A2B Receptors Mediate the Antimitogenic Effects of Adenosine in Cardiac Fibroblasts

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Abstract—Adenosine inhibits growth of cardiac fibroblasts; however, the adenosine receptor subtype that mediates this antimitogenic effect remains undefined. Therefore, the goals of this study were to determine which adenosine receptor subtype mediates the antimitogenic effects of adenosine and to investigate the signal transduction mechanisms involved. In rat left ventricular cardiac fibroblasts, PDGF-BB (25 ng/mL) stimulated DNA synthesis (³H-thymidine incorporation), cellular proliferation (cell number), collagen synthesis (³H-proline incorporation), and MAP kinase activity. The adenosine receptor agonists 2-chloroadenosine and 5′-N-methylcarboxamidoadenosine, but not N⁶-cyclopentyladenosine, 4-aminobenzyl-5′-N-methylcarboxamidoadenosine, or CGS21680, inhibited the growth effects of PDGF-BB, an agonist profile consistent with an A2B receptor-mediated effect. The adenosine receptor antagonists KF17837 and 1,3-dipropyl-8-p-sulfophenylxanthine, but not 8-cyclopentyl-1,3-dipropylxanthine, blocked the growth-inhibitory effects of 2-chloroadenosine and 5′-N-methylcarboxamidoadenosine, an antagonist profile consistent with an A2 receptor-mediated effect. Antisense, but not sense or scrambled, oligonucleotides to the A2B receptor stimulated basal CF growth is regulated by several autocrine/paracrine factors, including adenosine, which has long been known as a "retaliatory" metabolite, particularly in the heart, where it induces cardioprotective effects. The biological effects of adenosine are mediated by means of adenosine receptors, which exist in multiple subtypes (A1, A2A, A2B, and A3 receptors). Within the heart, the standard view is that mainly A1 and A2A adenosine receptors are cardioprotective. For example, activation of A1 receptors attenuates the sympathetic nervous system, inhibits renin release from juxtaglomerular cells and opens cardiac K⁺ channels. By means of activation of A2A receptors, adenosine causes vasodilation, inhibits platelet aggregation, diminishes neutrophil adhesion to vascular endothelial cells, attenuates neutrophil-induced endothelial cell damage, and stimulates nitric oxide release from vascular endothelial cells and vascular smooth muscle cells. Although the standard view is that A1 and A2A receptors are the most important with regard to adenosine-mediated cardioprotection, indirect evidence suggests that adenosine inhibits CF growth by means of activation of A2B receptors. However, due to lack of availability of selective A2B receptor antagonists or agonists, the inference that A2B receptors

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mediate the growth inhibiting effects of adenosine is inconclusive. Accordingly, in the present study, we developed antisense oligonucleotides against rat A\textsubscript{2B} receptors and used them to test the role of A\textsubscript{2B} receptors in regulating CF growth and MAP kinase activity.

**Methods**

All tissue culture ware and reagents were purchased from Gibco Laboratories. Fetal calf serum (FCS) was obtained from HyClone Laboratories Inc. Adenosine, 2-chloroadenosine (Cl-Ad), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), and PDGF-BB were purchased from Sigma Chemical Co. N\textsuperscript{6}-cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), idotubercidin (IDO), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), 5'-N-ethylcarboxamido- doadenosine (NECA), 5'-N-methylcarboxamidoadenosine (MECA), and 4-aminobenzyl-5'-N-methylcarboxamidoadenosine (AB-MECA) were purchased from Research Biochemicals International. KF17837 was a gift from Kyowa Hakko Kogyo Co. Ltd. (Sunto, Shizuoka, Japan). H-Thymidine (specific activity, 11.8 Ci/mmol) and L-[3H]-proline (23 Ci/mmol) were purchased from NEN. All other reagents were of tissue culture or best grade available.

**Cardiac Fibroblast Cultures**

Hearts were surgically removed from anesthetized (50 mg/kg intraperitoneal injection of pentobarbital) Sprague-Dawley male rats (150 to 200 g; n=14), and left ventricular CFs cultured using enzymatic digestion with collagenase and selective plating as described by us previously.\(^9\) CF purity of >98% was confirmed by morphology (CFs are thin, triangular cells with light cytoplasm) and immunostaining (negative immunostaining against sarcomeric actin, desmin, von Willebrand factor VIII, and positive immunostaining with antivimentin).\(^9\) CFs in first or second passage were used for all experiments.

**Antisense Oligonucleotides for A\textsubscript{2B} Receptors**

We used the GenBank database to obtain the rat A\textsubscript{2B} receptor cDNA sequence and used MacVector 4.1 to select an antisense oligonucleotide with complementary sequences to the A\textsubscript{2B} receptor mRNA (5'-CTCTGTTTCCAGTGACCA-3'). Midland Certified Reagent Company custom synthesized and purified the phosphorothioated oligonucleotide with the above sequence. In a previous study, we have shown that this antisense oligonucleotide blocks the effects of adenosine on vascular smooth muscle cell growth.\(^9\) Sense (5'-TTGTCACGGAACACGAG-3') and scrambled (5'-GCAC- GCTTATAGCTGATG-3') oligonucleotides were used as controls.

**Growth Studies**

CFs were plated at a density of 5×10\textsuperscript{4} cells/well in 24-well tissue culture dishes and allowed to grow to subconfluence. Cells were then grown arrested by feeding DMEM containing 0.25% albumin for 48 hours in the presence or absence of 0.5 \mu mol/L of antisense, sense, or scrambled oligonucleotides. Collagen synthesis was initiated by treating growth-arrested cells for 48 hours with DMEM supplemented with fresh oligonucleotides, PDGF-BB (25 ng/mL), and \(\text{H}-\text{proline (1 µCi/mL)}\) in the presence or absence of various treatments. Experiments were terminated by washing the cells twice with PBS and twice with ice-cold TCA (10%). The precipitate was solubilized and counted in a liquid scintillation counter.\(^8\) To make sure that the inhibitory effects of the experimental agents on collagen synthesis were not due to changes in cell number, the experiments were conducted in confluent monolayers of cells in which changes in cell number were precluded. Additionally, cell counting was performed in cells treated in parallel to the cells used for the collagen synthesis studies, and the data were normalized to cell number.

**MAP Kinase Activity**

CFs grown to confluence in 35-mm\textsuperscript{2} culture dishes were made quiescent by feeding DMEM supplemented with 0.25% BSA and containing or lacking 0.5 \mu mol/L of antisense, sense, or scrambled oligonucleotides for 48 hours. Growth-arrested CFs were washed with PBS and pretreated for 24 hours without various test agents in the presence or absence of fresh oligonucleotides. Cells were then stimulated with PDGF-BB (25 ng/mL). After stimulation, cells were washed with ice-cold PBS and extraction buffer (50 mmol/L \(\beta\)-glycerophosphate, 1.5 mmol/L EGTA, 1 mmol/L dithiothreitol, 100 \mu mol/L Na\textsubscript{3}VO\textsubscript{4}, 10 \mu g/mL aprotinin, 5 \mu g/mL pepstatin, 20 \mu g/mL leupeptin, and 1 mmol/L benzamidine), scraped off the plates and sonicated for 20 seconds in 0.5 mL of extraction buffer. The extracts were collected, the cytosolic fraction were separated by centrifuging the extracts at 100 000g for 20 minutes at 4°C, and the supernatants were diluted to a concentration of 1 mg protein/mL and were stored at −70°C for MAP kinase activity assay. The MAP kinase activity in the cytosolic extracts was quantified by our previously described method.\(^9\) Briefly, cytosolic extracts (5 \mu L) were added to 30 \mu L of MAP kinase assay buffer (25 mmol/L \(\beta\)-glycerophosphate, 1.25 mmol/L EGTA, 0.5 mmol/L dithiothreitol, 150 \mu mol/L Na\textsubscript{3}VO\textsubscript{4}, 2 \mu mol/L peptide inhibitor for cAMP-dependent protein kinase (H-PTTAP-AGTGAAGAAA-NH\textsubscript{2}, Bachem Bioscience Inc), 1 mg/mL bovine serum albumin, 10 \mu mol/L calmidazolium, 0.33 mg/mL myelin basic protein (MBP), and 100 \mu mol/L of [\(\gamma\text{P}\)]ATP). After incubation for 15 minutes at 30°C, 25-\mu L aliquots of the reaction mixture were spotted onto phosphocellulose paper (Whatman), washed 4 times with 150 mmol/L phosphoric acid, and counted in 10 mL of scintillation fluid on a gamma counter. To calculate the MAP kinase activity, samples incubated in the absence of MBP were subtracted from the same samples incubated with MBP.

**cAMP Synthesis**

Confluent monolayers of CFs were treated every 24 hours for 48 hours with or without 0.5 \mu mol/L of antisense, sense, or scrambled oligonucleotide to A\textsubscript{2B} receptor. After 48 hours, the monolayers were washed with PBS and CFs treated for 30 minutes under standard tissue culture conditions with PBS (buffered with HEPES, 25 mmol/L; Na\textsubscript{2}CO\textsubscript{3}, 13 mmol/L) containing fresh oligonucleotides and supplemented with 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) and containing or lacking 1 \mu mol/L Cl-Ad. After treatment, the supernatants were collected and the cells were treated with 1 mL of ice-cold propanediol. The extracellular (in supernatant) and the intracellular (cellular fraction) cAMP levels were determined using high-performance liquid chromatography with a previously described method. In CFs grown in parallel and treated similarly, the monolayers were inspected microscopically for intactness, the protein content was measured, and the data were normalized to protein content.

**Statistics**

All growth experiments were performed in triplicates or quadruplicates with 3 to 4 separate cultures. Data are presented as mean±SEM. Statistical analysis was performed using ANOVA,
paired Students’ t test, or Fisher’s Least Significant Difference test as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

PDGF-BB significantly stimulated all measures of cell growth (DNA, collagen, and cell proliferation) by 7- to 10-fold. As shown in Figure 1, in PDGF-BB-treated cells, Cl-Ad inhibited in a concentration-dependent manner all measures of cell growth. The lowest concentration of Cl-Ad that significantly inhibited PDGF-BB-induced cell growth was 1 nmol/L, and at a concentration of 1 μmol/L, Cl-Ad inhibited cell growth by approximately 50%. High (10−6 mol/L), but not low, concentrations of CPA and CGS21680 inhibited PDGF-BB-induced cell growth (Figure 1). 5′-N-Ethylcarboxamidoadenosine (NECA) was more potent than AB-MECA, CPA, and CGS21680, but less potent than MECA or Cl-Ad in inhibiting PDGF-BB–induced increases in cell growth (Figure 1). The inhibitory effects of Cl-Ad on all measures of cell growth were significantly blocked by KF17837 and DPSPX, but not by DPCPX (data not shown).

EHNA plus IDO inhibited PDGF-BB–induced DNA synthesis, collagen synthesis, and cell proliferation (Figure 3). The inhibitory effects of EHNA plus IDO on PDGF-BB–induced cell growth were significantly attenuated by KF17837 and DPSPX, but not by DPCPX (Figure 3). Trypan blue exclusion tests indicated no loss in viability of cells treated with CPA, CGS21680, MECA, AB-MECA, KF17837, DPSPX, or DPCPX.

Antisense, but not sense or scrambled, oligonucleotides increased basal as well as PDGF-BB–induced DNA synthesis by 52% and 56%, respectively. Consistent with our previous findings in vascular SMCs,8 preliminary studies showed that the stimulatory effect of the antisense oligonucleotide was biphasic in nature, and maximal stimulatory effects were observed at 0.2 to 0.5 μmol/L. Trypan blue exclusion and MTT assay showed no cell toxicity at 0.5 μmol/L. The inhibitory effects of Cl-Ad, MECA, EHNA plus IDO and cAMP (a precursor of adenosine) on PDGF-BB–induced cell
proliferation, DNA synthesis, and collagen synthesis were completely abolished in CFs pretreated with antisense oligonucleotides, but not in CFs treated with sense or scrambled oligonucleotides (Figure 4).

Treatment of CFs with PDGF-BB increased MAP kinase activity from 0.476 ± 0.07 pmol/min per mg protein to 6.82 ± 0.4 pmol/min per mg protein, and the stimulatory effects of PDGF-BB were inhibited by the MAP kinase inhibitor PD98059 (10 μmol/L) to 1.5 ± 0.04 pmol/min per mg protein. The stimulatory effects of PDGF-BB on MAP kinase activity were inhibited in a concentration-dependent manner in CFs pretreated for 24 hours with 0.001 to 1 μmol/L of Cl-Ad. PDGF-BB-induced MAP kinase activity was also inhibited by 1 μmol/L of MECA, CPA, CGS21680, and AB-MECA, which are selective A1 and A2A receptor agonists, respectively, but not by CGS21680, AB-MECA, or CPA (Figure 5A). The inhibitory effects of Cl-Ad, MECA, and EHNA plus IDO on PDGF-BB–induced MAP kinase activity were completely abolished in CFs pretreated with antisense, but not sense or scrambled, oligonucleotides (Figure 5B).

Treatment of CFs with Cl-Ad induced intracellular and extracellular cAMP levels by 23.2-fold and 55.4-fold, respectively (Figure 6). The stimulatory effects of Cl-Ad on cAMP synthesis activity were completely abolished in CFs pretreated with antisense, but not sense or scrambled, oligonucleotides (Figure 6).

Discussion
The present study demonstrates that A2B receptors mediate the antimitogenic effects of adenosine in cardiac fibroblasts. Treatment of CFs with a stable adenosine analog (Cl-Ad) and with agents that elevate endogenous adenosine (EHNA plus IDO) inhibited PDGF-BB–induced CF growth (DNA synthesis, collagen synthesis, cell proliferation, and MAP kinase activity). The potency of MECA, an adenosine agonist with high affinity for A2 receptors, was similar to Cl-Ad. The adenosine agonists CPA and CGS21680, which are selective A1 and A2A receptor agonists, respectively, were only weakly inhibitory at high concentration. Moreover, AB-MECA, an A3 adenosine receptor agonist, was ineffective in inhibiting PDGF-BB–induced CF growth. The inhibitory effects of Cl-Ad, MECA, and EHNA plus IDO on PDGF-BB–induced MAP kinase activity were completely abolished in CFs pretreated with antisense, but not sense or scrambled, oligonucleotides (Figure 5B).

The stimulatory effects of Cl-Ad on cAMP synthesis activity were completely abolished in CFs pretreated with antisense, but not sense or scrambled, oligonucleotides (Figure 6).
growth were completely abolished by antisense oligonucleotides to A<sub>2B</sub> receptors (to inhibit A<sub>2B</sub> receptor synthesis), but not by sense or scrambled oligonucleotides. These findings provide evidence that exogenous as well as CF-derived adenosine inhibits PDGF-BB–induced growth and MAP kinase activity in an autocrine/paracrine manner and by means of the A<sub>2B</sub> receptor.

That CPA (an adenosine analog that is highly selective for A<sub>1</sub> receptors and mediates its effects at pharmacologically low doses [10<sup>-9</sup> mol/L and lower]) does not inhibit PDGF-BB–induced growth at low concentrations suggests that the inhibitory effects of adenosine are not mediated by means of A<sub>1</sub> receptors. This conclusion is further supported by our observation that DPCPX, an adenosine receptor antagonist that is a 700-fold selective for A<sub>1</sub> receptors, does not block the inhibitory effects of Cl-Ad on growth. That KF17837 and DPPSPX, but not DPCPX, attenuate the inhibitory effects of Cl-Ad suggest that the inhibitory effects of adenosine are A<sub>2B</sub> receptor mediated. That CGS21680 is ineffective in mimicking the inhibitory effects of adenosine and NECA is more effective than CGS21680 but less effective than Cl-Ad and MECA strongly supports the conclusion that the effects of adenosine are not mediated by means of A<sub>1</sub> receptors. That AB-MECA, an A<sub>3</sub> adenosine receptor agonist did not attenuate PDGF-BB–induced CF growth rules out the antimetabolic role for A<sub>1</sub> adenosine receptor. Rather, our data suggests that A<sub>2B</sub> receptors mediate the inhibitory effects of adenosine on cell growth.

The hypothesis that the inhibitory effects of adenosine on growth are mediated by means of A<sub>2B</sub> receptors is supported further by the observation that the inhibitory effects of MECA and Cl-Ad on growth are completely abolished by antisense oligonucleotides, which inhibit the synthesis of A<sub>2B</sub> receptors. Importantly, neither sense nor scrambled oligonucleotides affect the inhibitory effects of MECA and Cl-Ad, which rules out nonspecific actions of the antisense oligonucleotides.

The above findings provide the first evidence that exogenous adenosine inhibits PDGF-BB–induced growth of CFs and that the inhibitory effects of adenosine are mediated by means of activation of A<sub>2B</sub> receptors. However, whether endogenous adenosine also inhibits CF growth cannot be inferred from studies with agonists. Therefore, we examined the growth inhibiting effects of agents that elevate cellular adenosine levels by means of different mechanisms to assess the role of endogenous, i.e., CF-derived, adenosine on CF growth.

The physiological effects of adenosine are governed in part by the rapid rate of elimination of adenosine from the extracellular space. Metabolism of adenosine to inosine and AMP by adenosine deaminase and adenosine kinase, respectively, play a key role in the catabolism of active adenosine. Inhibition of the enzyme adenosine deaminase by EHNA and the enzyme adenosine kinase by IDO increases endogenous levels of adenosine. Treatment of CFs with EHNA and IDO increases endogenous adenosine levels by means of different mechanisms to assess the role of endogenous, i.e., CF-derived, adenosine on CF growth.

That the inhibitory effects of exogenous cAMP on PDGF-BB–induced growth are abolished by antisense, but not sense or scrambled oligonucleotides, suggests that A<sub>2B</sub> receptors also play an important role in mediating the antimitogenic effects of exogenous cAMP. This idea is supported further by the findings that cAMP is a precursor of adenosine and CFs metabolize cAMP by means of an extracellular cAMP-adenosine pathway involving ecto-5′-nucleotidases. cAMP-derived adenosine inhibits CF growth by means of A<sub>2B</sub> receptors, and cAMP is hydrophilic and exogenous cAMP should not penetrate cell membranes. Therefore, the inhibitory effects of EHNA plus IDO are mediated by means of generation of adenosine and that these effects are A<sub>2</sub> adenosine receptor mediated. Moreover, the observation that the growth inhibitory effects of EHNA plus IDO are completely abolished in CFs pretreated with antisense oligonucleotides to A<sub>2B</sub> receptors, but not by sense or scrambled oligonucleotides, provides strong evidence that inhibitory effects of endogenous CF-derived adenosine are A<sub>2B</sub> receptor mediated.

One common signaling pathway that is activated by multiple growth factors and that is implicated in the cardiac remodeling process is the MAP kinase pathway. The MAP kinase pathway is activated at sites of ischemia-induced cardiac remodeling, MECA and Cl-Ad, but not CPA, AB-MECA and CGS21680, inhibit PDGF-BB–induced MAP kinase activity, and these effects are blocked by KF17837 and DPPSPX, but not DPCPX, suggesting that inhibition of the MAP kinase pathway by means of A<sub>2B</sub> adenosine receptors contributes to the inhibitory effects of adenosine on cell growth. This idea is further supported by the observation that the inhibitory effects of MECA and Cl-Ad on PDGF-BB–induced MAP kinase activity are completely abolished by antisense oligonucleotides to A<sub>2B</sub> receptors, but not by sense or scrambled oligonucleotides. Moreover, treatment of CFs with inducers of endogenous adenosine (EHNA plus IDO) inhibits MAP kinase activity, and these effects are blocked by KF17837 and DPPSPX, but not by DPCPX, suggesting that CF-derived adenosine inhibits MAP kinase activity by means of A<sub>2B</sub> receptors. This hypothesis is further supported by the observation that the inhibitory effects of EHNA plus IDO are completely abolished by antisense to A<sub>2B</sub> receptors, but not by sense or scrambled oligonucleotides. Importantly, the basal growth and MAP kinase activity of the CFs was significantly induced in CFs treated with antisense, suggesting that endogenously produced adenosine has inhibitory effects on MAP kinase activity and CF growth.

Although the above findings provide evidence that adenosine inhibits overall MAP kinase activity, these results may not accurately represent the activity of the p42 and p44 isoforms of MAP kinase. Because these MAP kinase isoforms were not immunoprecipitated from the lysates, participation of other kinases remains a possibility. In this regard, it is interesting to note that, in contrast to SMCs and CFs in endothelial cells, mast cells, and embryonic kidney-293 cells, A<sub>2B</sub> receptors increase, rather than inhibit, MAP kinase activity. Additional studies are needed to elucidate the different signal transduction pathways that our regulated by A<sub>2B</sub> receptors in various cell types.

That the inhibitory effects of exogenous cAMP on PDGF-BB–induced growth are abolished by antisense, but not sense or scrambled oligonucleotides, suggests that A<sub>2B</sub> receptors also play an important role in mediating the antimitogenic effects of exogenous cAMP. This idea is supported further by the findings that cAMP is a precursor of adenosine and CFs metabolize cAMP by means of an extracellular cAMP-adenosine pathway involving ecto-5′-nucleotidases, cAMP-derived adenosine inhibits CF growth by means of A<sub>2B</sub> receptors, and cAMP is hydrophilic and exogenous cAMP should not penetrate cell membranes. Therefore, the inhibitory effects of
tory effects of exogenous cAMP are largely mediated by means of adenosine and A2B adenosine receptors.

That the growth inhibitory effects of exogenous and endogenous adenosine are abolished in CFs treated with antisense oligonucleotides, but not in CFs treated with sense or scrambled oligonucleotides, provides evidence that the A2B receptor expression is downregulated in CFs treated with antisense oligonucleotides. Because of unavailability of specific antibodies to A2B receptors, we were unable to confirm the downregulation by Western blotting or immunostaining. However, we did find that the effects of Cl-Ad on cAMP production, a signal transduction mechanism activated by A2B receptors, was abolished in CFs treated with antisense, but not in CFs treated with sense or scrambled oligonucleotides.

In contrast to antisense blockade of A3 receptors, neither DPSPX nor KF17837 increased basal or PDGF-BB–induced growth of CFs. There are several possible explanations for these results. DPSPX and KF17837 are not pure A2B receptor antagonists, but rather both also block A2A receptors, and DPSPX antagonizes A1 receptors as well. Antagonism of multiple adenosine receptors by DPSPX and KF17837 may explain the differential effects of antisense oligonucleotides versus receptor antagonists on basal cell growth. Alternatively, both DPSPX and KF17837 may have other nonspecific effects that affect cell growth. For example, DPSPX inhibits phosphodiesterase activity, and high concentrations (10^{-6} \text{mol/L}) of DPSPX directly inhibit cell growth. In contrast to receptor antagonists, antisense oligonucleotides may explain the differential effects of antisense oligonucleotides on basal cell growth.

In conclusion, we provide evidence that A2B, and not A1, A2A, or A3 receptors are responsible for mediating the antimitogenic effects of both exogenous and endogenous adenosine on PDGF-induced growth of vascular CFs. Thus, A2B receptors may play a vital role in regulating CF growth in health and disease. Moreover, abnormal/decreased expression of A2B receptors may contribute to abnormal synthesis and deposition of collagen and hypertrophy of CFs observed in CFs associated with hypertension, myocardial infarction, and reperfusion injury after ischemia.

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