Table 4. CT Findings in the Lobar Intracerebral Hemorrhage

	Hypertensive (n=49)	Non-hypertensive (n=32)
Hematoma volume (cm^3)	34.7	38.1
Ventricular enlargement	5 (10.2%)	4 (12.5%)
Mass effect	42 (85.7%)	25 (78.1%)
Ventricular extension	11 (22.4%)	10 (31.3%)
Subarachnoid extension	2 (4.1%)	11 (34.4%)*

*significant ($p<0.001$) compared with hypertensive lobar ICH.

Table 5. Underlying Causes in Non-hypertensive Lobar Intracerebral Hemorrhage with or without Subarachnoid Extension

	Subarachnoid extension	
	yes	no
Cerebral amyloid angiopathy	2	4
Aneurysm	2	3
Arteriovenous malformation	2	2
Anticoagulant use	0	2
Liver cirrhosis	0	2
Thrombasthenia	1	0
Unknown	4	8
Total	11	21

only 9 patients had vascular abnormality. These results indicate that patients with subarachnoid extension are more likely to have vascular abnormality than those without subarachnoid extension ($p<0.001$).

Discussion

The frequency of hypertension among patients with lobar ICH was reported to range from 20.0% to 47.5% in the 1980's (Table 6) (1, 15–18). The frequency of hypertension in lobar ICH (60.0%) in the present study was higher than previously reported. A similarly high frequency of hypertension was reported in the 1990's by Massaro et al (54.8%) (19) and by Broderick et al (67.0%) (20). These reports pointed out the importance of hypertension as a cause of lobar ICH, and may indicate an increasing trend towards hypertensive lobar ICH, which has become noteworthy in the 1990's (19, 20).

Previous studies have reported the preferential localization of lobar ICH in specific sites in the brain. The most frequent site was the parietal lobe as reported by Weisberg (16), the occipital lobe by Ropper and Davis (15), the temporoparietal lobes by Kase et al (1), and the temporal or temporoparietal lobes by Tanaka et al (17). In the study by Massaro et al, lobar ICH confined to one lobe was observed most frequently in the frontal lobe. Lobar ICH overlying two or more lobes was located predominantly in the parieto-temporal or parieto-occipital lobes (19). In the present study, the frontal lobe was most commonly affected regardless of the presence or absence of hypertension. More than half of the patients with hypertensive lobar ICH had a frontal or temporal lesion, and patients with non-hypertensive lobar ICH showed a preferential localization in the frontal lobe. These results may simply reflect the fact that the frontal lobe has the largest volume of all lobes. Taken together, it is likely that there are no specific lobes which are particularly susceptible to lobar ICH.

Lobar ICH is characterized by clinical features such as seizures, which are more frequent in lobar ICH than in deep ICH (21–23). Between the hypertensive and non-hypertensive lobar ICH groups, the present study failed to detect any difference in clinical features, indicating that the clinical features are not defined by the underlying causes. However, the association of subarachnoid extension with non-hypertensive lobar ICH was intriguing in terms of the radiographic features (Table 4). Since subarachnoid extension was observed in non-hypertensive ICH patients, one half of the patients was expected to have vascular abnormalities (Table 5). Wakai et al examined brain specimens with lobar ICH but without angiographic abnormalities, and found vascular abnormalities such as amyloid angiopathy or vascular malformations in all patients with subarachnoid extension (24). Cerebral amyloid angiopathy occurs almost exclusively in the cerebral cortex (25, 26), and this may also help to explain the association of subarachnoid extension with non-

Table 6. Frequency of Hypertensive Patients in Lobar Intracerebral Hemorrhage

	Reference No.	Number of hypertensive patients	(%)	Mean age (years)
Ropper and Davis (1980)	[15]	8/26	(30.5)	65.0
Kase et al (1982)	[1]	10/22	(45.0)	55.0
Weisberg (1985)	[16]	5/25	(20.0)	–
Tanaka et al (1986)	[17]	13/32	(41.0)	47.0
Lipton et al (1987)	[18]	20/42	(47.5)	–
Massaro et al (1991)	[19]	36/65	(54.8)	68.0
Broderick et al (1993)	[20]	44/66	(67.0)	–
The present study		49/81	(60.0)	63.4

hypertensive ICH. Taken together, it is highly likely that subarachnoid extension is characteristic of lobar ICH caused by vascular abnormalities.

It seems reasonable to assume that hypertensive lobar ICH is caused more often by hypertension *per se* than by vascular or coagulation abnormalities. However, subarachnoid extension in hypertensive lobar ICH patients also warrants an extensive search for the underlying causes, because vascular or coagulation abnormalities may coexist with hypertension. Furthermore, recent advances in molecular biology have demonstrated an association between genetic abnormalities and some types of cerebrovascular disease. ICH is occasionally found in hereditary hemorrhagic telangiectasia, hereditary cerebral cavernous malformation with genetic heterogeneity mapped to 7q22, and hereditary cerebral amyloid angiopathy of either Dutch or Icelandic type (27). These abnormalities should be further investigated in non-hypertensive lobar ICH patients.

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Men's Health Study
—— Epidemiology of Erectile Dysfunction and Cardiovascular Disease ——

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The present study collected data about 6,112 Japanese male patients from 447 outpatient clinics. Of those who underwent medical examination by a general practitioner on an outpatient basis, up to 81% had some degree of erectile dysfunction (ED), ranging from mild to severe. ED was noted to be predominant among patients affected by cardiovascular disease (CVD) or diabetes mellitus (DM), and the presence of CVD increased the risk of ED. In an aging society, patients undergoing treatment for ED as part of their routine medical care are highly likely to have concomitant CVD. As shown in the present survey, clinicians need to be aware of the high incidence of ED among such patients, because ED represents a symptom originating from damage to the vascular endothelium. A total of 41% of ED patients are either willing to receive pharmacotherapy for ED or will consider treatment. Active treatment of ED with sildenafil is suitable for patients with CVD. (*Circ J* 2003; **67:** 656–659)

Key Words: Cardiovascular disease; Comorbidity; Endothelium; Erectile dysfunction; Risk factors

Hypertension, diabetes mellitus (DM), hyperlipidemia, and smoking all represent significant risk factors for cardiovascular disease (CVD) because they are all considered to induce vascular endothelial damage, resulting in vascular obstruction, plaque rupture, thrombosis, arterial sclerosis, and also erectile dysfunction (ED). More specifically, ED can be considered a symptom of damage to the vascular endothelium (Fig 1). Therefore, it can be expected that ED will be concomitant with CVD, and that the presence of ED suggests the existence of CVD. Integrated medical care may be necessary for these disease states. However, epidemiological data on ED and information regarding the current status of ED treatment among general practitioners in Japan is limited[1] possibly because there are no established criteria for the diagnosis of ED, as well as the societal and psychological obstacles to the patient consulting a clinician regarding this condition.

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Since its launch in March 1999, sildenafil citrate (Viagra® Tablets) has become widely used by numerous patients with CVD. Sildenafil selectively inhibits the action of intracellular phosphodiesterase 5 (PDE5) in the corpus cavernosum of the penis, and ameliorates the effects of ED by enhancing the action of cyclic guanosine monophosphate (cGMP) on vascular smooth muscle. The activity of PDE in the ventricle and saphenous veins of humans has been precisely measured, and the magnitude of the action of sildenafil on the cardiovascular system and its high affinity for PDE5 have been clearly demonstrated[2] In vitro examination of the effects of sildenafil on the activity of PDE isozymes originating from the human corpus cavernosum also suggests a high selectivity of sildenafil for PDE5 in that specific tissue[3]

Since sildenafil became available, public interest in ED has increased substantially and we therefore conducted a survey of general practitioners setting, because understanding the consultation status of ED treatment is important for the promotion of the optimal treatment of ED.

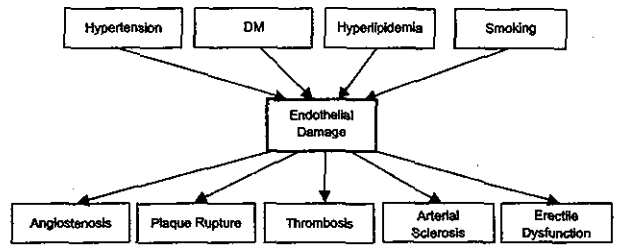
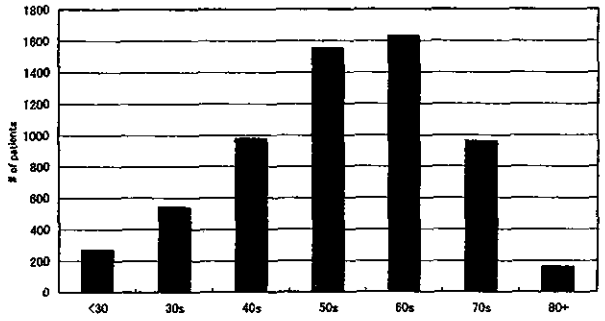


Fig 1. ED can be considered a symptom of damage to the vascular endothelium.



Missing=0.4%

Fig2. Age distribution of the survey respondents. Missing data=0.4%

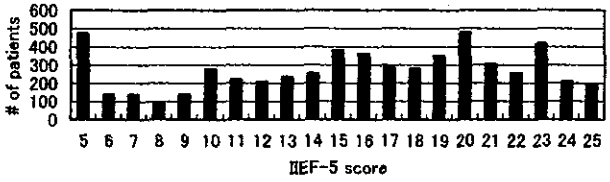
Study Objectives

To date, the Cardiovascular Issue Examining Committee of Viagra (organized by members affiliated to relevant academic societies in Japan, principally the Japanese Circulation Society) has issued 2 reports. The first reviewed the epidemiological studies of ED and existing statistics on CVD[4] In as well as describing the mechanism of action of sildenafil and clinical experience. The second report described the precise indications for sildenafil and the management of adverse events relevant to both general practice and cardiology[5] The present questionnaire survey of general practitioners was conducted as part of the activities of this committee, in order to investigate the current status of ED and its treatment in Japan.

Methods

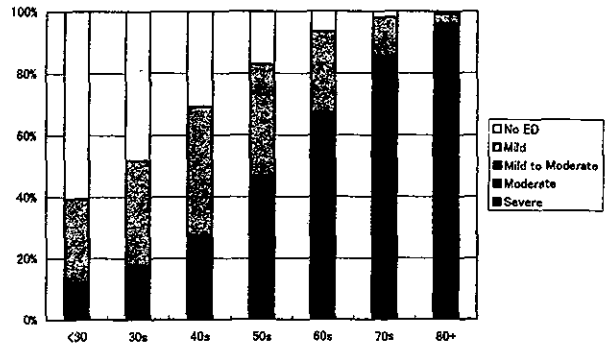
In the 4-month period from August to November 2001, a self-administered questionnaire survey was sent to general practitioners' offices and clinics all over Japan, because they represent the principal providers of medical care for lifestyle-related diseases. The level of patient satisfaction with their sex life was surveyed as a quality-of-life (QOL) profile, in addition to background information such as age, diseases under current medical treatment and health status, categorized into 5 levels. The level of satisfaction with sex life was evaluated by the question 'To what extent are you satisfied with your current sex life?' using 5 categories from 'very much' to 'very little'. The International Index of Erectile Function (IIEF)-5, which shortens the IIEF to 5 questions, was used to evaluate ED[6] The score of the IIEF-5 ranges from 5 to 25 points. In general evaluations using the IIEF-5, subjects are considered to have ED if they have a score of 5–21 points and are considered normal with a score of 22–25 points. Subjective evaluation of erectile function scored using IIEF-5 has been verified to suitably reflect objective measures of erectile function as determined by the nocturnal penile tumescence (NPT) test using the RigiScan Plus[7] In addition, psychological factors and urinary disturbance caused by prostate hyperplasia were evaluated using the Center for Epidemiologic Studies-Depression Scale (CES-D)[8] and the International Prostatic Symptoms Score (I-PSS)[9] respectively, as these are issues related to ED[10,11] Medical consultation for these health conditions, and satisfaction with the outcome, were also examined.

With regard to the method of administering the questionnaire, letters of intent were obtained from physicians throughout Japan who agreed to the purpose of the present survey. The self-administered questionnaires were then sent to the physicians and the reply forms were mailed either individually by the patients themselves or collectively by the physician in charge. Data were entered on an anonymous basis.



Missing=7.0%

Fig3. Severity of erectile dysfunction. Missing data=7.0%



Missing=7.3%

Fig4. Severity of erectile dysfunction with age. Missing data=7.3%

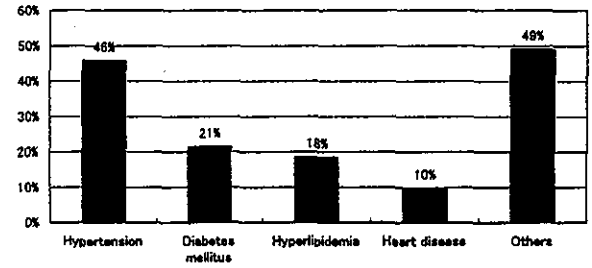
Statistical Analysis

The likelihood ratio test was conducted for concomitant CVD and severity of ED, and increased risk of ED with disease comorbidity. Furthermore, to calculate the risk of ED in the presence of any given disorder, subjects were categorized as either normal subjects or ED patients according to the IIEF-5 scores. A logistic model was then used to calculate the odds ratios (OR) and determine the magnitude of risk. Statistical significance was defined as $p<0.05$.

Results

Letters of interest were obtained from 700 outpatient clinics and 6,112 questionnaires were collected from 447 clinics. The mean (±SD) number of collected forms per medical institution was 14±9. The present survey was conducted in the outpatient setting of medical specialties including departments of Internal Medicine, Urology, Surgery, Orthopedics, Gastroenterology, Cardiology, and Radiology. Shin Joho Center, Inc collected and compiled the forms received from the participating medical institutions throughout Japan. Patients in the ages of 30–70 years accounted for 93% of the patients participating in the survey, with more than half (52.3%) of the total subject population represented by males aged in their 50s and 60s (Fig2).

A total of 4,609 of the 5,683 patients who answered the IIEF-5 questions in the present survey had an IIEF-5 score ≤21 points, which was 81% of the respondents (Fig3). According to the 5-level categorization of severity proposed by Rosen[12] patients with ED in the categories of 'severe' to



Number of patients=4,990

Fig 5. Patient background data. The category of 'others' includes prostatic disease, gastrointestinal ulceration, neurological disorder and/or psychiatric disorder. No. of patients=4,990

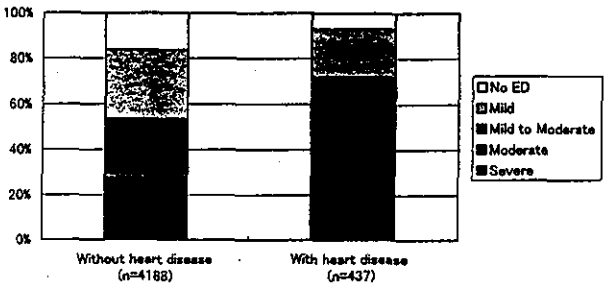


Fig 6. Severity of erectile dysfunction in patients with and without heart disease.

'mild to moderate' (IIEF-5 score: ≤16) accounted for 63.2% of all patients affected by ED (Fig 3). Severe ED was more pronounced among aged patients (Fig 4). Regarding the level of satisfaction with sex life, patients giving a reply of 'moderately dissatisfied' or 'very dissatisfied' comprised 30.2% of all replies.

CVD and ED

Subjects in the present survey displayed a mean of 1.45 concomitant diseases and the most frequently observed CVD was hypertension, accounting for 46% of valid replies (n=4,990) after excluding missing data (Fig 5). Furthermore, heart disease was observed in 10% of patients. In the present survey, patients over 70 years of age accounted for 40% of patients with heart disease who were undertaking medical treatment. These patients tended to be older than those with other conditions. ED was significantly more severe in the presence of heart disease ($p<0.0001$). Patients with severe or moderate ED comprised 46% of those with heart disease, but only 27% of those without (Fig 6).

Difference in Risk of ED According to Concomitant Disease

Among the concomitant lifestyle-related diseases, DM (21%) and hyperlipidemia (18%) predominated (Fig 5). Diabetes mellitus, heart disease, and hypertension displayed significant correlations with ED, with OR of 2.88, 2.82 and 1.79, respectively; however, the presence of hyperlipidemia did not affect the risk of ED (Table 1).

The risk of ED was calculated for an additional complication of an underlying disease. In the presence of heart disease, the risk of ED increased when DM was a complication ($p=0.0004$, p-value: likelihood ratio test) though it did not increase significantly when either hypertension ($p=0.2490$) or hyperlipidemia ($p=0.0917$) was the complication. Comparing the percentages of severe ED and moderate ED between patients with both heart disease and DM and those with only heart disease, the former was 65% and the latter was 42%.

Table 1 Concomitant Diseases and Correlation With Erectile Dysfunction

Estimated value of parameter	χ^2	*p value*	*OR*	*95% CI*
Intercept	*233.28*	*<0.0001*	-	-
Concomitant diseases				
Hypertension	*42.09*	*<0.0001*	*1.79*	*1.51–2.14*
Diabetes mellitus	*69.99*	*<0.0001*	*2.88*	*2.26–3.70*
Hyperlipidemia	*0.05*	*0.8313*	*0.98*	*0.80–1.20*
Heart disease	*27.66*	*<0.0001*	*2.82*	*1.95–4.23*

$R^2=0.0608$; Cutoff=21/22 (IIEF-5); logistic. OR, odds ratio.

Willingness to Receive Pharmacotherapy for ED

A total of 41% of patients did not respond to the question regarding willingness to receive pharmacotherapy for ED if it was available; however, 22.3% of the patients who replied did expressed such a willingness. The percentage of patients considering treatment for ED and of those unwilling to undergo treatment was 18.8% and 58.8%, respectively. The percentage of patients willing to receive pharmacotherapy did not differ among patients with hypertension, heart disease, or hyperlipidemia. However, patients with DM showed a significantly higher willingness to receive treatment ($p<0.0001$). In addition, patients affected by heart disease expressed less satisfaction with their sex life, compared with patients without the disease ($p<0.0001$). Conversely, the lower the satisfaction with sex life, the higher the willingness to accept pharmacotherapy for ED. However, patients who had actually undergone some form of treatment for ED comprised less than 40% of those patients who were willing to receive medication for ED.

Discussion

The Men's Health Study used the IIEF-5, and the ED evaluation scale revealed that as many as 81% of patients aged in the 30s to 70s who underwent a medical examination by general practitioners on an outpatient basis had some degree of ED.

The prevalence of ED reportedly increases with age[1] and the prevalence of ED by age of patients with an IIEF-5 score ≤16 in the present study closely resembled that of patients categorized as displaying Complete or Moderate ED in the study conducted by Shirai[1] In addition, ED was more prevalent among patients who had concomitant CVD or DM, which is a comparable result to other epidemiological studies conducted in Japan. Marumo et al identified hypertension, DM, heart disease, and cerebral infarction as risk factors associated with ED in males between the ages of 40 and 79 years[13] Another nationwide epidemiological study also demonstrated that ED was predominant in patients affected by CVD or DM[14] Similar results were obtained in an epidemiological survey conducted among males between the ages of 40 and 70 years in Massachusetts, USA[15] Concomitant CVD and DM can therefore be considered to increase the risk of ED, although hyperlipidemia does not appear to represent a significant risk factor, as indicated by both the present results and the findings of Marumo et al[13]

Patients undergoing treatment for ED in the course of routine medical care are highly likely to have CVD. Cur-

rently, the first choice for ED treatment among general practitioners in daily clinical practice is sildenafil. Although the effects of sildenafil on the cardiovascular system were of concern in the early stages of its development;[16] experience has demonstrated that sildenafil exerts positive effects on vascular endothelial cells and the safety of the drug has been established in the clinical setting. Safety information on sildenafil collected from Japanese general practitioners was reported for 3,152 cases in the Drug Use Investigation Study on sildenafil.[17] According to that study, the proportion of adverse drug reactions was 5.27% (166/3,152), and no serious adverse reactions were reported. In a study that examined the effects of sildenafil on coronary flow reserve in patients with serious coronary disease, baseline and post-administration data were compared. Systemic arterial pressure and pulmonary arterial pressure were shown to decrease slightly (<10%), and no effects on pulmonary capillary wedge pressure, right atrial pressure, heart rate, or cardiac output were observed.[18] In addition, coronary flow reserve was shown to be significantly augmented. The acute inhibitory activity of sildenafil on PDE 5 is demonstrated by increases in endothelium-dependent, bloodstream-mediated vasodilation in patients with chronic heart failure.[19] Those findings indicate that the inhibition of PDE 5 by sildenafil can rapidly improve endothelium-dependent vasodilation in patients with chronic heart failure. Sildenafil represents a safe therapeutic option when used appropriately, and does not add to the risk of cardiovascular dysfunction.[4,5] However, as revealed in the present survey, ED treatment is not adequately meeting the needs of patients. Clinicians should be encouraged to actively question patients regarding ED and provide an environment in which patients can comfortably and easily consult with clinicians regarding such issues.

Conclusion

The Men's Health Study shows that middle-aged Japanese men with CVD, DM or hypertension are commonly affected by ED. Cardiovascular specialists need to pay attention to treatment of sexual dysfunction as a real component of their patients' QOL.

Patients who were either willing to receive pharmacotherapy for ED or were considering treatment accounted for 41% of the ED patients in the present study. Sildenafil is a practical therapy for cardiovascular specialists to administer in daily practice, and both pharmacological and clinical evidence has been accumulated regarding the safety of the drug on the cardiovascular system. Based on these factors, active treatment of ED using sildenafil is suitable for patients with CVD.

Acknowledgments

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Treatment of acute inflammatory cardiomyopathy with intravenous immunoglobulin ameliorates left ventricular function associated with suppression of inflammatory cytokines and decreased oxidative stress

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Abstract

Although an autoimmune mechanism has been postulated for myocarditis and dilated cardiomyopathy, immunosuppressive agents had not been shown to be effective. Potential benefits of intravenous immunoglobulin (IVIg) in the therapy of patients with myocarditis and recent onset of dilated cardiomyopathy were reported. Also, experimental studies showed that IVIg is an effective therapy for viral myocarditis by antiviral and anti-inflammatory effects. Accordingly, in the current study, the effects of IVIg in the patients were investigated with the analyses of inflammatory cytokines and oxidative stress. Nine patients (six in myocarditis, three in acute dilated cardiomyopathy) were treated with high-dose intravenous IVIg (1–2 g/kg, over 2 days). All were hospitalized with New York Heart Association (NYHA) class III to IV heart failure, left ventricular ejection fraction (LVEF) <40%, and symptoms for <6 months at the time of presentation. Five patients were diagnosed using endomyocardial biopsy. LVEF determined by echocardiography improved from 19.0±7.5% (mean±S.D.) at baseline to 35.4±9.1% at follow up (12.2±5.8 days after the treatment) ($P<0.01$). C-reactive protein and plasma inflammatory cytokines (tumor necrosis factor-α and interleukin-6) were decreased by this treatment. In addition, plasma level of thioredoxin, which regulates the cellular state of oxidative stress, was decreased by the treatment. All nine patients improved functionally to NYHA class I to II, and were discharged without side-effects. There have been no subsequent hospitalizations for heart failure during the course of follow-up (3 months–4.5 years). LVEF improved 16% of EF in the patients with myocarditis and acute dilated cardiomyopathy with the reduction of cytokines associated with improvement of oxidative stress state by high-dose of IVIg. Thus, IVIg seems to be a promising agent in the therapy of acute inflammatory cardiomyopathy in view of not only suppression of inflammatory cytokines but a reduction of oxidative stress.

Keywords: Inflammatory cardiomyopathy; Intravenous immunoglobulin; Cytokines; Oxidative stress

1. Introduction

Limited success has been reported for the treatments of inflammatory myocardial diseases such as

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corticosteroid and cytotoxic drugs [1,2]. Furthermore, the need for continuing treatment exposed the patient to the risks of long-term immunosuppressive therapy.

Cardiomyopathy is defined as diseases of the myocardium associated with cardiac dysfunction [3]. Myocarditis, especially viral myocarditis, is considered to be a cause of dilated cardiomyopathy [4]. Immune or autoimmune mechanisms may be involved in the pathogenesis of myocarditis and subsequent cardiomyopathy. It was reported that T cells play a role in the development of myocarditis [4]; the severity of myocarditis is regulated not by B cells but by T cells.

Intravenous immunoglobulin (IVIg) has been used in several autoimmune conditions, including Kawasaki disease, idiopathic thrombocytopenic purpura, systemic vasculitis [5,6], but the mechanisms for its effect are unclear and may vary between conditions. Previous studies [5,6] showed the several mechanisms; neutralization of pathogens, attenuation of complement-mediated tissue damage, anti-inflammatory activities through the inhibitory Fc receptor, and acceleration of pathogenic immunoglobulin catabolism. We have previously shown that immunoglobulin therapy suppresses experimental myocarditis not only by the antiviral effects but also by the reduction of inflammatory cytokines [7,8]. Also, an increase in oxidative stress is thought to be involved in the progression of heart failure [9,10]. Here, we report the therapeutic effect of a single course of IVIg in patients with active myocarditis and acute dilated cardiomyopathy with the analyses of inflammatory cytokines and thioredoxin, which regulates the cellular state of oxidative stress [9].

2. Methods

The nine patients (five male, four female; aged 19–75 years) were included (Table 1). Six were active myocarditis (cases 2, 4, 5, 6, 7 and 8), and three acute dilated cardiomyopathy (cases 1, 3 and 9). The histological diagnosis of myocarditis was done by Dallas criteria [11]. The mean duration of disease before IVIg was 2.2 months (range; 1–6). Patients with dilated cardiomyopathy were eligible for this study if they presented with signs or symptoms of congestive heart failure of <6 months duration and a left ventricular ejection fraction (LVEF) of 40%. LVEF was assessed by echocardiography. Five patients underwent a pretreatment catheterization with an endomyocardial biopsy.

After baseline LVEF, and plasma cytokine [tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)] and thioredoxin (TRX) levels were assessed, IVIg was administered over a period of 48 h. Cytokines and TRX were measured using commercially available sandwich enzyme-linked immunosorbent assay kits [9]. The patients received a total dose of 1–2 g/kg. Immunoglobulin preparations used in this study were all intact types (having the Fc portion; Venilon for three cases, Polyglobin-N for three cases, and

Table 1
Patient characteristics

Case	Age	Sex	Months	Biopsy	LVDd	LVEF (%)		NYHA	
					(cm)	Before	After[a]	Before	After
1	43	M	6	Nonspecific	6.8	20	35	IV	II
2	75	M	6	Myocarditis	6.2	38	48	III	II
3	38	M	1	Not performed	5.8	20	30	III	I
4	60	M	1	Myocarditis	6.0	15	25	IV	II
5	19	F	1	Myocarditis	6.4	14	35	IV	II
6	22	F	2	Not performed	6.6	15	25	IV	II
7	24	M	1	Not performed	7.2	16	30	IV	II
8	57	F	1	Not performed	6.6	15	42	IV	II
9	31	F	1	Myocarditis	6.0	18	49	IV	I
Mn±S.D.	41.0±19.4		2.2±2.2		6.4±0.4	19.0±7.5	35.4±9.1[b]		

Months indicates duration of symptoms at the time of therapy; LVDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction.
[a] 12.2±5.8 days after the treatment.
[b] $P<0.01$ vs. before (mean±S.D.).

Venoglobulin-IH for three cases). All patients received continuous cardiac monitoring during therapy. LVEF and cytochemical analyses were reassessed 12.2±5.8 (S.D.) days after therapy. Patients were followed up every month.

Written informed consent was obtained from all the study patients, and the study protocol was approved by the ethics committee of institutes. Statistical analysis was performed with a paired *t*-test. Values are represented as mean±S.D.

3. Results

There were no clinically significant viruses detected is the sera of all patients.

All patients were hospitalized with New York Heart Association (NYHA) class III or IV symptoms. Hemodynamic evaluation revealed a mean pulmonary capillary wedge pressure of 24.3±5.3 mmHg, right atrial pressure of 12.0± 4.8, left ventricular end-diastolic pressure of 19.1±4.8, and cardiac output of 4.8±0.8 l/min. Histological analysis of endomyocardial biopsy samples in five biopsy-proven cases revealed definite myocarditis (by Dallas criteria [11]) in four patients of myocarditis (Fig. 1) and non-specific findings in one of acute dilated cardiomyopathy. One patient with myocarditis was diagnosed without endomyocardial biopsy findings. Five of nine patients received intravenous inotropic agents during the hospitalization, and five of nine required support with an intra-aortic balloon pumping at the time of IVIg therapy.

As shown in Table 1, all patients demonstrated an improvement of LVEF; mean LVEF for the entire population improved significantly, from 19.0±7.5% to 35.4±9.1% ($P<0.01$).

C-reactive protein (CRP), which was markedly raised in all patients before the therapy (9.1±5.1 mg/ml), fell to 3.7±2.6 mg/ml (Table 2). Plasma cytokines (TNF-α and IL-6) were significantly decreased after IVIg therapy compared with those before therapy. In addition, plasma TRX level was decreased significantly by the treatment.

All patients tolerated the therapy without serious complications, and were followed up for a median of 3.5 months after therapy (range, 3 to 54 months). All patients received an angiotensin converting enzyme inhibitor, digoxin, and diuretics at the time of discharge, and three of nine patients received a β-

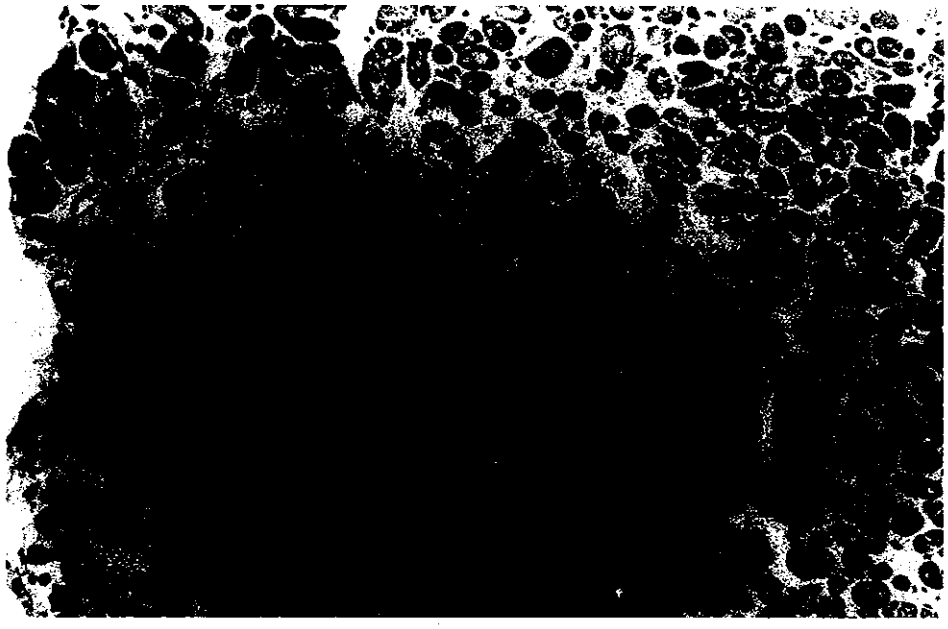

Fig. 1. Endomyocardial biopsy findings of Case 5. There are myocardial cell damages with cellular infiltrates and interstitial edema. The sample was obtained from the interventricular septum of the right ventricle. Hematoxylin–Eosin ×180.

Table 2
Changes of CRP and cytokines by immunoglobulin treatment

Case	CRP (mg/dl)		TNF-α (pg/ml)		IL-6 (pg/ml)		TRX (ng/ml)	
	Before	After	Before	After	Before	After	Before	After
1 43 M	15.2	7.4	12.5	8.8	–	–	–	–
2 75 M	9.5	2.1	19.6	5.2	16.3	4.5	52.5	30.5
3 38 M	3.2	1.0	16.2	8.0	18.8	8.5	48.5	21.1
4 60 M	5.2	1.5	18.3	6.6	11.9	4.7	55.5	18.0
5 19 F	6.5	3.0	8.6	5.0	–	–	–	–
6 22 F	18.5	5.6	–	–	36.5	15.2	–	–
7 24 M	11.5	6.6	32.3	17.7	61.4	12.5	38.8	18.5
8 57 F	7.2	5.3	35.2	17.0	38.0	7.0	66.2	25.0
9 31 F	4.8	0.5	–	–	47.1	7.2	80.5	43.2
Mn±S.D.	9.1±5.1	3.7±2.6*	20.4±9.9	9.8±5.4*	32.9±18.1	8.5±4.0**	57.0±14.6	26.1±9.6**

*$P<0.05$, **$P<0.01$ vs. before (mean±S.D.). Normal CRP levels were less than 0.6 mg/dl. Normal TNF-α, IL-6 and TRX levels were 1.2±0.3 pg/ml, 3.5±1.2 pg/ml and 14.0±4.6 ng/ml, respectively (each $n=7$, mean±S.D.).

blocker. All patients demonstrated functional improvement of NYHA (Table 1). No patients have been rehospitalized for congestive heart failure.

4. Discussion

The therapeutic efficacy of immunoglobulin in inflammatory and autoimmune diseases has been reported [5,6,12]. The mechanisms responsible for the efficacy of this treatment are, however, unknown. The prophylactic administration of immunoglobulin was reported to be of clinical value against some virus infections. This effect was due to the capacity of immunoglobulin to neutralize the viruses. The successful treatment of idiopathic thrombocytopenic purpura with immunoglobulin appears to result from the blockade of Fc receptors. One possible mechanism of action of IVIg in Kawasaki disease is the neutralization of a microbial toxin by immunoglobulin [13], that binds nonspecifically to certain variable regions of the T-cell-antigen receptor.

Drucker et al. reported potential benefits of IVIg in the therapy of children with a recent onset of myocarditis [13]. Subsequently, McNamara et al. [14] reported successful treatment of adult patients with acute cardiomyopathy by IVIg, which was associated with improved recovery of left ventricular function. Most recently, some investigators reported that IVIg may be useful for patients with chronic cardiomyopathy of left ventricular dysfunction [15–19]. In addition, Gullest et al. reported that IVIg was effective for patients with heart failure by modulating cytokine balance [19]. Accordingly, clarification of the mechanisms underlying this treatment in patients with inflammatory cardiomyopathy complicated with heart failure is warranted.

We have previously demonstrated that immunoglobulin therapy suppressed murine myocarditis induced by coxsackievirus B3 [7], the most cardiotropic agent in humans. Also, we have examined the effects of immunoglobulin upon experimental murine myocarditis induced by encephalomyocarditis virus, which is not pathogenic to men, and analyzed the behavior of inflammatory cytokines [8]. As a result immunoglobulin therapy had the potential to suppress myocarditis by reducing inflammatory cytokines.

In the present study, the use of IVIg was associated with a significant increase in left ventricular performance after the therapy, an absence of mortality in a population that presented with class III or IV heart failure, and a marked reduction of inflammatory cytokines associated with reduction of TRX.

It has been suggested that cytokines exert an important role in the development of inflammatory myocardial disease. For example, TNF-α inhibits the response of isolated myocytes to β-adrenergic stimuli [20]. Furthermore, TNF and interferon-γ are important mediators in the pathogenesis of myocardial inflammation in myosin-induced myocarditis and coxsackievirus B3 myocarditis. IL-6 is a well known Th 2 inflammatory cytokine. The reduction of cytokines by immunoglobulin administration was reported as a therapeutic possible mechanism in some

inflammatory diseases, because immunoglobulin itself contains antibodies against various cytokines [5,6]. Recently, suppression of cytokine-dependent T-cell proliferation by immunoglobulin treatment in in vitro experiments and in inflammatory disorders was demonstrated [6]. Most recently, anti-inflammatory activity of immunoglobulin via Fc portion was demonstrated [21,22], and the anti-inflammatory mechanisms of IVIg might be due to the inhibitory Fc receptor [21,22]. Accordingly, the suppression of cytokines after IVIg therapy obtained into the current study may be due to the active action of IVIg, but not to the associated results of the decrease of inflammation. These immunomodulatory effects of immunoglobulin may be significant for its therapeutic actions in immune-mediated diseases.

TRX is a ubiquitous protein that contains a redox-active disulfide/dithiol within the conserved active site [23–25]. TRX is stress-inducible, which protects cells from various types of stresses. We already reported that plasma levels of TRX were high in patients with heart failure [9], suggesting an oxidative stress overload in this condition. In this study, decreased oxidative stress in the patients after IVIg therapy was clearly shown, suggesting the improvement of inflammatory and redox environment in the patients with acute inflammatory cardiomyopathy. The similar therapeutic results of decreased oxidative stress was reported by the treatment of immunoglobulin adsorption [26]. Accordingly, the reduction of oxidative stress by immunoglobulin modulating therapy in patients with inflammatory cardiomyopathy may be promising for the treatment.

Toxicity and limited efficacy are serious disadvantages of existing immunosuppressive therapy for myocarditis and acute dilated cardiomyopathy. IVIg is safer, and experience with it has been gained in autoimmune or inflammatory conditions. Most recently, it was reported that IVIg treatment ameliorated myocarditis to some extent in an Intervention in Myocarditis and Acute Cardiomyopathy with Immune Globulin (IMAC) trial of human myocarditis [27]. A main reason for this limited efficacy may be that patients with dilated cardiomyopathy with noninflammatory causes occupied a large part of that trial. Although our study was uncontrolled, the improvement of redox state by IVIg might be of great value for the treatment of the patients, and the follow-up period was substantial. IVIg seems to be a promising agent in the therapy of acute inflammatory cardiomyopathy in view of the suppression of inflammatory cytokines associated with the reduction of oxidative stress.

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A calcium channel blocker activates both ecto-5′-nucleotidase and NO synthase in HUVEC

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Abstract

Since amlodipine, a long-acting Ca channel blocker, increases both NO and adenosine production in canine hearts, we investigated that amlodipine activates both ecto-5′-nucleotidase responsible for adenosine production and NO synthase (NOS) for NO production in human umbilical venous endothelial cells (HUVECs), and its cellular signaling. We measured activities of ecto-5′-nucleotidase and NOS in HUVECs in the condition with additions of xanthine (100 μM) + xanthine oxidase (1.6×10^{-3} U/ml) in the presence or absence of amlodipine (1×10^{-9}–1×10^{-6} M). Amlodipine increased both ecto-5′-nucleotidase and NOS activities. Xanthine + xanthine oxidase deactivated both NOS and ecto-5′-nucleotidase, and amlodipine increased both activities of NOS and ecto-5′-nucleotidase by 117 ± 33% and 48 ± 6%, respectively. Amlodipine phosphorylated p38MAP kinase and that an inhibitor of p38MAP kinase inhibited the amlodipine-induced activation of both NOS and ecto-5′-nucleotidase. Furthermore, amlodipine increased both adenosine and NO production in the canine ischemic hearts. We concluded that amlodipine activates both NOS and ecto-5′-nucleotidase via p38MAP kinase in vitro and enhances both NO and adenosine production in vivo.

Keywords: Ca channel blockers; Adenosine; NO; Endothelial cells; Canine hearts; p38MAP kinase

Ca channel blockers are often used for the treatment of ischemic heart diseases because of coronary vasodilation [1] via inhibition of Ca^{2+} entry into the smooth muscle cells [2]. Long-acting dihydropyridine Ca channel blockers were reported to protect the endothelial function of renal resistance arteries in hypertensive rats [3] and mesenteric arteries in circulatory shock rats [4]. Interestingly, amlodipine increases NO production in coronary arterial cells [5]; we have reported that long-acting Ca channel blockers have a potential to increase NO production in the hearts and this effect is enhanced in ischemic hearts relative to non-ischemic hearts [6,7]. Since adenosine is known to activate NO synthase (NOS), we hypothesized that amlodipine activates both ecto-5′-nucleotidase that is responsible for adenosine production and NOS.

We aimed to determine whether amlodipine activates either ecto-5′-nucleotidase or NOS in human umbilical venous endothelial cells (HUVEC) with or without oxidative stress. Furthermore, we investigated the cellular signaling pathways to activate either ecto-5′-nucleotidase or NOS.

Materials and methods

In the in vitro and in vivo studies, we used HUVEC and canine hearts, respectively. We used HBD (hybrid dogs mated with the Beagle, the American Fox Hound and the Labrador retriever, and bred for the laboratory use (Kitayama Labes, Yoshiki Farm, Gifu, Japan)). HBD (body mass, 15–21 kg) were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg body mass), intubated, and

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ventilated with room air mixed with oxygen (100% O_2 at a flow rate of 1.0–1.5 L/min). The methods for the experimental set-up were described previously [8].

All studies conformed to the "Position of the American Heart Association on Research Animal Use" adopted by the Association in November 1984.

Experimental protocols

Protocol I: effects of amlodipine on ecto-5′-nucleotidase and NOS activities. In HUVEC with or without xanthine (1×10^{-4} M) and xanthine oxidase (1.6×10^{-3} U/ml), the time courses of the changes in ecto-5′-nucleotidase and NOS activities were observed following an exposure to amlodipine of 1×10^{-7} M for 60 min. We also observed the time courses of the changes in ecto-5′-nucleotidase and NOS activities following an exposure to xanthine and/or xanthine oxidase for 60 min.

Protocol II: activities of the dose–response relationship between amlodipine and ecto-5′-nucleotidase and NOS. Since we obtained amlodipine which maximized the activities of ecto-5′-nucleotidase and NOS at 30 min in Protocol I, we examined the dose–response relationship between amlodipine (0, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} M) and the activity of the enzymes in the presence and absence of xanthine and xanthine oxidase. We also checked the phosphorylation of PKC or p38MAP kinase at the dose of, 1×10^{-6} or 1×10^{-7} M of amlodipine because either PKC or p38MAP kinase activates either ecto-5′-nucleotidase or NOS. Quantitative analysis was performed when we found the phosphorylation levels increased. Densitometry was performed on each sample and analyzed using NIH image software.

We examined whether SB203580 (1×10^{-6} M) attenuates the activation of these enzymes.

Protocol III: effects of amlodipine on both adenosine and NO release in canine hearts. In the open chest dogs, we sampled coronary arterial and venous blood for blood gas analysis and the measurements of lactate, nitrate + nitrite [9], and adenosine levels [8] by which we calculated coronary arteriovenous difference of nitrate + nitrite ($VAD(NO_x)$) or adenosine (VAD(Ado)). Lactate extraction ratio (LER) was obtained as the coronary arteriovenous difference of the lactate concentration multiplied by 100 and divided by the arterial lactate concentration.

We used 5 dogs in Protocol I. Hemodynamic parameters, i.e., systolic and diastolic aortic blood pressure as well as heart rate, were monitored. To examine whether amlodipine increases cardiac NO or adenosine levels in the ischemic hearts, after we reduced coronary perfusion pressure (CPP) so that coronary blood flow (CBF) decreased to 50% of the baseline for 5 min, we infused amlodipine (2 μg/kg/min, an infusion rate: 0.0167 ml/kg/min, a concentration of the solution: 0.12 mg/ml) for 60 min keeping CBF at the initial reduced value. In a preliminary study, the dose of amlodipine (2 μg/kg/min, an intracoronary infusion) was determined as the minimum dose that caused maximal coronary vasodilation.

Chemical analysis

The methods to measure plasma adenosine [8], NO_x [9], and lactate levels, and myocardial ecto-5′-nucleotidase [10], and NOS [11] activities have been reported previously.

For the measurement of NOS activity, we employed the Sigma's Fluorometric Cell Associated NOS Detection System (FCANOS-1, Sigma). Using the 5×10^5/ml HUVECs in the suspension buffer, we used 0.2 ml of cultured HUVECs for each well of 96-well plate (i.e., 1×10^5 HUVEC cells/well). In these wells with HUVEC and the reaction buffer, we added 1 μM diaminofluorescein-2 diacetate (DAF-2 DA). We added 1 mM arginine and 3.0 μM NADPH, which made the final volume of 0.2 ml. We measured the fluorescence using spectrofluorometer with excitation filter at 492 nM and an emission filter at 515 nM 2 h after the incubation. Since we used intact cell cultures, we did not add flavin mononucleotides, tetrahydrobiopterin or calmodulin. In fact, the addition of each component increased non-specific background or decreased the signals without alteration of the NOS activity. Since diphenylene iodonium chloride (DPI) did not affect NOS activity in HUVEC in the preliminary study, we did not use DPI in the present study.

Statistical analysis

Statistical analysis was performed using ANOVA [12] when data were compared among the groups. When ANOVA indicated a significant difference, we compared paired data using the Bonferroni test. Changes of the hemodynamic and metabolic parameters over time were compared by ANOVA for repeated measures. Results were expressed as means ± SEM, with $p < 0.05$ being considered significant.

Results and discussion

Ecto-5′-nucleotidase (control; 24.7 ± 2.2, 30 min; 32.6 ± 2.7 ($p < 0.05$), 60 min 33.8 ± 3.0 ($p < 0.05$) nmol/mg protein/min, $n = 6$ each) and NOS (control; 100%, 30 min; 136 ± 2% ($p < 0.01$), 60 min; 134 ± 3% ($p < 0.01$), $n = 6$ each) activities of HUVEC increased after the exposure to 1×10^{-7} M amlodipine. On the other hand, xanthine + xanthine oxidase decreased ecto-5′-nucleotidase (control; 25.2 ± 3.0, 15 min; 16.8 ± 3.0 ($p < 0.01$), 60 min; 16.1 ± 2.1 ($p < 0.01$) nmol/mg protein/min, $n = 6$ each) and NOS (control; 100%, 15 min; 81 ± 3% ($p < 0.01$), 60 min; 78 ± 6% ($p < 0.01$), $n = 6$ each) activities. Both enzyme activities became stable 30 min after the amlodipine exposure and 15 min after the exposure to xanthine + xanthine oxidase. Either xanthine or xanthine oxidase did not affect ecto-5′-nucleotidase activity (control; 23.9 ± 2.5, 60 min; 24.7 ± 1.9 or 25.2 ± 2.4 nmol/mg protein/min, $n = 6$ each) or NOS activity (control; 100%, 60 min; 102 ± 4% or 97 ± 3%, $n = 6$ each). Therefore, we examined the dose–response relationship at these time points. Table 1 shows that both ecto-5′-nucleotidase and NOS activities were activated by amlodipine in dose-dependent manner in the presence or absence of xanthine + xanthine oxidase. Table 1 further shows that (1) xanthine + xanthine oxidase deactivated both enzymes and (2) the concomitant administration of amlodipine in the presence of xanthine + xanthine oxidase restored both enzyme activities to the levels of the condition without xanthine + xanthine oxidase. Fig. 1 shows the phosphorylation of p38MAP kinase and several subtypes of PKC. Amlodipine phosphorylated p38MAP kinase, but it did not affect PKC phosphorylation. Quantitative analysis using densitometric analysis showed that amlodipine phosphorylated p38MAP kinase dose- and time-dependently compared with each control (10^{-7} M amlodipine; 30 min; 1.4 ± 0.2 (NS), 60 min; 1.6 ± 0.1 ($p < 0.01$), 60 min; 10^{-6} M amlodipine; 30 min; 2.0 ± 0.2 ($p < 0.01$), 60 min; 2.1 ± 0.3 ($p < 0.01$)). Table 1 shows that SB203580 blunts the amlodipine-induced activation of both ecto-5′-nucleotidase and NOS.

Table 1
The effects of amlodipine on the activities of ecto-5′-nucleotidase and NO synthase

		Control	Doses of amlodipine (M)			
			1×10^{-9}	1×10^{-8}	1×10^{-7}	1×10^{-6}
Without SB203580						
Ecto-5′-nucleotidase activity	Without X + XO	25.8 ± 3.5	25.5 ± 3.4	27.6 ± 3.2	30.6 ± 3.1*	31.0 ± 3.3*
(pmol/mg protein/min)	With X + XO	15.6 ± 3.1	17.2 ± 2.3	23.4 ± 3.2*	28.1 ± 3.5*	30.9 ± 3.5*
NOS activity	Without X + XO	100	108 ± 6	118 ± 4*	126 ± 6*	125 ± 5*
(% of control)	With X + XO	83 ± 4	88 ± 5	109 ± 3*	119 ± 3*	122 ± 4*
With SB203580						
Ecto-5′-nucleotidase activity	Without X + XO	25.1 ± 2.9	25.9 ± 3.1	24.8 ± 2.5	26.9 ± 2.2	26.5 ± 2.2
(pmol/mg protein/min)	With X + XO	18.7 ± 3.2	19.7 ± 2.1	17.3 ± 3.0	18.1 ± 2.9	17.1 ± 3.0
NOS activity	Without X + XO	100	97 ± 3	93 ± 3	101 ± 4	103 ± 4
(% of control)	With X + XO	91 ± 5	92 ± 4	90 ± 5	87 ± 4	90 ± 4

Values are means ± SEM, $n = 6$ each. *Abbreviations:* X, xanthine; XO, xanthine oxidase.
* $p < 0.01$ vs. the control value.

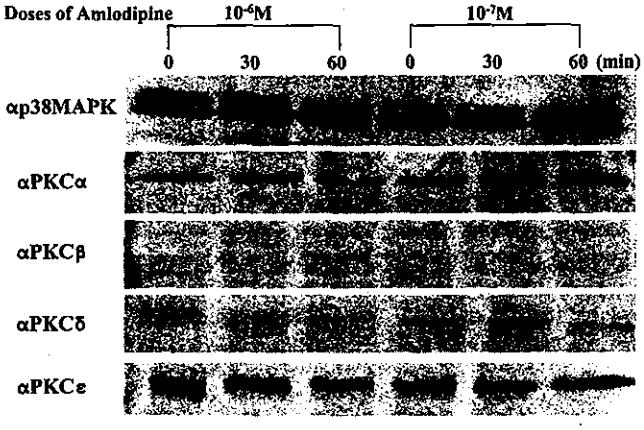

Fig. 1. Immunoblot of p38MAP kinase and several subtypes of PKC. P38MAP kinase is phosphorylated by amlodipine in 30–60 min. Immunoblot is performed using antibody of anti-p38MAPK or subtypes of PKC.

In the five ischemic canine hearts (CPP; 53 ± 2 from 103 ± 3 mm Hg, $n = 5$), we observed that an intracoronary administration of amlodipine increased VAD(Ado) (control, 16 ± 2; ischemia, 99 ± 7 ($p < 0.01$ vs. a control); and ischemia + amlodipine, 311 ± 21 ($p < 0.01$ vs. ischemia) pmol/ml, $n = 5$) and VAD(NO_x) (control, 4.2 ± 0.3; ischemia, 9.1 ± 1.1 ($p < 0.05$ vs. a control); and ischemia + amlodipine, 16.7 ± 2.0 ($p < 0.05$ vs. ischemia) nmol/ml, $n = 5$) despite controlled low CBF (control, 91 ± 1; ischemia, 46 ± 2 ($p < 0.01$ vs. a control); and ischemia + amlodipine, 46 ± 2 (NS vs. ischemia) ml/100 g/min, $n = 5$) and LER (control, 25.9 ± 2.3; ischemia, −47.4 ± 3.2 ($p < 0.01$ vs. a control); and ischemia + amlodipine, −43.9 ± 2.2 (NS vs. ischemia) ml/100 g/min, $n = 5$). Amlodipine deceased CPP (53 ± 2 from 46 ± 1 mm Hg, $p < 0.05$, $n = 5$), which indicates that amlodipine decreases coronary vascular resistance. Either systemic blood pressure (106 ± 2 from 102 ± 4 mm Hg, $n = 5$) or heart rate (139 ± 3 from 137 ± 3/min, $n = 5$) was not altered by amlodipine administration.

The present study revealed that amlodipine, a long-acting Ca channel blocker, activated both ecto-5′-nucleotidase and NOS in HUVEC via p38MAP kinase-dependent pathway. The effects of amlodipine on ecto-5′-nucleotidase and NOS were enhanced in the condition with oxidative stress. Furthermore, the effects of amlodipine on both adenosine and NO production were also seen in the in vivo canine hearts.

We have revealed novel aspects of amlodipine on ecto-5′-nucleotidase and NO synthase. First, as for the mechanisms of these novel aspects, although amlodipine is related to the inhibition of the Ca^{2+} inward, since HUVEC lacks voltage-dependent Ca^{2+} channels, we speculate Ca^{2+}-dependent pathways may not be likely to explain the present phenomenon. Since either NOS or ecto-5′-nucleotIdase is activated by PKC, we tested whether amlodipine activates PKC and we observed the negative results for PKC. On the other hand, we found that amlodipine directly affects p38MAP kinase and that SB203580, an inhibitor of p38MAP kinase, blunted the activation of both NOS and ecto-5′-nucleotidase. Since amlodipine is reported to enter the lipid bilayer of the cell membrane, it is likely that amlodipine affects the intracellular signal transduction pathway of p38MAP kinase, and the activation of p38MAP kinase activates both ecto-5′-nucleotidase and NOS. PKC, which can activates both enzymes are not related to the present observation.

Second, since oxygen-derived free radicals attenuate both ecto-5′-nucleotidase [13] and NOS, the elimination of oxidative stress may restore the reduction of these two enzymatic activities. Since amlodipine is reported to reduce oxidative stress [14], the reduction of oxidative stress due to amlodipine may explain the enhanced effects of amlodipine on the activation of ecto-5′-nucleotidase and NOS. We also excluded the possibility that the non-specific effects of either xanthine or xanthine oxidase alter ecto-5′-nucleotidase and NOS activities in HUVEC. Interestingly, this in vitro phenomenon is

completely reproduced in the in vivo canine hearts. Amlodipine increased both adenosine and NO production in the canine hearts.

Although amlodipine increased both adenosine and NO production, and thus decreased coronary resistance, the cellular signal transduction for coronary vasodilation due to either adenosine or NO is completely different. Indeed, both adenosine and NO elevate cyclic AMP and GMP levels, respectively, which independently cause coronary vasodilation. The former mainly causes detachments of actin–myosin interaction and the latter mainly causes the re-uptake of Ca^{2+} into sarcoplasmic reticulum. Therefore, the increased adenosine and NO levels independently cause coronary vasodilation. Since both adenosine and NO are believed to attenuate the severity of myocardial ischemia [15], both adenosine and NO increase CBF, attenuate myocardial anaerobic metabolism, inhibit platelet aggregation and leukocyte activation, and attenuate the activation of sympathetic nerve activity in an ischemic heart.

The present study suggests that amlodipine contributes to the activation of both ecto-5′-nucleotidase and NOS, and contributed to the elevation of the cardiac adenosine/NO levels, coronary vasodilation, and the attenuation of the severity of myocardial ischemia. Therefore, it is likely that the beneficial effects of amlodipine on the ischemic hearts seen in the present study are attributable to combination of the anti-oxidant effects, and adenosine-/NO-induced effects and Ca channel blocking effects of amlodipine.

Acknowledgments

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Activation of Adenosine A_1 Receptor Attenuates Cardiac Hypertrophy and Prevents Heart Failure in Murine Left Ventricular Pressure-Overload Model

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Abstract—Sympathomimetic stimulation, angiotensin II, or endothelin-1 is considered to be an essential stimulus mediating ventricular hypertrophy. Adenosine is known to protect the heart from excessive catecholamine exposure, reduce production of endothelin-1, and attenuate the activation of the renin-angiotensin system. These findings suggest that adenosine may also attenuate myocardial hypertrophy. To verify this hypothesis, we examined whether activation of adenosine receptors can attenuate cardiac hypertrophy and reduce the risk of heart failure. Our in vitro study of neonatal rat cardiomyocytes showed that 2-chloroadenosine (CADO), a stable adenosine analogue, inhibits protein synthesis of cardiomyocytes induced by phenylephrine, endothelin-1, angiotensin II, or isoproterenol, which were mimicked by the stimulation of adenosine A_1 receptors. For our in vivo study, cardiac hypertrophy was induced by transverse aortic constriction (TAC) in C57BL/6 male mice. Four weeks after TAC, both heart to body weight ratio (6.80±0.18 versus 8.34±0.33 mg/g, $P<0.0001$) as well as lung to body weight ratio (6.23±0.27 versus 10.03±0.85 mg/g, $P<0.0001$) became significantly lower in CADO-treated mice than in the TAC group. Left ventricular fractional shortening and left ventricular dP/dt_{max} were improved significantly by CADO treatment. Similar results were obtained using the selective adenosine A_1 agonist N^6-cyclopentyladenosine (CPA). A nonselective adenosine antagonist, 8-(*p*-sulfophenyl)-theophylline, and a selective adenosine A_1 antagonist, 8-cyclopentyl-1,3-dipropylxanthine, eliminated the antihypertrophic effect of CADO and CPA, respectively. The plasma norepinephrine level was decreased and myocardial expression of regulator of G protein signaling 4 was upregulated in CADO-treated mice. These results indicate that the stimulation of adenosine receptors attenuates both the cardiac hypertrophy and myocardial dysfunction via adenosine A_1 receptor–mediated mechanisms. (*Circ Res.* **2003;93:759-766.**)

Key Words: adenosine ■ cardiomyopathy ■ echocardiography ■ heart failure ■ myocytes

Patients with pressure-overload diseases such as systemic hypertension exhibit left ventricular hypertrophy (LVH), a major determinant of mortality and morbidity in cardiovascular diseases. It is well-known that many neurohumoral factors such as angiotensin II (Ang II),[1,2] endothelin-1 (ET-1),[3] catecholamines,[2,4] growth factors,[5,6] and tumor necrosis factor-α (TNF-α)[7] cause LVH via the activation of intracellular signal transduction mediated by calcineurin[8,9] or mitogen-activated protein kinases.[10,11]

Adenosine, a nucleoside abundantly produced by cardiac cells, is known to inhibit norepinephrine release from presynaptic vesicles,[12] reduce production of ET-1,[13] attenuate the activation of the renin-angiotensin system,[14] and counteract TNF-α.[15] Because norepinephrine, ET-1, Ang II, and TNF-α are believed to be involved in cardiac hypertrophy and remodeling,[1–4] we hypothesized that adenosine may reduce cardiac hypertrophy and improve subsequent cardiac dysfunction. Indeed, myocardial concentration of adenosine was found to markedly increase in the hypertrophied heart,[16] whereas exogenous or endogenous adenosine has been shown to inhibit the growth of rat cardiac fibroblasts in vitro.[17] We also demonstrated that the plasma concentration of adenosine increased in patients with chronic congestive heart failure (CHF)[18] and that an increase in plasma adenosine levels ameliorated CHF.[19] The enhancement of adenosine metabolism is therefore thought to improve the pathology of cardiac hypertrophy and subsequent heart failure.

Taking these findings into consideration, we postulated that sustained stimulation of adenosine receptors would be beneficial for attenuation of LVH and improvement of heart

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function. As far as we know, however, the role of adenosine on myocardial hypertrophy and heart function in pressure-overload state remains poorly understood. The study presented here was therefore undertaken to determine whether administration of 2-chloroadenosine (CADO), a stable analogue of adenosine, would have beneficial effects on the LV structure and heart function in a murine model of transverse aortic constriction (TAC) and, if so, to clarify the potential underlying mechanisms involved.

Materials and Methods

Agents

CADO, 8-sulfophenyltheophylline (8-SPT), phenylephrine (PE), ET-1, Ang II, isoproterenol (Iso), forskolin, N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamino adenosine hydrochloride (CGS21680), 5-ethylcarboxamidoadenosine (NECA), and N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine (IB-MECA) were purchased from Sigma Chemical Company.

Cell Culture for the In Vitro Study

Neonatal rat ventricular myocytes were isolated as described previously.[20] Cardiac myocytes were cultured in DMEM (Sigma) supplemented with 10% FBS (Equitech-Bio Inc). Culture media were changed to serum-free at 72 hours. Cardiomyocytes were cultured in serum-free conditions for 48 hours before experiments. Protein synthesis in cultured cells was evaluated by analysis of [^{3}H]leucine incorporation as described.[6] For cell surface area measurement, cardiomyocytes were stained with rhodamine-phalloidin and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI); confocal microscopic images (×400) were captured and surface area was measured using Scion image software (Scion Corporation).

Surgical Procedures for the In Vivo Study

Mice (C57BL/6, male, 8 to 9 weeks old, weight 18 to 25 g) were anesthetized with a mixture of pentobarbital (50 mg/kg IP) and ketamine (25 mg/kg IP). The animal model of pressure overload was created, and invasive measurement of trans-stenosis pressure gradient and left ventricular dP/dt_{max} was performed as described previously.[21–23]

Experimental Protocols I (In Vitro Study)

Cardiomyocytes were exposed to PE (10^{-4} mol/L), ET-1 (10^{-8} mol/L), Ang II (10^{-7} mol/L), Iso (10^{-5} mol/L), or forskolin (10^{-5} mol/L) for 30 hours in the presence or absence of CADO (10^{-6} mol/L), and the extent of increase in [^{3}H]leucine uptake was examined. We studied the effects of A_1 (CPA), A_{2A} (CGS21680), and A_3 (IB-MECA) receptor selective agonists and the nonselective agonist (NECA) for A_1, A_{2A}, and A_{2B} on cardiac myocyte hypertrophy.

Experimental Protocol II (In Vivo Study)

Determination of the Dosage of the Agents

Preliminary experiments were performed to determine the dosage of agents used in vivo studies. All of the agents were delivered by minipump infusion for 4 weeks (Alzet micro-osmotic pump model 1002, replaced at 2 weeks).

Roles of Stimulation of Adenosine Receptors

We treated mice with saline (TAC group), CADO alone (2 mg/kg per day), CADO (2 mg/kg per day) plus 8-SPT (10 mg/kg per day), 8-SPT alone (10 mg/kg per day), CPA (5 mg/kg per day), or CPA (5 mg/kg per day) plus DPCPX (5 mg/kg per day), respectively. Tail-cuff BP and HR measurements (BP-98A, Softron) were done at 1, 2, and 4 weeks, and the echocardiographic assessments were performed at 4 weeks after TAC. Mice were euthanized to obtain the organs for morphometric analysis. All procedures were performed in accordance with the guiding principles of Osaka University Graduate School of Medicine with regard to animal care.

Measurements of 5′-Nucleotidase Activity and the Levels of Norepinephrine and Renin

To examine whether the enzyme to produce adenosine via AMP is activated in the myocardial hypertrophic mice, we measured the myocardial 5′-nucleotidase (5′-ND) activity[24] in a time course. Plasma norepinephrine and renin levels were determined as described.[25,26]

Determination of the Expression of B Natriuretic Peptide and Regulator of G Protein Signaling 4 Using Quantitative Polymerase Chain Reaction

Total RNA was extracted from whole heart by using TRIzol reagent (GIBCO/BRL) as described by the manufacturer. Primers for quantitative polymerase chain reaction (PCR) were designed using Gene Express software (Applied Biosystems). Expression levels of natriuretic peptide precursor type B (BNP) and regulator of G protein signaling 4 (RGS-4) were determined using Quantitect SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer's instruction.

Results

Chloroadenosine Inhibits Myocyte Hypertrophy Induced by the Agonists of G-Protein–Coupled Receptor

Treatment with CADO alone did not affect the basal [^{3}H]leucine uptake of myocytes when the concentration of CADO was not higher than 10^{-5} mol/L, but CADO decreased [^{3}H]leucine uptake at concentrations higher than 10^{-5} mol/L (Figure 1A). Thus, we used CADO at the concentrations of $\leq 10^{-5}$ mol/L to assess its effects on myocyte hypertrophy. Figure 1B showed that CADO inhibited PE-induced cardiomyocyte hypertrophy in a concentration-dependent fashion. Myocyte cross-sectional area was also decreased by CADO (Figures 1C and 1D). In addition, the exposure to ET-1 or Ang II induced cardiomyocyte hypertrophy, as was gauged by changes in [^{3}H]leucine incorporation, and cotreatment with CADO (10^{-6} mol/L) inhibited these G-protein–coupled receptor agonist–induced increase in [^{3}H]leucine uptake (Figure 1E).

Chloroadenosine Also Blocks Protein Kinase A–Dependent Hypertrophic Signal Pathway

Treatment of cardiomyocytes with Iso (10^{-5} mol/L) increased protein synthesis, and cotreatment with CADO dose-dependently inhibited the increase of [^{3}H]leucine uptake (Figure 2A). Cellular enlargement induced by Iso was also attenuated in CADO-treated myocytes (Figures 2B and 2C). Furthermore, treatment with forskolin, a stimulator of adenylate cyclase, also increased [^{3}H]leucine uptake, which was abolished completely by CADO at the concentration of 10^{-5} to 10^{-6} mol/L (Figure 2A).

Antihypertrophic Effect of Chloroadenosine Is Mediated by the Stimulation of Adenosine A_1 Receptors

CPA, an A_1 selective agonist, and NECA, a nonselective agonist for A_1, A_{2a}, and A_{2b} receptors, significantly inhibited the PE-induced increase of cardiac myocyte protein synthesis, but neither CGS21680, an A_{2a} receptor agonist, nor IB-MECA, an A_3 selective receptor agonist, affected the PE-

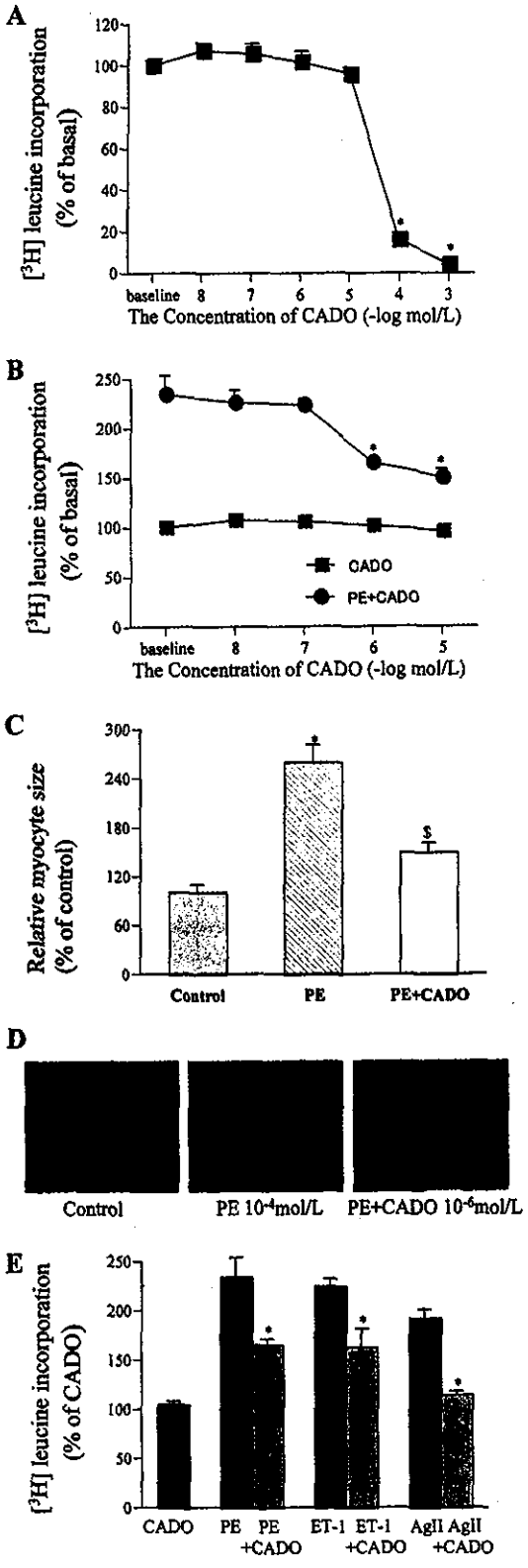

Figure 1. Inhibition of protein synthesis of myocytes by CADO. A, Concentration-dependent effects of CADO on [^{3}H]leucine incorporation in myocytes without adding stimulators. *P<0.01 compared with the value at baseline. B, Concentration-dependent effects of CADO on [^{3}H]leucine incorporation induced by PE (10^{-4} mol/L). *P<0.01 compared with the value at baseline. C, Enlargement of myocyte cross-sectional area induced by PE (10^{-4} mol/L) was decreased in the presence of CADO (10^{-6} mol/L), *P<0.01 compared with the value at control, \$$P$<0.01 vs PE (n=200 cells in every group). D, Representative confocal microscopic images of myocytes with rhodamine-phalloidin staining of actin and DAPI staining of the nucleus; CADO reduced PE (10^{-4} mol/L)-induced enlargement of myocyte cross-sectional area. E, Effects of CADO (10^{-6} mol/L) on protein synthesis stimulated by PE (10^{-4} mol/L), ET-1 (10^{-8} mol/L), and Ang II (10^{-7} mol/L). *P<0.01 vs the corresponding stimulator alone. All values are expressed as mean±SEM. Every experiment was repeated at least 3 times.

induced increase of [^{3}H]leucine uptake (Figure 3A). Similar results were obtained in forskolin-induced cardiac myocyte hypertrophy (Figure 3B). Therefore, we conclude that it is A_1, not A_{2a} or A_3 receptors, that mediates the antihypertrophic effect.

Activation of Adenosine A_1 Receptors Attenuates Myocardial Hypertrophy In Vivo

Myocardial 5′-ND activity in TAC mice increased from 2 weeks after surgery and achieved significant difference at 4 weeks compared with sham-operated mice (Figure 4A). Treatment with CADO in TAC mice and plasma concentrations of norepinephrine, renin, and a molecular marker of hypertrophy BNP were significantly reduced, whereas gene expression of RGS-4, an inhibitory factor of hypertrophy, was markedly upregulated (Figures 4B and 4C). The question is whether these changes are associated with attenuated cardiac hypertrophy. Interestingly, our preliminary study showed a dose-response attenuation of cardiac hypertrophy by 1 week of treatment with CADO (Figure 5A). Along with this preliminarily study, we determined that CADO of 2 mg/kg per day is the minimal dose that exerts the maximal effects. In 4-week chronic studies, the degree of cardiac hypertrophy in CADO-treated mice was significantly lower than in TAC mice receiving vehicle treatment (P<0.0001; Figures 5B through 5F), whereas TAC led to a 74% increase in heart weight at 4 weeks after the surgery. CADO attenuated the heart weight to body weight ratio by 41% and decreased the left ventricular posterior wall thickness by 52% (Table). No significant difference was found on body weight between CADO-treated and vehicle-treated TAC mice (Table). CADO also reduced myocardial (Figure 5G) and perivascular fibrosis (Figure 5H). Meanwhile, a selective adenosine A_1 receptor agonist CPA markedly attenuated cardiac hypertrophy, and this effect was abolished by a selective A_1 receptors antagonist DPCPX (Figures 5B and 5C). Treatment with 8-SPT alone did not additionally increase cardiac hypertrophy, but cotreatment with CADO abrogated the effects of CADO on attenuating cardiac hypertrophy, as determined by the heart weight to body weight ratio and the left ventricular posterior wall thickness (Figures 5B and 5C and Table). Similarly, 8-SPT alone did not deteriorate the heart function of TAC mice, but it reversed the effects of CADO on the improvement of heart function (Figure 6A and Table).

One, two, and four weeks after the pharmaceutical treatment, systolic blood pressure and heart rate were not significantly different among all the groups, except that systolic blood pressure was slightly higher in sham group. These

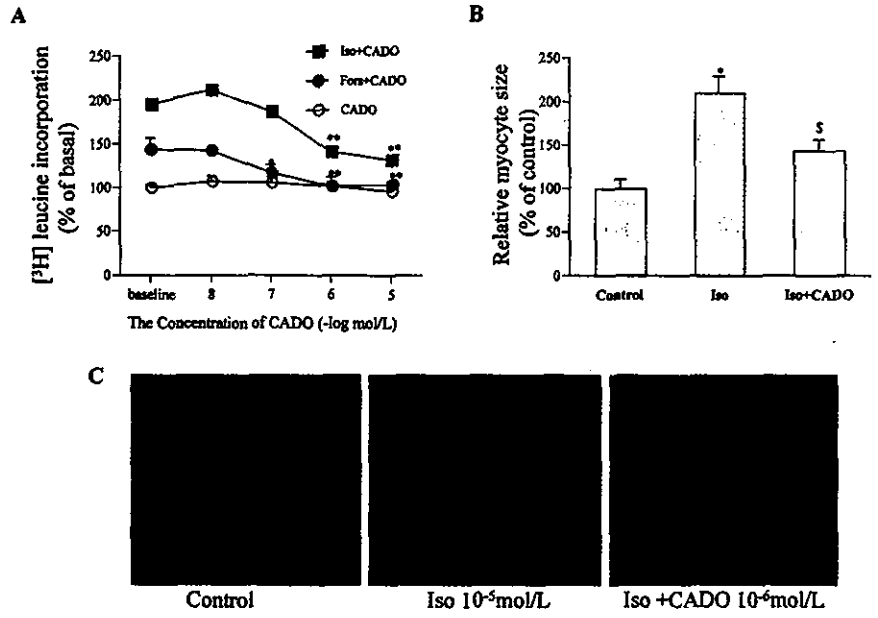

Figure 2. Inhibitory effects of CADO on protein synthesis stimulated by Iso (10^{-5} mol/L) or forskolin (Fors, 10^{-5} mol/L). A, Concentration-dependent effects of CADO on [³H]leucine incorporation induced by Iso or Fors. *$P<0.05$, **$P<0.01$ compared with the value at baseline. B, Enlargement of myocyte cross-sectional area induced by Iso (10^{-5} mol/L) was decreased in the presence of CADO (10^{-6} mol/L), *$P<0.01$ compared with the value at control, \$$P<0.01$ vs PE (n=200 cells in every group). C, Representative confocal microscopic images of myocytes with rhodamine-phalloidin staining of actin and DAPI staining of the nucleus. Values are expressed as mean±SEM. Every experiment was repeated at least 3 times.

results may be attributed to the use of minipump to deliver the drugs in a stable and low concentration that did not significantly affect hemodynamics and also suggest that the antihypertrophic effect of CADO is independent of blood pressure change. The trans-stenosis pressure gradients were similar in all the mice that received TAC treatment. The results of hemodynamics at 4 weeks are shown in the Table.

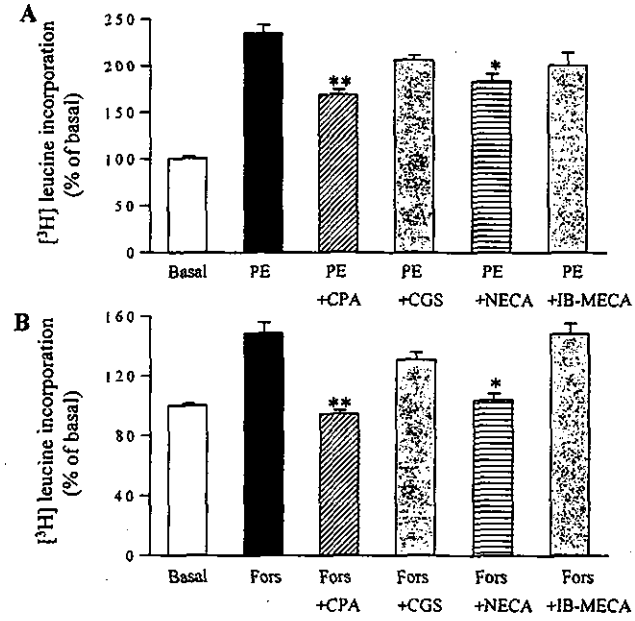

Figure 3. Effects of adenosine receptor agonists on myocyte hypertrophy induced by PE (A) or forskolin (B). Values are expressed as mean±SEM, *$P<0.05$, **$P<0.01$ compared with PE or forskolin alone. Every experiment was repeated at least 3 times. Concentration of PE and forskolin was 10^{-4} and 10^{-5} mol/L, respectively; concentration of other agents is 10^{-6} mol/L; CGS indicates 2-p-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamino adenosine hydrochloride.

Activation of Adenosine A_1 Receptors Prevents Heart Failure In Vivo

Pressure overload induced CHF manifested by increases in the lung weight and reduction in fractional shortening (FS) and LV dP/dt_{max}. In TAC mice, the lung weight to body weight ratio increased by an average of 93%, the treatment with CADO markedly ameliorated pulmonary congestion by ≈80%, and even no significant difference was found on the lung weight to body weight ratio between CADO-treated TAC mice and sham-operated mice (Figures 6A and 6B). Comparable results are also observed in CPA-treated TAC mice (Figure 6A). We defined lung weight to body weight ratio higher than mean±4 SD in sham mice as the criteria for pulmonary congestion; consequently, the incidence of pulmonary congestion was 62% (16 of 29) in saline-treated TAC mice, which is dramatically higher relative to 15% (3 of 20) in CADO-treated TAC mice ($P=0.0013$). FS and LV dP/dt_{max} also increased in either CADO- or CPA-treated mice compared with saline-treated TAC mice (Figures 6C and 6D). Linear correlation analysis noted a significant positive correlation between the heart weight to body weight ratio and the lung weight to body weight ratio ($r=0.857$, $P<0.001$).

Discussion

In this study we were able to demonstrate for the first time that the stimulation of adenosine receptors can effectively attenuate myocyte hypertrophy in vitro and in vivo and improve functioning of the pressure-overloaded heart. Our findings also suggest that these beneficial effects on cardiac hypertrophy and heart function are mediated by adenosine A_1 receptors.

As shown in this study, the stimulation of adenosine receptors attenuated G-protein–coupled receptor–induced cardiac hypertrophy in vitro, which suggests that adenosine receptor–induced intracellular signaling may interfere with the cardiac hypertrophic signaling. To clarify this issue, we examined what type of adenosine receptors is involved in this