Cardiac Fibroblasts Express the cAMP-Adenosine Pathway

Raghvendra K. Dubey, Delbert G. Gillespie, Zaichuan Mi, Edwin K. Jackson

Abstract—The extracellular “cAMP-adenosine pathway” refers to the local production of adenosine mediated by cAMP egress into the extracellular space, conversion of cAMP to AMP by ecto-5′-nucleotidase, and the metabolism of AMP to adenosine by ecto-5′-nucleotidase. The goal of this study was to assess whether the cAMP-adenosine pathway limits cardiac fibroblast growth. Studies were conducted in ventricular cardiac fibroblasts maintained in 3-dimensional cultures. Addition of exogenous cAMP to cardiac fibroblasts increased extracellular levels of AMP, adenosine, and inosine in a concentration-dependent and time-dependent manner. This effect was attenuated by blockade of total phosphodiesterase activity (3-isobutyl-1-methylxanthine), ectophosphodiesterase activity (high concentration of 1,3-dipropyl-8-p-sulphophenylxanthine), or ecto-5′-nucleotidase (α, β-methylene-adenosine-5′-diphosphate). Treatment with exogenous cAMP inhibited cell growth as assessed by DNA synthesis (³H-thymidine incorporation), cell proliferation (cell counts), and protein synthesis (³H-leucine incorporation). Antagonism of A₂ (KF17837) or A₁/A₂ (low concentration of 1,3-dipropyl-8-p-sulphophenylxanthine), but not A₁ (8-cyclopentyl-1,3-dipropylxanthine), adenosine receptors blocked the growth-inhibitory effects of exogenous cAMP, but not the growth inhibitory effects of 8-bromo-cAMP (stable cAMP analogue). The growth-inhibitory effects of exogenous cAMP were enhanced by the combined inhibition of adenosine deaminase [erythro-9-(2-hydroxy-3-nonyl) adenine] and adenosine kinase (iodotubercidin). In conclusion, the extracellular cAMP-adenosine pathway exists in cardiac fibroblasts and attenuates cell growth. Pharmacological augmentation of this pathway could abate pathological cardiac remodeling in heart disease.

Key Words: adenosine ■ cyclic AMP ■ cardiac fibroblast ■ myocardial infarction ■ cardiac remodeling

adenosine plays an important role in cardiac physiology. However, the biochemical mechanisms regulating adenosine levels within the heart and the cell types involved are incompletely understood. Our previous studies in noncardiac cells demonstrate that cAMP is an important determinant of adenosine production via a biochemical mechanism we refer to as the cAMP-adenosine pathway.۱—۵ This pathway involves the conversion of cAMP to AMP and hence to adenosine by the enzymes phosphodiesterase (PDE) and 5′-nucleotidase (5′-NT), respectively. As shown in Figure 1, the cAMP-adenosine pathway has both an intracellular and an extracellular arm, i.e., adenosine may be formed within the cell and transported to the extracellular space or may be formed directly in the extracellular space. The purpose of the present study was to determine whether the cAMP-adenosine pathway exists in and regulates growth of cardiac fibroblasts (CFs). CFs were selected for study because they constitute 60% of the total heart cells and because CFs are importantly involved in tissue remodeling, hypertrophy, and fibrosis, processes that could be modulated by adenosine.

Methods

CF Cell Culture

Hearts were obtained from anesthetized male Sprague-Dawley rats. Ventricular CFs were grown in 3-dimensional (3-D) cultures by the method of Mio et al,7 with minor modifications. All studies were conducted in 2nd or 3rd passaged cultures of CFs. All chemicals used for tissue culture were from Gibco Laboratories.

cAMP Metabolism Studies

CFs were washed twice with HBSS (HEPES-buffered Hanks’ balanced salt solution) and treated with 0.5 mL of Dulbecco’s PBS buffered with HEPES (25 mmol/L) and NaHCO₃ (13 mmol/L) in the presence and absence of various treatments. After the indicated incubation time, the supernatant was collected and frozen at −70°C until adenosine, inosine, and AMP levels were measured. The remaining cells were dislodged by treating cultures with 0.5 mL of a mixture of collagenase (1 mg/mL) and trypsin (0.25%), and the number of cells in each well was counted with a Coulter counter. To ensure that the various treatments caused no toxic effects or cell death, trypan blue exclusion assays were used to evaluate the viability of CFs treated in parallel. Adenosine, AMP, and inosine levels in the samples were analyzed by high-pressure liquid chromatography with our previously described method.8 The concentration of each purine in the samples was calculated from a standard curve and normalized to cell number.

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Figure 1. Schematic representation of the hypothesized role of the cAMP-adenosine pathway in CFs. Adenosine generated from cAMP by the sequential actions of PDE and 5′-NT provides adenosine to the A2 receptor, which induces anti-mitogenic effects via activation of adenylyl cyclase-dependent or -independent pathways. Hormone indicates adenylyl cyclase-stimulating hormone; Gs, stimulatory G protein; AC, adenylyl cyclase; ADO, adenosine; Tr, adenosine transporter; and DDA, 2′, 5′-dideoxyadenosine (adenylyl cyclase inhibitor). IBMX is a cell-permeable phosphodiesterase inhibitor; AMPPCP is an ecto-5′-NT inhibitor; DPSPX is an inhibitor of extracellular PDE at high concentrations and blocks A1/A2 adenosine receptors at low concentrations; and KF17837 is a selective A2 receptor antagonist.

Results

Growth Studies

3H-Thymidine and 3H-leucine incorporation studies were performed to investigate the effects of treatments on FCS-induced DNA and total protein synthesis, respectively. CFs (10⁵ cells per well) were plated in 24-well tissue culture dishes and allowed to grow in DMEM/F12 containing 10% FCS under standard tissue culture conditions. For 3H-thymidine incorporation, subconfluent CFs were growth arrested with culture medium containing 0.25% FCS for 48 hours and subsequently treated for 20 hours with culture medium supplemented with 2.5% FCS in the presence and absence of various treatments. After 20 hours the treatments were repeated with freshly prepared solutions but supplemented with 3H-thymidine (1 μCi/mL) for an additional 4 hours. For 3H-leucine incorporation, confluent CFs were growth arrested for 48 hours and treated for 48 hours with culture medium containing 2.5% FCS in the presence and absence of various treatments. Cells were washed twice with Dulbecco’s PBS, dislodged by digesting with 0.5 mL of a mixture of collagenase (1 mg/mL) and trypsin (0.25%), and treated with 10% ice-cold trichloroacetic acid (TCA). The TCA-precipitated cell pellet was obtained by centrifugation and was solubilized in 0.5 mL of 0.3 N NaOH and 0.1% sodium dodecyl sulfate. Aliquots from 4 wells for each treatment were counted in a liquid scintillation counter. Experiments were conducted in quadruplicate and repeated 3 to 5 times.

To evaluate the effects of treatments on cell proliferation, growth-arrested cells were incubated in the presence and absence of various treatments in DMEM supplemented with 2.5% FCS. Treatment was repeated after 48 hours, and cell counts were assayed after 4 days of treatment by dislodging cells with 0.5 mL of a mixture of collagenase (1 mg/mL) and trypsin (0.25%) and counting cells in a Coulter counter.

Statistical Analysis

Results are presented as mean±SEM of CF preparations from rat ventricles (n=number of ventricles). Statistical analyses were performed with ANOVA and paired or unpaired Student’s t test as appropriate. A value of P<0.05 was considered statistically significant.

Results

Metabolism of cAMP by Ventricular CFs

As shown in Figure 2A, addition of cAMP (30 μmol/L) to CFs caused a time-related increase in the extracellular levels of AMP, adenosine, and inosine. Compared with CFs not treated with cAMP, the levels of AMP, adenosine, and inosine increased significantly in samples incubated for 1 to 30 minutes. The maximal increases in AMP and adenosine were observed after 1 and 10 minutes of incubation, respectively. The increase in inosine levels did not reach a plateau even after 30 minutes of incubation.

The metabolism of cAMP to AMP, adenosine, and inosine was also concentration dependent (Figure 2B). Compared with the untreated controls, AMP, adenosine, and inosine levels were significantly different in CFs incubated for 60 minutes with concentrations of cAMP ≥1 μmol/L. Significant levels of inosine were present in CFs incubated with 0.01 μmol/L of cAMP, and at concentrations >1 μmol/L, the levels of inosine were greater than those for AMP and adenosine, and the levels of AMP were greater than those for adenosine.

Figure 3 illustrates the effects of various inhibitors on the metabolism of cAMP to purines. Compared with CFs treated with PBS alone (vehicle), the extracellular (medium) levels of AMP, adenosine, and inosine increased significantly in CFs treated with 30 μmol/L of cAMP. In vehicle-treated CFs, the levels (nmol/L per 10⁶ cells) of AMP and adenosine were near or below the assay detection limit, whereas the levels of inosine were 58±5. In cAMP-treated cells, the levels (nmol/L per 10⁶ cells) of AMP, adenosine, and inosine were 960±108, 302±42, and 1192±141, respectively (P<0.05 versus vehicle-treated CFs).

Metabolism of cAMP into AMP, adenosine, and inosine was significantly inhibited by 3-isobutyl-1-methylxanthine (IBMX; 1 mmol/L; Figure 3), a PDE inhibitor that crosses cell membranes. The levels of adenosine and inosine were...
but is restricted to the extracellular compartment. In CFs (DPSPX; 0.1 mmol/L; Figure 3), a xanthine that inhibits PDE 6, and 546 decreased by DPSPX, the levels of AMP, adenosine, and inosine were near or below the detection limit. In CFs treated with DPSPX alone, the levels of AMP, adenosine, and inosine in the medium were analyzed by high-performance liquid chromatography. Values are mean ± SEM from 3 to 4 separate experiments, conducted in quadruplicate, using separate cultures. *P < 0.05 compared with control CFs treated with cAMP.

Figure 3. Metabolism of cAMP to AMP, adenosine, and inosine by ventricular CFs in 3-D culture in the presence and absence of various inhibitors. Cells were treated for 60 minutes under standard tissue culture conditions with buffered Dulbecco’s PBS (Veh; n = 6) or cAMP (30 μmol/L; n = 6) in the absence or presence of IBMX (1 mmol/L; PDE inhibitor; n = 6), AMPCP (0.1 mmol/L; ecto-5'-NT inhibitor; n = 6), or DPSPX (ecto-PDE inhibitor; 0.1 mmol/L; n = 6), and AMP, adenosine, and inosine in the medium were detectable in the media of IBMX-treated cells, and AMP, adenosine, and inosine were due to their inhibitory effects on specific biochemical pathways and not due to cell toxicity, viability tests using trypan blue exclusion were conducted in CFs treated similarly and parallel to the metabolic studies. The viability of IBMX-treated cells compared with control cells. The increase in AMP levels induced by cAMP was markedly attenuated in IBMX-treated CFs treated with IBMX alone and were not significantly increased by cAMP (30 μmol/L) in CFs treated with IBMX. AMP levels were detectable in the media of IBMX-treated cells, and compared with CFs treated with IBMX alone, the levels were marginally, but significantly, increased in CFs treated with IBMX plus cAMP. However, the increase in AMP levels induced by cAMP was markedly attenuated in IBMX-treated cells compared with control cells.

Metabolism of cAMP to AMP, adenosine, and inosine was also attenuated by 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; 0.1 mmol/L; Figure 3), a xanthine that inhibits PDE but is restricted to the extracellular compartment. In CFs treated with DPSPX alone, the levels of AMP, adenosine, and inosine were near or below the detection limit. In CFs treated with cAMP in the presence of DPSPX, the levels of AMP, adenosine, and inosine (nmol/L per 10^6 cells) were 263 ± 24, 45 ± 6, and 546 ± 51, respectively. However, compared with control CFs treated with cAMP (30 μmol/L) in the absence of DPSPX, the levels of AMP, adenosine, and inosine were decreased by >50% in CFs treated with cAMP plus DPSPX (P < 0.05), indicating that DPSPX attenuated the metabolism of cAMP to AMP, adenosine, and inosine.

Treatment of CFs with cAMP in the presence of the ecto-5'-NT inhibitor α,β-methylene-adenosine-5'-diphosphate (AMPCP; 0.1 mmol/L) inhibited the metabolism of cAMP to adenosine and inosine but not to AMP (Figure 3). The levels of adenosine and inosine in CFs treated with AMPCP alone or with AMPCP plus cAMP were near or below the detection limit, whereas the levels of AMP (nmol/L per 10^6 cells) were 274 ± 42 and 2683 ± 262, respectively (P < 0.05).

To determine whether the effects of IBMX, AMPCP, and DPSPX on cAMP metabolism to AMP, adenosine, and inosine were due to their inhibitory effects on specific biochemical pathways and not due to cell toxicity, viability tests using trypan blue exclusion were conducted in CFs treated similarly and parallel to the metabolic studies. The viability in CFs incubated with PBS alone was >97%. No loss in cell viability was observed in cells treated with IBMX, AMPCP, or DPSPX in the absence and presence of cAMP.

Effects of cAMP on Growth of Ventricular CFs
FCS significantly increased [3H]thymidine and [3H]leucine incorporation and cell number in growth-arrested ventricular CFs. Treatment of growth-arrested CFs with exogenous cAMP (0.001 to 10 μmol/L) significantly inhibited FCS-induced [3H]thymidine (Figures 4A and 5A) and [3H]leucine (Figure 5C) incorporation and cell number (Figure 5B). To assess whether the effects of cAMP on [3H]thymidine and [3H]leucine incorporation and cell number were in part mediated via generation of adenosine, the effects of exogenous cAMP were evaluated in the presence and absence of (E)-1,3-dipropyl-7-methyl-8-(3,4-dimethoxytiryll)xanthine (KF17837) (a selective A2 receptor antagonist), DPSPX (a nonselective adenosine receptor antagonist), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (a selective A1 receptor antagonist). The inhibitory effects of cAMP on FCS-induced [3H]thymidine and [3H]leucine incorporation and cell number were significantly reduced by KF17837 (10 nmol/L) and DPSPX (10 nmol/L) but not by DPCPX (Figures 4 and 5). Similar to cAMP, treatment of CFs with 8-bromo-
cAMP (10 μmol/L), a nonmetabolizable analogue of cAMP, inhibited FCS-induced ³H-thymidine incorporation (Figure 4B). However, in contrast to cAMP, the inhibitory effect of 8-bromo-cAMP on FCS-induced ³H-thymidine was not reduced by KF17837 or DPSPX (Figure 4B).

Treatment of growth-arrested CFs with exogenous cAMP (10 μmol/L) inhibited FCS-induced ³H-thymidine incorporation by 38±6% (Figure 6A; P<0.001). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; 10 μmol/L), which elevates endogenous levels of adenosine by inhibiting adenosine deaminase, together with iodotubercidin (IDO; 0.1 μmol/L), which increases endogenous adenosine by inhibiting adenosine kinase, inhibited FCS-induced ³H-thymidine incorporation by 46±4% (Figure 6A; P<0.001). The inhibitory effects of cAMP on FCS-induced ³H-thymidine incorporation were significantly enhanced in the presence of EHNA + IDO (Figure 6A; P<0.001). The incorporation of ³H-thymidine was 100% in controls, 62±4% in presence of cAMP (P<0.01 versus control), 52±5% in presence of EHNA + IDO (P<0.01 versus control), and 21±2% in presence of cAMP plus EHNA plus IDO (P<0.05 versus cAMP and EHNA + IDO). Similarly, cAMP and EHNA + IDO inhibited cell number, and the inhibitory effect of cAMP on cell number was further enhanced in the presence of EHNA + IDO (Figure 6B).

Discussion

Formation of adenosine occurs via 3 biochemical pathways. The intracellular ATP pathway involves intracellular dephosphorylation of ATP to adenosine when energy demand exceeds energy supply. The extracellular ATP pathway entails the metabolism of adenine nucleotides released from a variety of cell types, thus providing substrates for ectoenzymes (ecto-ATPases, ecto-ADPases, and ecto-5'-nucleotidase) that convert ATP to adenosine. In particular, the extracellular ATP pathway would be increased whenever adenine nucleotides are released during sympahtoadrenal activation, platelet aggregation, and activation of cardiovascular cells by clotting factors, neutrophil interactions, and catecholamines. The transmethylation pathway involves the hydrolysis of S-adenosyl-l-homocysteine (SAH) to l-homocysteine and adenosine by the enzyme SAH-hydrolase.

Since the intracellular and extracellular ATP pathways of adenosine production require crisis events and the transmethylation pathway is mostly constitutive, the 3 traditional routes of adenosine biosynthesis are not well suited for physiological modulation. However, we have proposed a fourth pathway, the cAMP-adenosine pathway, for adenosine production that would be more amenable to physiological modulation of adenosine levels by hormones. As shown in Figure 1, stimulation of adenylyl cyclase activates the cAMP-adenosine pathway, which has both intracellular and extracellular sites of adenosine production. Intracellular metabolism of cAMP to AMP and AMP to adenosine is catalyzed via cytosolic PDE and cytosolic 5'-NT, respectively, and the adenosine thus formed reaches the extracellular space by way of facilitated transport. However, intracellular formation of adenosine may be diminished by the competition of cytosolic 5'-NT and adenylate kinase for AMP and by the competition of transport mechanisms with adenosine kinase for...
adenosine. Therefore, the extracellular limb of the cAMP-adenosine pathway may be quantitatively more important.

Ecto-5'-NT is a ubiquitous enzyme that is tethered to the extracellular face of the plasma membrane via a lipid-sugar linkage. Ecto-5'-NT efficiently metabolizes AMP to adenosine, and vascular endothelial and smooth muscle cells, as well as heart cells (myocytes, endothelial cells, and fibroblast cells), are well equipped with ecto-5'-NT. Activation of adenylyl cyclase always causes egress of cAMP into the extracellular space. Therefore, provided that sufficient levels of ecto-PDE exist, activation of adenylyl cyclase would trigger the extracellular metabolism of cAMP to AMP and hence to adenosine. Because these reactions would take place in a highly localized environment, this newly formed adenosine could then act in an autocrine and/or paracrine fashion to amplify, inhibit, and/or expand the local response to hormonal stimulation of adenylyl cyclase. Importantly, relatively modest increases in cAMP production could give rise to significant concentrations of adenosine at the cell surface.

Our studies in theperfused rat renal vascular bed demonstrate that infusion of cAMP causes a concentration-dependent increase in the renal secretion rates of AMP, adenosine, and inosine, and the increases in AMP and adenosine secretion are inhibited by IBMX (PDE inhibitor) and DPSPX (ecto-PDE inhibitor at high concentrations), whereas the increases in adenosine, but not AMP, secretion are blocked by AMPCP (ecto-5'-NT inhibitor). Our studies in vascular smooth muscle cells provide evidence for a cAMP-adenosine pathway in smooth muscle cells.

Because cardiac cells contain both receptor-activated adenylyl cyclase and ecto-5'-NT, it is possible that CFs also represent an important cell type that supports a cAMP-adenosine pathway. The present study addresses this hypothesis in rat ventricular CFs in 3-D cultures, which maintain their in vivo phenotype and physiological characteristics. In CFs incubated with exogenous cAMP, extracellular levels (ie, levels in the medium) of AMP, adenosine, and inosine are increased several-fold, and the increases in AMP, adenosine, and inosine are blocked by inhibition of PDE with IBMX and ecto-PDE with DPSPX. In addition, AMPCP blocks the metabolism of exogenous cAMP to adenosine and inosine but not to AMP. These data are consistent with the hypothesis that the cAMP-adenosine pathway exists in CFs and contributes to the production of adenosine.

The finding that the levels of AMP, adenosine, and inosine are increased by addition of cAMP, whereas blockade of PDE by IBMX inhibits this process, is highly consistent with the existence of the cAMP-adenosine pathway. Several lines of evidence support the suggestion that the metabolism of exogenous cAMP to adenosine occurs mainly in the extracellular space. First, because cAMP is hydrophilic, exogenous cAMP should not penetrate cell membranes, and therefore its conversion to adenosine most likely takes place extracellularly. Second, because AMPCP only inhibits ecto-5'-NT, not endo-5'-NT, the blockade of cAMP metabolism to adenosine by AMPCP is consistent with an extracellular site of metabolism. Finally, because DPSPX has a negative charge at physiological pH and is restricted to the extracellular space, inhibition by DPSPX of the conversion of exogenous cAMP to AMP and adenosine further supports an extracellular site of metabolism.

The present study demonstrates that exogenous cAMP inhibits FCS-induced CF growth, and this response is significantly enhanced by EHNA+IDO. The increased inhibitory effect of cAMP in the presence of EHNA+IDO, agents that prevent the metabolism of adenosine, suggests that cAMP-derived adenosine inhibits CF proliferation. Direct evidence regarding the role of cAMP-derived adenosine in inhibiting CF proliferation comes from our observation that the inhibitory effects of cAMP, but not 8-bromo-cAMP, are significantly reversed in the presence of KFI7837 and DPSPX. With regard to cell growth, our previous studies demonstrate that the inhibitory effects of exogenous as well as endogenous adenosine on smooth muscle cell and CF proliferation are mediated via A1 receptors. Our observation that KFI7837 and DPSPX, but not DPCPX, block the inhibitory effects of cAMP on CF growth, whereas KFI7837, DPSPX, and DPCPX do not reverse the inhibitory effects of 8-bromo-cAMP, strongly suggest that the cAMP-adenosine pathway may contribute importantly to the regulation of CF proliferation via A1 receptors.

Abnormal growth of CFs contributes to structural changes in the heart (hypertrophy and remodeling) associated with hypertension and myocardial infarction, and these structural changes adversely affect heart performance. Numerous studies suggest that a cascade of events involving autocrine/paracrine factors generated by blood cells, endothelium, and cardiac myocytes contributes to cardiac hypertrophy and remodeling. In this regard, a balanced basal production of growth-promoting and growth-inhibiting factors maintains growth homeostasis within the heart. Our present studies in CFs suggest that the cAMP-adenosine pathway, by generating adenosine locally at the surface of CFs, may protect the heart against pathological hypertrophy and remodeling.

In summary, our findings provide evidence that adenosine generated from cAMP inhibits FCS-induced growth of ventricular CFs. Therefore, adenosine produced by the metabolism of cAMP by CFs may play a role in cardiac physiology/cell biology, and abnormalities in the cAMP-adenosine pathway may contribute to the abnormal proliferation of CFs observed in hypertension and myocardial infarction.

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