Activation of Adenosine A1 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 A1 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 A1 agonist N6-cyclopentyladenosine (CPA). A nonselective adenosine antagonist, 8-(p-sulphophenyl)-theophylline, and a selective adenosine A1 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 A1 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 calcineurin8,9 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

Original received March 27, 2003; revision received August 28, 2003; accepted August 28, 2003.
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Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000094744.88220.62
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, N6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-(p-(2-carboxyethyl)phenethylamino)-5'-ethylcarboxamido adenosine hydrochloride (CGS21680), 5-ethylcarboxamidoaenosine (NECA), and N6-(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. Cardiomyocytes 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 [3H]leucine incorporation as described. 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 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 in a time course. Plasma norepinephrine and renin levels were determined as described.

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 [3H]leucine uptake of myocytes when the concentration of CADO was not higher than 10^-5 mol/L, but CADO decreased [3H]leucine uptake at concentrations higher than 10^-5 mol/L (Figure 1A). Thus, we used CADO at the concentrations of 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 [3H]leucine incorporation, and cotreatment with CADO (10^-6 mol/L) inhibited these G-protein–coupled receptor agonist–induced increase in [3H]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 [3H]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 [3H]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 A1 Receptors
CPA, an A1 selective agonist, and NECA, a nonselective agonist for A1, A2A, and A2B receptors, significantly inhibited the PE-induced increase of cardiac myocyte protein synthesis, but neither CGS21680, an A2A receptor agonist, nor IB-MECA, an A1 selective receptor agonist, affected the PE-
induced increase of [3H]leucine uptake (Figure 3A). Similar results were obtained in forskolin-induced cardiac myocyte hypertrophy (Figure 3B). Therefore, we conclude that it is A1, not A2a or A3 receptors, that mediates the antihypertrophic effect.

**Activation of Adenosine A1 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 preliminary 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 A1 receptor agonist CPA markedly attenuated cardiac hypertrophy, and this effect was abolished by a selective A1 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 results were obtained in forskolin-induced cardiac myocyte hypertrophy (Figure 3B). Therefore, we conclude that it is A1, not A2a or A3 receptors, that mediates the antihypertrophic effect.
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.

**Activation of Adenosine A<sub>1</sub> 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<sub>max</sub>. 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<sub>max</sub> 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<sub>1</sub> 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
phenomenon. Considering that the EC50 of CADO on adenylate cyclase activity mediated by A1, A2A, and A2B receptors is 100, 460, and 15,000 nmol/L, respectively, and the inhibitory effects of CADO are mimicked by CPA (A1 receptor selective agonist) and NECA (A1, A2A, and A2B receptor agonist) but not CGS21680 (A2A receptor selective agonist), the inhibitory effects of adenosine on protein synthesis are most likely mediated via A1 receptors rather than A2A receptors. However, mediation via the A2B receptor cannot be completely ruled out because the low affinity of CADO for the A2B receptor makes it unlikely that the stimulation of A2B receptors mediates myocardial antihypertrophy produced by CADO. Furthermore, our in vivo studies using a selective adenosine A1 agonist, CPA, and an antagonist, DPCPX, clearly showed that the stimulation of A1 receptors mainly mediates the antihypertrophic effect of CADO. Moreover, there is a substantial
body of evidence suggesting that the stimulation of A₁ receptors mediates antigrowth effect. Adenosine-induced inhibition of norepinephrine release from adrenergic nerves in the heart is also mediated via the A₁ receptor and this stimulation leads to the activation of adenylate cyclase, which therefore may be an alternative mechanism for CADO-induced attenuation of hypertrophy. To clarify this point, we used forskolin, the most potent AC stimulator, to stimulate protein synthesis of myocytes. As expected, CADO completely eliminated any increase in the protein synthesis induced by forskolin. We therefore posit that the signaling of A₁ receptor activation is linked with both Gₐ and Gᵦ proteins and that inhibition of adenylate cyclase may suppress the hypertrophic signaling pathways in cardiomyocytes.

It has become clear that the development of cardiac hypertrophy is a multigenic, integrative response involving signal integration of multiple pathways. The antihypertrophic effect of CADO could also be attributable, in part, to a reduction of [Ca²⁺], in myocytes, because [Ca²⁺] can stimulate the calcineurin-mediated cardiac hypertrophic pathway. Even more interesting is that RGS, which attenuates G protein-mediated signaling, may also be an alternative pathway through which CADO can inhibit Gᵦ protein-induced hypertrophy, because it is reported that an increase in cAMP level resulted in a reduction of the RGS-4 message by nearly 50%. We also confirmed in this study that expression of RGS-4 was upregulated by CADO treatment. Thus, CADO may exert an antihypertrophic effect mediated by increasing RGS-4 via the inhibition of adenylate cyclase.

Our results indicate that CADO not only attenuates cardiac hypertrophy but also dramatically improves heart function, which is consistent with our findings in patients with heart failure as previously reported. The antihypertrophic effect of CADO is likely to protect the heart in a way similar to that of the adrenergic β blocker by reducing either contractility or heart rate. Moreover, CADO may be superior to β blocker because it can attenuate the activation of renin-angiotensin system and counteract TNF-α. On the other hand, we also should notice that A₁ adenosine antagonist BG9719 was reported to preserve renal function via promoting natriuresis during treatment for heart failure. Reducing glomerular filtration rate should be considered one of the adverse effects of adenosine A₁ agonists, but it is not enough to deny the potential beneficial effects of adenosine receptor activation on heart failure mediated by their well-known antihypertrophic effect. Nevertheless, a recent study revealed that no significant difference on glomerular filtration rate was noted between A₁ receptor deficiency mice and wild-type mice. In this study, we have noted a positive correlation between cardiac hypertrophy and pulmonary congestion, indicating that the beneficial effect of CADO on preventing heart failure at least partially should be attributed to its inhibitory effect on myocardial hypertrophy. Although this seems paradoxical to the traditional notion that hypertrophy is a needed compensatory mechanism, recent studies on murine TAC model suggest that cardiac hypertrophy is not a required compensatory response to pressure overload.

We have characterized the time course of LVH and heart function changes in a previous study and showed that significant LVH and impaired LV heart function (FS) appeared at 4 weeks after TAC. In this study, we noted that myocardial 5'-ND activity was also increased significantly at 4 weeks, a time course in good accordance with FS, suggesting that declined heart function is associated with increased 5'-ND activity. This observation is in agreement with the clinical investigation. Although in this study we did not find that 8-SPT additionally increases cardiac hypertrophy and decelerates the heart function in the TAC model, the cardioprotection of adenosine cannot be excluded because the concentration of endogenous adenosine may be far below the
level to be able to act at the receptor level in pressure-overload state. Therefore, it would be of importance to clarify whether augmentation of endogenous adenosine is beneficial to cardiac hypertrophy.

In conclusion, the data in this study indicate that the activation of adenosine A1 receptors attenuates both the cardiac hypertrophy and myocardial dysfunction mediated by combined mechanisms of antiadrenergic effect and upregulation of RGS-4.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (Nos. 12470153 and 12877107) from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Human Genome, Tissue Engineering and Food Biotechnology (H13-Genome-011) in Health and Labor Sciences Research Grants, and Comprehensive Research on Aging and Health (H13-21seiki [seikatsu]-23) in Health and Labor Sciences Research Grants from the Ministry of Health and Labor and Welfare, Japan. This work was also supported by Research on Health Technology Assessment (H14-iryo-025) in Health and Labor Sciences Research Grants from the Ministry of Health and Labor and Welfare, Japan. The authors would like to thank Tomi Fukushima and Junko Yamada for expert technical assistance.

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_Circ Res._ 2003;93:759-766; originally published online September 11, 2003; doi: 10.1161/01.RES.0000094744.88220.62

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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