Adenosine-Stimulated Atrial Natriuretic Peptide Release Through A1 Receptor Subtype

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Abstract—Adenosine acts as an important protector of ischemic myocardium through coronary vasodilation and the depression of cardiac contractility. The protective effect of adenosine may partly relate to the cardiac hormone atrial natriuretic peptide (ANP). The aim of the present study was to investigate the effects of adenosine and the adenosine receptor subtype on atrial hemodynamics and ANP release using isolated perfused beating rat atria. Adenosine, a nonselective adenosine receptor agonist, increased the ANP release with negative inotropism in a dose-dependent manner. Adenosine-stimulated ANP release was attenuated by a selective A1 antagonist but not A2A antagonist or A1 antagonist. The order of potency of the various agonists for the ANP release was A1 agonists >> A3 agonist = adenosine > A2A agonist. The order of potency for the negative inotropy was A1 agonists >> adenosine = A2A agonist > A1 agonist. The negative inotropism and ANP release by a specific A1 agonist (N6-cyclopentyl-adenosine) were also attenuated by A1 antagonist but not A2A antagonist or A1 antagonist. Treatment with A1 agonist resulted in a decrease of cAMP contents in atria and perfusates. The agonist-stimulated ANP release was significantly attenuated in the presence of forskolin, isoproterenol 8-Br-cAMP, or an adenylyl cyclase inhibitor. These results suggest that the A1 receptor subtype is responsible for the adenosine-induced ANP release and negative inotropism through adenylyl cyclase–cAMP pathway. (Hypertension. 2005;46:1381-1387.)

Key Words: adenosine • natriuretic peptides • cyclin AMP • heart • hypertension, experimental • receptor • kidney

Adenosine is recognized as an important signaling molecule in heart. Adenosine originates from the hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase during normal condition or from the hydrolysis of AMP by 5′-nucleotidase during ischemic or hypoxic conditions.1 Adenosine effects are mediated through 4 distinct receptors, A1, A2A, A2B, and A3, initially defined pharmacologically based on their effects on adenyl cyclase (AC; inhibition or stimulation) and their selectivity for agonists and antagonists.2–4 All the receptor subtypes appear to be expressed in cardiomyocytes.4 In the cardiovascular system, adenosine causes coronary vasodilation, the reduction of heart rate and cardiac contractility, and the attenuation of stimulatory actions of catecholamines on heart.5 Endogenous release of adenosine during myocardial ischemia and hypoxia induces a potent protective effect in paracrine and autocrine ways.6 Adenosine produced during a brief period of ischemia or exogenously administered adenosine causes a cardioprotective effect manifested by a reduction of infarct size or decreased myocardial stunning during ischemic preconditioning7 via activation of A1 and A2 receptor subtypes.5,8 Myocardial adenosine and ADP levels are increased during hypoxia and in severely hypertrophied hearts. Therefore, adenosine can exert effects on the ischemic or overloaded heart that are of considerable scientific and potential therapeutic interest.

Studies have indicated that adenosine increases plasma concentration of atrial natriuretic peptide (ANP)9,10 and ventricular ANP gene expression.11 However, the molecular mechanisms of adenosine-mediated ANP secretion has not been fully addressed9,12 and is still controversial. In the present study, we hypothesized that the cardioprotective effect of adenosine is partly attributable to an increase in ANP secretion. We investigated effects of adenosine and its specific receptor agonists on the ANP release and the molecular mechanism using isolated perfused rat atria.

Methods

Isolated Perfused Beating Atrial Preparation

Male Sprague-Dawley rats weighing 300 to 320 g were used in this study. The investigation conformed to the animal care guidelines in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Isolated perfused beating atria were prepared by using a method described previously.13 In brief, the left atrium was dissected from the heart after killing and fixed into a Tygon cannula. The cannulated atrium was perfused immediately with oxygenated HEPES-buffered solution (HBS) at 36.5°C and paced at 1.2 Hz (duration 0.3 ms; voltage 40 V). The composition of HBS was as follows: 118 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl2, 1.2 mmol/L MgSO4, 25 mmol/L NaHCO3, 10 mmol/L HEPES, 10 mmol/L glucose, and 0.1% BSA. Intra-atrial pressure was recorded on
a Physiograph via a pressure transducer and contractility and calculated from the differences between systolic and diastolic pressures. To measure the translocation of extracellular fluid (ECF), [H]-inulin was used as described previously. The atrium was perfused with HBS for 100 minutes to stabilize the secretion of ANP and maintain a steady-state [H]-inulin level in the extracellular space. The perfusate was collected into prechilled tubes at 2-minute intervals at 4°C.

Experimental Protocols

Experiments were performed with 5 groups.

Group 1 included the adenosine-perfused atrium. Adenosine (1, 10, and 30 μmol/L; n=6 to 11) was introduced into the atrial lumen after a 10-minute control collection period, and perfusate was collected continuously for 50 minutes.

Group 2 included the purinoceptor antagonist–pretreated atrium. To define adenosine receptor subtype, a receptor antagonist for A1 (8-cyclopentyl-1,3-dipropylxanthine [DPCPX]; 0.1 μmol/L; n=8), A2A (ZM 241385; 1 μmol/L; n=7), or A3 (MRS 1220; 1 μmol/L; n=7), forskolin (AC activator; 1 μmol/L; n=10), or 8-Br-cAMP (AC inhibitor; 10 μmol/L; n=7) was administered. ANP secretion in terms of ECF translocation (ie, interstitial ANP concentration obtained from the mean of 5 control periods and last 3 experimental periods) was measured as a control period. Adenosine (1, 10, or 30 μmol/L) was then infused simultaneously with a modulator using the same protocol described for group 1.

Group 3 included the purinoceptor agonist–perfused atrium. To determine the specific effect of CPA (1 μmol/L) on atrial contractility and ANP secretion, DPCPX (1 μmol/L; n=9), ZM 241385 (1 μmol/L; n=8), or MRS 1220 (1 μmol/L; n=7) was administered as a pretreatment, and CPA was simultaneously infused using the same protocol described for group 1.

Group 4 included the purinoceptor antagonist–pretreated atrium. To examine the effector molecule of CPA (1 μmol/L) on atrial contractility and ANP secretion, MDL 12330 (AC inhibitor; 10 μmol/L; n=8), forskolin (AC activator; 1 μmol/L; n=9), isoproterenol (β-adrenoceptor agonist; 1 μmol/L; n=10), 6-chloro-N-(2-benzofuran-5-yl)-N-methyluronamide (CI-IB-MECA; A1 agonist; 10 μmol/L; n=8) was perfused using the same protocol described for group 1.

Group 5 included the AC modulator–pretreated atrium. To determine the specific effect of CPA (1 μmol/L) on atrial contractility and ANP secretion, DPCPX (1 μmol/L; n=9), ZM 241385 (1 μmol/L; n=8), or MRS 1220 (1 μmol/L; n=7) was administered as a pretreatment, and CPA was simultaneously infused using the same protocol described for group 2.

To measure the effect of CPA on ANP content, the atrium was blotted, frozen in liquid nitrogen, and kept at −70°C until use. The cAMP concentrations in perfusate in control 3 periods and last 3 experimental periods were measured as described below. All agonists and antagonists used in the study were purchased from Tocris Cookson Inc.

Radioimmunoassay of ANP Concentration

The concentration of immunoreactive ANP in the perfusate was measured with a specific radioimmunoassay (RIA). The molar concentration of ANP release was calculated as follows because ANP was secreted into the atrial lumen with the translocation of ECF. ANP released (μL/min)/ECF translocation (μL/min/g)/atrial wet weight (mg)×1000.

Measurement of ECF Translocation

A 2-step sequential mechanism of ANP secretion from the atrium was applied to determine ECF translocation as reported previously. The radioactivity of [H]-inulin in the perfusate was measured with a liquid scintillation counter. The amounts of ECF translocated through the atrial wall were calculated as: ECF translocation (μL/min/g)=total radioactivity in perfusate (cpm/min)/radioactivity in pericardial reservoir (cpm/μL)/atrial wet weight (mg)×1000.

RIA of cAMP Concentration

For measurement of cAMP concentration in perfusate, 100 μL of the perfusate was treated with trichloroacetic acid (TCA; 300 μL) to a final concentration of 6% for 15 minutes at room temperature and centrifuged at 4°C. The supernatant (100 μL) was extracted with water-saturated ether 3× and then dried using a SpeedVac concentrator (Savant). The dried samples were resuspended in 100 μL sodium acetate buffer (50 mmol/L; pH 4.8) and used for cAMP measurement.

For measurement of cAMP concentration in the atrium, the tissue was minced in 2 mL ice-cold TCA (6%) and homogenized at 4°C by 30-s bursts in a Polytron homogenizer. The homogenates were centrifuged at 1000 g for 10 minutes at 4°C, and the supernatant was extracted with ether and dried as described above. The pellet was treated with 500 μL NaOH (1 N), ultrasonicated, and used for protein determination.

Levels of cAMP were measured with a specific RIA, as described previously. Briefly, standards or samples were taken up in a final volume of 100 μL of 50 mmol/L sodium acetate buffer, pH 4.8, containing theophylline (8 mmol/L), and then 5 μL of a mixture of acetic anhydride and triethylamine (1:2) was added to the assay tube. One hundred microliters of diluted cAMP antisera (Calbiochem–Novabiochem) and iodinated 2'-O-monsucyclinyl-adenosine 3',5'- cyclic mono-phosphate tyrosyl methyl ester ([125I]ScAMP-TME) and 10 000 cpm/100 μL were added and incubated for 24 hours at 4°C. The bound form was separated from the free form by charcoal treatment. RIA of cAMP in perfusate was done on the day of the experiments, and all samples from 1 experiment were analyzed in a single assay.

Statistical Analysis

The results are given as the means±SEM. An ANOVA was followed by the Bonferroni’s comparison test or Duncan multiple range test. Student t test was also used. The statistical significance was taken at a value of P<0.05.

Results

Effects of Adenosine on Atrial Contractility and ANP Release

Figure 1A shows the effect of adenosine on contractility, ANP secretion, ECF translocation, and interstitial ANP concentration in beating rat atria. After 100 minutes of stabilization, perfusate was collected 5× every 2 minutes to serve as a control period. Adenosine (1, 10, or 30 μmol/L) was then administered. Atrial contractility was decreased dramatically in a dose-dependent manner as soon as adenosine was administered and maintained persistently during the infusion period. The ECF translocation was maintained relatively constant except the highest dose. The ANP secretion was increased gradually in a dose-dependent manner by adenosine. The ANP released from atrial myocytes into the interstitial space was translocated into atrial lumen, concomitantly with the ECF translocation. Therefore, the ANP secretion in terms of ECF translocation (interstitial ANP concentration) was markedly increased in a dose-dependent manner.

Figure 1B shows the relative percent changes in atrial contractility, ECF translocation, ANP secretion, and interstitial ANP concentration obtained from the mean of 5 control values and the last 5 experimental values for exposure to adenosine (1, 10, or 30 μmol/L). Adenosine decreased atrial contractility and increased the ANP secretion in a dose-dependent manner. The decrease in ECF translocation was only observed with the highest dose of adenosine. Because it has been shown that ANP was secreted into the atrial lumen with the translocation of ECF, the increase in ANP secretion in terms of ECF translocation (ie, interstitial ANP concentration) reflects an increase in ANP release from atrial myocardial cells.
myocytes. Adenosine, at a dose of 1, 10, and 30 μmol/L, caused increases in ANP concentration by 14%, 52%, and 132%, respectively, compared with control value.

Modification of Adenosine-Induced Increase in ANP Release and Negative Inotropy by Adenosine Receptor Antagonists

To define which receptors are involved in adenosine-induced augmentation of ANP release and negative inotropism, specific A1, A2A, or A3 receptor antagonist was pretreated, and then 10 μmol/L of adenosine was perfused simultaneously. Figure 1B shows the relative percent changes in pulse pressure, ECF translocation, ANP secretion, and interstitial ANP concentration by adenosine in the presence of adenosine receptor antagonist. DPCPX, an A1 receptor antagonist, completely blocked adenosine-induced augmentation of ANP release and attenuated negative inotropy. However, the presence of ZM 241385 (A2A receptor antagonist) or MRS 1220 (A3 receptor antagonist) did not block the effects of adenosine on ANP release and atrial contractility.

Effects of CPA on Atrial Contractility, ANP Release, and cAMP Amount

To compare the potency of adenosine with specific receptor agonist for A1, various doses of CPA (A1 agonist) were perfused. Figure 2 shows the relative percent changes in atrial
contractility, ECF translocation, ANP secretion, and interstitial ANP concentration obtained from the mean of 5 control values and the last 5 experimental values. CPA caused decreases in atrial contractility and ECF translocation and an increase in ANP secretion in a dose-dependent manner. The fact showing an increase in ANP secretion by CPA despite of decrease in ECF translocation means an increase in ANP release from atrial myocytes into interstitial space. In other words, CPA, at doses of 0.01, 0.1, 1, and 10 μmol/L, caused increases in interstitial ANP concentration by 140%, 253%, 398%, and 431%, respectively (Figure 2D). The amounts of cAMP in atrial tissues (Figure 2E) and perfusate (Figure 2F) were decreased in a CPA dose-dependent manner.

To compare the potency of adenosine with specific receptor agonist for A<sub>1</sub>, A<sub>2A</sub>, or A<sub>3</sub> receptor, CPA (A<sub>1</sub> agonist), CGS (A<sub>2A</sub> agonist), or CI-IB-MECA (A<sub>3</sub> agonist; all 10 μmol/L) was perfused. Figure 3 shows the relative percent changes in atrial contractility, ECF translocation, ANP secretion, and interstitial ANP concentration obtained from the mean of 5 control values and the last 5 experimental values for exposure to agonist compared with adenosine. CPA caused marked decreases in atrial contractility and ECF translocation and an increase in ANP secretion. The effects of CGS were similar to adenosine. CI-IB-MECA caused an increase in ANP secretion without changes in atrial contractility and ECF translocation. The order of potency for the increment of ANP release was CPA>A<sub>1</sub>-IB-MECA=adenosine>CGS. The order of potency for the decrement of atrial contractility was CPA>adenosine>CGS>CI-IB-MECA.

Figure 4 shows the blocking effects of receptor antagonists on atrial contractility, ECF translocation, ANP secretion, and interstitial ANP concentration by CPA. The CPA-induced augmentation of ANP release and negative inotropism were markedly attenuated by DPCPX but not ZM 241385 or MRS 1220.

**Effects of AC Modulator on CPA-Induced ANP Release**

Above results suggest that A<sub>1</sub> receptor subtype is responsible for the stimulation of ANF release. This receptor subtype couples with G<sub>i</sub> protein and inhibits AC, resulting in inhibition of cAMP production. To determine whether the effects of CPA on the ANF release are mediated by the A<sub>1</sub> receptor signaling, MDL 12330 (AC inhibitor), forskolin (AC activator), isoproterenol (β-adrenoceptor agonist), or 8-Br-cAMP (membrane permeable cAMP analog) was pretreated, and then 1 μmol/L of CPA was simultaneously perfused with a modulator. MDL 12330, forskolin, isoproterenol, and 8-Br-cAMP did not affect basal ANP secretion (data not shown). However, these AC modulators significantly attenuated CPA-induced increase in ANP secretion, whereas ECF translocation and negative inotropy were decreased (Figure 5). Interestingly, 8-Br-cAMP did not affect negative inotropy (Figure 5A). The basal level of cAMP was markedly increased by the pretreatment of forskolin and isoproterenol (Figure 5E). MDL...
12330 attenuated percent change in cAMP levels in perfusate by CPA, whereas forskolin and isoproterenol augmented the cAMP levels (Figure 5D).

**Discussion**

A relationship between adenosine and the cardiac hormone ANP is not fully studied despite the existence of adenosine receptors and their effects in the heart. Our study has revealed that adenosine increases an ANP release with negative inotropism via stimulation of A₁ receptor subtype and that cellular cAMP level regulates the ANP release.

Adenosine is well known as a physiological regulator of tissue function by increasing the energy supply and decreasing the energy demand. Adenosine also exerts a protective role against ischemic injury. In addition to cardioprotective effects, adenosine inhibits a release of vasopressor hormones. These hormonal effects may be expected to exert beneficial effects on the overloaded heart. We found that adenosine increases the ANP release directly from beating atria with negative inotropism. Our results agree with other reports that adenosine increases plasma concentration of ANP in vivo⁹,¹⁰ and in vitro experiments.¹¹ Elias et al⁹ found a marked increase in plasma ANP and antidiuretic hormone levels despite a marked reduction in arterial blood pressure. They suggest that adenosine promotes the release of ANP by either direct or through modification of atrial stretch via an effect on

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**Figure 3.** Comparison of relative changes in pulse pressure (A), ECF translocation (B), ANP secretion (C), and ANP concentration (D) by adenosine receptor agonists with adenosine (all 10 μmol/L). Abbreviations are the same as in Figure 1 legend. *P<0.05, **P<0.005 vs adenosine-perfused group; +P<0.05, ++P<0.005 vs the corresponding group.

**Figure 4.** Relative percent changes in pulse pressure (A), ECF translocation (B), ANP secretion (C), and ANP concentration (D) by CPA (1 μmol/L) in the presence of specific receptor antagonist. CONT, indicates CPA-perfused group in the absence of antagonist. Abbreviations are the same as in Figure 1 legend. **P<0.01, ***P<0.005 vs control group.
cardiac myocyte tone. It has also been reported that adenosine-stimulated ANP release is more potent than ATP and ADP. These findings demonstrate that adenosine plays a direct stimulatory role on the ANP release. However, Uusimaa et al. found an inhibition of the ANP release with negative chronotropy by adenosine in perfused whole rat heart instead of atria. They suggest that the effect of adenosine on ANP release is partly attributable to negative chronotropic effect, and that adenosine has a direct inhibitory effect on ANP release. This discrepancy may be attributable to a different experimental model in which atrium is not fully stretched and the effect of adenosine on ventricles is not completely ruled out.

Evidence for an involvement of A1 receptor in ANP release is that selective A1 agonists are the most potent ANP releasing agents compared with other subtype agonists. Our results have shown that the A1 agonist CPA increases 300-fold more potent than adenosine. These observations are also supported by a selective A1 antagonist. Adenosine-stimulated ANP release is blocked by the A1 receptor antagonist but not A2 or A3 receptor antagonist. It is well established that the A1 receptor couples with G-protein mediating the inhibition of AC, whereas the A2 subtypes couple with G-protein mediating AC stimulation. A previous study demonstrated that an increase of cAMP production inhibits the ANP release in isolated rabbit atria. Along the same lines, we found that CPA decreases the levels of cAMP in atrial tissue and perfusate, whereas the ANP release is increased in a dose-dependent manner. Moreover, the CPA-stimulated ANP release is inhibited in the presence of an AC inhibitor. We also found that agents for elevating cAMP level such as forskolin and isoproterenol blocked CPA-induced ANP release but did not affect CPA-induced decrease in cAMP. These observations suggest that adenosine-induced ANP release involves the A1 receptor–mediated inhibition of AC via G-protein.

Physiological roles of the A1 receptor in mediating preconditioning-induced myocardial protection against infarction, arrhythmias, or postischemic contractile dysfunction are well recognized. Overexpression of the A1 receptor increases myocardial resistance to ischemia. It has also been reported that antihypertrophic effects of adenosine are mediated by the A1 receptor in in vitro and in vivo studies. These A1 receptor–mediated responses are closely related to physiological effects of adenosine. Adenosine has been shown to play a protective role against ischemic injury, including coronary vasodilation, the reduction of heart rate, and cardiac contractility. Together with our findings that adenosine exhibits a direct stimulatory effect on ANP release with negative inotropism, ANP released from atria by adenosine signaling may support beneficial effects on the overloaded heart by decreasing cardiac workload and dilating coronary artery.
In conclusion, the A1 receptor subtype is responsible for adenosine-induced ANP release and negative inotropism through AC–cAMP pathway. Our findings support the beneficial effects of adenosine as well as the A1 agonist on the heart function, and these compounds may be a possible target for the treatment of heart diseases. However, an involvement of other signaling pathways for the regulation of ANP release should not be overlooked.

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