Original Article

Impact of Adenosine Receptor Signaling and Metabolism on Pathophysiology in Patients with Chronic Heart Failure

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Adenosine is well known to be a cardioprotective substance in ischemic heart disease. However, the modulation of adenosine receptors and the production and degradation of endogenous adenosine in chronic heart failure (CHF) are not fully understood. We analyzed the gene expression patterns of adenosine-related genes in human failing and nonfailing myocardium using DNA microarray analysis and quantitative real time–polymerase chain reaction (RT-PCR). DNA microarray analysis revealed that the gene expression of adenosine A2a, A2b, and A3 receptors (A2aR, A2bR, and A3R) as well as that of adenosine deaminase (ADA) decreased in failing myocardium. The down-regulation of these genes was verified by quantitative RT-PCR. We also measured the activities of these adenosine metabolism–related enzymes in failing myocardium and cardiac adenosine levels in patients with CHF. In CHF patients, we observed the decreased enzyme activity of ADA and the elevation of cardiac adenosine levels in CHF patients. To enhance the signaling of adenosine receptors, we increased plasma adenosine levels using dipyridamole, which decreased the severity of CHF. The gene expression of A2aR, A2bR, A3R, and ADA was decreased in the failing hearts, and this decrease may impair adenosine-related signal transduction. The activities of adenosine-related enzymes were altered, thus increasing the myocardial adenosine levels; this increase may compensate for the impairment of adenosine-related signal transduction in patients with CHF. The impairment of adenosine-related signal transmission contributes to the pathophysiology of CHF. (*Hypertens Res* 2007; 30: 781–787)

***Key Words*: DNA microarray, adenosine, single nucleotide polymorphism, heart failure, adenosine deaminase, adenosine A2a receptor**

Introduction

Chronic heart failure (CHF) represents the common characteristics secondary to various cardiac diseases, such as systemic hypertension, dilated cardiomyopathy, hypertrophic cardiomyopathy, ischemic heart disease, valvular heart disease, and myocarditis (*1*). Interestingly, catecholamine, angiotensin, aldosterone, and cytokines are known to be involved in the pathophysiology of CHF (*2–5*), as evidenced

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Table1. Patient Characteristics

Case	Age (years old)	Sex	Diagnosis	Operation	LAD (mm)	LVDd (mm)	EF (%)	MR	ANP (ng/mL)	BNP (ng/mL)
01	53	M	ICM	Batista	31	88	24	IV	25	90
02	45	M	DCM	Batista	63	81	39	IV	85	217
03	72	M	DCM	Batista	52	71	14	III	86	201
04	58	F	ICM	Dor	44	76	24	I	NA	NA
05	57	M	HCM	Dor	54	52	44	III	20	80
06	69	M	DCM	Batista	49	86	15	IV	100	465
07	40	M	AR	Dor	44	76	38	I	39	200
08	75	M	ICM	Dor	28	48	35	II	37	150
09	32	M	DCM	Batista	54	81	26	IV	170	403
10	51	F	Myocarditis	Dor	26	68	35	IV	70	196
11	54	M	ICM	Dor	47	64	27	I	84	302
12	58	M	Myocarditis	Dor	48	77	18	III	800	2,710

LAD, left atrial diameter; LVDd, diastolic left ventricular diameter; EF, ejection fraction; MR, severity of mitral regurgitation; ANP, the concentration of plasma atrial natriuretic peptide (ng/mL); BNP, the concentration of plasma brain natriuretic peptide; M, male; F, female; ICM, ischemic cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; AR, aortic valve regurgitation; NA, not available.

by the fact that β-adrenoceptor antagonists, angiotensin-converting enzyme (ACE) inhibitors, and aldosterone receptor antagonists are widely accepted as drugs for CHF (*6*, *7*). Adenosine has biological effects on various tissues (*8–10*). Since several lines of evidence (*9*, *10*) support the idea that adenosine is cardioprotective against deleterious sequels in CHF as well as ischemic heart disease, it is intriguing and important to analyze the adenosine receptor– or adenosine metabolism–related genes using DNA microarray analysis. Adenosine is known to be an endogenous nucleoside acting as a cardioprotective substance that modulates numerous physiological processes, including the regulation of coronary blood flow (*9*, *10*). Adenosine is produced or degraded by several enzymes, including 5′-nucleotidase, adenosine deaminase (ADA), and adenosine kinase (AK). Adenosine elicits its physiological actions by binding to four specific receptors: A1, A2a, A2b, and A3. A1 and A3 receptors are coupled through Gi protein to adenylate cyclase inhibition, while A2a and A2b receptors are coupled to adenylate cyclase activation through Gs protein. However, the adenosine metabolism and its receptor-mediated signaling in patients with CHF remain unclear.

In the present study, we first examined gene expression in failing and nonfailing myocardium by focusing on adenosine-related genes using DNA microarray analysis followed by quantitative real time–polymerase chain reaction (RT-PCR). Then, to examine whether or not the consequences of the altered gene expression are related to the pathophysiology of human CHF, we also measured cardiac adenosine levels and the activities of adenosine-related enzymes. Finally, we tested whether or not increased adenosine levels using dipyridamole, an adenosine uptake inhibitor, improves the pathophysiology of patients with CHF.

Table 2. The Comparison of Gene Expressions between the Nonfailing and Failing Hearts

Gene name	Fold change
Adenosine receptors	
A1 receptor	1.51±0.32
A2a receptor	0.29±0.04
A2b receptor	0.75±0.07
A3 receptor	0.61±0.04
Adenosine-related enzymes	
Adenosine deaminase	0.52±0.03
Adenosine kinase	1.14±0.16

Methods

RNA Samples from Human Heart Tissues

Tissue samples of human failing heart were obtained from 12 patients (average age 55 years [range 32–75 years]; 10 males and 2 females) who had undergone partial left ventriculectomy (the Batista or Dor procedure) for end-stage heart failure at Hayama Heart Center. All heart tissues were stored in RNA Later (Ambion, Austin, USA). Because of the difficulty of acquiring nonfailing heart tissues in Japan, we obtained total RNAs of nonfailing myocardium of Mongolian people from BioChain Institute Inc. (Hayward, USA). The collection and use of tissue were approved by independent ethics committees of the National Cardiovascular Center at Osaka University and of the Hayama Heart Center.

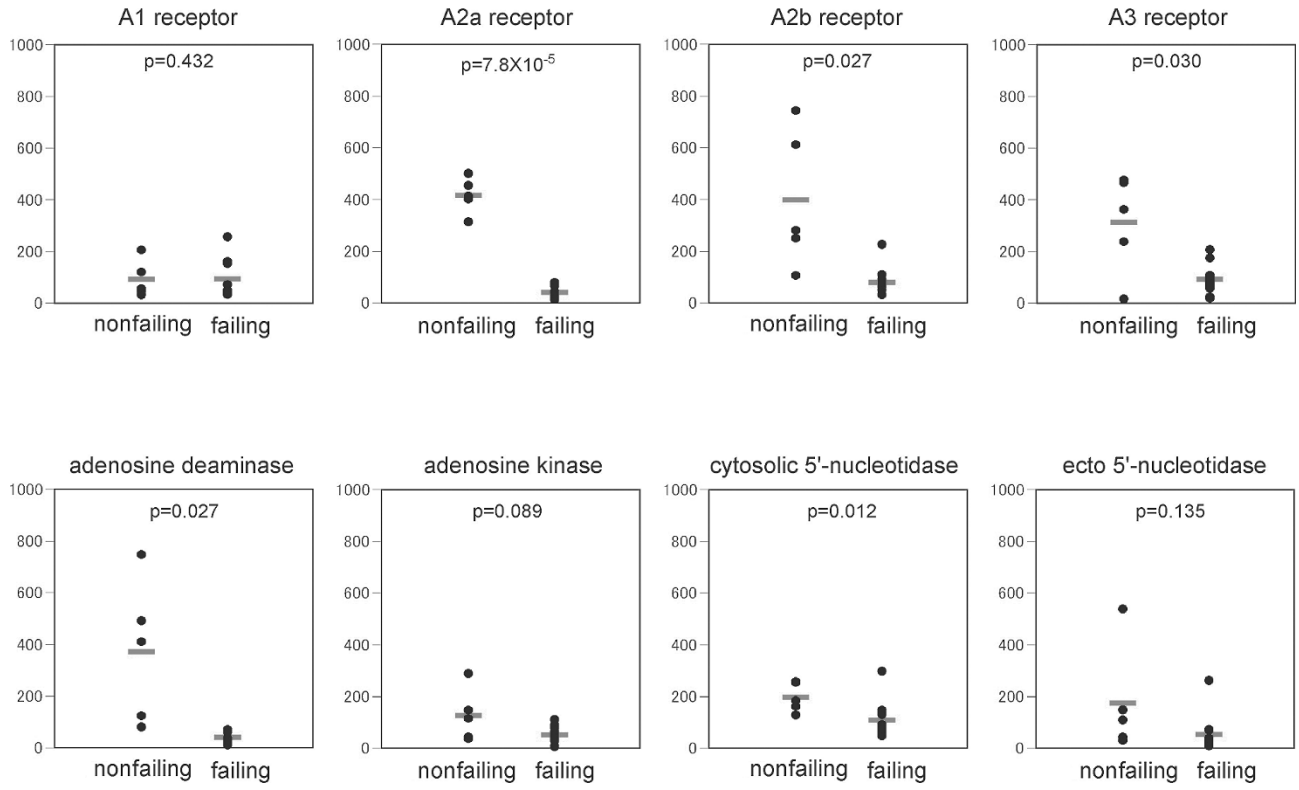

Fig. 1. *Quantitative real-time RT-PCR of genes related to adenosine. Expression of eight genes related to adenosine were verified by quantitative real-time RT-PCR. The expression levels of A2aR, A2bR, ADA, and cytosolic 5′-nucleotidase were significantly down-regulated in failing hearts compared to nonfailing hearts. The levels of gene expression of the other four genes did not differ significantly between failing and nonfailing hearts. The relative expression levels are normalized by GAPDH expression as 100.*

RNA Isolation and DNA Microarray Hybridization

Total RNA was extracted from 12 failing human heart tissues (Table 1) by TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The integrity of the RNA was verified with an RNA 6000 Nano LabChip Kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). DNA microarray analysis was performed according to the Affymetrix GeneChip expression analysis protocol. Biotinylated cRNA was generated and was applied to Affymetrix oligonucleotide array GeneChip Human Genome U95 sets (Affymetrix, Santa Clara, USA). Expression differences between the nonfailing and failing hearts were analyzed by MAS ver 4.0 (Affymetrix).

Quantitative Real-Time RT-PCR

Eight RT-PCR products ADA, AK, cytosolic and ecto 5′-nucleotidase, and adenosine receptors (A1, A2a, A2b, and A3) from 5 nonfailing heart tissues and 12 failing heart tissues were used to confirm the DNA microarray data by quantitative real-time RT-PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, USA). The respective primers used in this study were designed according to the sequences available in GenBank using Primer Express Software (Applied Biosystems). We used GAPDH as the internal control gene because it showed similar expression levels in nonfailing and failing heart samples.

Measurements of Adenosine-Related Enzyme Activities and Plasma Adenosine Levels

The preparation of the myocardium for the measurement of adenosine-related enzymes, *i.e.*, 5′-nucleotidase, ADA, and AK, was reported previously (*10*). Failing myocardium were obtained from 12 patients who had undergone cardiac biopsy. Blood was sampled from either the ascending aorta near the ostium of the coronary artery or the coronary sinus vein using an NIH catheter. Plasma adenosine levels were determined by radioimmunoassay as previously reported (*11*).

Dipyridamole Treatment

Twenty-one patients judged to be in functional classification II or III of the New York Heart Association (NYHA) were

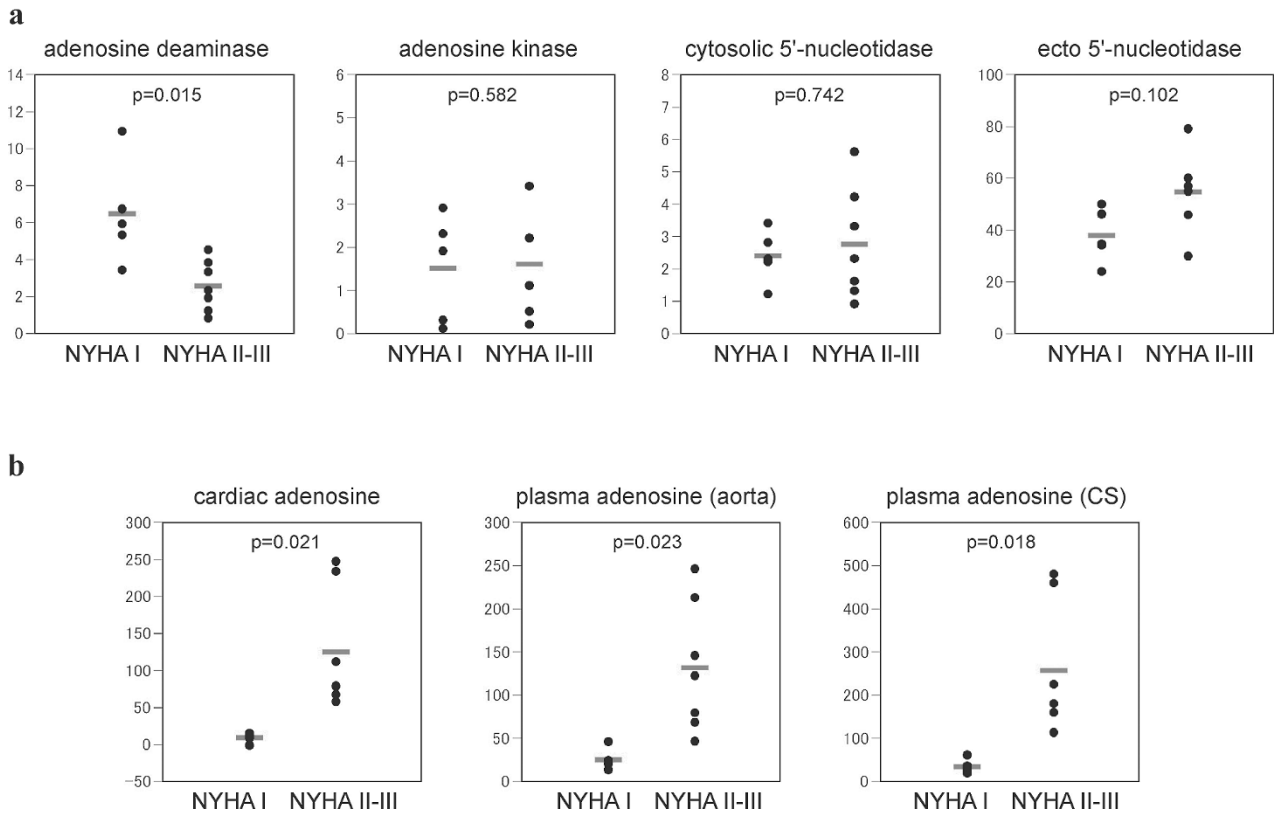

Fig. 2. *Enzyme activities of adenosine-related enzymes and cardiac adenosine levels. a: The enzyme activity of ADA was repressed in patients with NYHA II-III compared to those with NYHA I. The enzyme activity of ecto 5′-nucleotidase was elevated in patients with NYHA II-III compared to those with NYHA I. The activities of AK and cytosolic 5′-nucleotidase did not differ between patients with NYHA I and those with NYHA II-III. The unit of activity of each enzyme is nmol/kg protein/min. b: Cardiac adenosine levels were elevated in patients with NYHA II-III compared to those with NYHA I. The unit of plasma adenosine level is nmol/L.*

examined. There were 11 patients with dilated cardiomyopathy, 5 patients with ischemic cardiomyopathy, 3 patients with valvular heart disease, and 2 patients with hypertensive heart disease. We administered dipyridamole at 75 mg/day ($n=8$) or 300 mg/day ($n=6$) for 6 months. At the onset and again 6 months after the onset of administration we assessed NYHA classification, ejection fraction (EF), and fractional shortening (FS) using echocardiography, and we measured maximal oxygen uptake using an ergometer.

Statistical Analysis

Statistical analyses were performed using ANOVA when the data were compared among the groups. When ANOVA reached a significant level, we compared pairs of data using the Bonferroni test. The values are expressed as means±SD, with $p<0.05$ considered significant.

Results

Expression of Adenosine-Related Genes in Human Failing Hearts

We examined the expression levels of six adenosine-related genes in failing myocardium using DNA microarray analysis. Table 2 shows that ADA expression was down-regulated in the failing hearts compared to the nonfailing hearts. Interestingly, the adenosine receptors were modulated in the myocardium of patients with CHF. Most of all, the expression of A2a receptors was markedly down-regulated in the failing myocardium to less than one-third the level in the nonfailing hearts.

We performed quantitative RT-PCR of these genes to confirm the expression patterns of these transcripts related to adenosine from DNA microarray analysis. The results showed that the mRNA levels of A2a receptor, A2b receptor, A3 receptor, ADA, and cytosolic 5′-nucleotidase were down-regulated in the failing hearts compared with the nonfailing

Table 3. The before and after Dipyridamole Treatment of CHF

	Pre-medication	6-month treatment	*p* value
Control group (n=7, average 66 years old, 6 male)			
LAD (mm)	47.7±10.5	47.8±10.6	n.s.
LVDd (mm)	58.6±7.1	58.2±7.5	n.s.
LVDs (mm)	49.7±9.0	49.9±9.1	n.s.
FS (%)	16.6±6.6	15.0±6.2	n.s.
EF (%)	34.6±8.4	34.0±9.7	n.s.
BNP (ng/mL)	211.4±172.5	227.3±178.9	n.s.
NYHA(I/II/III/IV)	0/3/4/0	0/3/4/0	n.s
V_{O_2} (mL/kg/min)	16.9±5.3	17.5±5.4	n.s.
Workload (Mets)	5.0±1.3	5.2±1.2	n.s.
Dypiridamol group (n=14, average 66 years old, 10 male)			
LAD (mm)	49.5±6.8	46±6.6	n.s.
LVDd (mm)	58.9±11.9	54.8±11.9	n.s.
LVDs (mm)	50.8±12.1	45.1±12.4	n.s.
FS (%)	14.4±5.1	18.7±6.1	0.02
EF (%)	34.1±9.9	45.4±10.5	0.01
BNP (ng/mL)	236.8±154.0	105.8±125.1	0.02
NYHA(I/II/III/IV)	0/1/13/0	1/7/6/0	0.001
V_{O_2} (mL/kg/min)	16.5±3.9	20.4±4.2	0.052
Workload (Mets)	5.6±1.4	6.4±1.2	n.s.

LAD, left atrial diameter; LVDd and LVDs, diastolic and systolic left ventricular diameters, respectively; FS, fractional shortenening; EF, ejection fraction; MR, severity of mitral regurgitation; BNP, the concentration of plasma brain natriuretic peptide; V_{O_2}, oxygen consumption. Values are expressed as the individual number or mean±SD. *p* values are obtained by the comparison between the conditions of pre-medication and 6 months medication.

hearts (Fig. 1). The expression of the A1 receptor, adenosine kinase, and ecto 5′-nucleotidase did not differ between the failing and the nonfailing hearts.

Enzyme Activity Assay and Adenosine Level

We examined the enzyme activities of the adenosine-related enzyme and the cardiac adenosine level to examine whether or not the altered gene expression reflects the change in adenosine metabolism in patients with CHF. We observed that ADA activity was lower in patients with NYHA II-III than in patients with NYHA I (Fig. 2), while cytosolic 5′-nucleotidase activity was unchanged. Cardiac adenosine levels were higher in patients with NYHA II-III than in those with NYHA I. Together, these results suggest that adenosine plays an important role in the pathophysiology of CHF.

An Adenosine Potentiator as a Therapy Target for CHF

In 21 patients with CHF, we administered dipyridamole at either 75 or 300 mg daily in 14 patients with CHF for 6 months. Table 3 shows the clinical data on the control and dipyridamole groups. Echocardiography showed that dipyridamole increased cardiac functions such as EF and fractional shortening. Dipyridamole decreased plasma brain natriuretic peptide (BNP) level in patients with CHF. This indicates that the enhancement of plasma adenosine levels compensates for the down-regulation of adenosine receptors and improves the pathophysiology of CHF.

Discussion

Impact of the Present Study on the Pathophysiology of CHF

Despite the recent advances in our knowledge of CHF, the complex pathophysiological events of CHF, especially at the molecular and genetic levels, remain to be fully elucidated (*12*). Microarray analysis has been expected to respond to the questions surrounding this complexity. In the present study, we demonstrated the expression of the genes related to adenosine in failing and nonfailing human heart tissues using microarray analysis and quantitative real-time RT-PCR. We clarified the downregulation of the A2a receptor, the A2b receptor, the A3 receptor, cytosolic 5′-nucleotide, and ADA genes. We also revealed the elevation of cardiac adenosine levels in CHF patients with NYHA II-III compared to the patients with NYHA I. Finally we suggested that the enhancement of adenosine level improves cardiac functions in patients with CHF. This report revealed that adenosine is involved in the pathophysiology of CHF, and the augmenta-

tion of endogenous adenosine can be a novel treatment for CHF.

The Down-Regulation of Adenosine Receptors and the Pathophysiology of CHF

Since adenosine is known to be cardioprotective, the down-regulation of adenosine receptors as shown in the present study is speculated to be a cause of CHF. Indeed, the activation of A2a and A2b receptors increases myocardial contractility *via* cyclic AMP–independent pathways (*13*) and increases coronary blood flow *via* K_{ATP} channel–dependent mechanisms (*14*). Since decreases in myocardial contractility or abnormal coronary perfusion are thought to be potential causes of CHF, these functional abnormalities of A2a and A2b receptors may be responsible for CHF. Furthermore, the lack of A2a and A2b receptor function facilitates platelet aggregation and leukocyte activation, both of which damage the myocardium and coronary microcirculation (*15*).

Importantly, it is reported that A2a receptors are up-regulated in the peripheral circulating cells of patients with end-stage CHF compared with control subjects (*16*). The difference between the results of that study and those of ours remains unknown, but may be attributable to the differences in 1) the severity of CHF, 2) the causes of CHF, and 3) sampling sites for assessing A2a receptor expression. First, Varani *et al*. investigated patients with very severe CHF who have had heart transplants, and we investigated moderate to severe CHF patients who have undergone either the Batista or Dor operation. Secondly, the causes of CHF in the present study were mainly non-ischemic heart diseases such as dilated cardiomyopathy, whereas Varani *et al*. did not discuss the causes of CHF in their patients. Finally, the regulation of A2a receptor in lymphocytes may differ from that in cardiomyocytes and the up-regulation of A2a receptor in lymphocytes as reported by Varani *et al*. does not necessarily indicate the upregulation of A2aR in the myocardia seen in the present study (*16*).

We also observed the down-regulation of A3 receptors in the myocardia of CHF patients. Since the activation of A3 receptors also provides cardioprotection (*17*), the down-regulation of A3 receptor expression may contribute to the severity of CHF.

Using rat myocardial infarction (MI) models, we recently reported that long-term stimulation of A2b receptors attenuates cardiac fibrosis in non-infarcted myocardium and improves cardiac function (*18*). These results suggest that the down-regulation of A2b receptors might be involved in cardiac fibrosis in patients with CHF.

The Down-Regulation of ADA and the Pathophysiology of CHF

What are the roles of ADA expression down-regulation and of the modulated activities of ADA? The reduced activity of cardiac adenosine deaminase in the myocardia of patients with CHF enhances intracellular adenosine levels by inhibiting adenosine degradation. Enhanced adenosine is released extracellularly and acts on adenosine receptors of various other cells in the heart and vessels. Since adenosine is believed to be cardioprotective, the changes are thought to compensatee for the down-regulation of adenosine receptors and the pathophysiology of CHF. Although the expression level of cytosolic 5′-nucleotidase decreased, the activities of ecto and cytosolic 5′-nucleotidase were not modulated, and the adenosine degradation capability *via* ADA decreased without modulation of the adenosine production capability. We do not attempt to clarify the reason why cytosolic 5′-nucleotidase activity was not changed despite the decreased expression level of cytosolic 5′-nucleotidase. One possibility is that cytosolic 5′-nucleotidase is phosphorylated and activated by neurohumoral factors such as angiotensin II, preventing any change in the activity of cytosolic 5′-nucleotidase. As a whole, we found that cardiac adenosine levels are elevated in patients with CHF, and that further increases in adenosine levels by dipyridamole administration restored cardiac function. Since the patients with CHF (NYHA II-III) were enrolled in this study, it is unclear whether or not this result can be applied to patients with more severe CHF. Our observation that the plasma adenosine level was high in patients with NYHA class III or IV implied that dipyridamole treatment might improve cardiac dysfunction in patients with class IV CHF.

Conclusions

We found that the gene expression of the A2a receptor, the A2b receptor, the A3 receptor, ADA and cytosolic 5′-nucleotidase was down-regulated in human failing myocardia. This result implies that the downregulation of adenosine plays an important role in causing heart failure by impairing adenosine signal transduction. We also found elevated cardiac adenosine levels and the repression of ADA enzyme in patients with severe heart failure. The enhancement of adenosine levels by dipyridamole improved cardiac functions in a small population of patients with CHF. These results from our basic and clinical research imply that adenosine therapy might be a promising approach to treat CHF, although we need to perform either medium- or large-scale trials to confirm this.

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