the renin-angiotensin-aldosterone system and left ventricular mass. Heart 1997;77:24-31.

6. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. Circulation 1991;83:1849-65.
7. Young M, Fullerton M, Dilley R, et al. Mineralocorticoids, hypertension, and cardiac fibrosis. J Clin Invest 1994;93:2578-83.
8. Robert V, Van Thiem N, Cheav SL, et al. Increased cardiac types I and III collagen mRNAs in aldosterone-salt hypertension. Hypertension 1994;24:30-6.
9. Campbell SE, Janicki JS, Weber KT. Temporal differences in fibroblast proliferation and phenotype expression in response to chronic administration of angiotensin II or aldosterone. J Mol Cell Cardiol 1995;27:1545-60.
10. Guder G, Bauersachs J, Frantz S, et al. Complementary and incremental mortality risk prediction by cortisol and aldosterone in chronic heart failure. Circulation 2007;115:1754-61.
11. Pitt B, Zannad F, Remme WJ, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med 1999;341:709-17.
12. Hunt SA, Abraham WT, Chin MH, et al. ACC/AHA 2005 guideline update for the diagnosis and management of chronic heart failure in the adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation 2005;112:e154-235.
13. Dickstein K, Cohen-Solal A, Filippatos G, et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur Heart J 2008;29:2388-442.
14. Bozkurt B, Agoston I, Knowlton AA. Complications of inappropriate use of spironolactone in heart failure: when an old medicine spirals out of new guidelines. J Am Coll Cardiol 2003;41:211-4.
15. Tsutsui H, Tsuchihashi-Makaya M, Kinugawa S, et al. Clinical characteristics and outcome of hospitalized patients with heart failure in Japan. Rationale and Design of Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD). Circ J 2006;70:1617-23.
16. Hamaguchi S, Tsuchihashi-Makaya M, Kinugawa S, et al. Chronic kidney disease as an independent risk for long-term adverse outcomes in patients hospitalized with heart failure in Japan. Report from the Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD). Circ J 2009;73:1442-7.
17. Hamaguchi S, Tsuchihashi-Makaya M, Kinugawa S, et al. Anemia is an independent predictor of long-term adverse outcomes in patients hospitalized with heart failure in Japan. A Report From the Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD). Circ J 2009;73:1901-8.
18. Hamaguchi S, Yokoshiki H, Kinugawa S, et al. Effects of atrial fibrillation on long-term outcomes in patients hospitalized for heart failure in Japan. Circ J 2009;73:2084-90.
19. Tsuchihashi-Makaya M, Hamaguchi S, Kinugawa S, et al. Characteristics and outcomes of hospitalized patients with heart failure and reduced vs preserved ejection fraction. Report From the Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD). Circ J 2009;73:1893-900.
20. Tsuchihashi-Makaya M, Furumoto T, Kinugawa S, et al. Discharge use of angiotensin receptor blockers provides comparable effects with angiotensin-converting enzyme inhibitors on outcomes in patients hospitalized for heart failure. Hypertens Res 2010;33:197-202.
21. Pitt B, Remme W, Zannad F, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med 2003;348:1309-21.
22. Ezekowitz JA, McAlister FA. Aldosterone blockade and left ventricular dysfunction: a systematic review of randomized clinical trials. Eur Heart J 2009;30:469-77.
23. Jessup M. Aldosterone blockade and heart failure. N Engl J Med 2003;348:1380-2.
24. Juurlink DN, Mamdani MM, Lee DS, et al. Rates of hyperkalemia after publication of the Randomized Aldactone Evaluation Study. N Engl J Med 2004;351:543-51.
25. McMurray JJ, O'Meara E. Treatment of heart failure with spironolactone—trial and tribulations. N Engl J Med 2004;351:526-8.
26. Tsutamoto T, Wada A, Maeda K, et al. Effect of spironolactone on plasma brain natriuretic peptide and left ventricular remodeling in patients with congestive heart failure. J Am Coll Cardiol 2001;37:1228-33.
27. Cicoira M, Zanolla L, Rossi A, et al. Long-term, dose-dependent effects of spironolactone on left ventricular function and exercise tolerance in patients with chronic heart failure. J Am Coll Cardiol 2002;40:304-10.
28. Chan AK, Sanderson JE, Wang T, et al. Aldosterone receptor antagonism induces reverse remodeling when added to angiotensin receptor blockade in chronic heart failure. J Am Coll Cardiol 2007;50:591-6.
29. Zannad F, Alla F, Dousset B, et al. Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with congestive heart failure: insights from the randomized aldactone evaluation study (RALES). RALES Investigators. Circulation 2000;102:2700-6.
30. Macdonald JE, Kennedy N, Struthers AD. Effects of spironolactone on endothelial function, vascular angiotensin converting enzyme activity, and other prognostic markers in patients with mild heart failure already taking optimal treatment. Heart 2004;90:765-70.

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