A2B Receptors Mediate Antimitogenesis in Vascular Smooth Muscle Cells

Raghvendra K. Dubey, Delbert G. Gillespie, Holly Shue, Edwin K. Jackson

Abstract—Adenosine inhibits growth of vascular smooth muscle cells. 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 aortic vascular smooth muscle cells, platelet-derived growth factor–BB (PDGF-BB) (25 ng/mL) stimulated DNA synthesis ([3H]thymidine incorporation), cellular proliferation (cell number), collagen synthesis ([3H]leucine incorporation), and mitogen-activated protein (MAP) kinase activity. The adenosine receptor agonists 2-chloroadenosine and 5′-N-methylcarboxamidoadenosine, but not N′-cyclopentyladenosine 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-sulphophenylxanthine, 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 and PDGF-induced DNA synthesis, cell proliferation, and MAP kinase activity. Moreover, the growth-inhibitory effects of 2-chloroadenosine, 5′-N-methylcarboxamidoadenosine, and erythro-9-(2-hydroxy-3-nonyl)adenine plus iodotubericidin (inhibitors of adenosine deamination and adenosine kinase, respectively) were abolished by antisense, but not scrambled or sense, oligonucleotides to the A2B receptor. Our findings strongly support the hypothesis that adenosine causes inhibition of vascular smooth muscle cell growth by activating A2B receptors coupled to inhibition of MAP kinase activity. Pharmacological or molecular biological activation of A2B receptors may prevent vascular remodeling associated with hypertension, atherosclerosis, and restenosis following balloon angioplasty. (Hypertension. 2000;35[part 2]:267-272.)

Key Words: adenosine ■ muscle, smooth ■ receptors, adenosine ■ vascular remodeling ■ hyperplasia

Adenosine is an important cardioprotective autacoid. Although adenosine activates multiple receptor subtypes (A1, A2A, A2B, and A3 receptors), 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. Via activation of A2 receptors, adenosine causes vasodilation, inhibits platelet aggregation, diminishes neutrophil adhesion to vascular endothelial cells, stimulates nitric oxide release from vascular endothelial cells, attenuates neutrophil-induced endothelial cell damage, and stimulates nitric oxide release from vascular endothelial cells and vascular smooth muscle cells (SMCs).1,2

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 vascular SMC growth via activation of A2 and more specifically A2B receptors.2-6 However, the inference that A2B receptors mediate the growth-inhibiting effects of adenosine is weak because to date researchers do not have available agonists or antagonists selective for A2B receptors. Moreover, recent data suggest that in contrast to SMCs, A2 receptors induce growth in vascular endothelial cells5,8 and stimulate mitogen-activated protein (MAP) kinase activity in several cell types.9,10

Blockade of A2B receptor synthesis is an alternative approach to investigate the participation of A2B receptors in adenosine-mediated inhibition of vascular SMC growth. Accordingly, in the present study we developed antisense oligonucleotides against rat A2B receptors and used them to test the role of A2B receptors in regulating SMC growth and MAP kinase activity.

Methods

SMC Cultures

Arterial SMCs were cultured as explants from the abdominal aortas, obtained from anesthetized (50 mg/kg IP injection of pentobarbital) Sprague-Dawley male rats (n=14; Charles River, Wilmington, Mass) and as described previously.3,4 SMC purity was characterized.

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by immunofluorescence staining with smooth muscle–specific anti-smooth muscle α-actin monoclonal antibodies and by morphological criteria specific for SMC, as described in detail previously.\textsuperscript{3,4}

**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 2 antisense oligonucleotides with complementary sequences to the A\textsubscript{2B} receptor mRNA: (1) 5'-CTTATTCGTTGATGCCATCC-3' and (2) 5'-CTCGTTGTCAGTCAGCCAA-3'. Midland Certified Reagents Company custom synthesized and purified the phosphorothioated oligonucleotides with the above sequences. Preliminary studies revealed that the antisense oligonucleotides were equipotent in modulating the effects of adenosine on SMC growth. Hence, we selected the antisense oligonucleotide 5'-CTCGTTGTCAGTCAGCCAA-3' for all the experiments. Sense (5'-TTGTTCACTGGACACACGAG-3') and scrambled (5'-GCACGCCTCTATACTGCTAG-3') oligonucleotides were used as controls.

**Growth Studies**

SMCs were plated at a density of 5×10\textsuperscript{4} cells per well in 24-well tissue culture dishes and allowed to grow to subconfluence. Cells were then grown arrested by feeding DMEM containing 0.4% albumin for 48 hours in the presence or absence of 0.2 μmol/L of antisense, sense, or scrambled oligonucleotides. For [\textsuperscript{3}H]thymidine incorporation (index of DNA synthesis), growth was initiated by treating growth-arrested cells for 20 hours with DMEM supplemented with fresh oligonucleotides and containing platelet-derived growth factor–BB (PDGF-BB) (25 ng/mL) in the presence or absence of various treatments. Experiments were terminated by washing the cells twice with ice-cold PBS and pretreating for 24 hours with or without various test agents in the presence or absence of fresh oligonucleotides. Cells were then stimulated for 10 minutes 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 diethiothreitol, 100 μmol/L Na\textsubscript{2}VO\textsubscript{4}, 10 μg/mL aprotinin, 5 μg/mL pepstatin, 20 μg/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 was separated by centrifuging the extracts at 100 000 g for 20 minutes at 4°C, and the supernatants were diluted to a concentration of 1 mg protein per milliliter and stored at –70°C for MAP kinase activity assays. The MAP kinase activity in the cytosolic extracts was quantified by the method of Bornfeld et al.,\textsuperscript{12} with minor modifications as previously described by us.\textsuperscript{13} Briefly, cytosolic extracts (5 μL) were added to 30 μL of MAP kinase assay buffer (25 mmol/L \( \beta \)-glycerophosphate, 1.25 mmol/L EGTA, 0.5 mmol/L dithiothreitol, 150 μmol/L Na\textsubscript{2}VO\textsubscript{4}, 2 μmol/L peptide inhibitor for cAMP-dependent protein kinase [H-\textsuperscript{T}TTAAPISGTAGAAAAn-NH\textsubscript{2}; Bachem Bioscience Inc], 1 mg/mL bovine serum albumin, 10 μmol/L calmidazolium, 0.33 mg/mL myelin basic protein, and 100 μmol/L (\( \gamma \)P\textsuperscript{32}P)ATP). After incubation for 15 minutes at 30°C, 25-μL aliquots of the reaction mixture were spotted onto Whatman 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 presence of myelin basic protein were subtracted from the same samples incubated with myelin basic protein.

**Statistical Analysis**

All growth experiments were performed in triplicate or quadruplicate with 3 to 4 separate cultures. Data are presented as mean±SEM. Statistical analysis was performed with ANOVA, paired Student’s 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 total protein synthesis and cell proliferation) by 6- to 9-fold. As shown in Figure 1, in PDGF-BB–treated cells, 2-chloroadenosine inhibited in a concentration-dependent manner all measures of cell growth (protein synthesis data not shown). The lowest concentration of 2-chloroadenosine that significantly inhibited PDGF-BB–induced cell growth was 1 nmol/L; at a concentration of 1 μmol/L, 2-chloroadenosine inhibited cell growth by \( \approx 50\% \). High (10\textsuperscript{–6} mol/L), but not low, concentrations of \( N\textsuperscript{9}-\text{cyclopentyladenosine} \) (CPA) and CGS21680 inhibited PDGF-BB–induced cell growth (Figure 1). \( N\textsuperscript{9}-\text{Ethylcarboxamidoadenosine} \) (NECA) was more potent than CPA and CGS21680 inhibited PDGF-BB–induced cell growth (Figure 1). \( N\textsuperscript{9}-\text{Methylcarboxamido} \) (MECA) or 2-chloroadenosine in inhibiting PDGF-BB–induced increases in cell growth (Figure 1). KF17837 and 1,3-dipropyl-8-(p-sulfophenyl)xanthine (DPSX), but not 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), significantly reversed the inhibitory effects of 2-chloroadenosine as well as MECA on all measures of cell growth (Figure 2; protein synthesis data not shown).

Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) plus idotubericidin inhibited PDGF-BB–induced DNA, collagen, and total protein synthesis and cell proliferation (Figure 3; protein synthesis data not shown). The inhibitory effects of EHNA plus idotubericidin on PDGF-BB–induced cell growth were significantly attenuated by KF17837 and DPSX but not by DPCPX (Figure 3). Trypan blue exclusion tests indicated no loss in
viability of cells treated with CPA, CGS21680, MECA, DPSPX, KF17837, or DPCPX (data not shown).

Antisense, but not sense or scrambled, oligonucleotides increased basal as well as PDGF-BB–induced DNA synthe-

sis, and the growth effects of PDGF-BB were enhanced in a concentration-dependent fashion by antisense, but not sense or scrambled, oligonucleotides (Figure 4). The stimulatory effect of the antisense oligonucleotide on DNA synthesis was biphasic in nature, and maximal stimulatory effects were observed at 0.2 μmol/L. Trypan blue exclusion and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay showed no cell toxicity at 0.2 μmol/L, but at concentrations of ≥1 μmol/L, cell shrinkage and apoptotic death were evident. Concentrations of antisense that maximally increased
DNA synthesis also stimulated basal as well as PDGF-BB–induced cell proliferation. Compared with untreated cells, the basal and PDGF-BB–induced growth (cell number) was stimulated by 63\% and 48\% respectively, in cells treated with 0.2 \mu mol/L of the antisense for 4 days. The inhibitory effects of 2-chloroadenosine, MECA, EHNA plus iodotubericidin, and cAMP (a precursor of adenosine) on PDGF-BB–induced cell proliferation and DNA, collagen, and total protein synthesis were completely abolished in SMCs pretreated with antisense oligonucleotides but not in SMCs treated with sense and scrambled oligonucleotides (Figure 5; protein synthesis data not shown).

SMCs pretreated with antisense, but not sense or scrambled, oligonucleotides (Figure 6B).

**Discussion**

The present study demonstrates that A2B receptors mediate the antimitogenic effects of adenosine in vascular SMCs. Treatment of SMCs with a stable adenosine analogue (2-chloroadenosine) and with agents that elevate endogenous adenosine (EHNA plus iodotubericidin) inhibited PDGF-BB–induced SMC growth (DNA, collagen, and total protein synthesis, cell proliferation, and MAP kinase activity). The potency of MECA, an adenosine agonist with high affinity for A2 receptors, was similar to that of 2-chloroadenosine. The adenosine agonists CPA and CGS21680, which are selective A1 and A2A receptor agonists, respectively, were only weakly inhibitory at high concentrations. The inhibitory effects of 2-chloroadenosine, MECA, and EHNA plus iodotubericidin were significantly reversed by KF17837, a selective A2 receptor antagonist, and by DPSPX, a nonselective A2 receptor antagonist, but not by DPCPX, a selective A1 receptor antagonist. Moreover, the inhibitory effects of MECA, 2-chloroadenosine, and EHNA plus iodotubericidin on growth were completely abolished by antisense oligonucleotides to A2B receptor (to inhibit A2B receptor synthesis) but not by sense or scrambled oligonucleotides. These findings provide evidence that exogenous as well as SMC-derived adenosine inhibits PDGF-BB–induced growth and MAP ki-
nase activity in an autocrine/paracrine fashion and via the A2B receptor.

That CPA (an adenosine analogue that is highly selective for A1 receptors and mediates its effects at pharmacologically low doses [≤10^{-7} mol/L]) does not inhibit PDGF-BB–induced growth at low concentrations suggests that the inhibitory effects of adenosine are not mediated via A1 receptors. This conclusion is further supported by our observation that DPCPX, an adenosine receptor antagonist that is 700-fold selective for A1 receptors, does not block the inhibitory effects of 2-chloroadenosine on growth. That KFI7837 and DPSPX, but not DPCPX, attenuate the inhibitory effects of 2-chloroadenosine suggests that the inhibitory effects of adenosine are A2 receptor mediated. That CPGS21680 is ineffective in mimicking the inhibitory effects of adenosine and NECA is more effective than CPGS21680 but less effective than 2-chloroadenosine and MECa strongly supports the conclusion that the effects of adenosine are not mediated via A2 receptors. Since rat A3 receptors are resistant to blockade by xanthine antagonists, the fact that DPSPX blocks the inhibitory effects of 2-chloroadenosine suggests that A1 receptors do not mediate the growth-inhibitory effects of adenosine. Rather, our data suggest that A2 receptors mediate the inhibitory effects of adenosine on cell growth. The hypothesis that the inhibitory effects of adenosine on growth are mediated via A2 receptors is supported further by the observation that the inhibitory effects of MECa and 2-chloroadenosine in growth are completely abolished by antisense oligonucleotides to inhibit the synthesis of A2 receptors. Importantly, neither sense nor scrambled oligonucleotides affect the inhibitory effects of MECa and 2-chloroadenosine, which rules out nonspecific actions of the antisense oligonucleotides.

The aforementioned findings provide the first evidence that exogenous adenosine inhibits PDGF-BB–induced growth of SMCs and that the inhibitory effects of adenosine are mediated via activation of A2 receptors. However, whether endogenous adenosine also inhibits SMC growth cannot be inferred from studies with agonists. Therefore, we examined the growth-inhibiting effects of agents that elevate cellular adenosine levels via different mechanisms to assess the role of endogenous, ie, SMC-derived adenosine on SMC growth.

The physiological effects of adenosine are governed in part by the rapid rate of elimination of adenosine from the extracellular space. Conversion of adenosine to inosine and AMP by adenosine deaminase and adenosine kinase, respectively, plays a key role in the catabolism of active adenosine. Inhibition of the enzyme adenosine deaminase by EHNA and the enzyme adenosine kinase by iodotubericidin increases endogenous levels of adenosine. Treatment of SMCs with EHNA and iodotubericidin inhibits PDGF-BB–induced SMC growth. That the inhibitory effects of EHNA plus iodotubericidin on SMC growth are significantly reversed by KFI7837 and DPSPX, but not DPCPX, provides evidence that the inhibitory effects of EHNA plus iodotubericidin are mediated via generation of adenosine and that these effects are A2 adenosine receptor mediated. Moreover, the observation that the growth-inhibitory effects of EHNA plus iodotubericidin are completely abolished in SMCs pretreated with antisense oligonucleotides to A2 receptors, but not by sense or scrambled oligonucleotides, provides strong evidence that inhibitory effects of endogenous SMC-derived adenosine are A2 receptor mediated.

One common signaling pathway that is activated by multiple growth factors and that is implicated in the vascular remodeling process is the MAP kinase pathway. The MAP kinase pathway is activated at sites of balloon injury–induced neointima formation. MECa and 2-chloroadenosine, but not CPA and CPGS21680, inhibit PDGF-BB–induced MAP kinase activity, and these effects are blocked by DPSPX but not DPCPX, suggesting that inhibition of the MAP kinase pathway via A2 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 2-chloroadenosine on PDGF-BB–induced MAP kinase activity are completely abolished by antisense oligonucleotides to A2 receptors but not by sense or scrambled oligonucleotides. Moreover, treatment of SMCs with inducers of endogenous adenosine (EHNA plus iodotubericidin) inhibits MAP kinase activity, and these effects are blocked by DPSPX but not by DPCPX, suggesting that SMC-derived adenosine inhibits MAP kinase activity via A2 receptors. This hypothesis is further supported by the observation that the inhibitory effects of EHNA plus iodotubericidin are completely abolished by antisense to A2 receptors but not by sense or scrambled oligonucleotides. Importantly, the basal growth and MAP kinase activity of the SMCs were significantly induced in SMCs treated with antisense, suggesting that endogenously produced adenosine has inhibitory effects on MAP kinase activity and SMC growth. Although our data provide evidence that antisense treatment induces cell proliferation, it is feasible that polyploidy may also contribute to the increase in DNA synthesis in response to antisense.

In our study we used a peptide inhibitor of protein kinase A, a calmodulin antagonist, and a calcium chelator in the MAP kinase assay. Thus, kinase activity due to protein kinase A, calmodulin-dependent protein kinases, and calcium-dependent forms of protein kinase C were excluded. In addition, stimulation of kinase activity as measured by our assay was abolished by PD98059, a selective MAP kinase kinase (MEK) inhibitor. Since MEK selectively activates p42MAPK and p44MAPK, the results with PD98059 suggest that the kinase activity measured with our assay was due to p42MAPK and/or p44MAPK. Nonetheless, since we did not immunoprecipitate p42MAPK and p44MAPK from the lysates, the participation of other kinases remains a possibility.

It is interesting to note that in contrast to SMCs, A2 receptors induce growth of endothelial cells and activate MAP kinase activity in several cell types, including mast cells and embryonic kidney-293 cells. Hence, future studies should be focused on dissecting the various signal transduction pathways that are regulated via A2 receptors in the SMCs versus endothelial cells.

That the inhibitory effects of exogenous cAMP on PDGF-BB–induced growth are abolished by antisense, but not sense or scrambled, oligonucleotides suggests that A2 receptors also play an important role in mediating the antimitogenic
effects of exogenous cAMP. This idea is supported further by the following findings: cAMP is a precursor of adenosine, and SMCs metabolize cAMP via an extracellular cAMP-adenosine pathway involving ecto-5′-nucleotidases. cAMP-derived adenosine inhibits SMC growth via A2B receptors; cAMP is hydrophilic, and exogenous cAMP should not penetrate cell membranes. Therefore, the inhibitory effects of exogenous cAMP are largely mediated via adenosine and A2B adenosine receptors.

That the growth-inhibitory effects of exogenous and endogenous adenosine are abolished in SMCs treated with antisense oligonucleotides, but not in SMCs treated with sense or scrambled oligonucleotides, provides evidence that the A2B receptor expression is downregulated in SMCs treated with antisense oligonucleotides. Because of the 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 2-chloroadenosine and MECA on cAMP production, a signal transduction mechanism activated by A2B receptors, were abolished in SMCs treated with antisense oligonucleotides but not in SMCs treated with sense or scrambled oligonucleotides. In this regard, the basal and MECA (0.1 μmol/L)–stimulated levels of cAMP in control SMCs were 7.5±0.4 and 29.1±3.2 nmol/L, respectively, and in antisense-treated cells were 5.6±0.5 and 7.9±0.7 nmol/L, respectively.

Our findings provide the first evidence that abolishing A2B receptors with antisense oligonucleotides induces SMC growth. However, in contrast to antisense oligonucleotides, antagonism of A1 receptors with either DPSPX or KF17837 does not increase basal or PDGF-BB–induced growth of SMCs, even though both antagonists block the effects of exogenous adenosine and its analogues. A possible explanation for this discrepancy is that adenosine receptor antagonists exert other nonspecific effects that inhibit cell growth. In this regard, we have observed inhibition of cell growth with concentrations of either DPSPX or KF17837 ~10–fold higher than used in the present study (R.K. Dubey, et al, unpublished data, 1997). Adenosine receptor antagonists are well known to inhibit phosphodiesterase activity, and it is possible that this mechanism masks the ability of adenosine receptor antagonists to stimulate cell growth. Clearly, further studies using pure A2B receptor antagonists and/or molecular approaches are required to further explore the role of A2B receptors in regulating SMC growth.

In conclusion, we provide evidence that A2B, and not A1 or A2A, receptors are responsible for mediating the antimitogenic effects of both exogenous and endogenous adenosine on PDGF-induced growth of vascular SMCs. Thus, A2B receptors may play a vital role in regulating SMC growth in health and disease.

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