室原豊明	Ohshima S, Isobe S, Izawa H, Nanasato M, Ando A, Yamada A, Yamada K, Kato T, Obata K, Noda A, Nishizawa T, Kato K, Nagata K, Okumura K, Murohara T, Yokota M	Cardiac sympathetic dysfunction correlates with abnormal myocardial contractile reserve in dilated cardiomyopathy patients.	J Am Coll Cardiol	46	2061	2005
	Amano T, Matsubara T, Izawa H, Torigoe M, Yoshida T, Hamaguchi Y, Ishii H, Miura M, Hayashi Y, Ogawa Y, Murohara T	Impact of plasma aldosterone levels for prediction of in-stent restenosis	Am J Cardiol	97	785	2006
	Hirashiki A, Izawa H, Somura F, Obata K, Nishizawa T, Yamada A, Asano H, Ohshima S, Noda A, Iino S, Nagata K Okumura K, Murohara T, Yokota M	Prognostic value of pacing-induced mechanical alternans in patients with mild to moderate idiopathic dilated cardiomyopathy in sinus rhythm	J Am Coll Cardiol	47	1382	2006
今中恭子	Nishioka T, Suzuki M, Onishi K, Takakura N, Inada H, Yoshida T, Hiroe M, and Imanaka-Yoshida K	Eplerenone attenuates myocardial fibrosis in the angiotensin II induced hypertensive mouse: Involvement of tenascin-C induced by aldosterone-mediated inflammation	J Cardiovasc Pharmacol	in press		2007
	Terasaki F, Okamoto H, Onishi K, Sato A, Shimomura H, Tsukada B, Imanaka-Yoshida K, Hiroe M, Yoshida T, Kitaura Y, Kitabatake A, and Study Group for Intractable Diseases by a Grant from the Ministry of Health, Labor and Welfare of Japan	Higher serum tenascin-C levels reflect the severity of heart failure, left ventricular dysfunction and remodeling in patients with dilated cardiomyopathy	Circ J	in press		2007

今中恭子	Sato A, Aonuma K, Imanaka-Yoshida K, Yoshida T, Isobe M, Kawase D, Kinoshita N, Yazaki Y, Hiroe M	Serum tenascin-C might be a novel predictor of left ventricular remodeling and prognosis after acute myocardial infarction	J Am Coll Cardiol	47	2319-25	2006
	Hayashi M, Imanaka-Yoshida K, Yoshida T, Wood M, Fearns C, Tatake RJ, Lee JD	A crucial role of mitochondrial Hsp40 in preventing dilated cardiomyopathy	Nat Med	12	128-32	2006
	Watanabe N, Nakagawa M, Hanato T, Takeuchi Y, Hara M, Yoshida T, Imanaka-Yoshida K	In vitro model for mouse coronary vasculogenesis	Anat Rec A Discov Mol Cell Evol Biol	288	714-22	2006
北浦泰	寺崎文生，北浦泰	心臓サルコイドーシスの病理形態	呼吸と循環	54	947-954	2006
	Terasaki F, Okamoto H, Onishi K, et al	Higher serum tenascin-C levels reflect the severity of heart failure, left ventricular dysfunction and remodeling in patients with dilated cardiomyopathy	Circulation Journal	71	in press	2007
	Otsuka K, Terasaki F, Iimori A, et al	Right atrial blood cyst with total occlusion of the right coronary artery	Heart and Vessels	in press		2007
	Suzuki S, Kamihata H, Hata T, et al	Success rate of implantation and mid-term outcomes of the sirolimus-eluting stent	Circulation Journal	71	15-19	2007
	Kurumazuka D, Mori T, Matsumoto N, et al	Gender difference of atorvastatin's vasoprotective effect in balloon-injured rat carotid arteries	Eur J Pharmacol	553	263-268	2006
	Tanaka T, Sohmiya K, Kono T, et al	Thiamine attenuates the hypertension and metabolic abnormalities in CD36-defective SHR: Uncoupling of glucose oxidation from cellular entry accompanied with enhanced protein O-GlcNAcylation in CD36 deficiency	Mol Cell Biochem	in press		2006
	Ito T, Suwa M, Tonari S, et al	Regional postsystolic shortening in patients with hypertrophic cardiomyopathy: Its incidence and characteristics assessed by strain imaging	J Am Soc Echocardiogr	19	987-993	2006
	Tanaka T, Sohmiya K, Kitaura Y, et al	Clinical evaluation of point-of-care-testing of heart-type fatty acid-binding protein (H-FABP) for the diagnosis of acute myocardial infarction	J Immunoassay Immunochem	27	225-238	2006
	Inamoto S, Hayashi T, Tazawa N, et al	Angiotensin-II receptor blocker exerts cardioprotection in diabetic rats exposed to hypoxia	Circulation Journal	70	787-792	2006

北浦 泰	Hozumi T, Ito T, Suwa M, et al	Effects of dual-chamber pacing on regional myocardial deformation in patients with hypertrophic obstructive cardiomyopathy	Circulation Journal	70	63-68	2006
齊藤能彦	Imagawa K, et al	Inhibitory effect of efonidipine on aldosterone synthesis and secretion in human adrenocarcinoma(H295R)cells	J Cardiovasc Pharmaco	47	133-138	2006
	Iwama H, et al	Cardiac expression of placental growth factor predicts the improvement of chronic phase left ventricular function in patients with acute myocardial infarction	J Am Coll Cardiol	47	1559-1567	2006
	Takaoka M, et al	Inflammatory response to acute myocardial infarction augments neointimal hyperplasia after vascular injury in a remote artery	Arterioscler Thromb Vasc Biol	26	2083-2089	2006
	Watanabe M, et al	Usefulness of 16-slice multislice spiral computed tomography for follow-up study of coronary stent implantation	Circ J	70	691-697	2006
	Okayama S, et al	Blocking T-type Ca^{2+} channels with efonidipine decreased plasma aldosterone concentration in healthy volunteers	Hypertens Res	29	493-497	2006
	Nakagawa Y, et al	Class II HDACs mediate CaMK-dependent signaling to NRSF in ventricular myocytes	J Mol Cell Cardiol	41	1010-1022	2006
島田俊夫	Fujiwaki T, et al	Quantitative evaluation of sphingolipids using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry with sphingosylphosphorylcholine as an internal standard Practical application to cardiac valves from a patient with Fabry disease	J Chromatogr B Analyt Technol Biomed Life Sci	832	97 - 102	2006
	Matsumori A, et al	Myocarditis and Heart Failure Associated With Hepatitis C Virus Infection	Journal of Cardiac Failure	12	293-298	2006

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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) ——

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Background Heart failure (HF), defined as a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood, is a leading cause of mortality and hospitalization for adults older than 65 years in the industrialized countries. The characteristics and outcome of patients with HF have been described by several epidemiological studies and large scale clinical trials, performed mainly in the United States and Europe. Very little information is available on this issue in Japan.
Methods and Results The Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD) is designed to prospectively study the characteristics, treatment, and outcomes of a broad sample of patients hospitalized with HF at teaching hospitals throughout Japan between January 2004 to June 2005 and the outcomes, including death and hospital readmission, will be followed through 2006 (mean follow-up at least 1 year). Participating cardiologists identify patients admitted for worsening of HF symptoms. Demographics, medical history, severity, treatment, and outcome data are collected and entered into a database via secure web browser technology. As of June 2005, baseline data for 2,676 patients with HF have been registered from 164 participating hospitals.
Conclusions The JCARE-CARD will provide important insights into the management of patients with HF in routine clinical practice in Japan, thus providing the framework for improved management strategies for these patients. (*Circ J* 2006; **70:** 1617–1623)

Key Words: Heart failure; Management; Outcome; Registry

Heart failure (HF) is defined as a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood, according to the guidelines for the diagnosis and treatment of chronic heart failure of American College of Cardiology/American Heart Association and European Society of Cardiology (ESC)[1,2] The manifestations of HF are dyspnea and fatigue, which may limit exercise tolerance, and fluid retention, which may lead to pulmonary congestion and peripheral edema[1,2] HF is a leading cause of morbidity and mortality in the industrialized countries[3] and is a growing public health problem, mainly because of the aging of the population and the increased prevalence of HF in the elderly[4] The clinical characteristics, treatment, and outcome of these patients have been well described by a number of both community-based[5–7] and hospital-based studies[8–11] as well as by clinical trials of HF treatment[12–14] However, information derived from clinical trials is not necessarily representative of "real world" patients with HF and, moreover, these studies have been performed mainly in the United States and Europe.

Very limited information is available on the characteristics and outcome of patients with HF in Japan[15–17] Our previous studies were the first detailed analysis of the clinical characteristics, management, and outcome, including mortality and HF-related readmission, in Japan[18–20] They demonstrated that HF patients were elderly, comprised more women, especially at higher ages, and had a higher incidence of overt HF despite a relatively normal ejection fraction (EF). As many as 35% of hospitalized patients with HF were readmitted within 1 year of hospital discharge. These characteristics are consistent with those of patient populations in community-based studies reported previously[21,22]

The Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD) has been developed to provide a national prospective registry database describing the clinical characteristics, treatment, and outcomes of patients hospitalized for worsening of HF symptoms. It will also establish the framework for future initiatives to improve the outcomes of these patients. Specifically, this study aimed to determine the influence of clinical characteristics on patient outcomes and further identify the predictive risk of adverse outcomes. This report presents a detailed de-

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Table 1 Framingham Criteria for Heart Failure (HF)

Major criteria
Paroxysmal nocturnal dyspnea
Neck vein distension
Rales
Radiographic cardiomegaly (increased heart size on chest X-ray)
Acute pulmonary edema
S3 gallop
Increased central venous pressure (>16cm water at right atrium)
Circulation time ≥25 s
Hepatojugular reflux
Pulmonary edema, visceral congestion, or cardiomegaly at autopsy
Minor criteria
Bilateral ankle edema
Nocturnal cough
Dyspnea on ordinary exertion
Hepatomegaly
Pleural effusion
Decrease in vital capacity by one-third from maximum value recorded
Tachycardia (rate ≥120 beats/min)
Major or minor criteria
Weight loss ≥4.5 kg in 5 days in response to treatment

The diagnosis of HF was established by the simultaneous presence of at least 2 major criteria or 1 major criterion in conjunction with 2 minor criteria.

scription of the rationale and design of JCARE-CARD.

Methods

Study Design

JCARE-CARD is a multicenter registry designed to compile a large clinical database on the characteristics, management, and outcomes of patients hospitalized for the worsening of HF in Japan. Baseline data are collected during the episode of index hospitalization from January 2004 to June 2005. Follow-up data will be collected at least 1 year after the index admission.

Study Objectives

The specific objectives of the JCARE-CARD include the following: (1) to describe the demographic and clinical characteristics of patients hospitalized with HF in Japan; (2) to describe the in-hospital and long-term outcomes; and (3) to identify the factors, including specific treatments, associated with improved or worsened outcomes.

Study Hospitals

The study hospitals include the cardiology units serving as primary, secondary, and tertiary referral medical centers for cardiovascular patients across Japan. They are authorized as teaching hospitals by the Japanese Circulation Society.

Study Patients

For this registry, HF is defined as a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood. The presence of HF is confirmed by using the Framingham criteria (Table 1)[5] Patients readmitted to hospital during the study period are included only by the first hospitalization (index admission). Patients must be at least 15 years old at the time of hospital admission. Eligibility is not contingent on the use of any particular therapeutic agent or regimen.

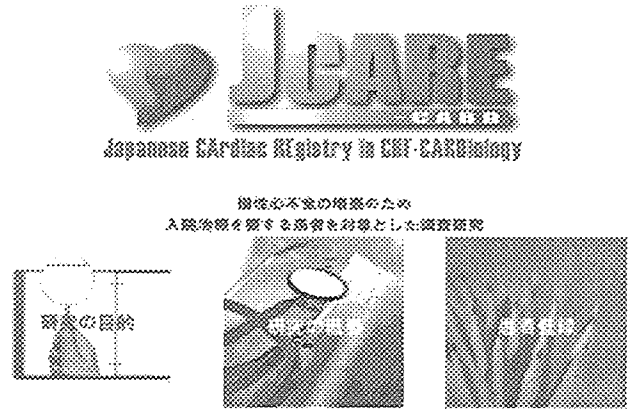

Fig 1. Screen-shot of the top page of the Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD) web site (www.jcare-card.jp).

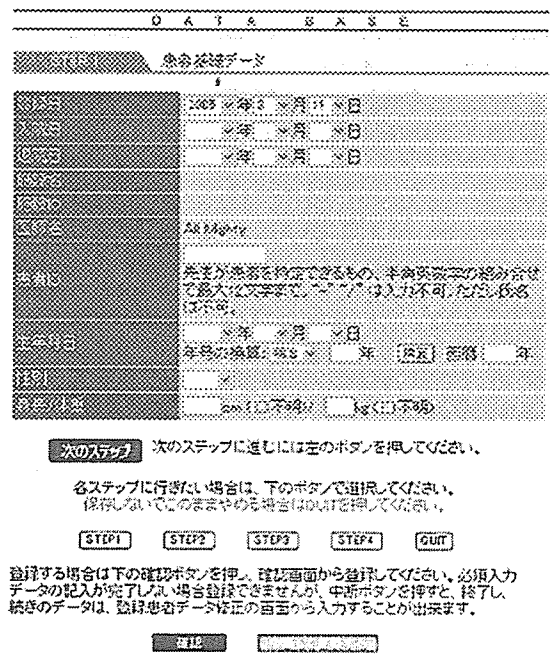

Fig 2. Sample screen-shot of a page of the electronic case report form with sample pull-down menus from the Japanese Cardiac Registry of Heart Failure in Cardiology web site (www.jcare-card.jp).

Data Collection and Processing

Data are entered using a web-based electronic data capture (EDC) system licensed by the JCARE-CARD (www.jcare-card.jp). The EDC system was chosen because of perceived advantages over the traditional, paper-based data entry process, including the ability to inform participating hospitals of missing or illogical data fields at the time of data submission. A study web site has been created with a public area providing general information regarding this study and a registry-site-only area that provides information concerning data registry (Figs 1,2). The study hospitals are encouraged to register the patients as consecutively as possible. The diagnosis of HF is established by the simultaneous presence of at least 2 major criteria or 1 major criterion in conjunction with 2 minor criteria of the Framingham criteria (Table 1). Compliance with these

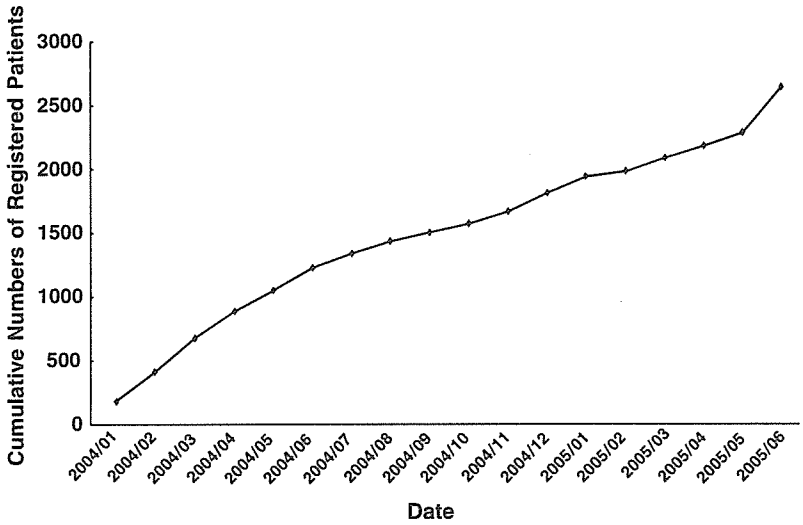

Fig 3. The Japanese Cardiac Registry of Heart Failure in Cardiology cumulative number of registered patients from January 2004 to June 2005.

methods of registry is not strictly monitored.

For each case, baseline data recorded on the form include (1) demography; (2) cause of HF; (3) precipitating cause; (4) comorbidities; (5) complications; (6) clinical status; (7) electrocardiographic and echocardiographic findings; and (8) treatment including discharge medications.

The status of all patients is surveyed at least 1 year after admission and the following information is obtained: (1) survival, (2) cause of death, and (3) hospital readmission because of exacerbation of HF that required more than continuation of the usual therapy on prior admission.

Patient Confidentiality

The JCARE-CARD protocol was organized to ensure compliance with the Guidelines for the Epidemiological Research published by the Japanese Ministry of Health, Labour and Welfare. The original study protocol was approved by the Institutional review board (IRB) at Kyushu University. IRB approval from each participating hospital is also required for participation in this registry. Informed consent is given by each patient. The study does not include any protocol-specified alteration of treatment or any other aspect of hospital care. Patient confidentiality is preserved because direct patient identifiers, such as name, address, and identification number, are not collected. Access to the EDC system at each hospital is carefully controlled by the data management office.

Statistical Analysis

Descriptive statistics are used to summarize baseline characteristics, treatment, and outcomes for the patients and for specific subgroups of interest.

Results

The JCARE-CARD enrolled HF patients from January 2004 to June 2005. As of June 2005, baseline data on 2,676 patients with HF have been registered from 164 participating hospitals (Fig 3, Table 2).

Table 2 Number of Participating Hospitals and Registered Patients Among 8 Regions in Japan

Region	*No. of participating hospitals*	*No. of registered patients*
Hokkaido	*8*	*143*
Tohoku	*7*	*140*
Kanto	*44*	*728*
Hokuriku	*10*	*55*
Tokai	*20*	*499*
Kinki	*31*	*491*
Chugoku · Shikoku	*18*	*239*
Kyushu	*26*	*381*
Total	*164*	*2,676*

Discussion

The characteristics and outcomes of Japanese patients with HF are poorly defined despite the public health importance of this disease. The JCARE-CARD, which aimed to better characterize this population, is the first diverse, large-scale, prospective multicenter database of patients hospitalized for HF in Japan.

We have previously reported the characteristics and outcomes of patients admitted to urban cardiology departments in Fukuoka, Japan[18–20] Those studies highlighted several important features of Japanese patients with HF. One key feature was their advanced age: the mean age of HF patients was 69 years (70% were ≥65 years of age). In particular, women were mostly over 70 years of age, which is consistent with results from previous community-based studies[21,22] Another important feature was the high proportion of patients with relatively preserved EF; that is, half of the patients with definite HF who had echocardiography had normal EF (≥50 %), indicating the contribution of diastolic dysfunction in the pathogenesis of HF[20] A most interesting and important finding was a relatively good survival prognosis for the study patients; the 1-year mortality rate was 8.3 %. Survival prognosis for patients with decreased EF (<40%) was still good; their 1-year mortality rate was 9.1%. At the first glance, this finding appears to contradict the generally held notion that advanced age and more comorbidity are related to poor survival[18] In contrast to the relatively low mortality, rates of readmission for HF were as high as 40% within 1 year after discharge. This is comparable to the rates found in prior studies (3–6-month readmission rate of 30–50%)[23,24] and the most commonly identified cause for hospital readmission was lack of compliance with

medical and dietary treatment (48%)[19]

Even though our previous studies gave a valuable insight into the clinical characteristics, outcomes, and the potential effective treatment strategies for HF patients in Japan,[18–20] generalization of these results is questioned because our investigation involved a small number of patients (n=230). Therefore, it is of critical importance to analyze the data of HF patients in routine clinical practice on a national basis and to form a database for future investigations. For this purpose, JCARE-CARD is designed to focus on the demographic and clinical characteristics, treatment strategies, and outcomes of patients admitted to hospitals throughout Japan. It is important to consider the JCARE-CARD in the context of other large-scale databases such as the Acute Decompensated Heart Failure National Registry (ADHERE) or EuroHeart that have been established to evaluate epidemiologic and clinical aspects of HF[8,10,11] These administrative data sets have provided important insights concerning the prognostic and public health role of a number of classic epidemiologic factors, as well as information on medication use. The JCARE-CARD is expected to provide us with important information regarding the characteristics, treatment, and outcomes of HF patients in Japan, which may be complementary to that gathered from the studies in Europe and the USA. This information is often critical to our understanding of the clinical characteristics of HF, including independent prognostic predictors.

There have been 2 large-scale registries of HF reported: the EuroHeart Failure Survey from Europe and ADHERE from the USA. The EuroHeart Failure Survey registered 11,304 HF patients in departments of cardiology, cardiovascular surgery, general internal medicine and geriatrics at 115 hospitals, including both general hospitals and university centers from 24 ESC member countries over a 6-week period during March 2000 and May 2001[9–11] Patients were enrolled as HF if they fulfilled at least 1 of the following criteria: (1) clinical diagnosis of HF during the admission; (2) diagnosis of HF recorded at any time in the last 3 years; (3) administration of a loop diuretic for any reason other than renal failure during the 24 h prior to death or discharge; (4) pharmacological treatment for HF or ventricular dysfunction within 24 h of death or discharge. The Euro Heart Failure Survey described the quality of care, and the diagnostic and therapeutic management of patients with HF in Europe. Outcome was further assessed by repeat interviews in 6–12 months[25,26]

The ADHERE is a registry designed to study the characteristics, management, and outcomes in a broad sample of patients hospitalized with acute decompensated HF throughout the USA[8] Participating hospitals identify patients with a primary or secondary discharge diagnosis of HF. Medical history, management, treatment, and outcome data are collected through review of medical records and entered into a database via secure web browser technology. Of available data (105,388 patients from 274 hospitals), the mean age was 72.4 years old, and 52% were women. The most common comorbid conditions were hypertension (73%), coronary artery disease (57%), and diabetes (44%). Evidence of mild or no impairment of systolic function was found in 46% of patients. In-hospital mortality was 4.0%. The ADHERE data provided important insights into the clinical characteristics and patterns of care of these patients. Similar to our previous studies,[20] the ADHERE demonstrated that many patients hospitalized with HF had mild or no impairment of systolic ventricular function[27] These registry data demonstrate significant differences in the definition of HF between patients hospitalized for HF and those enrolled in randomized clinical trials[28]

Even though JCARE-CARD and ADHERE share many similarities in their design and rationale, there are several important differences between them. Follow-up data were not obtained in the ADHERE, so the subsequent clinical outcomes, including death and readmission of patients after the index hospitalization, are unknown. Data are gathered retrospectively after hospital discharge in the ADHERE, which may preclude prospective analysis of particular treatments in these patients.

Study Limitations

Several crucial limitations inherent in the design of the JCARE-CARD should be considered. First, the data are based on the decisions made by the participating cardiologists. The lack of a precise, universal definition of HF makes this type of registry open to many criticisms. However, it is not the objective of this survey to restrict enrollment to the narrowly defined population of HF usually included in clinical trials, but rather to include a broad range of patients reflecting the current reality of clinical practice. All participating hospitals are authorized as teaching hospitals by the Japanese Circulation Society. In addition, the information regarding the study protocol was regularly provided at national as well as local meetings and also via monthly e-mail notice. Second, this survey relies on the hospitals to volunteer their support, which almost certainly biased the study towards larger centers that can support research staff. In addition, we excluded specialist wards other than cardiology from this survey.

Conclusions

The JCARE-CARD will be the first survey to provide valuable information on current patient characteristics, management, and outcomes in a broad sample of Japanese patients who are hospitalized with HF as routine clinical practice. These data may indicate that there are substantial opportunities to improve the management of these patients. By helping to better characterize this disease state, it will ultimately have a significant impact on public health at the national level in Japan.

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References

1. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, 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–e235.
2. Swedberg K, Cleland J, Dargie H, Drexler H, Follath F, Komajda M, et al. Guidelines for the diagnosis and treatment of chronic heart failure: Executive summary (update 2005): The Task Force for the Diagnosis and Treatment of Chronic Heart Failure of the European

Society of Cardiology. *Eur Heart J* 2005; **26:** 1115–1140.
3. Jessup M, Brozena S. Heart failure. *N Engl J Med* 2003; **348:** 2007–2018.
4. Massie BM, Shah NB. Evolving trends in the epidemiologic factors of heart failure: Rationale for preventive strategies and comprehensive disease management. *Am Heart J* 1997; **133:** 703–712.
5. Ho KK, Anderson KM, Kannel WB, Grossman W, Levy D. Survival after the onset of congestive heart failure in Framingham Heart Study subjects. *Circulation* 1993; **88:** 107–115.
6. Schocken DD, Arrieta MI, Leaverton PE, Ross EA. Prevalence and mortality rate of congestive heart failure in the United States. *J Am Coll Cardiol* 1992; **20:** 301–306.
7. Rodeheffer RJ, Jacobsen SJ, Gersh BJ, Kottke TE, McCann HA, Bailey KR, et al. The incidence and prevalence of congestive heart failure in Rochester, Minnesota. *Mayo Clin Pro* 1993; **68:** 1143–1150.
8. Adams KF Jr, Fonarow GC, Emerman CL, LeJemtel TH, Costanzo MR, Abraham WT, et al. Characteristics and outcomes of patients hospitalized for heart failure in the United States: Rationale, design, and preliminary observations from the first 100,000 cases in the Acute Decompensated Heart Failure National Registry (ADHERE). *Am Heart J* 2005; **149:** 209–216.
9. Cleland JG, Swedberg K, Cohen-Solal A, Cosin-Aguilar J, Dietz R, Follath F, et al. The Euro Heart Failure Survey of the EUROHEART survey programme: A survey on the quality of care among patients with heart failure in Europe: The Study Group on Diagnosis of the Working Group on Heart Failure of the European Society of Cardiology: The Medicines Evaluation Group Centre for Health Economics University of York. *Eur J Heart Fail* 2000; **2:** 123–132.
10. Cleland JG, Swedberg K, Follath F, Komajda M, Cohen-Solal A, Aguilar JC, et al. The EuroHeart Failure survey programme: A survey on the quality of care among patients with heart failure in Europe. Part 1: Patient characteristics and diagnosis. *Eur Heart J* 2003; **24:** 442–463.
11. Komajda M, Follath F, Swedberg K, Cleland J, Aguilar JC, Cohen-Solal A, et al. The EuroHeart Failure Survey programme: A survey on the quality of care among patients with heart failure in Europe. Part 2: Treatment. *Eur Heart J* 2003; **24:** 464–474.
12. Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure: The SOLVD Investigators. *N Engl J Med* 1991; **325:** 293–302.
13. Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, et al. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure: US Carvedilol Heart Failure Study Group. *N Engl J Med* 1996; **334:** 1349–1355.
14. Pfeffer MA, Swedberg K, Granger CB, Held P, McMurray JJ, Michelson EL, et al. Effects of candesartan on mortality and morbidity in patients with chronic heart failure: The CHARM-Overall programme. *Lancet* 2003; **362:** 759–766.
15. Itoh A, Saito M, Haze K, Hiramori K, Kasagi F. Prognosis of patients with congestive heart failure: Its determinants in various heart diseases in Japan. *Intern Med* 1992; **31:** 304–309.
16. Koseki Y, Watanabe J, Shinozaki T, Sakuma M, Komaru T, Fukuchi M, et al. Characteristics and 1-year prognosis of medically treated patients with chronic heart failure in Japan. *Circ J* 2003; **67:** 431–436.
17. Shiba N, Watanabe J, Shinozaki T, Koseki Y, Sakuma M, Kagaya Y, et al. Poor prognosis of Japanese patients with chronic heart failure following myocardial infarction: Comparison with nonischemic cardiomyopathy. *Circ J* 2005; **69:** 143–149.
18. Tsuchihashi M, Tsutsui H, Kodama K, Kasagi F, Takeshita A. Clinical characteristics and prognosis of hospitalized patients with congestive heart failure: A study in Fukuoka, Japan. *Jpn Circ J* 2000; **64:** 953–959.
19. Tsuchihashi M, Tsutsui H, Kodama K, Kasagi F, Setoguchi S, Mohr M, et al. Medical and socioenvironmental predictors of hospital readmission in patients with congestive heart failure. *Am Heart J* 2001; **142:** E7.
20. Tsutsui H, Tsuchihashi M, Takeshita A. Mortality and readmission of hospitalized patients with congestive heart failure and preserved versus depressed systolic function. *Am J Cardiol* 2001; **88:** 530–533.
21. Kannel WB, Belanger AJ. Epidemiology of heart failure. *Am Heart J* 1991; **121:** 951–957.
22. Cowie MR, Wood DA, Coats AJ, Thompson SG, Poole-Wilson PA, Suresh V, et al. Incidence and aetiology of heart failure: A population-based study. *Eur Heart J* 1999; **20:** 421–428.
23. Krumholz HM, Parent EM, Tu N, Vaccarino V, Wang Y, Radford MJ, et al. Readmission after hospitalization for congestive heart failure among Medicare beneficiaries. *Arch Intern Med* 1997; **157:** 99–104.
24. Chin MH, Goldman L. Correlates of early hospital readmission or death in patients with congestive heart failure. *Am J Cardiol* 1997; **79:** 1640–1644.
25. Lenzen MJ, Scholte op Reimer WJ, Boersma E, Vantrimpont PJ, Follath F, Swedberg K, et al. Differences between patients with a preserved and a depressed left ventricular function: A report from the EuroHeart Failure Survey. *Eur Heart J* 2004; **25:** 1214–1220.
26. Lenzen MJ, Boersma E, Reimer WJ, Balk AH, Komajda M, Swedberg K, et al. Under-utilization of evidence-based drug treatment in patients with heart failure is only partially explained by dissimilarity to patients enrolled in landmark trials: A report from the Euro Heart Survey on Heart Failure. *Eur Heart J* 2005; **26:** 2706–2713.
27. Yancy CW, Lopatin M, Stevenson LW, De Marco T, Fonarow GC. Clinical presentation, management, and in-hospital outcomes of patients admitted with acute decompensated heart failure with preserved systolic function: A report from the Acute Decompensated Heart Failure National Registry (ADHERE) Database. *J Am Coll Cardiol* 2006; **47:** 76–84.
28. Heiat A, Gross CP, Krumholz HM. Representation of the elderly, women, and minorities in heart failure clinical trials. *Arch Intern Med* 2002; **162:** 1682–1688.

Appendix 1

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Appendix 3

Patient Data Form for JCARE-CARD

Step 1. Demographic Data

1. Date of registry
2. Date of admission
3. Date of discharge
4. Date of birth
5. Age
6. Sex
7. Height
8. Body weight
9. Body mass index

Step 2. Clinical Data (Medical History)

1. Causes of heart failure
   1. Ischemic
   2. Hypertensive
   3. Cardiomyopathic, dilated
   4. Cardiomyopathic, hypertrophic
   5. Cardiomyopathic, dilated phase of hypertrophic cardiomyopathy
   6. Valvular heart disease
   7. Congenital heart disease
   8. Others
   9. Unknown
2. Precipitating causes of heart failure
   1. Lack of compliance with sodium and fluid restriction
   2. Lack of compliance with drugs
   3. Overactivity
   4. Infection
   5. Arrhythmias
   6. Ischemia
   7. Uncontrolled hypertension
   8. Other
   9. Unknown
3. Comorbidity
   1. Hypertension (Blood pressure >140/90 mmHg)
   2. Diabetes mellitus (Fasting blood sugar ≥125 mg/dl or 2-h blood sugar ≥200 mg/dl)
      Insulin treatment
   3. Hyperlipidemia (Total cholesterol ≥220 mg/dl or LDL ≥140 mg/dl)
   4. Renal failure (Serum creatinine 2.5 mg/dl or dialysis)
      Serum creatinine: [ ] mg/dl
      Hemodialysis
   5. Hyperuricemia (Serum uric acid >7.0 mg/dl)
      Serum uric acid: [ ] mg/dl
   6. Cerebrovascular disease
      (Brain infarction, brain hemorrhage, transient ischemic attack)
   7. Anemia (Hemoglobin ≤10 g/dl)
      Hemoglobin: [ ] g/dl
   8. COPD
   9. Smoking
4. Complications
   1. Prior myocardial infarction
   2. Atrial fibrillation or flutter
   3. Sustained ventricular tachycardia or ventricular fibrillation
5. Medical history
   1. First-time diagnosis of HF
   2. Interval after the initial diagnosis of HF (months)
   3. Prior hospitalization for heart failure
   4. Percutaneous coronary intervention
   5. Coronary artery bypass surgery
   6. Valve surgery

Step 3. Clinical Data (Medical Status)

1. New York Heart Association (NYHA) functional class on admission and at discharge
2. Heart rate (beats/min)
3. Blood pressure (mmHg)
4. Left bundle branch block
   QRS duration: [ ] ms
5. Left ventricular hypertrophy (SV_1+RV_5 or $V_6 \geq 3.5$ mV or RV_5 or $V_6 > 2.6$ mV)
6. Echocardiographic data on admission and at discharge
   1. Left ventricular end-diastolic and end-systolic diameters (mm)
   2. Left ventricular ejection fraction (%)
   3. Left ventricular wall thickness (mm)
   4. Mitral regurgitation
   5. Transmitral velocity (E/A ratio, deceleration time of E wave)
7. Serum BNP levels at admission and discharge

Step 4. Discharge Status and Treatment

1. Discharge status
   1. In-hospital death
      Autopsy
   2. Discharge to home
   3. Transfer to another ward for heart failure treatment
   4. Transfer to another ward to treat other diseases
2. Discharge medications
   1. Angiotensin-converting enzyme inhibitors
      [ ] Enalapril [ ] Lisinopril [ ] Perindopril
      [ ] Imidapril [ ] Captopril [ ] Cilazapril
      [ ] Temocapril [ ] Other [ ] No
   2. Angiotensin II receptor blockers
      [ ] Losartan [ ] Valsartan [ ] Candesartan
      [ ] Telmisartan [ ] Other [ ] No
   3. Beta-blockers
      [ ] Carvedilol: daily dosage [ ] mg/dl
      [ ] Bisoprolol: daily dosage [ ] mg/dl
      [ ] Metoprolol: daily dosage [ ] mg/dl
      [ ] Others: daily dosage [ ] mg/dl
      [ ] No
   4. Diuretics
      [ ] Thiazide [ ] Furosemide [ ] Azosemide
      [ ] Spironolactone [ ] Eplerenone [ ] Other
      [ ] No
   5. Digitalis
      [ ] Yes [ ] No
   6. Oral inotropic agents
      [ ] Pimobendan [ ] Docarpamine [ ] Other
      [ ] No
   7. Calcium channel blockers
      [ ] Amlodipine [ ] Nefedipine [ ] Diltiazem
      [ ] Other [ ] No
   8. Alpha-blockers
      [ ] Doxazosin [ ] Other [ ] No
   9. Nitrates
      [ ] Yes [ ] No
   10. Antiarrhythmic agents
      [ ] Amiodarone [ ] Sotalol [ ] Bepridil
      [ ] Disopyramide [ ] Aprindine [ ] Mexiletine
      [ ] Flecainide [ ] Pilsicainide [ ] Cibenzoline
      [ ] Other [ ] No
   11. Aspirin
      [ ] Yes [ ] No
   12. Antiplatelet agents
      [ ] Ticlopidine [ ] Cilostazol [ ] Other
      [ ] No
   13. Warfarin
      [ ] Yes [ ] No
   14. Statins
      [ ] Pravastatin [ ] Fluvastatin [ ] Atorvastatin
      [ ] Simvastatin [ ] Other [ ] No
   15. Participation in clinical trial
      [ ] J-CHF [ ] Bisoprolol
      [ ] Other
      [ ] No
3. Non-pharmacological therapy
   1. Permanent pacemaker
   2. Cardiac resynchronization therapy
   3. Implantable cardioverter defibrillator
   4. Left ventricular assist device
   5. Cardiac transplantation

Step 5. Long-Term Outcomes

1. Date of survey
2. Death
   1. Date of death
   2. All-cause death
   3. Cause of death
      [ ] Cardiac death [ ] Non-cardiac death [ ] Unknown
   4. Autopsy
3. Hospital readmission because of exacerbation of heart failure
   1. Date of readmission
   2. Date of discharge
4. Sustained ventricular tachycardia or ventricular fibrillation

Cardiac-Specific Overexpression of Diacylglycerol Kinase ζ Prevents Gq Protein–Coupled Receptor Agonist–Induced Cardiac Hypertrophy in Transgenic Mice

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Background—Diacylglycerol is a lipid second messenger that accumulates in cardiomyocytes when stimulated by Gqα protein–coupled receptor (GPCR) agonists such as angiotensin II, phenylephrine, and others. Diacylglycerol functions as a potent activator of protein kinase C (PKC) and is catalyzed by diacylglycerol kinase (DGK) to form phosphatidic acid and inactivated. However, the functional roles of DGK have not been previously examined in the heart. We hypothesized that DGK might prevent GPCR agonist–induced activation of diacylglycerol downstream signaling cascades and subsequent cardiac hypertrophy.

Methods and Results—To test this hypothesis, we generated transgenic (DGKζ-TG) mice with cardiac-specific overexpression of DGKζ. There were no differences in heart size and heart weight between DGKζ-TG and wild-type littermate mice. The left ventricular function was normal in DGKζ-TG mice. Continuous administration of subpressor doses of angiotensin II and phenylephrine caused PKC translocation, gene induction of atrial natriuretic factor, and subsequent cardiac hypertrophy in WT mice. However, in DGKζ-TG mice, neither translocation of PKC nor upregulation of atrial natriuretic factor gene expression was observed after angiotensin II and phenylephrine infusion. Furthermore, in DGKζ-TG mice, angiotensin II and phenylephrine failed to increase cross-sectional cardiomyocyte areas and heart to body weight ratios. Phenylephrine-induced increases in myocardial diacylglycerol levels were completely blocked in DGKζ-TG mouse hearts, suggesting that DGKζ regulated PKC activity by controlling cellular diacylglycerol levels.

Conclusions—These results demonstrated the first evidence that DGKζ negatively regulated the hypertrophic signaling cascade and resultant cardiac hypertrophy in response to GPCR agonists without detectable adverse effects in in vivo hearts. (***Circulation.*** **2006;113:60-66.)**

Key Words: angiotensin ■ hypertrophy ■ enzymes ■ signal transduction

Cardiac hypertrophy is an initially adaptive response in several forms of cardiac disease, whereas sustained hypertrophy is a powerful independent risk factor for cardiac morbidity and mortality.[1] Therefore, to identify the critical molecular mechanisms involved in cardiac hypertrophy is an important challenge of cardiovascular biology and medicine. Multiple lines of experimental and clinical evidence have suggested the importance of the Gqα-phosphoinositide signaling system in the development of pathological cardiac hypertrophy and heart failure.[2–6] Gqα protein–coupled receptor (GPCR) agonists such as angiotensin II,[7] endothelin-1,[8] and phenylephrine[9] activate phospholipase C–mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, which produces inositol 1,4,5-trisphosphate and diacylglycerol (DAG). DAG functions as a potent activator of protein kinase C (PKC). The binding of DAG to the C1 domain of PKC induces an active conformation, and activated PKC regulates a variety of cellular functions including cell growth and differentiation. We and others have previously demonstrated that PKC plays an important role in the development and progression of cardiac hypertrophy.[10–12]

One major route for terminating DAG signaling is thought to be its phosphorylation and inactivation by DAG kinase (DGK), producing phosphatidic acid.[13–16] A previous study

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has shown that of the α, ε, and ζ isoforms of DGK expressed in the myocardium, DGKζ is the predominant isoform.[17] We have recently demonstrated using cultured rat neonatal cardiomyocytes that DGKζ blocks endothelin-1–induced activation of PKC, extracellular signal–regulated kinase (ERK), and activator protein-1.[18] DGKζ also inhibits gene induction of atrial natriuretic factor (ANF), increases in protein synthesis, and resultant cardiomyocyte hypertrophy in response to endothelin-1 in neonatal cardiomyocytes. However, the in vivo role of DGKζ has not been previously investigated in the heart.[19]

We hypothesized that DGKζ may act as a negative regulator for GPCR agonist–induced activation of the DAG-PKC signaling cascade and subsequent cardiac hypertrophy in vivo. To test this hypothesis, we generated transgenic mice with cardiac-specific overexpression of DGKζ using an α-myosin heavy chain (MHC) promoter. We examined the functional role of DGKζ to interfere with hypertrophic responses by GPCR agonists such as angiotensin II and phenylephrine in transgenic mouse hearts.

Methods

Generation of DGKζ Transgenic Mice

All experimental procedures were performed according to the animal welfare regulations of Yamagata University School of Medicine, and the study protocol was approved by the Animal Subjects Committee of Yamagata University School of Medicine. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Transgenic mice with cardiac-specific overexpression of DGKζ (DGKζ-TG) were created in Yamagata University by standard techniques.[10,12,20] Briefly, a 5.5-kb fragment of murine α-MHC gene promoter (a kind gift from Dr J. Robbins, Children's Hospital Research Foundation, Cincinnati, Ohio) and 3.4-kb rat DGKζ cDNA[13] were subcloned into pBsIISK(+) plasmids. The plasmid was digested with *Spe*I to generate a 9.5-kb DNA fragment composed of the α-MHC gene promoter, DGKζ cDNA, and a poly A tail of the human growth hormone, as illustrated in Figure 1A. We microinjected the construct into the pronuclei of single-cell fertilized mouse embryos to generate transgenic mice as previously described.[10,12,20] To detect the exogenous DGKζ gene, genomic DNA was extracted from the tail tissues of 3- to 4-week-old pups, and polymerase chain reaction (PCR) was performed with one primer specific for the α-MHC gene promoter and another primer specific for the DGKζ, as shown in Figure 1A.

DGK Activity

DGK activity in the left ventricle was measured by octyl glucoside mixed-micelle assay as described previously.[21,22] The 1,2-dioleoyl-*sn*-glycerol (18:1/18:1 DAG) and 1-stearoyl-2- linoleoyl-*sn*-glycerol (18:0/18:2 DAG) were used as substrates for kinase assays. Phosphatidic acid separated by thin layer chromatography was scraped and counted by liquid scintillation.[21,22]

Western Blotting

Total protein was extracted from the left ventricle with ice-cold lysis buffer as described previously.[23–25] Protein concentration of myocardial samples was carefully determined by the protein assay, and equal amounts of protein extracts were loaded on each gel lane. To ensure equivalent protein loading and quantitative transfer efficiency of proteins, membranes were stained with Ponceau S before incubating with primary antibodies. Western blotting was performed as reported previously, and DGKζ protein levels in TG mice were expressed as fold increase over wild-type (WT) mice. Membrane and cytosolic fractions were also prepared from left ventricular myocardium as previously reported.[10,12,25] Membrane/cytosol ratios of immunoreactivity with the use of isoform-specific antibodies (mouse monoclonal anti-PKCα, β, δ, and ε; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) were used as indices for the extent of translocation of PKC isoforms.[10,12,25]

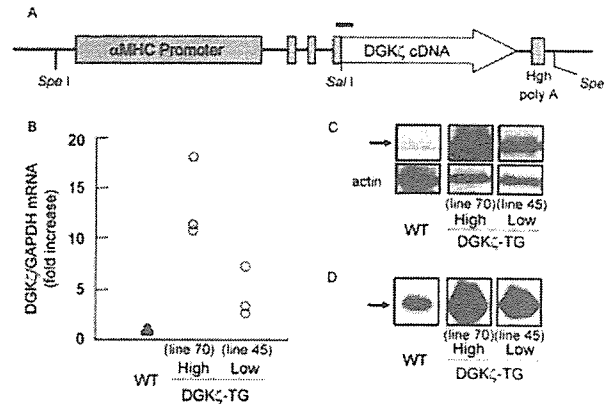

Figure 1. A, Diagram of the transgene construct used for the generation of DGKζ-TG mice. The construct contains the α-MHC gene promoter, the full-length rat DGKζ cDNA clone, and a human growth hormone (Hgh) polyadenylylation sequence. The solid line indicates the region amplified by PCR for genotyping. B, Quantitative analysis of DGKζ mRNA expression in the left ventricle of DGKζ-TG and WT mice. Left ventricular myocardium of 8-week-old mice was examined by real-time PCR analysis and normalized with the use of GAPDH mRNA. Data are reported as fold increase over WT and obtained from 3 mice for each. C, Representative Western blot analysis of DGKζ protein from the left ventricle of DGKζ-TG-High, DGKζ-TG-Low, and WT mice. D, Representative autoradiograms of DGK assay of DGKζ-TG-High, DGKζ-TG-Low, and WT mice.

Extraction of Total RNA and Real-time Reverse Transcriptase–PCR

Total RNAs were extracted from the left ventricle, and first-strand cDNA was synthesized as previously described.[26,27] To examine mRNA levels of DGKζ and ANF quantitatively, real-time reverse transcriptase–PCR (RT-PCR) amplification was performed.[18] Amplification was performed with the use of LightCycler and analyzed with the use of LightCycler Software Version 3.5 (Roche Diagnostics Japan). Standard curves of DGKζ and ANF were generated by full sequence plasmid of known concentrations. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold increase over WT mice. Primers were designed on the basis of GenBank sequences (DGKζ, BC049228; ANF, K02781; and GAPDH, NM_001001303).

Isolated Cardiomyocyte Function and Ca^{2+} Transient

Ventricular myocytes were isolated from hearts from WT mice and mouse lines with the high expression of transgene mRNA (DGKζ-TG-High), and cardiomyocyte mechanical properties were examined with the use of a computerized edge-detection analyzer as previously reported.[28] Cells were paced at 0.5 Hz throughout the experiments. Same isolated cells were used for measurements of cytosolic free Ca^{2+} by indo-1 with a previously described method.[28] Data from at least 5 to 6 cardiomyocytes were averaged for each mouse heart, and the statistical analysis was performed on the basis of the number of hearts studied (n=6 for each group).

Histological Examinations

Coronary arteries were retrogradely flushed with saline, and the heart was fixed with 4% paraformaldehyde at 4°C for 24 hours and then embedded in paraffin.[26,27] Three sections were stained with

hematoxylin-eosin or elastica Goldner stain. Transverse sections were captured digitally, and cardiomyocyte cross-sectional area was measured with the use of a Scion imaging system (Scion Corporation).[29] At least 300 cardiomyocytes were examined in each heart, and the data were averaged.

Hemodynamic Measurements and Echocardiography

Heart rate (bpm) and blood pressure (mm Hg) were determined with animals in the conscious state with the use of a computerized tail-cuff manometer, MK-1030 (Muromachi Kikai Co, Ltd), as reported previously.[12] Echocardiography was performed as described previously[26,27] with an FFsonic 8900 (Fukuda Denshi Co) equipped with a 13-MHz phased-array transducer. Left ventricular wall thickness and internal dimensions at end-systole and end-diastole were measured digitally on the M-mode tracings and averaged for 3 cardiac cycles. Left ventricular fractional shortening was calculated as previously reported.[26,27]

Lipid Extraction and Measurements of Myocardial DAG Levels

Myocardial lipid extract was prepared from the left ventricle, and DAG levels were measured as previously reported.[30] Briefly, with the use of the DAG within myocardial lipid extract as substrate and with the use of [γ-^{32}P]ATP, myocardial DAG level was quantified by production of [^{32}P]phosphatidic acid. Lipid extract was solubilized in 50 μL of 0.6% (wt/vol) Triton X/288 μmol/L phosphatidylserine. The reaction mixture contained 0.3% (wt/vol) Triton X, 144 μmol/L phosphatidylserine, 50 mmol/L imidazole/HCl, pH 6.6, 50 mmol/L NaCl, 12.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L dithiothreitol, 0.5 mmol/L ATP (1 μCi of [γ-^{32}P]ATP), and 5 m-unit of DGK (*Escherichia coli*). After 30 minutes of incubation, the reaction was terminated, and the radiolabeled product was separated by TLC on silica plates. The [^{32}P]phosphatidic acid was identified by autoradiography. Silica corresponding to phosphatidic acid was scraped and counted by liquid scintillation counting.[30]

Subcutaneous Implantation of Osmotic Minipump

A subpressor dose of angiotensin II (100 ng/kg per minute) or phenylephrine (20 mg/kg per day) dissolved in saline or saline alone (control) was continuously infused into mice subcutaneously via an osmotic minipump (ALZET Osmotic Pumps, DURECT Corporation) for 14 or 3 days, respectively.[2,3,31,32] Heart rate and blood pressure were measured before and after subcutaneous infusion of angiotensin II or phenylephrine.

Statistical Analysis

All values are reported as mean±SD. Comparisons of hemodynamic data and gravimetric and echocardiographic measurements at basal conditions among WT, DGKζ-TG-High, and DGKζ-TG-Low mice were made by the Kruskal-Wallis test. Isolated cardiomyocyte mechanical properties and calcium transient data between WT and DGKζ-TG-High mice were compared by the Mann-Whitney *U* test. Effects of angiotensin II and phenylephrine on body weight, blood pressure, heart rate, heart weight, left ventricular weight, PKC translocation, DAG level, ANF expression, and cardiomyocyte surface area in animal groups were compared by 2-way ANOVA followed by multiple comparisons with the Bonferroni test. Probability values of <0.05 were considered statistically significant.

Results

Generation of DGKζ Transgenic Mice

After microinjection and embryo implantation, 2 lines of transgenic mice (lines 70 and 45) were successfully established. Figure 1B shows the real-time PCR results of DGKζ in the left ventricle of TG and WT mice. Heterozygous mouse lines with the high expression of transgene mRNA (DGKζ-TG-High) and the low expression of transgene mRNA (DGKζ-TG-Low) in the left ventricle were characterized in detail in the following experiments. RNA was extracted from brain, heart, lungs, liver, kidney, spleen, intestine, and skeletal muscle tissues of DGKζ-TG mice, and cardiac-specific expression of transgene was confirmed by RT-PCR (data not shown). Protein levels of DGKζ were augmented 21- and 5.5-fold in DGKζ-TG-High and DGKζ-TG-Low hearts compared with control WT littermates, respectively (Figure 1C). Additionally, we confirmed that kinase activities of DGK in the heart were also augmented in both DGKζ-TG mouse lines (Figure 1D). No neonatal and adult deaths were observed in DGKζ-TG mice.

TABLE 1. Gravimetric Data and In Vivo Cardiac Function of DGKζ-TG Mice at Basal Condition

	WT	DGKζ-TG-High	DGKζ-TG-Low
BW, g	22.6±1.1	21.7±2.8	23.5±3.8
BP, mm Hg	96±13	99±6	95±7
HR, bpm	673±52	643±71	650±68
HW, mg	117±9	122±16	122±18
LVW, mg	77±6	78±15	80±14
HW/BW, mg/g	4.92±0.24	5.07±0.41	4.94±0.33
LVW/BW, mg/g	3.25±0.14	3.22±0.44	3.24±0.38
Echocardiography			
LVEDD, mm	3.31±0.17	3.21±0.17	3.24±0.28
LVESD, mm	1.60±0.15	1.50±0.19	1.45±0.15
LVFS, %	52±4	53±5	53±5
IVS, mm	0.64±0.07	0.64±0.05	0.63±0.05
PW, mm	0.66±0.05	0.65±0.05	0.65±0.05

Data were analyzed by the Kruskal-Wallis test and are reported as mean±SD (n=8). BW indicates body weight; BP, blood pressure; HR, heart rate; HW, heart weight; LVW, left ventricular weight; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVFS, left ventricular fractional shortening; IVS, interventricular septum; and PW, posterior wall.

Gravimetric Data, Cardiac Function, and Isolated Cardiomyocyte Mechanical Properties of DGKζ-TG Mice at Basal Condition

To characterize mouse phenotype, all experiments were performed with age- and sex-matched (8- to 10-week-old) DGKζ-TG and WT littermate mice. Body weight, blood pressure, and heart rate were similar between DGKζ-TG and WT mice (Table 1). There was no evidence of fibrosis on microscopic examinations of multiple histological sections (data not shown). The absolute heart weight, ratio of heart to body weight, and ratio of the left ventricle to body weight were not different between DGKζ-TG and WT mice (Table 1). Echocardiography demonstrated that cardiac dimensions, wall thickness, and fractional shortening were normal in DGKζ-TG mice, as shown in Table 1. Isolated cardiomyocyte mechanical properties and Ca^{2+} transients were examined with the use of 6 WT mice and 6 DGKζ-TG-High mice. Cell shortening (4.10±0.47% versus 4.71±0.49%), time to peak shortening (52±4 versus 54±6 ms), and time to 80% relaxation (105±17 versus 89±17 ms) were not different between

TABLE 2. Changes in Hemodynamic and Gravimetric Parameters in WT and DGKζ-TG Mice After Angiotensin II or Phenylephrine Infusion

	WT			DGKζ-TG-High			DGKζ-TG-Low		
	Saline	Ang II (14 d)	PE (3 d)	Saline	Ang II (14 d)	PE (3 d)	Saline	Ang II (14 d)	PE (3 d)
BW, g	21.5±2.1	23.5±2.9	23.9±2.5	24.1±3.4	22.0±3.0	21.7±2.8	25.8±3.5	23.8±4.0	24.0±1.9
BP, mm Hg	99±14	101±14	102±6	99±7	105±13	110±6	96±8	94±8	107±17
HR, bpm	665±47	663±38	641±29	632±99	631±41	624±61	632±70	663±33	670±61
HW/BW, mg/g	4.96±0.29	5.51±0.27*	5.76±0.81*	5.03±0.23	5.07±0.11†	4.95±0.39§	4.93±0.34	4.98±0.57†	5.19±0.37‡
LVW/BW, mg/g	3.22±0.20	3.98±0.18*	3.91±0.63*	3.21±0.38	3.34±0.20†	3.38±0.38§	3.24±0.36	3.22±0.34†	3.38±0.41‡

Data were analyzed by 2-way ANOVA followed by multiple comparisons with the Bonferroni test and are presented as mean±SD (n=6). Ang II indicates angiotensin II; PE, phenylephrine; BW, body weight; BP, blood pressure; HR, heart rate; HW, heart weight; and LVW, left ventricular weight.
*$P<0.01$ vs saline-infused WT mice; †$P<0.01$ vs Ang II–infused WT mice; ‡$P<0.05$ and §$P<0.01$ vs PE-infused WT mice.

DGKζ-TG-High and WT mice. The amplitude of the Ca^{2+} signal (0.084±0.011 versus 0.077±0.007) and half-life decay of the Ca^{2+} signal (156±13 versus 162±13 ms) were also same between DGKζ-TG-High and WT mice.

Effects of DGKζ on GPCR Agonist–Induced Activation of DAG-PKC Signaling

DGKζ-TG and WT mice were assessed with respect to their susceptibility to hypertrophic response to subpressor doses of subcutaneous angiotensin II[2,3] and phenylephrine[31,32] administration. No significant changes in body weight, heart rate, and blood pressure were observed between WT and DGKζ-TG mice after subcutaneous infusion of angiotensin II or phenylephrine, as shown in Table 2.

In the present study there were no significant changes in total protein abundance of PKC isoforms after angiotensin II or phenylephrine infusion (data not shown). We have previously demonstrated angiotensin II–induced translocation of PKC isoforms through pathways involving phospholipase C in the guinea pig ex vivo heart.[5] As shown in Figure 2, the membrane-associated immunoreactivities of PKCα and PKCε, but not PKCβ and PKCδ, were significantly increased in angiotensin II–treated WT mice compared with saline-infused WT mice ($P<0.05$). However, angiotensin II–induced translocation of PKCα and PKCε was blocked in both DGKζ-TG-High and DGKζ-TG-Low mice ($P<0.05$ versus angiotensin II–infused WT mice).

Next, we examined effects of another GPCR agonist, phenylephrine, in TGKζ-TG mice. Phenylephrine induced translocation of the PKCε isoform ($P<0.01$), but not α, β, and δ isoforms, in WT mouse hearts, as shown in Figure 3. However, in both DGKζ-TG-High and DGKζ-TG-Low mice, translocation of the PKCε by phenylephrine was completely blocked ($P<0.01$ versus phenylephrine-infused WT mice). These data suggested that DGKζ had an inhibitory effect on GPCR agonist–induced translocation of PKC isoforms in in vivo mouse hearts.

Lipid extracts were then prepared from the left ventricular myocardium, and we quantified myocardial DAG levels in WT and DGKζ-TG-High mouse hearts (n=6 for each group). At basal condition, DAG levels were similar between WT and DGKζ-TG-High mice (51±15 versus 66±18 pmol/mg tissue). In WT mouse hearts, myocardial DAG level increased significantly after continuous administration of phenylephrine for 3 days (from 51±15 to 103±27 pmol/mg tissue; $P<0.001$). On the other hand, this effect of phenylephrine on myocardial DAG levels was completely suppressed in DGKζ-TG-High mouse hearts (from 66±18 to 34±7 pmol/mg tissue; P=NS). These data suggest that DGKζ regulates PKC activity by controlling cellular DAG levels.

Effects of DGKζ on Hypertrophic Programs in Response to GPCR Agonists

Ventricular hypertrophy induced by continuous infusion of angiotensin II or phenylephrine is accompanied by the induction of several specific genes such as ANF.[2,18] As shown in Figure 4, the mRNA expression of ANF was increased in WT mice given angiotensin II and phenylephrine compared with saline-infused WT mice ($P<0.01$). However, in DGKζ-TG-High and DGKζ-TG-Low mice, angiotensin II failed to cause gene induction of ANF ($P<0.01$). Phenylephrine-induced ANF gene induction was significantly blocked in DGKζ-TG-High mice ($P<0.01$), but this inhibitory effect was not

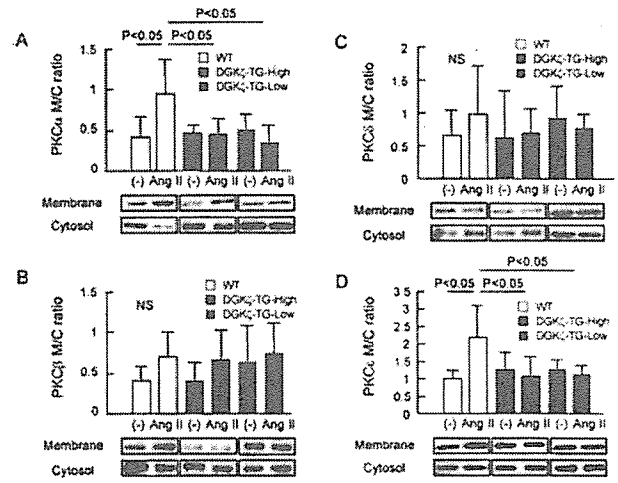

Figure 2. Translocation of PKCα (A), PKCβ (B), PKCδ (C), and PKCε (D) in DGKζ-TG-High, DGKζ-TG-Low, and WT mice in response to angiotensin II (Ang II). A subpressor dose of angiotensin II (100 ng/kg per minute) was continuously infused with an osmotic minipump for 14 days.[2,3] Membrane/cytosol (M/C) ratio of immunoreactivity was used as an index of PKC activation. Data were analyzed by 2-way ANOVA followed by multiple comparisons with the Bonferroni test and are reported as mean±SD obtained from 6 to 7 mice for each group.

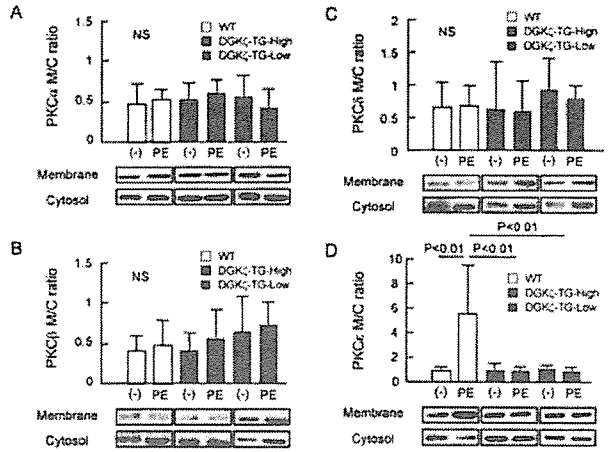

Figure 3. Translocation of PKCα (A), PKCβ (B), PKCδ (C), and PKCϵ (D) and in DGKζ-TG-High, DGKζ-TG-Low, and WT mice in response to phenylephrine (PE). A dose of phenylephrine (20 mg/kg per day) was infused into mice subcutaneously via an osmotic minipump for 3 days.[31,32] Membrane/cytosol (M/C) ratio of immunoreactivity was used as an index of PKC activation. Data were analyzed by 2-way ANOVA followed by multiple comparisons with the Bonferroni test and are reported as mean±SD obtained from 6 to 7 mice for each group.

statistically significant in DGKζ-TG-Low mice (P=0.0704). These data suggested that DGKζ blocked hypertrophic gene induction by angiotensin II and phenylephrine in in vivo mouse hearts.

As shown in Table 2, heart weight and left ventricular weight corrected for body weight were not significantly different between saline-infused WT and saline-infused DGKζ-TG mice. Subcutaneous infusion of angiotensin II and phenylephrine caused significant increases in the ratio of heart to body weight and ratio of the left ventricle to body weight in WT mice (P<0.01). However, in both DGKζ-TG-High and DGKζ-TG-Low mice, neither angiotensin II nor phenylephrine produced increases in the ratio of heart to body weight and ratio of the left ventricle to body weight (Table 2).

Microscopic observations revealed that no significant difference in cardiomyocyte cross-sectional area was seen between saline-infused WT and saline-infused DGKζ-TG mice

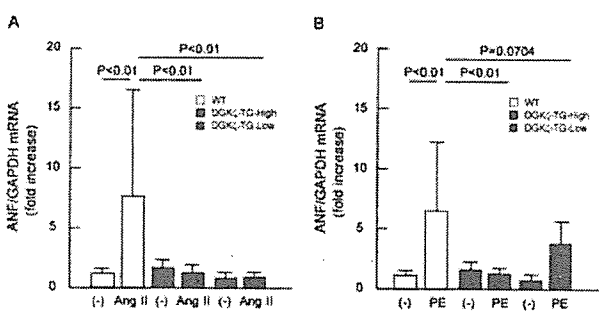

Figure 4. Cardiac ANF gene expressions in DGKζ-TG-High, DGKζ-TG-Low, and WT mice in response to angiotensin II (Ang II) (A) and phenylephrine (PE) (B). Mean value in saline-infused WT mice is presented as 1.0. Data were analyzed by 2-way ANOVA followed by multiple comparisons with the Bonferroni test. Data reported are mean±SD obtained from 6 to 8 mice for each group.

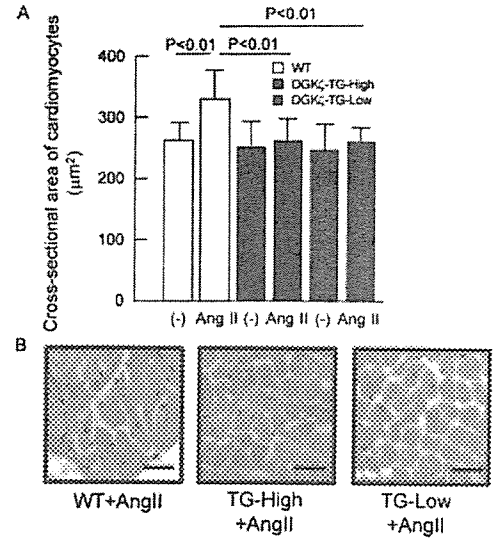

Figure 5. Histological analysis in DGKζ-TG-High, DGKζ-TG-Low, and WT mice after angiotensin II (AngII) infusion. Bottom panels show representative images of hematoxylin-eosin micrographs of cardiomyocyte cross sections. (Magnification ×200, bar=20 μm). Top bar graph shows quantitative analysis of cardiomyocyte cross-sectional area from the left ventricle. Data were analyzed by 2-way ANOVA followed by multiple comparisons with the Bonferroni test. Data reported are mean±SD obtained from 7 mice for each group.

(Figures 5 and 6). In WT mice, cardiomyocyte cross-sectional area was significantly increased by angiotensin II and phenylephrine infusion (P<0.01 and P<0.05, respectively). However, in both DGKζ-TG-High and DGKζ-TG-Low mice, neither angiotensin II nor phenylephrine caused increases in

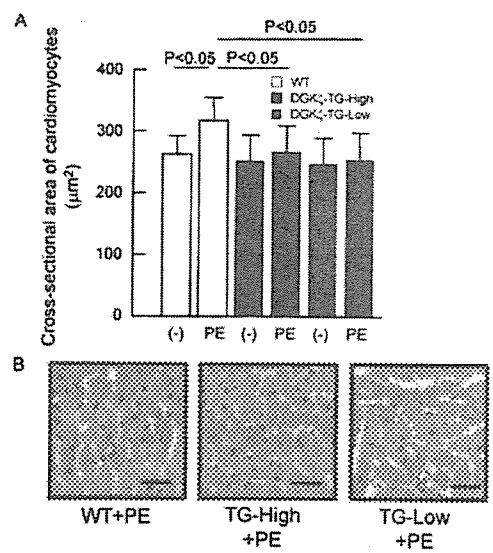

Figure 6. Histological analysis in DGKζ-TG-High, DGKζ-TG-Low, and WT mice after phenylephrine (PE) infusion. Bottom panels show representative images of hematoxylin-eosin micrographs of cardiomyocyte cross sections. (magnification ×200, bar=20 μm). Top bar graph shows quantitative analysis of cardiomyocyte cross-sectional area from the left ventricle. Data were analyzed by 2-way ANOVA followed by multiple comparisons with the Bonferroni test. Data are reported as mean±SD obtained from 7 mice for each group.

cardiomyocyte cross-sectional area ($P<0.01$ versus angiotensin II–infused WT and $P<0.05$ versus phenylephrine-infused WT mice). Obvious fibrosis in the myocardium was not observed in DGKζ-TG and WT mice given angiotensin II or phenylephrine (data not shown). Taken together, these data clearly demonstrated that DGKζ might interfere with GPCR agonist–induced cardiac hypertrophy.

Discussion

This is the first report characterizing a functional role of DGKζ in the in vivo mouse heart. Molecular, gravimetric, and morphological analyses clearly showed that cardiac-specific overexpression of DGKζ abrogated cardiac hypertrophy in response to GPCR agonists such as angiotensin II and phenylephrine by regulating the DAG-PKC signaling in transgenic mouse hearts.

DGKζ-TG mice were indistinguishable in appearance from WT mice, and no baseline cardiac effects were observed in physiological or histological analyses. In addition, we found that isolated cardiomyocyte function and in vivo cardiac function evaluated by echocardiography were normal in DGKζ-TG mice. Blood pressure and heart rate after subcutaneous infusion of angiotensin II and phenylephrine were not different among DGKζ-TG-High, DGKζ-TG-Low, and WT mice, indicating that the overexpression of DGKζ does not affect hemodynamic regulations in response to angiotensin II and phenylephrine. Consistent with previous works,[2,3,31,32] the hypertrophic response in this study occurred independently of the hemodynamic effects of angiotensin II and phenylephrine because systemic blood pressure was not elevated after infusion.

Previous studies in human heart failure and animal models of heart failure have clearly demonstrated that activation of PKC isoforms plays a critical role in the development of cardiac hypertrophy and progression to heart failure.[5,6,10–12] GPCR agonists increase production of DAG, resulting in sustained PKC activation in the cardiomyocyte.[7,33] DGK is an enzyme that is responsible for controlling the cellular levels of DAG by converting it to phosphatidic acid, and thus is thought to be acting as an endogenous regulator of PKC activity. Continuous infusion of phenylephrine for 3 days increased myocardial DAG levels in WT mice, but this effect was completely abolished by overexpression of DGKζ. These data suggest that DGKζ regulates PKC activity by controlling cellular DAG levels.

Phosphatidic acid is yielded not only by DGK but also by the action of phospholipase D. Phospholipase D hydrolyzes phosphatidylcholine to form phosphatidic acid, and phosphatidic acid itself also has signaling function, stimulates DNA synthesis, and modulates activity of several enzymes, including phosphatidylinositol 5-kinases, ERK, and others.[34] However, because the bulk of the signaling pool of phosphatidic acid is mainly derived from the action of phospholipase D in cardiomyocytes,[35] overexpression of DGKζ may not affect phosphatidic acid pool and its signaling function. We previously demonstrated that activation of downstream ERK and protein synthesis by endothelin-1 were abolished by DGKζ in isolated neonatal rat cardiomyocytes.[18] These results suggested the importance of inhibiting the DAG-PKC signaling pathway by DGKζ to prevent cardiomyocyte hypertrophy.

The in vitro studies have reported that DGK isoforms modulate the DAG-PKC signaling in several types of cells.[36,37] In particular, Luo et al[38] have recently showed that DGKζ spatially regulates PKCα activity by attenuating local accumulation of DAG in HEK293 cells. It has been reported that the DGKε isoform reduces cellular DAG levels in aortic endothelial cells.[36] We have recently found that adenovirus-mediated overexpression of the DGKζ isoform blocked endothelin-1–induced activation of the DAG-PKC signaling and resultant cardiomyocyte hypertrophy in cultured rat neonatal cardiomyocytes.[18] To further elucidate these issues obtained from in vitro studies, isoform-specific regulation of DGK in an in vivo study with the use of a transgenic approach, as we employed in the present study, is necessary. In our present study, angiotensin II– and phenylephrine-induced translocation of PKC was blocked in DGKζ-TG mice (Figures 2 and 3). These data suggest that DGKζ has an inhibitory effect on PKC translocation, which depends on kinase activity, in the left ventricular myocardium. Furthermore, angiotensin II– and phenylephrine-induced hypertrophic programs determined by gene induction of ANF, increases in heart weight, and enlargements of cardiomyocyte surface area were abolished in DGKζ-TG mice (Table 2 and Figures 4 to 6). These data suggest that DGKζ functions as a negative regulator of the DAG-PKC signaling and prevents subsequent cardiomyocyte hypertrophy. Because this study used only the overexpression approach, future experiments of a loss of DGK function with the use of knockout mice are necessary to elucidate further the role of DGKζ in signaling cascade in vivo.

In conclusion, our study provides the first in vivo evidence that DGKζ blocks cardiac hypertrophy in response to GPCR agonists by regulating DAG-PKC signaling. Further studies will be necessary to examine whether and to what extent DGKζ may prevent pressure overload–induced cardiac hypertrophy.

Acknowledgments

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Disclosures

None.

References

1. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med.* 1990;322:1561–1566.
2. Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation.* 1998;97:1952–1959.
3. Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR, Doetschman T. TGF-β1 mediates the hypertrophic cardio-

myocyte growth induced by angiotensin II. *J Clin Invest.* 2002;109: 787–796.
4. Izumiya Y, Kim S, Izumi Y, Yoshida K, Yoshiyama M, Matsuzawa A, Ichijo H, Iwao H. Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II–induced cardiac hypertrophy and remodeling. *Circ Res.* 2003;93:874–883.
5. Takeishi Y, Jalili T, Ball NA, Walsh RA. Responses of cardiac protein kinase C isoforms to distinct pathological stimuli are differentially regulated. *Circ Res.* 1999;85:264–271.
6. Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med.* 1999;341:1276–1283.
7. Sadoshima J, Izumo S. Signal transduction pathways of angiotensin II–induced c-fos gene expression in cardiac myocytes in vitro: roles of phospholipid-derived second messengers. *Circ Res.* 1993;73:424–438.
8. Shubeita HE, McDonough PM, Harris AN, Knowlton KU, Glembotski CC, Brown JH, Chien KR. Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes: a paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem.* 1990;265:20555–20562.
9. Otani H, Otani H, Das DK. α1-Adrenoceptor-mediated phosphoinositide breakdown and inotropic response in rat left ventricular papillary muscles. *Circ Res.* 1988;62:8–17.
10. Takeishi Y, Chu G, Kirkpatrick DL, Wakasaki H, Li Z, Kranias EG, King GL, Walsh RA. In vivo phosphorylation of cardiac troponin I by PKCβ2 decreases cardiomyocyte calcium responsiveness and contractility in transgenic mouse heart. *J Clin Invest.* 1998;102:72–78.
11. Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, Sabbah HN, Mizrahi JL, Gromo G, King GL, Vlahos CJ. Increased protein kinase C activity and expression of Ca^{2+}-sensitive isoforms in the failing human heart. *Circulation.* 1999;99:384–391.
12. Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA. Transgenic overexpression of constitutively active protein kinase C-ϵ causes concentric cardiac hypertrophy. *Circ Res.* 2000;86:1218–1223.
13. Goto K, Kondo H. A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus. *Proc Natl Acad Sci U S A.* 1996;93:11196–11201.
14. Topham MK, Bunting M, Zimmerman GA, McIntyre TM, Blackshear PJ, Prescott SM. Protein kinase C regulates the nuclear localization of diacylglycerol kinase-ζ. *Nature.* 1998;394:697–700.
15. Topham MK, Prescott SM. Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J Biol Chem.* 1999;274: 11447–11450.
16. Goto K, Kondo H. Diacylglycerol kinase in the central nervous system: molecular heterogeneity and gene expression. *Chem Phys Lipids.* 1999; 98:109–117.
17. Takeda M, Kagaya Y, Takahashi J, Sugie T, Ohta J, Watanabe J, Shirato K, Kondo H, Goto K. Gene expression and in situ localization of diacylglycerol kinase isozymes in normal and infarcted rat hearts: effects of captopril treatment. *Circ Res.* 2001;89:265–272.
18. Takahashi H, Takeishi Y, Seidler T, Arimoto T, Akiyama H, Koyama Y, Shishido T, Tsunoda Y, Niizeki T, Hozumi Y, Abe J, Hasenfuss G, Goto K, Kubota I. Adenovirus-mediated overexpression of diacylglycerol kinase ζ inhibits endothelin-1–induced cardiomyocyte hypertrophy. *Circulation.* 2005;111:1510–1516.
19. Arimoto T, Takahashi H, Shishido T, Niizeki T, Koyama Y, Nakajima O, Goto K, Takeishi Y. Cardiac-specific overexpression of diacylglycerol ζ prevents angiotensin II–induced cardiac hypertrophy in transgenic mice. *Circulation.* 2004;110:III-226. (Abstract)
20. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci U S A.* 1980;77:7380–7384.
21. Goto K, Kondo H. Molecular cloning and expression of a 90-kDa diacylglycerol kinase that predominantly localizes in neurons. *Proc Natl Acad Sci U S A.* 1993;90:7598–7602.
22. Goto K, Funayama M, Kondo H. Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum. *Proc Natl Acad Sci U S A.* 1994;91: 13042–13046.
23. Takeishi Y, Abe J, Lee JD, Kawakatsu H, Walsh RA, Berk BC. Differential regulation of p90 ribosomal S6 kinase and big mitogen-activated protein kinase-1 by ischemia/reperfusion and oxidative stress in perfused guinea pig hearts. *Circ Res.* 1999;85:1164–1172.
24. Takeishi Y, Huang Q, Abe J, Glassman M, Che W, Lee JD, Kawakatsu H, Lawrence EG, Hoit BD, Berk BC, Walsh RA. Src and multiple MAP kinase activation in cardiac hypertrophy and congestive heart failure under chronic pressure-overload: comparison with acute mechanical stretch. *J Mol Cell Cardiol.* 2001;33:1637–1648.
25. Takahashi H, Takeishi Y, Miyamoto T, Shishido T, Arimoto T, Konta T, Miyashita T, Ito M, Kubota I. Protein kinase C and extracellular signal regulated kinase are involved in cardiac hypertrophy of rats with progressive renal injury. *Eur J Clin Invest.* 2004;34:85–93.
26. Shishido T, Nozaki N, Yamaguchi S, Shibata Y, Nitobe J, Miyamoto T, Takahashi H, Arimoto T, Maeda K, Yamakawa M, Takeuchi O, Akira S, Takeishi Y, Kubota I. Toll-like receptor-2 modulates ventricular remodeling after myocardial infarction. *Circulation.* 2003;108: 2905–2910.
27. Nozaki N, Shishido T, Takeishi Y, Kubota I. Modulation of doxorubicin-induced cardiac dysfunction in toll-like receptor-2 knockout mice. *Circulation.* 2004;110:2869–2874.
28. Kubota I, Tomoike H, Han X, Sakurai K, Endoh M. The Na^+-Ca^{2+} exchanger contributes to β-adrenoceptor mediated positive inotropy in mouse heart. *Jpn Heart J.* 2002;43:399–407.
29. Bendall JK, Cave AC, Heymes C, Gall N, Shah AM. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II–induced cardiac hypertrophy in mice. *Circulation.* 2002;105:293–296.
30. Paterson A, Plevin R, Wakelam MJO. Accurate measurement of *sn*-1,2-diradyl-glycerol mass in cell lipid extracts. *Biochem J.* 1991;280: 829–836.
31. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med.* 2002;8:35–40.
32. Roman BB, Geenen DL, Leitges M, Buttrick PM. PKC-beta is not necessary for cardiac hypertrophy. *Am J Physiol.* 2001;280:H2264–2270.
33. Ohanian J, Ollerenshaw J, Collins P, Heagerty A. Agonist-induced production of 1,2-diacylglycerol and phosphatidic acid in intact resistance arteries: evidence that accumulation of diacylglycerol is not a prerequisite for contraction. *J Biol Chem.* 1990;265:8921–8928.
34. Dhalla NS, Xu YJ, Sheu SS, Tappia PS, Panagia V. Phosphatidic acid: a potential signal transducer for cardiac hypertrophy. *J Mol Cell Cardiol.* 1997;29:2865–2871.
35. Exton JH. Phospholipase D: enzymology, mechanisms of regulation, and function. *Physiol Rev.* 1997;77:303–320.
36. Pettitt TR, Wakelam MJ. Diacylglycerol kinase ϵ, but not ζ, selectively removes polyunsaturated diacylglycerol, inducing altered protein kinase C distribution in vivo. *J Biol Chem.* 1999;274:36181–36186.
37. Verrier E, Wang L, Wadham C, Albanese N, Hahn C, Gamble JR, Chatterjee VK, Vadas MA, Xia P. PPARγ agonists ameliorate endothelial cell activation via inhibition of diacylglycerol-protein kinase C signaling pathway: role of diacylglycerol kinase. *Circ Res.* 2004;94:1515–1522.
38. Luo B, Prescott SM, Topham MK. Association of diacylglycerol kinase ζ with protein kinase C α: spatial regulation of diacylglycerol signaling. *J Cell Biol.* 2003;160:929–937.

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ß2-Adrenergic Agonists Suppress Rat Autoimmune Myocarditis: Potential Role of ß2-Adrenergic Stimulants as New Therapeutic Agents for Myocarditis

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β2-Adrenergic Agonists Suppress Rat Autoimmune Myocarditis

Potential Role of β2-Adrenergic Stimulants as New Therapeutic Agents for Myocarditis

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Background—The therapeutic potential of β2-adrenergic receptor (AR) agonists in the treatment of autoimmune diseases has been reported. However, the role of these drugs in the myocardial structure–induced autoimmune process, which is thought to play a crucial role in the progression of myocarditis to subsequent complications, has not been elucidated.

Methods and Results—Experimental autoimmune myocarditis (EAM) was induced in rats by immunization with cardiac myosin. On daily administration from day 0 after immunization, the β2-selective AR agonists formoterol or salbutamol ameliorated EAM on day 21 and increased myocardial interleukin-10/interferon-γ mRNA levels. Propranolol, a nonselective β-AR antagonist, aggravated EAM on day 21 and decreased mRNA levels, whereas metoprolol, a β1-selective AR antagonist, showed no effect. These results were reflected in vivo by the proliferation of cardiac myosin–primed lymph node cells from drug-treated rats. In vitro addition of β2-selective AR agonists inhibited the activation of cardiac myosin fragment–specific myocarditogenic T lymphocytes, and this effect was reversed by ICI118,551, a β2-selective AR antagonist. Furthermore, treatment with 2 different β2-selective AR agonists starting on day 14 also ameliorated EAM on day 21.

Conclusions—β2-AR stimulation suppressed the development of EAM by inhibiting cardiac myosin–specific T-lymphocyte activation in lymphoid organs and by shifting the imbalance in Th1/Th2 cytokine toward Th2 cytokine. Furthermore, it also ameliorated established myocardial inflammation. β2-AR–stimulating agents may represent important immunomodulators of the cardiac myosin–induced autoimmune process and have potential as a new therapy for myocarditis. (***Circulation.* 2006;114:936-944.**)

Key Words: immune system ■ myocarditis ■ receptors, adrenergic, beta

Apart from those with fulminant cases requiring mechanical circulatory support for severely deteriorated circulatory collapse, most patients with acute myocarditis recover rapidly to an uncomplicated status, with cessation of myocardial inflammation and a generally favorable outcome.[1] Some patients, however, progress to persistent myocardial inflammation and subsequent dilated cardiomyopathy.[2,3] Although chronic viral infection has long been recognized as a candidate causative factor for these pathophysiological mechanisms,[3] a number of experimental models have demonstrated the crucial role[4,5] of myocardial structure–mediated autoimmune processes, which follow the myocardial damage provoked by the initial viral infection.[6,7] The presence of autoantibodies against myocardial structure in patients with myocarditis and dilated cardiomyopathy[8,9] supports the involvement of myocardial structure–mediated autoimmune processes in these settings in humans.

Clinical Perspective p 944

Investigations using rat experimental autoimmune myocarditis (EAM) have shown that Th1 cytokines such as interferon-γ (IFN-γ) and interleukin-12 (IL-12) are major promoters of these autoimmune processes.[10,11] On the other hand, given reports that β2-adrenergic receptors (β2-ARs) are present on Th1 T lymphocytes and antigen-presenting cells and that their activation suppresses the production of Th1 cytokines such as IFN-γ and IL-12,[12,13] β2-AR has been investigated as a potential immunomodulator in Th1 cytokine–induced autoimmune disease.[14] However, the role of β2-AR–stimulating agents on myocardial structure–mediated

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autoimmune processes remains unknown. In this study we compared the effects of β-AR agents on EAM.

Methods

In Vivo Experiments

Induction of Rat Autoimmune Myocarditis

EAM was induced by immunizing 5-week-old female Lewis rats (Charles River Laboratory, Tsukuba, Ibaraki, Japan) with 0.25 mg of porcine cardiac myosin conjugated with complete Freund's adjuvant containing 0.25 mg of *Mycobacterium tuberculosis* H73RA (Difco, Detroit, Mich), as previously reported.[15] All experimental procedures and protocols used in this study conformed to the institutional guidelines of Kitasato University School of Medicine for the care and use of animals.

Therapeutic Protocols

Protocol I

Groups of 18 healthy animals each received intraperitoneal administration of propranolol (Sigma, St Louis, Mo) at 10 mg/kg per day as a nonselective β-AR antagonist, metoprolol at 30 mg/kg per day as a β1-selective AR antagonist (Novartis Pharmaceutical Co, Tokyo, Japan), formoterol at 22.5 μg/kg per day as a β2-selective AR agonist[16] (Yamanouchi Pharmaceutical Co, Tokyo, Japan), salbutamol at 200 μg/kg per day as a β2-selective AR agonist[13,14] (Sigma Chemical Co, St Louis, Mo), or an equal volume of phosphate-buffered saline vehicle containing 0.5% methylcellulose daily from immunization with myosin, on day 0 until euthanasia on day 21. These drugs were also administered to control groups of 8 healthy animals each without immunization with myosin for 3 weeks. Doses were selected on the basis of previous findings[17] to ensure a near-equipotent β1-AR blocking effect.

Protocol II

Groups of 12 healthy animals each were given formoterol at 22.5 μg/kg per day, salbutamol at 200 μg/kg per day, or an equal volume of vehicle by intraperitoneal administration from day 14 until day 21 after immunization with myosin.

Hemodynamic Analysis

Blood pressure (BP), heart rate (HR), and fractional shortening were determined in healthy (without myosin immunization) and diseased rats (with immunization) treated with β-AR–modulating agents or vehicle by the tail-cuff method with the use of a photoelectric tail-cuff detection system (Softron, Tokyo, Japan) and echocardiographic study (SSD-6500SV, Aloka, Tokyo, Japan) just before euthanasia on day 21. All measurements were averaged over at least 3 consecutive cardiac cycles.

Assessment of Severity of Myocarditis

All rats were killed under ether anesthesia on day 21. The ratio of heart weight to body weight (HW/BW) was calculated, and macroscopic scores were classified according to a 5-grade scoring system as previously reported.[18] The cardiac ventricles were then divided transversely into 2 sections. The ratio of the area of inflammatory infiltrates to that of the whole myocardium on a sliced half-transverse section was calculated with a microscope, as previously reported,[18] by 2 blinded observers. Interobserver and intraobserver variance was <5%.

Measurement of Cytokine Expression in Hearts

Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed to measure myocardial expression of IFN-γ or interleukin-10 (IL-10) mRNA in the other half of the hearts. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with the use of an ABI PRISM 7700 Sequence Detection System (PE Biosystems). Positive-stranded and negative-stranded primers for mRNA amplification were ATCTGGAGGAACTGGCAAAAGGACG and CCTTAGGCTAGATTCTGGTGACAGC for IFN-γ,[19] ACTGCTCTGTTGCCTGCTCTTACT and GAATTCAAATGCTCCTTGATTTCT for IL-10,[19] and ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA for glyceraldehyde phosphate dehydrogenase.[19] A standard curve was calculated with the use of the ABI PRISM 7700 System, from which the absolute copy numbers of mRNA in the samples were obtained.

In Vitro Experiments

β-AR–Modulating Agents on Myocarditogenic T-Lymphocyte Activities

A myocardiogenic CD4-positive Th1-phenotype T-lymphocyte line specific for the cardiac myosin fragment CM2 (a.a. 1539–1555)[20] was established as previously reported.[18] This T-lymphocyte line (5×10^4 per well) was cultured in triplicate supplemented with CM2 (10 μg/mL) and irradiated (5000 rad) syngeneic thymocytes as antigen-presenting cells (1×10^6 per well). Formoterol (10^{-10} to 10^{-4} mol/L), salbutamol (10^{-10} to 10^{-4} mol/L), or denopamine (10^{-10} to 10^{-4} mol/L; Tanabe Pharmaceutical Co, Tokyo, Japan) as a β1-selective AR agonist or vehicle was added to the cell-suspension culture solution, with or without ICI118,551 (10^{-8} to 10^{-6} mol/L; Tocris, Ellisville, Mo) as a β2-selective AR antagonist. After incubation, proliferation of cardiac myosin–specific T lymphocytes and levels of IFN-γ and IL-12 in each well were determined as previously described.[18] Three series of experiments were performed for each investigation.

Proliferation Assay Using Myosin-Primed Lymph Node Cells From Treated Rats

Popliteal lymph nodes were removed from Lewis rats killed 11 days after immunization with porcine cardiac myosin under daily administration of metoprolol at 30 mg/kg per day, propranolol at 10 mg/kg per day, formoterol at 22.5 μg/kg per day, salbutamol at 200 μg/kg per day, or vehicle (n=9 each). Viable mononuclear cells (5×10^4 per well) from the lymph nodes in single-cell suspension were cultured for 48 hours in triplicate with or without 10 μg/mL of cardiac myosin. Cell proliferation and levels of IFN-γ and IL-12 in each well were then determined as described previously.[18]

Intracellular cAMP Measurement

Myosin-primed lymph node cells (5×10^4 per well) from drug-treated rats (n=9 each) were cultured in triplicate with cardiac myosin as described above. Cells were pelleted by centrifugation at 1400*g* for 5 minutes followed by the addition of lysis buffer for 10 minutes. Intracellular cAMP levels were then measured with an enzyme-linked immunosorbent assay (ELISA) kit (Amersham, Piscataway, NJ).

Statistical Analysis

Data are expressed as mean±SEM. Statistical analyses were performed by 1-way ANOVA, followed by a post hoc test (Bonferroni multiple comparison test). RT-PCR analysis was performed as follows. The copy number of IFN-γ or IL-10 mRNA was normalized for GAPDH mRNA, and the myocardial expression of cytokine in each sample from EAM rats that received treatment with the β-AR agent or vehicle was then expressed as fold increase over the average level in the control group, composed of 9 EAM rats, on day 21 with no treatment. The balance of Th1 and Th2 cytokines was expressed as the ratio of IL-10 mRNA to IFN-γ mRNA: IL-10/IFN-γ. IL-10/IFN-γ level in each sample with therapy was also expressed as fold increase over that of the control group. Levels of IFN-γ, IL-10, or IL-10/IFN-γ, as well as histological and hemodynamic variables in the vehicle and control groups, were approximately equal (data not shown). To examine the effects of β-AR agents on the expression of cytokine, fold increase was compared across therapeutic groups. Probability values <0.05 were considered statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.