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【好評発売中】



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当直医実戦くすりマニュアル

●著 梅田悦生

「当直医実戦マニュアル」の姉妹本。時間外の薬物治療トラの巻。症状ごとに時間外治療のコンセプト、処方例と主な薬剤名を収載。薬効別便覧では「選び方と使い方のコツ」「当直現場で必要とされる安全性情報」、小児薬用量、FDAなどを解説。併用禁忌薬剤は、すべての記載に薬効分類、一般名、商品名を補った。効果がシャープな注射薬を数多く収載した。

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Identification of genes related to heart failure using global gene expression profiling of human failing myocardium

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ABSTRACT

Although various management methods have been developed for heart failure, it is necessary to investigate the diagnostic or therapeutic targets of heart failure. Accordingly, we have developed different approaches for managing heart failure by using conventional microarray analyses. We analyzed gene expression profiles of myocardial samples from 12 patients with heart failure and constructed datasets of heart failure-associated genes using clinical parameters such as pulmonary artery pressure (PAP) and ejection fraction (EF). From these 12 genes, we selected four genes with high expression levels in the heart, and examined their novelty by performing a literature-based search. In addition, we included four G-protein-coupled receptor (GPCR)-encoding genes, three enzyme-encoding genes, and one ion-channel protein-encoding gene to identify a drug target for heart failure using *in silico* microarray database. After the *in vitro* functional screening using adenovirus transfections of 12 genes into rat cardiomyocytes, we generated gene-targeting mice of five candidate genes, namely, *MYLK3*, *GPR37L1*, *GPR35*, *MMP23*, and *NBC1*. The results revealed that systolic blood pressure differed significantly between *GPR35*-KO and *GPR35*-WT mice as well as between *GPR37L1*-Tg and *GPR37L1*-KO mice. Further, the heart weight/body weight ratio between *MYLK3*-Tg and *MYLK3*-WT mice and between *GPR37L1*-Tg and *GPR37L1*-KO mice differed significantly. Hence, microarray analysis combined with clinical parameters can be an effective method to identify novel therapeutic targets for the prevention or management of heart failure.

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Introduction

Heart failure is a multi-factorial condition with increasing prevalence worldwide; further, a significant increase has been observed in the mortality rate and economic impact associated with this condition. In the last 20 years, substantial development of treatment for heart failure, including angiotensin-converting-enzyme inhibitors [1] and beta-blockers [2,3], has greatly improved the

prognosis of the patients with heart failure. However, despite these rapid advancements in the management of heart failure, effective treatment of end-stage heart failure without providing ventricular assistance or heart transplantation is still difficult. Investigation of new and unexplored targets for the prevention or treatment of heart failure is warranted. Global gene expression analysis using microarray technique has been used in the last decade to identify biomarkers or drug targets for heart failure [4–10]. Several gene expression signatures of heart failure have been identified by analyzing independent microarray datasets [11,12]. However, most of these analyses did not consider the severity of heart failure. Because the severity of heart failure may quantitatively reflect the expression levels of genes such as the natriuretic

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peptide-encoding gene, expression analysis combined with the severity of heart failure could be an appropriate method to identify heart failure-related genes. However, microarray analysis of genes expressed in failing myocardium while considering the severity of heart failure has not yet been reported.

Hence, we investigated the genes whose expression level correlated with clinical parameters such as pulmonary artery pressure (PAP), left ventricular ejection fraction (EF), and brain natriuretic peptide (BNP) mRNA level. Using this approach, we identified cardiac myosin light chain kinase as a novel heart failure-related gene [13]. Here, we describe newly identified several genes whose expression correlated with clinical parameters and additional genes encoding G-protein-coupled receptor genes (GPCRs), other enzymes and ion-channel proteins, and performed the functional analysis of these heart failure-related genes. This novel strategy involving the use of clinical parameters might find potential applications for the identification of disease-associated genes that could not be detected using conventional microarray techniques.

Materials and methods

Patient characteristics. We recruited 12 patients (11 males and 1 female) with heart failure and obtained written informed consent from them. The patients were diagnosed with severe chronic heart failure due to various cardiac diseases such as dilated cardiomyopathy and myocardial infarction [13]. The average age of patients was 55 ± 13 years. The plasma level of BNP, which is the best marker for the severity of heart failure, ranged from 80 to 2710 pg/ml. The mean PAP measured using a Swan-Ganz catheter 1–4 weeks before the operation varied between 16 and 59 mmHg. The average of EF determined by echocardiography on the day before the operation was $32.5\% \pm 12.4\%$.

Microarray analysis and subsequent in silico functional analysis. RNA was extracted from myocardium samples of 12 heart failure patients who had undergone either Batista or Dor surgeries. RNA samples of non-failing hearts were purchased from Biochain, Inc. Complementary RNA (cRNA) was prepared from RNA samples and hybridized to HG-U95 Affymetrix GeneChip (Affymetrix, US). The expression data were analyzed using Microarray Analysis Suite version 5.0 software. Among all the genes detected on the microarray, we selected the genes whose expression was significantly different in the failing and non-failing myocardial samples ($p < 0.005$). From these genes, we selected genes whose expression was correlated with PAP, EF, and BNP mRNA level, with 0.7 being the cutoff value of the correlation coefficient. The values of PAP, EF, and BNP mRNA level used for the correlation analysis were normalized to their median during the measurements. Subsequently, the functional analysis of datasets was performed using Ingenuity Pathway Analysis (Ingenuity® Systems; www.ingenuity.com), and the biological functions most significant to the dataset were identified.

Cell culture. Cardiomyocytes were harvested before the experiments from 2- to 3-day-old neonatal rats and cultured as described in previous studies [14]. Briefly, primary cardiomyocytes isolated from neonatal rats were grown in Dulbecco's modified Eagle medium/F12 (Gibco) supplemented with 10% fetal calf serum for 72 h, and then cultured in a serum-free condition for 24 h.

Adenovirus generation and transfection. Adenovirus constructs encoding the genes of interest were generated using the ViraPower Adenoviral Expression System (Invitrogen, US) according to the manufacturer's method. Adenovirus vectors were transfected to cultured cardiomyocytes for 12 h according to the published protocol.

In vitro functional analysis of genes. Cultured rat cardiomyocytes were infected by adenovirus vectors. After 24 h, hypertrophic

reaction, cell viability, and cellular morphology were assessed. Hypertrophic reaction was determined by estimating the incorporation of [3 H]phenylalanine. In brief, [3 H]phenylalanine was added to the culture medium at the final concentration of 0.1 μ Ci/ml, and the cells were incubated for an additional 24 h. Then, the incorporation of [3 H]phenylalanine was determined by counting the radioactivity of each sample with a liquid scintillation counter. The viability of cardiomyocytes was evaluated by the Alamar blue assay according to the manufacturer's method. The morphology of cardiomyocytes was evaluated 24 h after adenovirus transfections.

Generation of transgenic and knockout mice. To generate transgenic mice, open reading frame of each gene, namely, *Mylk3*, *Gpr3711*, or *Nbc1* was amplified from mouse cDNA by PCR, with Sal I site linker on each end, and cloned into Sal I site of alpha-MHC clone 26 vector. Then the DNAs used in the microinjections were released from the vector by digestion with NotI and were microinjected into fertilized eggs of mouse. Founder mice were identified by PCR analysis with appropriate primers. To develop *Gpr3711* knockout mice, the targeting vector was assembled to replace the exon 1 and 2 by neomycin selection cassette resulting in the absence of *Gpr3711* protein. W9.5 ES cells were electroporated with linearized targeting vector. ES cell clones with successful homologous recombination was determined by the PCR and subsequent direct sequence. From these targeted ES cells, the chimera mice were bred to C57 BL/6 females to generate F1 and F2 offsprings were obtained. The *Gpr3711* null mice were determined by PCR genotyping of F2 offsprings. The knockout mice of *Gpr35* and *Mmp23* (the mouse ortholog of MMP23B) were purchased from Deltagen, Inc. (California, US).

Invasive blood pressure measurement. The phenotype of the gene-targeted mice was examined. Before sacrificing the mice, their hemodynamic parameters were evaluated. The mice were anesthetized and ventilated, and a Millar catheter was inserted via right carotid artery. The left-ventricular systolic and end-diastolic pressures were measured. Then, the mice were sacrificed and the weight of the whole body and heart was determined.

Statistical analysis. Unpaired Student's *t*-test was used for comparing the two groups. Results are expressed as means \pm SEM, and *p* value less than 0.05 was considered statistically significant.

Results

Identification of heart failure-related genes by expression analysis using clinical parameters

We performed microarray analysis of the genes expressed in failing myocardium obtained from 12 patients with heart failure and the genes expressed in non-failing myocardium from two normal objects whose characteristics were reported in the previous study [13]. Although all patients were diagnosed with chronic heart failure, the plasma BNP level, which is an index of the severity of heart failure, ranged from 80 to 2710 pg/ml, suggesting that the severity of heart failure varied extensively among the patients. This marked difference in the severity of heart failure reflects the fact that the gene expression patterns in the 12 patients were not uniform, as shown in Fig. 1A. Thus, we analyzed gene expression profiles of failing myocardium using clinical parameters representing the severity of heart failure. We identified 166 and 194 genes whose expressions were correlated with PAP and BNP mRNA level, respectively (Fig. 1B and Supplementary Tables S1, S2). Among these, 49 genes correlated with both PAP and BNP mRNA level (Fig. 1C). The expression of only two genes, namely, *FMO2* and *LMAN1L*, correlated with the EF. We investigated the functional categories of these genes by performing Ingenuity Pathway Analysis. The number of genes in each group, functional categories, and

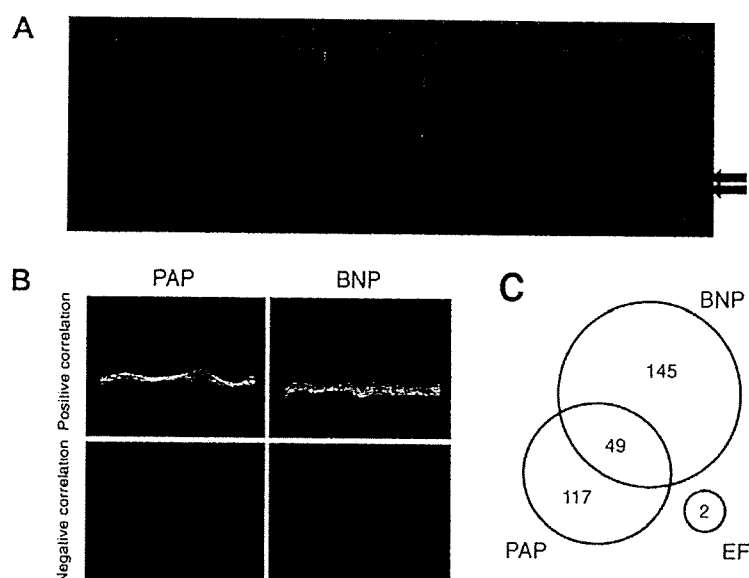


Fig. 1. The gene expression profile of human failing or non-failing myocardium. Gene expression levels of myocardial samples from 12 patients with severe heart failure and from two normals were analyzed using microarray. (A) Heat maps showing the genes with differential expression between the 12 failing 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
<i>Positive correlation</i>			
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, SERPINF1 CKS1B, DDR2, FCGR2B, FNI
<i>Negative correlation</i>			
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, PRKARIA, 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 disease-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
<i>Genes relevant to clinical parameters</i>							
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	Peroxisome oxidin 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
<i>Genes encoding orphan GPCRs</i>							
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
<i>Genes encoding interesting enzymes or ion-channels</i>							
38950_at	MMP23B	Matrix metalloproteinase 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 [³H]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 asso-

ciated 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

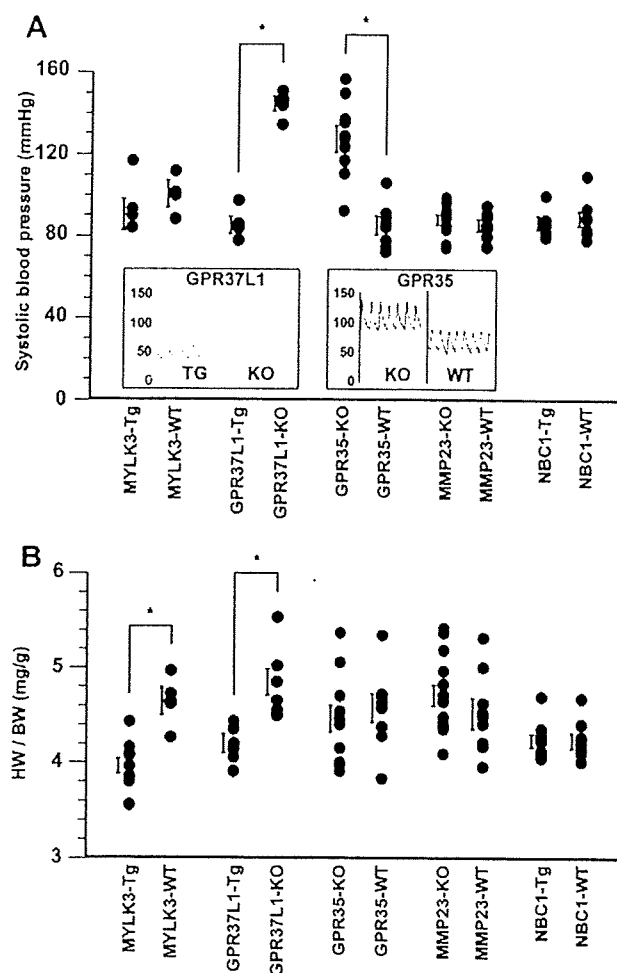


Fig. 2. *In vivo* functional analysis using gene-targeting mice of the *Mylk3*, *Gpr37l1*, *Gpr35*, *Mmp23*, and *Nbc1* genes. Blood pressure and heart weight (HW)/body weight (BW) of transgenic (Tg), knockout (KO) and their wild type (WT) littermate mice of each gene were investigated. Values are means \pm 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 indoleamine 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.

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Natriuretic Peptides Enhance the Production of Adiponectin in Human Adipocytes and in Patients With Chronic Heart Failure

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Objectives	We investigated the functional relationship between natriuretic peptides and adiponectin by performing both experimental and clinical studies.
Background	Natriuretic peptides are promising candidates for the treatment of congestive heart failure (CHF) because of their wide range of beneficial effects on the cardiovascular system. Adiponectin is a cytokine derived from adipose tissue with various cardiovascular-protective effects that has been reported to show a positive association with plasma brain natriuretic peptide (BNP) levels in patients with heart failure.
Methods	The expression of adiponectin messenger ribonucleic acid (mRNA) and its secretion were examined after atrial natriuretic peptide (ANP) or BNP was added to primary cultures of human adipocytes in the presence or absence of HS142-1 (a functional type A guanylyl cyclase receptor antagonist). Changes of the plasma adiponectin level were determined in 30 patients with CHF who were randomized to receive intravenous ANP (0.025 $\mu\text{g}/\text{kg}/\text{min}$ human ANP for 3 days, $n = 15$) or saline ($n = 15$).
Results	Both ANP and BNP dose-dependently enhanced the expression of adiponectin mRNA and its secretion, whereas such enhancement was inhibited by pre-treatment with HS142-1. The plasma adiponectin level was increased at 4 days after administration of human ANP compared with the baseline value (from $6.56 \pm 0.40 \mu\text{g}/\text{ml}$ to $7.34 \pm 0.47 \mu\text{g}/\text{ml}$, $p < 0.05$), whereas there was no change of adiponectin in the saline group (from $6.53 \pm 0.57 \mu\text{g}/\text{ml}$ to $6.55 \pm 0.56 \mu\text{g}/\text{ml}$).
Conclusions	Natriuretic peptides enhance adiponectin production by human adipocytes in vitro and even in patients with CHF, which might have a beneficial effect on cardiomyocytes in patients receiving recombinant natriuretic peptide therapy for heart failure. (J Am Coll Cardiol 2009;53:2070-7) © 2009 by the American College of Cardiology Foundation

Plasma natriuretic peptide levels are increased in patients with congestive heart failure (CHF), and the measurement of these peptides is used widely to assess the presence,

severity, and prognosis of CHF (1,2). Both atrial natriuretic peptide and brain natriuretic peptide (ANP and BNP, respectively) have a beneficial effect in patients with heart failure because of their various biological actions (3-5).

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Adiponectin is a circulating cytokine derived from adipose tissue that has attracted considerable interest because of its identification as a risk factor for cardiovascular disease (6,7) and CHF (8). Adiponectin production is down-regulated in patients with coronary risk factors that are associated with the development of heart failure (9,10).

Recently, adiponectin was reported to have a cardioprotective effect against ischemia-reperfusion injury (11) and hemodynamic stress (12,13) in mice. Interestingly, it has been reported that the level of N-terminal pro-brain natriuretic peptide shows a positive correlation with the plasma adiponectin concentration in patients with chronic heart failure (14).

Given these experimental and clinical observations, we hypothesized that natriuretic peptides might increase adiponectin production in patients with heart failure to protect the cardiovascular system. Accordingly, in the present study, we investigated whether natriuretic peptides could directly increase adiponectin production by these adipocytes (and the cellular mechanisms involved) and confirmed this effect on adiponectin in the clinical setting.

Methods

Agents. Both human ANP and BNP were purchased from Sigma-Aldrich (St. Louis, Missouri). HS142-1, a functional guanylyl cyclase-A type receptor antagonist, was provided by Kyowa Hakko Kogyo Co., Ltd. (Mishima, Japan). A cGMP analog (8-pCPT-cGMP) and a selective cGMP-dependent protein kinase G (PKG) inhibitor (R_p -8-Br-PET-cGMP-S) were obtained from Biolog Life Science Institute (Bremen, Germany). An antibody directed against mouse adiponectin (MAB3608) was purchased from Chemicon International, Inc.

Primary culture and in vitro study of human adipocytes. Subcutaneous adipocytes derived from the adipose tissue of 6 women were obtained commercially together with culture medium from Zen-Bio, Inc. (Research Triangle Park, North Carolina). The donors were nonsmokers with a mean body mass index of 27.0 kg/m² (range 25.9 to 29.1 kg/m²) and an average age of 47 years (range 29 to 63 years). Cells were maintained in adipocytes maintenance medium (i.e., AM-1) containing Dulbecco's modified Eagle medium/Ham's F-12 (1:1, v/v), 3% fetal calf serum, 15 mmol/l HEPES (pH 7.4), biotin, pantothenate, human insulin, 1 μ mol/l dexamethasone, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B at 37°C in a humidified atmosphere of 95% air/5% CO₂. The medium was changed every 2 days. Primary cultures of the adipocytes were used to examine the effects of natriuretic peptides (ANP or BNP) on the expression of adiponectin.

Before these experiments, the cells were plated in adipocyte basal medium (i.e., BM-1) containing Dulbecco's modified Eagle medium/Ham's F-12 (1:1, volume/volume), 15 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), biotin, and pantothenate for 24 h. Then the indicated concentrations of either natriuretic peptide (from 10⁻¹¹ to 10⁻⁹ mol/l) were added to the BM-1 medium. After 24 h of incubation, the medium was harvested for Western blotting to measure the secretion of adiponectin, and the cells were also harvested for ribonucleic acid (RNA) analysis. The effect of each natriuretic peptide on adiponectin messenger ribonucleic acid (mRNA) levels

was determined by quantitative real-time polymerase chain reaction (PCR).

Measurement of adiponectin. In patients with CHF, the plasma adiponectin concentration was measured by the use of an ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. Adiponectin secretion by primary cultured human adipocytes was assessed by Western blotting of the culture medium, as previously described (15), and the immunoreactive bands were quantified by densitometry (Molecular Dynamics, Sunnyvale, California).

Reverse transcriptional-PCR. Total RNA was extracted from adipocytes derived from human white fat with the use of RNA-Bee-RNA Isolation Reagent (Tel-Test, Inc., Gainesville, Florida). Then, 200 ng of total RNA was reversed transcribed and amplified by the use of an Omniscript RT kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The forward primers for type A guanylyl cyclase receptor (GC-A) and natriuretic peptide receptor (NPR)-C were 5'-CCAGTTC AAGTCTTTGCCAA-GACAGCA and 5'-GGAAGACATCGTGCGCAATA, respectively, and the reverse primers for GC-A and NPR-C were 5'-CATTGTGTAGAAACAGCATGCCCTTGA-CGA and 5'-TGCTCCGGATGGTGTCACT, respectively. As a positive control, we used the samples of human cardiac tissue under the protocol approved by the institutional review board of the National Cardiovascular Center (No. 14-18) (16).

Quantitative real-time PCR analysis. Quantitative real-time PCR was performed as described previously (17). Oligonucleotide primers and TaqMan probes for human adiponectin and glyceraldehyde 3-phosphate dehydrogenase were purchased from Applied Biosystems (Foster City, California).

Subjects and design of the clinical study. We prospectively studied 30 consecutive CHF patients who were admitted to the emergency department of the National Cardiovascular Center between April and July 2006. The exclusion criteria were as follows: age >80 years, cardiogenic shock or hypotension (systolic blood pressure <100 mm Hg), and renal failure (serum creatinine >2.0 mg/dl). This study was approved by the Committee on Human Investigation of the National Cardiovascular Center, and all patients who participated gave informed consent. The 30 patients were randomized to 2 groups, a human atrial natriuretic peptide (hANP) group consisting of 15 patients who received administration of hANP and a control group consisting of 15 patients who were administered saline. In the hANP group, from immediately after the diagnosis of

Abbreviations and Acronyms

ANP = atrial natriuretic peptide

BNP = brain natriuretic peptide

CHF = congestive heart failure

GC-A = type A guanylyl cyclase receptor

hANP = human atrial natriuretic peptide

NPR = natriuretic peptide receptor

PKG = protein kinase G

acute exacerbation of CHF, hANP (0.025 $\mu\text{g}/\text{kg}/\text{min}$) was infused intravenously for 3 days. The study protocol did not restrict or specify any other diagnostic or therapeutic strategies. Blood for measuring the plasma adiponectin level was sampled before and 1 and 7 days after finishing the administration of hANP or saline (days 1, 4, and 10, respectively) (Fig. 3A).

Statistical analysis. For analysis of differences between the various treatments of adipocytes, analysis of variance was performed, followed by the appropriate post-hoc test. The differences in adiponectin levels between days 1 and 4 in each group were tested with a paired *t* test. The changes in adiponectin levels from day 1 to 4 between ANP group and saline group was tested with an unpaired *t* test. Results are expressed as the mean \pm SEM, and *p* values of <0.05 were considered significant.

Results

Effect of natriuretic peptides on the expression and secretion of adiponectin by primary cultured human adipocytes. First, we checked the expression of GC-A and NPR-C mRNA by using reverse transcriptional-PCR. As shown in Figure 1A, both GC-A and NPR-C mRNA was detectable in primary cultured human adipocytes. To investigate the effects of natriuretic peptides on the regulation of adiponectin production in adipocytes, we incubated primary cultured human adipocytes with recombinant ANP. When ANP was used at a concentration of 10^{-10} mol/l (pathological plasma concentration), it increased adiponectin mRNA expression after 6 h of incubation and reached a maximum after 12 h (Fig. 1B). Next, we incubated human adipocytes with ANP at the concentration of from 10^{-11} mol/l (normal plasma concentration) to 10^{-9} mol/l (pharmacological plasma concentrations) and demonstrated enhanced adiponectin mRNA expression and adiponectin secretion into the medium in a dose-dependent manner, whereas these changes were completely inhibited by pretreatment with HS142-1 (Figs. 1C and 1D). Incubation of adipocytes with BNP also increased the expression of adiponectin mRNA in a dose-dependent manner and this effect was completely blocked by pretreatment with HS142-1 (Figs. 1E and 1F).

Involvement of cGMP/PKG signaling in natriuretic peptide-induced synthesis of adiponectin. Because both ANP and BNP exert their biological effects by promoting cGMP production, to investigate the role of the GC-A/cGMP/PKG signaling pathway in adiponectin production, we measured the changes of cGMP in ANP-treated primary cultured human adipocytes. We found that incubation with ANP increased the cGMP level and that this effect was blunted by co-treatment with HS142-1 (data not shown). Next, we treated human adipocytes with the cGMP analog 8-pCPT-cGMP and the PKG inhibitor (R_p)-8-Br-PET-cGMP-S. The activation of PKG by 8-pCPT-cGMP (50 $\mu\text{mol}/\text{l}$ for 12 h) produced an increase of adiponectin

mRNA expression similar to that observed after incubation with ANP. The effect of ANP on adiponectin mRNA expression was abolished in the presence of (R_p)-8-Br-PET-cGMP-S (100 nmol/l) (Fig. 2A). Consistent with these findings, adiponectin secretion into the culture medium also was increased by stimulation of the cGMP/PKG-dependent pathway (Fig. 2B). These results suggested that natriuretic peptides promote adiponectin synthesis via the GC-A/cGMP/PKG-dependent pathway.

Increase of plasma adiponectin levels in CHF patients treated with hANP. To confirm the effect of natriuretic peptides on the production of adiponectin, we conducted the clinical study. Thirty consecutive patients who met the inclusion criteria were enrolled in this clinical study. Fifteen patients were randomized to the ANP group, and 15 were assigned to the saline group. Baseline variables and treatments of the 2 groups are shown in Table 1. There were no differences in baseline clinical characteristics, hemodynamics, biochemical data, or medications. There was also no significant difference in the baseline plasma level of adiponectin between the 2 groups. As shown in Figure 3B, the plasma level of adiponectin did not change throughout the study in the saline group. On the other hand, the plasma adiponectin level at 1 day after finishing the administration of hANP (day 4) was significantly increased compared with the baseline value (day 1) in the ANP group, and it returned to baseline by 7 days after the completion of hANP infusion (day 10). These results suggested that hANP infusion led to an increase of the plasma adiponectin level in patients with CHF.

Discussion

In the present study, we demonstrated a novel effect of natriuretic peptides (ANP and BNP) on the production of adiponectin by adipocytes in both experimental and clinical studies. First, we clearly demonstrated that pathophysiological and pharmacological concentrations of either ANP or BNP increased adiponectin synthesis by primary cultured human adipocytes. Second, we showed that administration of recombinant ANP increased the plasma adiponectin level in patients with CHF.

ANP and BNP play an important role in the regulation of cardiovascular homeostasis. Their actions are primarily mediated via GC-A, which is expressed in various tissues and organs, including the kidneys, blood vessels, adrenal glands, and heart (18). Consistent with a previous report (19), we demonstrated that GC-A and NPR-C are expressed by human adipocytes. In the present study, we demonstrated a novel effect of both ANP and BNP on primary cultured human adipocytes, which was that pathophysiological or pharmacological concentrations of both peptides augmented adiponectin production by human adipocytes, with this effect being inhibited by treatment with HS142-1. Furthermore, we demonstrated that natriuretic peptides augment the production of adiponectin via a cGMP-dependent

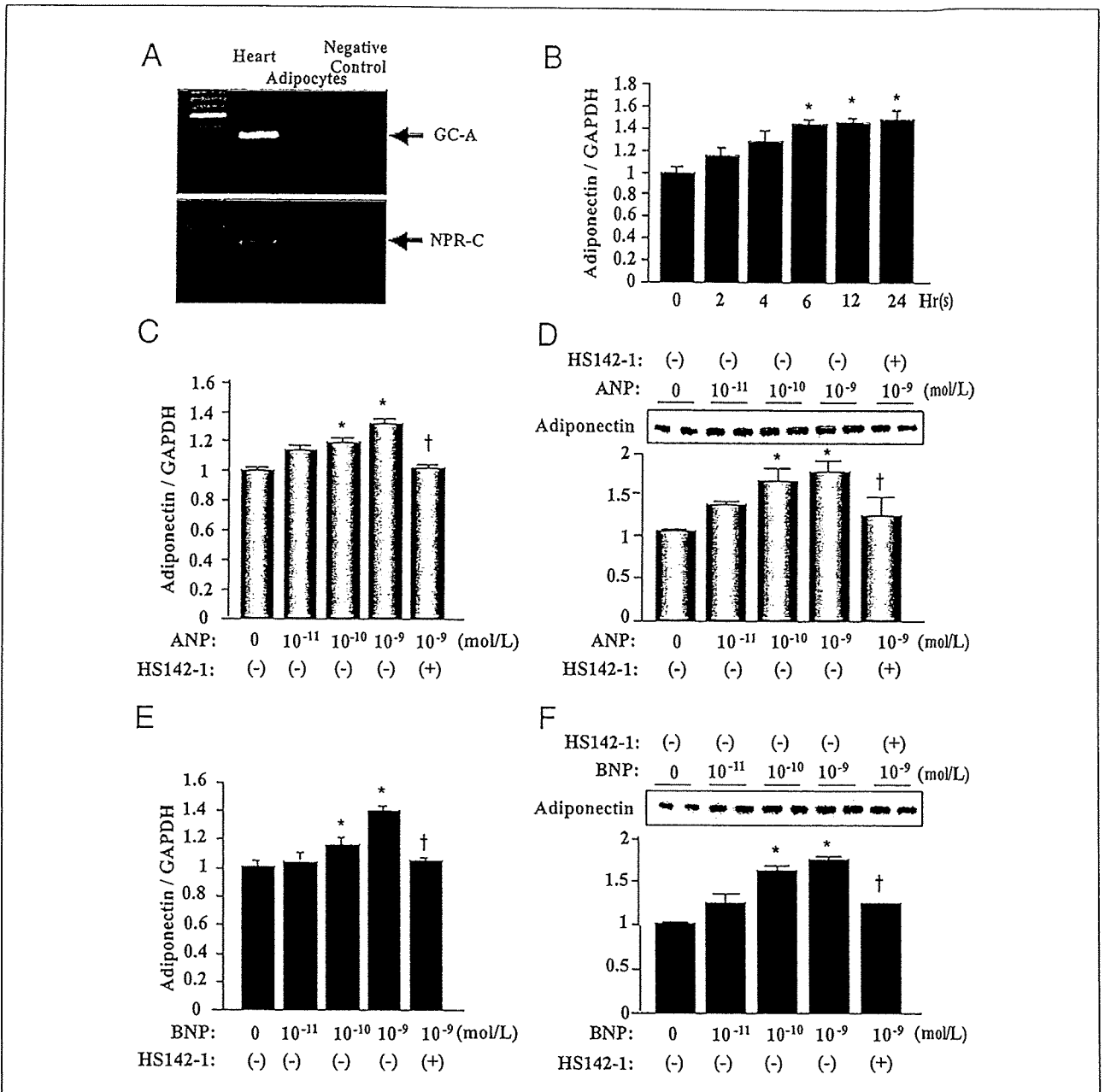


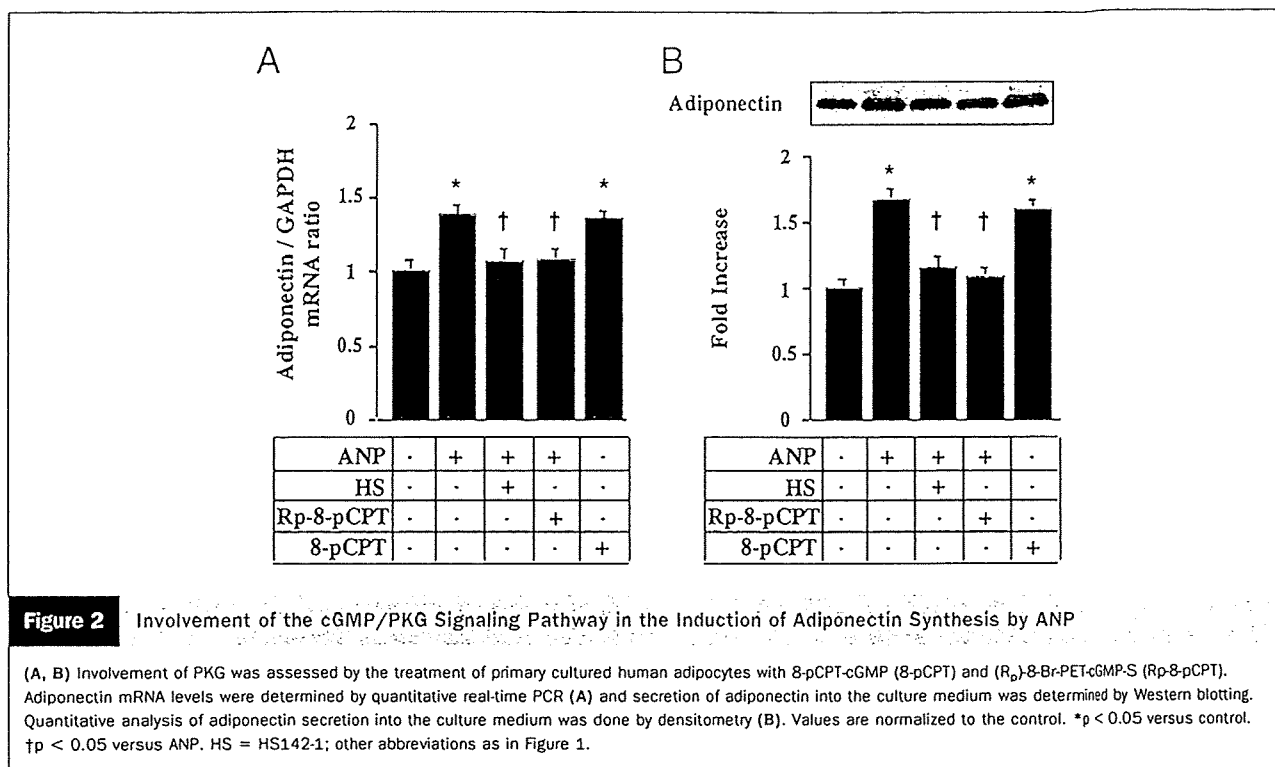
Figure 1 Effect of Natriuretic Peptides on the Expression and Secretion of Adiponectin by Primary Human Adipocytes

(A) Expression of GC-A receptors (top) and NPR-C (bottom) mRNA by primary cultured human adipocytes. Reverse-transcription PCR revealed expression of both GC-A receptors and NPR-C by human adipocytes. (B) Effect of ANP (10⁻¹⁰ mol/l) on the expression of adiponectin mRNA as determined by quantitative real-time PCR. (C) Dose-dependent effect of ANP on adiponectin mRNA expression, as determined by quantitative real-time PCR. Human adipocytes were treated with the indicated concentrations of ANP for 24 h. (D) Dose-dependent effect of ANP on adiponectin secretion into the culture medium. (Top) A representative Western blot of adiponectin. (Bottom) Quantitative analysis of adiponectin by densitometry. Values are normalized to the control. *p < 0.05 versus control, †p < 0.05 versus ANP 10⁻⁹ mol/l. (E) Dose-dependent effect of BNP on adiponectin mRNA expression, as determined by quantitative real-time PCR. (F) Dose-dependent effect of BNP on adiponectin secretion into the culture medium as determined by Western blotting. (Top) Representative Western blot of adiponectin. (Bottom) Quantitative analysis of adiponectin by densitometry. Values are normalized to the control. *p < 0.05 versus control, †p < 0.05 versus BNP 10⁻⁹ mol/l. ANP = atrial natriuretic peptide; BNP = brain natriuretic peptide; GC-A = type A guanylyl cyclase receptor; mRNA = messenger ribonucleic acid; NPR-C = natriuretic peptide receptor C; PCR = polymerase chain reaction.

pathway. These findings are important evidence that ANP and BNP regulate adiponectin production by human adipocytes.

Intravenous infusion of nesiritide (recombinant human BNP) has been reported to have beneficial hemodynamic

effects in patients with CHF (4,5). The use of ANP also has been reported to have beneficial effects in patients with acute myocardial infarction (20,21). These beneficial effects have been attributed to the cardiovascular-protective actions of natriuretic peptides, including diuresis, natriuresis, vaso-



dilation, and reduction of activity of the sympathetic nervous system and the renin-angiotensin-aldosterone system (3-5). In the present study, we administered recombinant ANP to patients with CHF and observed the changes of plasma adiponectin. The plasma adiponectin level of the ANP group was increased at 1 day after the finish of ANP administration compared with that in the control group, and then returned to baseline by 7 days after the completion of administration in patients with CHF.

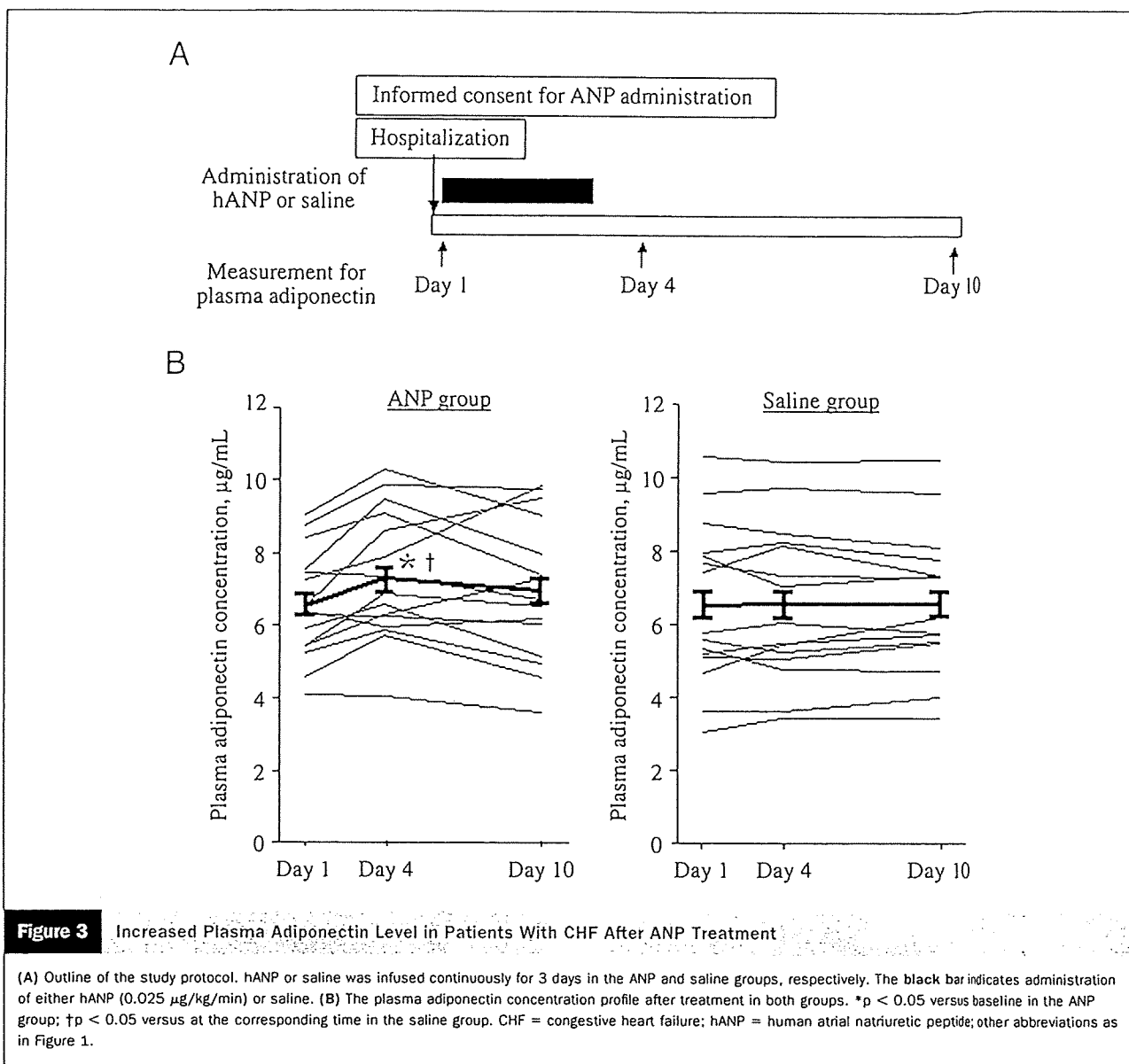
Importantly, Moro et al. (22) showed that ANP did not affect the secretion of adiponectin in human abdominal

adipose tissue from overweight women. This result may appear contradict ours, but we believe that is not the case. First, the concentration of ANP they used (10^{-6} mol/l) in the experiment of cultured adipocytes was greater than our concentration. Second, our data that recombinant ANP increased the plasma adiponectin levels were drawn from patients with heart failure, whereas the data of Moro et al. (22) were from cultured fat tissues of overweight women who underwent plastic surgery. However, they also demonstrated the potential stimulatory effect of ANP on adiponectin production from human adipose tissue in the presence of

Table 1 Clinical Characteristics of the 2 Groups

	hANP Group (n = 15)	Saline Group (n = 15)	p Value
Age (yrs)	60 ± 19	59 ± 19	NS
Sex (male/female)	9/6	10/5	NS
Heart rate (beats/min)	62 ± 11	66 ± 7	NS
Body mass index (kg/m ²)	21.4 ± 1.1	21.1 ± 1.7	NS
Systolic blood pressure (mm Hg)	116 ± 9	113 ± 9	NS
Diastolic blood pressure (mm Hg)	76 ± 12	74 ± 6	NS
NYHA functional class (II/III)	14/1	10/5	NS
LVEF by echocardiography (%)	32 ± 2	31 ± 8	NS
Plasma BNP (pg/ml)	506 ± 39	537 ± 33	NS
Other medications n (%)			
Loop diuretics	9 (60)	10 (67)	NS
Spironolactone	5 (33)	8 (53)	NS
ACEI or ARB	12 (80)	11 (80)	NS
Beta-blockers	13 (86)	12 (80)	NS

ACEI = angiotensin-converting enzyme inhibitors; ARB = angiotensin II receptor blockers; BNP = brain natriuretic peptide; hANP = human atrial natriuretic peptide; LVEF = left ventricular ejection fraction; NS = not significant; NYHA = New York Heart Association.



hormone-sensitive lipase inhibitor, which inhibits the formation of lipolysis-derived byproducts by ANP-induced lipolysis (22).

Recently, Yu et al. (23) demonstrated the increased ANP-induced lipolysis rates in large adipocytes compared with small adipocytes. Thus, the difference of adipocyte size between patients with CHF and obesity might contribute to the different pattern of adiponectin secretion. Finally, catecholamines also are involved in the control of lipolysis in humans (24). Thus, the prolonged exposure of high plasma level of catecholamines or the treatment with beta-adrenergic receptor blockers in patients with CHF also might affect the distinct pattern of adiponectin secretion from adipocytes. Although precise mechanisms are unknown, the human adipocytes could secrete adiponectin when the certain stress was loaded. However, it remains possible that factors such as tumor necrosis factor- α (25)

and alpha-adrenergic stimulation (26), both of which are increased in patients with CHF, may influence the expression of adiponectin or that adiponectin levels are affected by medical treatment, so further investigations are needed.

It is not clear whether ANP augments the plasma adiponectin levels in healthy subjects because of the ethical problems. However, we have reported that the plasma adiponectin level increased along with an increase of plasma BNP levels in 1,538 healthy subjects (27). These results suggest that an increase of natriuretic peptides augments the plasma adiponectin levels and exerts a cardioprotective effect in clinical settings.

Under normal conditions the adult heart utilizes predominantly fatty acids to derive the majority of its energy (28). However, metabolic remodeling such as a marked shift in substrate preference away from fatty acids toward glucose is observed in hypertrophic and failing hearts and the decrease

in fatty acid oxidation is not fully compensated for by an increase in glucose oxidation (29). Thus, the failing heart suffers from chronic energy starvation (30). Insulin resistance also is common in patients with heart failure (31). Adiponectin improves both glucose metabolism and insulin resistance via the AMPK signaling pathway (32). Therefore, we believe that the administration of recombinant natriuretic peptide has beneficial effects on cardiac energy metabolism via adiponectin in patients with CHF.

Interestingly, the plasma adiponectin level was reported to be decreased in patients with risk factors for heart failure (9,33-35) and increased along with BNP after the onset of heart failure (14). Although approximately 10% increase in adiponectin levels in the ANP group seems relatively small, this would not be the case because there was about a 20% reduction in plasma adiponectin levels in patients with coronary artery disease compared with those in control subjects (35), which leads us to believe that the 10% increase in adiponectin is important from the viewpoint of pathophysiology of heart diseases. Therefore, we hypothesized that ANP and/or BNP regulates the plasma level of adiponectin in patients with CHF and conducted this study.

Conclusions

We demonstrated that natriuretic peptides increase the production of adiponectin by human adipocytes, as well as in patients with CHF. These findings may help to shed more light on the pathophysiology of heart failure.

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Key Words: adiponectin ■ natriuretic peptides ■ heart failure ■ adipose tissue.

PRE-CLINICAL RESEARCH

Prolonged Targeting of Ischemic/ Reperfused Myocardium by Liposomal Adenosine Augments Cardioprotection in Rats

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Objectives	The purpose of this study was to investigate whether liposomal adenosine has stronger cardioprotective effects and fewer side effects than free adenosine.
Background	Liposomes are nanoparticles that can deliver various agents to target tissues and delay degradation of these agents. Liposomes coated with polyethylene glycol (PEG) prolong the residence time of drugs in the blood. Although adenosine reduces the myocardial infarct (MI) size in clinical trials, it also causes hypotension and bradycardia.
Methods	We prepared PEGylated liposomal adenosine (mean diameter 134 ± 21 nm) by the hydration method. In rats, we evaluated the myocardial accumulation of liposomes and MI size at 3 h after 30 min of ischemia followed by reperfusion.
Results	The electron microscopy and ex vivo bioluminescence imaging showed the specific accumulation of liposomes in ischemic/reperfused myocardium. Investigation of radioisotope-labeled adenosine encapsulated in PEGylated liposomes revealed a prolonged blood residence time. An intravenous infusion of PEGylated liposomal adenosine ($450 \mu\text{g}/\text{kg}/\text{min}$) had a weaker effect on blood pressure and heart rate than the corresponding dose of free adenosine. An intravenous infusion of PEGylated liposomal adenosine ($450 \mu\text{g}/\text{kg}/\text{min}$) for 10 min from 5 min before the onset of reperfusion significantly reduced MI size ($29.5 \pm 6.5\%$) compared with an infusion of saline ($53.2 \pm 3.5\%$, $p < 0.05$). The antagonist of adenosine A_1 , A_{2a} , A_{2b} , or A_3 subtype receptor blocked cardioprotection observed in the PEGylated liposomal adenosine-treated group.
Conclusions	An infusion as PEGylated liposomes augmented the cardioprotective effects of adenosine against ischemia/reperfusion injury and reduced its unfavorable hemodynamic effects. Liposomes are promising for developing new treatments for acute MI. (J Am Coll Cardiol 2009;53:709-17) © 2009 by the American College of Cardiology Foundation

Liposomes are now widely used for drug delivery in cancer treatment to target specific organs actively or passively and to prevent the degradation of chemotherapy agents (1). However, the application of liposomes for cardiovascular diseases is still limited. In ischemic/reperfused myocardium,

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cellular permeability is enhanced and vascular endothelial integrity is disrupted (2,3), suggesting that nanoparticles

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**Abbreviations
and Acronyms**8-SPT = 8-(*p*-sulfophenyl)
theophylline

EM = electron microscopy

MI = myocardial infarction

PEG = polyethylene glycol

RI = radioisotope

TTC = triphenyltetrazolium
chloride

such as liposomes may be a promising drug delivery system for targeting damaged myocardium with cardioprotective agents. Additionally, coating liposomes with polyethylene glycol (PEG) prolongs their residence time in the circulation (1). Because enhanced microvascular permeability persists for at least 48 h after the occurrence of myocardial infarction (MI) (2), drugs delivered in PEGylated li-

posomes should be able to display their maximum beneficial effects on myocardial damage after MI.

Adenosine has multiple physiological functions that are mediated via the adenosine A₁, A_{2a}, A_{2b}, and A₃ receptors (4,5). Although large-scale clinical trials suggested the potential value of adenosine therapy for patients with acute MI (6,7), this agent has an extremely short half-life (1 to 2 s) and causes hypotension and bradycardia because of vasodilatory and negative chronotropic effects (4). Because a high dose of adenosine is required to exert cardioprotective effects, it is difficult to use clinically because of the associated hemodynamic consequences. Therefore, we hypothesized that adenosine encapsulated in PEGylated liposomes would cause less hemodynamic disturbance and might also specifically accumulate in ischemic/reperfused myocardium, leading to augmented cardioprotective effects. To test this hypothesis, we created PEGylated liposomal adenosine by the hydration method and investigated: 1) whether liposomal adenosine accumulated in ischemic/reperfused myocardium and prolonged blood residence time; 2) whether liposomal adenosine caused less severe hypotension and bradycardia than free adenosine; and 3) which adenosine receptor subtype was involved in mediating the cardioprotective effects of liposomal adenosine against ischemia/reperfusion injury.

Methods

Materials. The materials for preparing PEGylated liposomes, including hydrogenated soy phosphatidyl choline (HSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000), and cholesterol were obtained from Nissei Oil Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Co., Ltd. (Osaka, Japan). [³H]-adenosine was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Other materials were obtained from Sigma (St. Louis, Missouri), including 8-(*p*-sulfophenyl)theophylline (8-SPT; a nonselective adenosine receptor antagonist), 1,3-diethyl-8-phenylxanthine (DPCPX; a selective adenosine A₁ receptor antagonist), 5-amino-7-(phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261; a selective adenosine A_{2a} receptor antagonist), 8-[4-[(4-cyanophenyl)carbamoylmethyl]oxy]phenyl]-1,3-di-(*n*-propyl)xanthine (MRS1754; a selective

adenosine A_{2b} receptor antagonist), and 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523, a selective adenosine A₃ receptor antagonist).

Animals. Male Wistar rats (9 weeks old and weighing 250 to 310 g, Japan Animals, Osaka, Japan) were used. The animal experiments were approved by the National Cardiovascular Center Research Committee and were performed according to institutional guidelines.

Preparation of PEGylated liposomes. The PEGylated liposomes were prepared by the hydration method. Briefly, adenosine was added to the lipid solution. After mixture of lipid and adenosine, DSPE-PEG2000 was added and incubated. The final composition of PEGylated liposomes was HSPC:cholesterol:DSPE-PEG2000 = 6.0:4.0:0.3 (molar ratio). After ultracentrifugation several times, the pellet of liposomal adenosine was resuspended in sodium lactate at each required concentration for use in the experimental protocols. Some samples of final liposomal adenosine were disrupted by dilution with 50% methanol (1.5 ml per 30- μ l of liposomes). After 10 min of ultracentrifugation, the concentration of adenosine in the supernatant was measured by high-performance liquid chromatography.

To prepare fluorescent-labeled liposomes, 0.5 mol% tetramethylrhodamine isothiocyanate (rhodamine) was added to the lipid mixture. To prepare radioisotope (RI)-labeled adenosine encapsulated in liposomes, [³H]-radiolabeled adenosine (Daiichi Pure Chemicals, Tokyo, Japan) was diluted with free adenosine and was encapsulated in liposomes as described above.

Characterization of PEGylated liposomal adenosine. The characterization of the liposomes was performed by the dynamic scatter analysis (Zetasizer Nano ZS, Malvern, Worcestershire, United Kingdom). The analyses were performed 10 times per sample, and results represented analyses of 4 independent experiments.

Experimental protocols. **PROTOCOL 1: EFFECTS OF PEGYLATED LIPOSOMAL ADENOSINE ON HEMODYNAMICS IN RATS.** Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Catheters were advanced into a femoral artery and vein for the measurement of systemic blood pressure and infusion of drugs, respectively. Both blood pressure and heart rate were monitored continuously during the study using a Power Lab (AD Instruments, Castle Hill, Australia). After hemodynamics became stable, we intravenously administered empty PEGylated liposomes (*n* = 8), free adenosine (*n* = 8), or PEGylated liposomal adenosine (*n* = 8) for 10 min. Either PEGylated liposomal or free adenosine was infused at an initial dose of 225 μ g/kg/min (0.1 ml/min) for 10 min. After a 5-min interval, either PEGylated liposomal adenosine or free adenosine was infused at 450 μ g/kg/min (0.1 ml/min) for 10 min. In the same manner, PEGylated liposomal adenosine or free adenosine was then infused at 900 μ g/kg/min (0.1 ml/min).

PROTOCOL 2: EFFECTS OF PEGYLATED LIPOSOMAL ADENOSINE ON INFARCT SIZE IN RATS. The MI was induced by transient ligation of the left coronary artery as described previously (8). In the first series of experiments, to examine the dose-dependent effects of liposomal adenosine on MI size, PEGylated liposomal adenosine was infused intravenously at 50, 150, or 450 $\mu\text{g}/\text{kg}/\text{min}$ for a 10-min period starting from 5 min before the onset of reperfusion. In the second series of experiments, to determine the adenosine receptor subtype involved in cardioprotective effects by the liposomal adenosine, the antagonist of adenosine subtype receptor was intravenously injected as a bolus followed by the infusion of liposomal adenosine for 10 min. The MI size was evaluated at 3 h after the start of reperfusion. The doses of adenosine receptor subtype antagonists were determined according to the previous reports (9-11).

Measurement of infarct size. At 3 h after the onset of reperfusion, the area at risk and the infarcted area were determined by Evans blue and triphenyltetrazolium chloride (TTC) staining, respectively, as previously described (8). Infarct size was calculated as $[\text{infarcted area}/\text{area at risk}] \times 100(\%)$ in a blind manner. The area at risk was composed of border (TTC staining) and infarcted (TTC nonstaining) areas.

Electron microscopy (EM). Myocardial samples for EM were obtained from the central and peripheral areas in ischemic/reperfused myocardium, which roughly corresponded to the infarcted and border areas, respectively, after the left coronary artery was occluded for 30 min of ischemia followed by 3 h of reperfusion. Samples were prepared as previously reported (12). Liposomes, whose major membrane component is unsaturated phospholipids, were visualized as homogenous dark dots with a diameter of 100 to 150 nm (13).

Accumulation of fluorescent-labeled PEGylated liposomes in ischemic/reperfused myocardium. Unlabeled or fluorescent-labeled PEGylated liposomes were infused intravenously at a dose of 0.1 ml/min as liposomal adenosine was infused in protocol 2. At 3 h after reperfusion, hearts were quickly removed and cut into 4 sections parallel to the axis from base to apex. Then *ex vivo* bioluminescence imaging was performed with an Olympus OV 100 imaging system (Olympus, Tokyo, Japan) and signals were quantified using WASABI quantitative software (Hamamatsu Photonics K.K., Shizuoka, Japan). Fluorescent intensity in the region of interest was measured as previously reported (14). Control intensity indicated the fluorescent intensity in the nonischemic area of the individual rat.

Time-course changes of free and PEGylated liposomal RI-labeled adenosine in plasma and myocardium. Free or PEGylated liposomal [^3H]-adenosine (83 kBq per rat) was infused intravenously at a dose of 0.1 ml/min as liposomal adenosine was infused in protocol 2. At the time indicated, rat hearts were harvested for counting of radioactivity (LSC-3100, Aloka Co., Tokyo, Japan). Results are expressed as a percentage of the injected dose per 1 ml of blood or 1 g of wet tissue weight.

Statistical analysis. The parameters of the liposomes were expressed as the average \pm SD, whereas other data were expressed as the average \pm SEM. Comparison of time-course changes in hemodynamic parameters between groups was performed by 2-way repeated-measures analysis of variance (ANOVA) followed by a post-hoc Bonferroni test. For comparison of RI activity between groups, statistical analysis was done with the Mann-Whitney *U* test. To address the differences in infarct size among groups, we performed a nonparametric (Kruskal-Wallis) test followed by evaluation with the Mann-Whitney *U* test. Resulting *p* values were corrected according to the Bonferroni method. To compare parameters of liposomes, an unpaired *t* test was performed. In all analyses, *p* < 0.05 was considered to indicate statistical significance.

Results

Characterization of liposomes by dynamic light scatter analysis. The dynamic light scatter analysis showed no significant difference in mean diameter, polydispersity index, or zeta-potential distribution between empty and adenosine-loaded PEGylated liposomes (Table 1).

Liposomes in ischemic/reperfused myocardium. The EM revealed the intact vascular endothelial cells and cardiomyocytes in the nonischemic myocardium (Figs. 1A and 1B). There were no homogenous dark dots indicating liposomes in the nonischemic myocardium of rats that received either saline (Fig. 1A) or liposomes (Fig. 1B). In the border area, many homogenous dark dots indicating liposomes were accumulated in rats that received liposomes, but not saline (Figs. 1C and 1D). In this area, significant structural damage was not observed in endothelium, but slight swelling of mitochondria was often observed. In the infarcted area, numerous liposomes were detected in rats that received liposomes, but not saline (Figs. 1E and 1F). In this area, the disrupted endothelial integrity and marked swelling of mitochondria were often observed.

Table 1 Characterization of Liposomes by Dynamic Light Scatter Analysis

	Mean Diameter (nm)	Polydispersity Index	Zeta Potential (mV)
PEGylated liposomes (empty liposomes)	126 \pm 12	0.035 \pm 0.003	-1.7 \pm 0.4
PEGylated liposomal adenosine	134 \pm 21	0.094 \pm 0.002	-2.3 \pm 1.1

Results represented analysis of 4 independent experiments. Values are expressed as mean \pm SD.
PEG = polyethylene glycol.

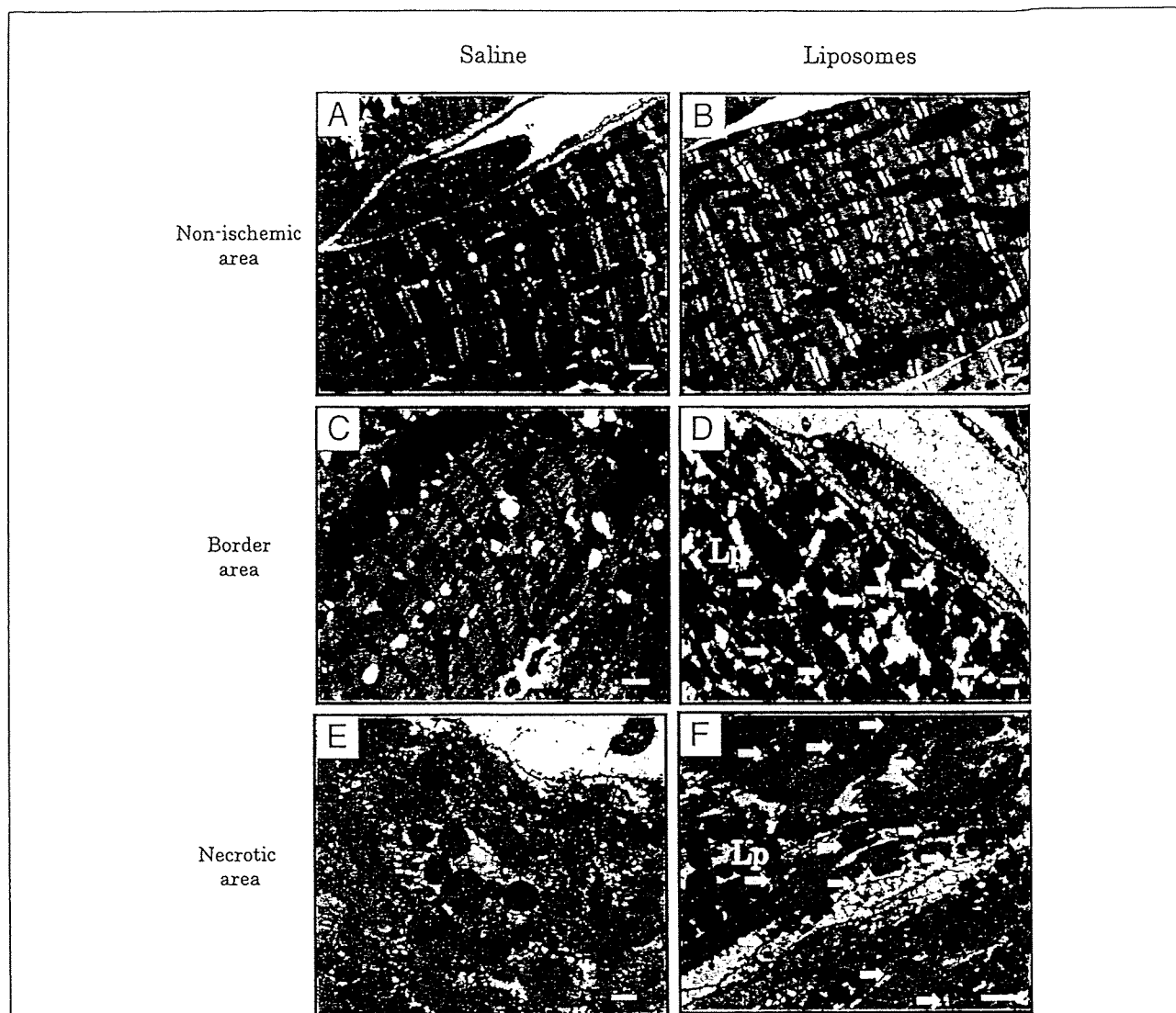


Figure 1 Liposomes in Ischemic/Reperused Myocardium

(A, B) Representative electron micrographs of the nonischemic area in rats that received saline (A) or liposomes (Lp) (B). (C, D) Representative electron micrographs of border area at 3 h after myocardial infarction (MI). Many dark dots accumulated in this area in the rat that received liposomes but not saline. (E, F) Representative electron micrographs of infarcted areas at 3 h after MI. Numerous dark dots accumulated in this area in the rat that received liposomes but not saline. Scale bars represent 1 μm .

Fluorescent-labeled PEGylated liposomes in ischemic/reperused myocardium. Quantitative analysis by bioluminescence ex vivo bioluminescence imaging revealed that the target to control fluorescent intensity ratio was higher in the border (noninfarcted area at risk) as well as infarcted areas compared with a nonischemic one, suggesting that fluorescent-labeled liposomes were accumulated in the border as well as infarcted areas. Since there was no high-intensity area when unlabeled liposomes were infused, it was suggested that this was not a nonspecific phenomenon to MI by the ex vivo bioluminescence imaging system (Fig. 2). The Evans blue staining was unrelated to the fluorescence intensity (data not shown).

Plasma radioactivity of RI-labeled adenosine was markedly higher in the PEGylated liposomal adenosine group at 10 min and 3 h after the intravenous infusion than in the free adenosine group (Fig. 3A). Encapsulation within PEGylated liposomes also augmented the accumulation of adenosine in ischemic/reperused myocardium compared with that of free adenosine (Fig. 3B).

Hemodynamic effects of PEGylated liposomal adenosine. Baseline hemodynamic parameters did not differ among the groups. An intravenous infusion of free adenosine at doses of 225, 450, and 900 $\mu\text{g}/\text{kg}/\text{min}$ decreased the mean blood pressure by 14.8%, 25.4%, and 33.7%, respectively, compared with the effect of empty PEGylated liposomes.