Endogenous Cyclic AMP-Adenosine Pathway Regulates Cardiac Fibroblast Growth

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

Abstract—Our previous studies show that cardiac fibroblasts express the extracellular “cAMP-adenosine pathway,” that is, the generation of adenosine from extracellular cAMP. The goal of this study was to assess whether activation of the cAMP-adenosine pathway by stimulation of endogenous cAMP synthesis regulates cardiac fibroblast growth. Cardiac fibroblasts in 3D cultures were used as the model system. Treatment of cardiac fibroblasts with forskolin, isoproterenol, or norepinephrine increased cAMP production and extracellular levels of adenosine, and these effects were prevented by inhibition of adenyl cyclase (2',5'-dideoxyadenosine). Treatment with forskolin, isoproterenol, or norepinephrine for 24 hours inhibited DNA synthesis (H-thymidine incorporation), and this effect was enhanced by combined inhibition of adenosine deaminase (erythro-9-[2-hydroxy-3-nonyl] adenine) plus adenosine kinase (iodotubercidin). Inhibition of adenyl cyclase or adenosine receptors (1,3-dipropyl-8-p-sulfophenylxanthine or KF17837) prevented the effects of forskolin, isoproterenol, and norepinephrine on DNA synthesis. Forskolin also inhibited protein synthesis (H-leucine incorporation) and cell proliferation, and these effects were blocked by adenosine receptor antagonism. Treatment of cardiac fibroblasts with norepinephrine for >48 hours but not <48 hours increased DNA synthesis, protein synthesis, and cell number. However, blockade of adenyl cyclase or antagonism of adenosine receptors caused norepinephrine to induce proliferation in <48 hours. Our findings indicate that the endogenous cAMP-adenosine pathway regulates cardiac fibroblast growth. (Hypertension. 2001;37:1095-1100.)

Key Words: adenosine ■ cyclic AMP ■ fibroblasts ■ myocardial infarction ■ hypertension, experimental ■ remodeling

Recent studies in our laboratory demonstrate that extracellular cAMP is an important determinant of adenosine production in cardiac fibroblasts (CFs) through a biochemical mechanism we refer to as the cAMP-adenosine pathway. This pathway involves the conversion of extracellular cAMP to AMP and hence to adenosine by the enzymes ecto-phosphodiesterase (ecto-PDE) and ecto-5'-nucleotidase (ecto-5'-NT), respectively. Our previous studies also indicate that exogenous adenosine and exogenous cAMP, through metabolism to adenosine, potently inhibit CF growth. However, whether stimulation of the endogenous cAMP-adenosine pathway inhibits CF growth through adenosine formation is unknown. The purpose of this study was to assess whether CF-derived cAMP regulates CF growth through activation of the cAMP-adenosine pathway. To achieve our goal, we evaluated the effects of forskolin, isoproterenol, and norepinephrine, agents that stimulate adenyl cyclase, on fetal calf serum (FCS)-induced DNA synthesis, cell proliferation, and protein synthesis in 3D cultures of ventricular CFs. Moreover, we evaluated whether the effects of these agents on cell growth were reduced by inhibition of adenyl cyclase and antagonism of adenosine receptors and enhanced by inhibition of adenosine metabolism. Moreover, we assessed whether the growth effects of these agents were accompanied with changes in cAMP and adenosine levels.

Methods

CF Cell Culture
Hearts were harvested from pentobarbital-anesthetized (50 mg/kg IP), male Sprague-Dawley rats; the left ventricular CFs were isolated and cultured as previously described by us. For all the studies, the method of Mio et al, with minor modifications, was then used to grow ventricular CFs in 3D cultures. Briefly, 0.8 mL of 2 mg/mL collagen (type I from rat tail; Sigma Chemical Co) solution in 0.5% acetic acid was mixed at 4°C with 0.21 mL of dilution medium (455 μL 10× basal medium, 112 μL of 7.5% NaHCO3, 50 μL of 200 mmol/L L-glutamine, 13 μL of FCS, 25 to 50 μL of 0.142 mol/L NaOH to adjust pH to 7.5, and 425 μL [minus vol of NaOH] of H2O). Subsequently, 0.3 mL of collagen mixture, devoid of air bubbles, was plated in each well of a 24-well culture plate and allowed to gel for 1 hour at 37°C. CFs were then plated on the top layer and allowed to adhere by incubation under standard tissue culture conditions. After overnight incubation, the medium was

Received May 25, 2000; first decision June 21, 2000; revision accepted September 29, 2000.
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Adenosine and cAMP Synthesis Studies

CFs were washed twice with HEPES-buffered Hank’s 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 30 minutes, 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; Sigma) 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, trypsin 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.4 The concentration of each purine in the samples was calculated from a standard curve and normalized to cell number.

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 (104 cells/well) were plated in 24-well tissue culture dishes and allowed to grow in DMEM/F-12 containing 10% FCS under standard tissue culture conditions. For 3H-thymidine incorporation, subconfluent CFs were growth-arrested with culture medium containing 2.5% FCS and 3H-thymidine (1 Ci/mL, NEN) for 48 hours before treatment 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, NEN) 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 and 3H-leucine (1 μCi/mL, ICN Biochemicals) in the presence and absence of various treatments. Cells were washed twice with Dulbecco’s PBS, dislodged by digestion with 0.5 mL of a mixture of collagenase (1 mg/mL) and trypsin (0.25%), and treated with 10% ice-cold trichloroacetic acid. The trichloroacetic acid–precipitated cell pellet was obtained by centrifugation and was solubilized in 0.5 mL of 0.3N NaOH and 0.1% sodium dodecyl sulfate. Aliquots from 4 wells for each treatment, with 10 mL scintillation fluid, were counted in a liquid scintillation counter. Each experiment was conducted in triplicate or 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.

Statistics

All experiments were conducted in triplicate or quadruplicate and repeated 3 to 4 times with separate cultures. Results are presented as mean±SEM. Statistical analyses were performed with ANOVA. When evaluating a treatment-dependent effect and/or concentration-dependent effect within a group, data were analyzed by 1-factor ANOVA followed by Fisher’s least significant difference test for multiple comparisons. As appropriate, a Bonferroni t test or Dunnett multiple comparison test was applied to compare differences between groups. All treatment-related effects within a group at a specific time point were compared by Student’s unpaired t test. A value of P<0.05 was considered statistically significant.

Results

Forskolin and isoproterenol significantly inhibited FCS-induced 3H-thymidine incorporation in a concentration-dependent manner (Figure 1), and the inhibitory effects of forskolin and isoproterenol were significantly attenuated by 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX, A1/A2 adenosine receptor antagonist) and KF17837 (A2 adenosine receptor antagonist) but not by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, A1 adenosine receptor antagonist). Forskolin significantly inhibited FCS-induced cell proliferation, and this effect also was attenuated significantly by DPSPX and KF17837 but not DPCPX (Figure 2). Treatment of CFs for 24 hours with norepinephrine inhibited FCS-induced 3H-thymidine incorporation, but supplemented with 3H-thymidine (1 Ci/mL, NEN) for 20 minutes at 37°C. Cells were treated with 1 mL of DMEM/F-12 supplemented with 10% FCS; cells were allowed to grow to subconfluence for growth studies or to confluence for the cAMP and adenosine synthesis studies. Experiments were conducted in CFs in the second or third passage. All chemicals used for tissue culture were from Gibco Laboratories. KF17837 was kindly provided by Dr Fumio Suzuki of Hakko Kagyo Co Ltd (Sunto, Shinzuoka, Japan). Forskolin, isoproterenol, and norepinephrine were purchased from Sigma, and the source of all other chemicals was as previously described.3

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thymidine incorporation, and this effect was attenuated by DPSPX (Figure 3 and 4). 2',5'-Dideoxyadenosine (DDA, adenylyl cyclase inhibitor) significantly attenuated the inhibitory effects of forskolin, isoproterenol, and norepinephrine on 3H-thymidine incorporation (Figure 3) and the effects of forskolin on cell proliferation (Figure 2). Forskolin inhibited 3H-leucine incorporation by 47%, and this effect was attenuated significantly by DPSPX and DDA (Figure 3). Both norepinephrine and isoproterenol significantly stimulated FCS-induced 3H-thymidine incorporation in CFs, and this effect was significantly enhanced by DPSPX and DDA (Figure 3). Norepinephrine and isoproterenol inhibited 3H-thymidine incorporation but stimulated 3H-leucine incorporation. A possible explanation for these contrasting effects of norepinephrine and isoproterenol is that 3H-thymidine incorporation was assessed after 24 hours of treatment, whereas 3H-leucine incorporation was determined after 48 hours of treatment. To investigate this possibility, the time course of norepinephrine effects on growth were studied by treating CFs with norepinephrine for 24 to 96 hours. Treatment with norepinephrine (100 nmol/L) induced time-dependent effects on CF growth (Figure 4). Treatment of CFs with norepinephrine for 48 hours and 96 hours inhibited and stimulated, respectively, FCS-induced 3H-thymidine incorporation and cell number (Figure 4). Both DDA and DPSPX reversed the early inhibitory effects of norepinephrine and significantly enhanced the late stimulatory effects of norepinephrine (Figure 4).

Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; 10 μmol/L; adenosine deaminase inhibitor) plus iodotubercidin (IDO; 0.1 μmol/L; adenosine kinase inhibitor) significantly inhibited FCS-induced 3H-thymidine incorporation and enhanced the effects of forskolin and isoproterenol on 3H-thymidine incorporation (Figure 5). Furthermore, DPSPX but not DPCPX significantly reversed the inhibitory effects of forskolin and isoproterenol in the presence and absence of EHNA plus IDO (Figure 5). FCS-induced 3H-leucine incorporation in CFs, and this effect was significantly enhanced by DPSPX and DDA (Figure 3).

Norepinephrine and isoproterenol inhibited 3H-thymidine incorporation but stimulated 3H-leucine incorporation. A possible explanation for these contrasting effects of norepinephrine and isoproterenol is that 3H-thymidine incorporation was assessed after 24 hours of treatment, whereas 3H-leucine incorporation was determined after 48 hours of treatment. To investigate this possibility, the time course of norepinephrine effects on growth were studied by treating CFs with norepinephrine for 24 to 96 hours. Treatment with norepinephrine (100 nmol/L) induced time-dependent effects on CF growth (Figure 4). Treatment of CFs with norepinephrine for <48 hours and >48 hours inhibited and stimulated, respectively, FCS-induced 3H-thymidine incorporation and cell number (Figure 4). Both DDA and DPSPX reversed the early inhibitory effects of norepinephrine and significantly enhanced the late stimulatory effects of norepinephrine (Figure 4).

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Figure 6. Synthesis of cAMP and adenosine by CFs in response to isoproterenol (ISO; 10−6 mol/L), forskolin (FOR; 10−5 mol/L), and norepinephrine (NE; 10−5 mol/L) in presence and absence of EHNA (10−5 mol/L), plus IDO (10−5 mol/L), EHNA+IDO+DPSPX (10−5 mol/L), and DDA (10−5 mol/L). Results are expressed as nmol/L/10^6 cells; each value represents mean±SEM from 6 experiments conducted with separate cultures. *P<0.01 vs cells treated with vehicle; †P<0.05 vs cells treated with ISO, FOR, or IDO.

plus IDO. Treatment of CFs with EHNA+IDO increased the levels of adenosine and cAMP in the medium from 0.6±0.18 to 241±10 nmol/L/10^6 cells and below detection limit to 0.543±0.13 nmol/L/10^6 cells. Also, in the presence of EHNA+IDO, isoproterenol, forskolin and norepinephrine increased both cAMP and adenosine levels in the medium. Forskolin-, isoproterenol-, and norepinephrine-induced cAMP synthesis was significantly inhibited in the presence of DDA. A low concentration of DPSPX (Figure 6) did not inhibit cAMP and adenosine synthesis induced by forskolin, isoproterenol, or norepinephrine (Figure 6). Also, forskolin-, isoproterenol- and norepinephrine-induced cAMP and adenosine synthesis were not blocked by DPCPX or KF17837. The cAMP and adenosine levels in CFs treated in presence of EHNA+IDO with forskolin, forskolin+DPCPX, and forskolin+KF17837 were 498±20, 488±26, and 513±18 nmol/L/10^6 cells, respectively, for cAMP and 388±23, 394±18, and 367±26 nmol/L/10^6 cells, respectively, for adenosine. The cAMP and adenosine levels in cells treated with isoproterenol and norepinephrine were 164±14 and 275±24 nmol/L/10^6 cells, respectively, for isoproterenol and 191±12 and 355±20 nmol/L/10^6 cells, respectively, for norepinephrine. Similar to forskolin, the stimulatory effects of isoproterenol and norepinephrine on cAMP and adenosine synthesis were not blocked by DPCPX or KF17837 and influenced by <2% to 4% (P>0.05).

Discussion

Several lines of evidence suggest that adenosine plays a major role as an endogenous nucleoside that protects the heart and the vasculature against cardiovascular disease. Formation of adenosine within the heart and/or near the blood vessel wall is believed to occur through three biochemical pathways. The intracellular ATP pathway and extracellular ATP pathway entail dephosphorylation of ATP to adenosine inside and outside the cell, respectively. The transmethylation pathway involves the hydrolysis of S-adenosyl-L-homocysteine to L-homocysteine and adenosine by the enzyme S-adenosyl-L-homocysteine—hydrolase. Because the intracellular and extracellular ATP pathways of adenosine production require crisis events and the transmethylation pathway is mostly constitutive, the three traditional routes of adenosine biosynthesis are not well suited for physiological modulation. More recently, we have identified a fourth pathway, the cAMP-adenosine pathway, for adenosine production. In contrast to the other pathways for adenosine formation, the cAMP-adenosine pathway may be more amenable to physiological modulation by hormones. Because a large amount of adenosine is synthesized in the heart, we hypothesize that the cAMP-adenosine pathway may also be present in cardiac cells and more specifically in CFs, which constitute 60% of the total heart cells.

The activation of adenyl cyclase turns on the cAMP-adenosine pathway, which has both intracellular and extracellular sites of adenosine production. Intracellular or extracellular metabolism of cAMP to AMP and AMP to adenosine is catalyzed through cytosolic PDE and ecto-PDE, respectively, and cytosolic 5′-nucleotidase and ecto-5′-nucleotidase, respectively. However, intracellular formation of adenosine may be diminished by the competition of cytosolic 5′-nucleotidase 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.

In a recent study, we provided evidence for the physiological relevance of the cAMP-adenosine pathway in regulating CF growth. In this regard, our previous study shows that the inhibitory effects of cAMP on FCS-induced CF growth are significantly enhanced in the presence of EHNA, which prevents the metabolism of adenosine to inosine, plus IDO, which inhibits the metabolism of adenosine to AMP. Moreover, the inhibitory effects of cAMP but not Br-cAMP (stable cAMP analog) are significantly reversed in the presence of KF17837 and DPSPX. Because the inhibitory effects of exogenous as well as endogenous adenosine on smooth muscle cell and CF proliferation are mediated by A2 receptors, the fact that KF17837 and DPSPX but not DPCPX block the inhibitory effects of cAMP on CF growth, whereas KF17837, DPSPX, and DPCPX do not reverse the inhibitory effects of Br-cAMP, indicates that the cAMP-adenosine pathway may contribute importantly to the regulation of cardiac biology and more specifically CF proliferation.

Although our previous findings provide evidence that adenosine derived from exogenous cAMP inhibits CF growth, whether similar effects are exerted physiologically by endogenously generated cAMP is unclear. To address this issue, we evaluated the effects of agents known to induce cellular cAMP levels on the growth of CFs. The findings that forskolin increases cAMP and adenosine levels in the medium, inhibits FCS-induced DNA synthesis, cell proliferation, and protein synthesis, and that these effects are blocked by the adenyl cyclase inhibitor DDA.
as well as by A₂-adenosine receptor antagonists KF17837 and DPSPX provide convincing evidence that cAMP-derived adenosine inhibits CF growth and that these effects are A₂-adenosine receptor mediated. This notion is further strengthened by the observation that the effects of DDA are accompanied by decreases in the levels of cAMP and adenosine, whereas DPSPX and KF17837 do not reduce the exogenous levels of adenosine. Similar to forskolin, both iso-proterenol and norepinephrine induce cAMP and adenosine synthesis and inhibit FCS-induced DNA synthesis in CFs. Also like forskolin, the effects of iso-proterenol and norepinephrine on cAMP and adenosine synthesis and DNA synthesis are abolished by the adenylyl cyclase inhibitor DDA, and the effects of iso-proterenol and norepinephrine on DNA synthesis are attenuated by the A₂-adenosine receptor antagonists DPSPX and KF17837. The observation that in contrast to forskolin, iso-proterenol and norepinephrine induce protein synthesis, even though all three agents increase cAMP and adenosine levels and inhibit DNA synthesis, does not disprove the inhibitory role of cAMP on growth. Indeed, several studies report similar effects for norepinephrine and iso-proterenol on the growth of cardiac cells. In addition to inducing cAMP, catecholamines increase the synthesis of several mitogenic factors such as endothelin, angiotensin II, and transforming growth factor-α. Because the net result on growth depends on the balanced generation of growth inducers and growth inhibitors, it is feasible that as compared with cAMP, norepinephrine and iso-proterenol induce the generation of hypertrophic factors much more. In this regard, it is plausible that the observed increases in protein synthesis in response to 48-hour treatment with catecholamines are due to delayed mitogenic effects of catecholamines. This hypothesis is supported by our observations that (1) norepinephrine inhibits FCS-induced DNA synthesis and cell number in CFs treated for <48 hours but induces CF growth in CFs treated for >48 hours; and (2) the stimulatory effects of norepinephrine and iso-proterenol on protein synthesis and the delayed mitogenic effects of norepinephrine on DNA synthesis and cell number are significantly enhanced when cAMP generation is inhibited by DDA or when adenosine effects are blocked with DPSPX. Taken together, the above findings confirm that the net effects of cAMP on CF growth are inhibitory.

The above findings support the hypothesis that the cAMP-adenosine pathway is of physiological relevance in maintaining homeostasis within the cardiovascular system. The feasibility of this system is also evident from the fact that vascular endothelial and smooth muscle cells, as well as heart cells (myocytes, endothelial cells, and fibroblast cells), are well-equipped with ecto-5′-NT, a ubiquitous enzyme that efficiently metabolizes AMP to adenosine and constitutes a part of the extracellular limb of the cAMP-adenosine pathway. Because activation of adenyl cyclase always causes egress of cAMP into the extracellular space, provided that sufficient levels of ecto-PDE exist, activation of adenyl 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 modulate (amplify, inhibit, and/or expand) the local response to hormonal stimulation of adenylyl cyclase. It is important to note that relatively modest increases in cAMP production could give rise to significant concentrations of adenosine at the cell surface because adenosine would be synthesized by a series of spatially linked enzymatic reactions.

Abnormal growth of CFs contributes to the structural changes in the heart associated with hypertension and myocardial infarction, and this adversely affects the performance of the heart. A balanced basal production of growth-promoting and growth-inhibiting factors maintains homeostasis within the heart and prevents pathological cardiac remodeling. Our previous studies show that CFs and smooth muscle cells synthesize adenosine and that exogenous as well as endogenous (cell-derived) adenosine inhibits FCS-induced growth in these cells in an autocrine/paracrine fashion. On the basis of the observation that CFs have the capability of efficiently converting cAMP to adenosine, it is feasible that if the rate of cAMP production is increased in response to hormonal stimulation, CFs can effectively increase the extracellular levels of adenosine. Moreover, the increased generation of adenosine locally at the surface of CFs may be an important mechanism by which CFs protect themselves against factors generated to induce abnormal growth of CFs and cardiac remodeling.

Summary

These experiments provide the first evidence that CFs are capable of metabolizing endogenous cAMP to adenosine through the cAMP-adenosine pathway and that adenosine generated from endogenous cAMP inhibits FCS-induced growth of ventricular CFs in an autocrine/paracrine fashion. Moreover, our studies demonstrate that norepinephrine stimulates the cAMP-adenosine pathway and that this contributes to the delayed mitogenic effects of norepinephrine on ventricular CFs. Therefore, adenosine produced by the metabolism of cAMP by CFs may play a vital role in cardiac physiology/cell biology, and abnormalities in the cAMP-adenosine pathway may contribute importantly to the abnormal proliferation of CFs observed in hypertension and myocardial infarction.

Acknowledgments

This study was supported by the Swiss National Science Foundation grant 32-54172.98 and National Institutes of Health grants HL-55314 and HL-35909.

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Hypertension. 2001;37:1095-1100
doi: 10.1161/01.HYP.37.4.1095

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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