A2B Adenosine Receptors Stimulate Growth of Porcine and Rat Arterial Endothelial Cells

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

Abstract—The goal of this study was to determine which adenosine receptor subtype mediates growth stimulation by adenosine in arterial endothelial cells. In porcine coronary artery and rat aortic endothelial cells, 2-chloroadenosine (Cl-Ad), a metabolically stable analog of adenosine, stimulated DNA synthesis ([3H]-thymidine incorporation), cellular proliferation (cell number), collagen synthesis ([3H]-proline incorporation), and cell migration. The growth effects of adenosine and Cl-Ad were mimicked by the adenosine receptor agonist 5'-N-methylcarboxamidoadenosine but not by the adenosine receptor agonists N6-cyclopentyladenosine, 4-aminobenzyl-5'-N-methylcarboxamidoadenosine or CGS21680, 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-stimulatory effects of Cl-Ad and 5'-N-methylcarboxamidoadenosine, an antagonist profile consistent with an A2 receptor-mediated action. Treatment of endothelial cells with erythro-9-(2-hydroxy-3-nonyl) adenine plus iodotubericidin (inhibitors of adenosine deaminase and adenosine kinase, respectively) induced endothelial cell growth, and these effects were blocked by 1,3-dipropyl-8-p-sulphophenylxanthine and KF17837 but not 8-cyclopentyl-1,3-dipropylxanthine, suggesting that endothelial cell–derived adenosine induces growth via A2 receptors. The growth-stimulatory effects of Cl-Ad, 5'-N-methylcarboxamidoadenosine, and erythro-9-(2-hydroxy-3-nonyl) adenine plus iodotubericidin were abolished by antisense but not scrambled or sense oligonucleotides to the A2B receptor. Our findings strongly support the hypothesis that adenosine induces endothelial cell growth by activating A2B receptors. Thus, A2B receptors may play a critical role in regulating vascular remodeling associated with endothelial cell proliferation in angiogenesis, collateral vessel development, and recovery after vascular injury. Pharmacological or molecular biological activation of A2B receptors may be useful in modulating vascular remodeling. (Hypertension. 2002;39[part 2]:530-535.)

Key Words: adenosine • endothelium • receptors, adenosine • remodeling • microcirculation • vasculature • vascular diseases

Adenosine, a nucleoside long known as a “retaliatory” metabolite within the heart, has several anti–vaso-occlusive and vasoprotective properties.1 The biological effects of adenosine are mediated via adenosine receptors, which exist in multiple subtypes (A1, A2A, A2B, and A3 receptors).1 However, participation of A1 and A2 adenosine receptors seems to be more important in vascular biology, particularly with regard to reducing the risk and consequences of vaso-occlusive events associated with hypertension, atherosclerosis, and restenosis.2 For example, activation of A1 receptors by adenosine attenuates the sympathetic nervous system by inhibiting the release of norepinephrine and attenuates the renin-angiotensin system by inhibiting renin release from juxtaglomerular cells.1 Both of these pathways are involved in the vascular remodeling processes associated with hypertension and atherosclerosis. Furthermore, A1 receptor–induced opening of K+ channels has been suggested to protect the heart from myocardial ischemia/reperfusion injury. Via activation of A2 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.2,3

Adenosine is also a potent regulator of growth in both vascular and nonvascular cells.1,2,4 We have previously shown that adenosine inhibits growth of vascular smooth muscle cells,5 which play a key role in the vascular remodeling process that leads to vaso-occlusive disorders. Moreover, using receptor-specific agonists and antagonists, as well as antisense oligonucleotides, we demonstrated that the antimitogenic effects of adenosine are mediated via activation of...
A$_{2B}$ receptors. The role of A$_{2B}$ receptors in mediating the growth effects of adenosine are further supported by our recent findings that via this receptor, adenosine inhibits mitogen-induced growth of cardiac fibroblasts and glomerular mesangial cells (phenotypically similar to smooth muscle cells). In contrast to vascular smooth muscle cells, adenosine has been shown to induce mitogenic effects on endothelial cells via A$_3$ adenosine receptors. However, whether these effects are mediated via A$_3$ or A$_{2B}$ receptors remains unclear. One recent report by Grant et al implicates A$_{2B}$ receptor in mediating the mitogenic effects of adenosine on retinal microvascular endothelial cells; however, these observations remain unconfirmed. Moreover, whether A$_3$ receptors regulate growth of endothelial cells from conduit arteries (coronary arteries and aorta) is unknown.

Accordingly, the overall aims of the present study were to determine whether A$_{2B}$ receptors are responsible for mediating the stimulatory effects of adenosine on 4 major growth processes: DNA synthesis, collagen synthesis, cell proliferation, and cell migration. To accomplish our goals, we studied the effects of adenosine and its receptor-specific analogs and antagonists on the growth of porcine coronary artery and rat aortic endothelial cells. Moreover, blockade of A$_{2B}$ receptor synthesis with antisense oligonucleotides was used to provide direct evidence for a role of A$_{2B}$ receptors in stimulating endothelial cell growth.

### Methods

#### Materials

All tissue culture ware and reagents were purchased from GIBCO Laboratories. FCS was obtained from HyClone Laboratories Inc. Adenosine, 2-chloroadenosine (Cl-Ad), and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) were purchased from Sigma Chemical Co. N'-cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), idodotubericidin (IDO), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), and KF17837 (KF) were purchased from Research Biochemicals International. FCS was obtained from HyClone Laboratories Inc. N6-cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), idodotubericidin (IDO), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), and KF17837 (KF) were purchased from Research Biochemicals International. FCS was obtained from HyClone Laboratories Inc.

#### Pharmacology of Agents That Interact With the Adenosine System

<table>
<thead>
<tr>
<th>Agent</th>
<th>Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Chloroadenosine (Cl-Ad)</td>
<td>Nonselective but stable adenosine analog</td>
</tr>
<tr>
<td>5'-N-methylcarboxamidoadenosine (MECA)</td>
<td>Nonselective adenosine receptor agonist</td>
</tr>
<tr>
<td>4-aminobenzyl-5'-N-methylcarboxamidoadenosine (AB-MECA)</td>
<td>Selective A$_1$ receptor agonist</td>
</tr>
<tr>
<td>5'-N-ethylcarboxamidoadenosine (NECA)</td>
<td>Nonselective adenosine receptor agonist</td>
</tr>
<tr>
<td>N6-cyclopentyladenosine (CPA)</td>
<td>Selective A$_1$ receptor agonist</td>
</tr>
<tr>
<td>2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamino adenosine (CGS21680)</td>
<td>Selective A$_4$ receptor agonist</td>
</tr>
<tr>
<td>KF17837 (KF)</td>
<td>Selective A$_2$ receptor antagonist</td>
</tr>
<tr>
<td>1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX)</td>
<td>Nonselective adenosine receptor antagonist</td>
</tr>
<tr>
<td>8-cyclopentyl-1,3-dipropylxanthine (DPCPX)</td>
<td>Selective A$_3$ receptor antagonist</td>
</tr>
<tr>
<td>erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA)</td>
<td>Adenosine deaminase inhibitor</td>
</tr>
<tr>
<td>Iodotubericidin (IDO)</td>
<td>Adenosine kinase inhibitor</td>
</tr>
</tbody>
</table>

This list the abbreviations and summarizes the pharmacology of the compounds used in this study.

### Isolation and Culture of Porcine Coronary Artery and Rat Aortic Endothelial Cells

Hearts from female pigs were obtained from a local slaughter house and placed in ice-cold oxygenated DMEM containing antibiotics. The epicardial coronary arteries were isolated, and the fat and connective tissue were removed. Endothelial cells were isolated by collagenase/dispase digestion. The isolated cells were washed in DMEM/F12 medium supplemented with 10% FCS and endothelial cell growth supplement (Clonetics), and cells were grown to confluence under standard tissue culture conditions. Aortic endothelial cells, which had been isolated from aortas of Sprague-Dawley rats using anti-rat PECAM-1 tagged magnetic beads (Dyna Inc), were obtained from Dr. Christine Baylis (Department of Physiology, West Virginia University, Morgantown, WVa). The purity of the cultures was characterized by immunostaining with factor VIII antigen and by assaying the preferential uptake of the fluorescent probe Dil-acetylated low-density lipoprotein, which was >98%. Cells were passaged by trypsinization, and cells in second and third passage were used for all experiments.

#### Antisense Oligonucleotides for A$_{2B}$ Receptors

We used antisense oligonucleotide with complementary sequences to the A$_{2B}$ receptor mRNA (phosphorothioated 5'-CTCGTGTCG-CAGTGACCAA-3'; Midland Certified Reagent Company) and which we have previously shown effectively to block A$_{2B}$ receptor-mediated cAMP synthesis and antimitogenic effects in smooth muscle cells and cardiac fibroblasts. Sense (5'-TTTGTCATCGGAAAACAGG-3') and scrambled (5'-GCACGCCTCTATACGTGATG-3') oligonucleotides were used as controls.

#### Growth Studies

Endothelial cells were plated at a density of 5×10$^3$ cells/well in 24-well tissue culture dishes and allowed to grow to confluence. Cells were then growth arrested by feeding DMEM containing 0.5% albumin for 48 hours in the presence or absence of 0.5 μmol/L antisense, sense, or scrambled oligonucleotides. For 3H-thymidine incorporation (index of DNA synthesis), growth was initiated by treating growth-arrested cells for 20 hours with DMEM supplemented with fresh oligonucleotides in the presence or absence of various treatments and subsequently pulsed for 4 hours with 3H-thymidine (1 μCi/mL). 3H-thymidine incorporation in the acid-insoluble fraction was assayed as described previously. For cell number experiments, endothelial cells were allowed to attach overnight, were growth arrested for 48 hours and then treated every 24 hours for 5 days, and on day 6 cells were dislodged and counted on a Coulter counter.
\(^{1}\)H-proline incorporation studies were done to investigate the effects of various test agents on collagen synthesis. Confluent monolayers of endothelial cells were made quiescent by culturing the cells in DMEM containing 0.25% albumin for 48 hours in the presence or absence of 0.5 \(\mu\)mol/L antisense, sense, or scrambled oligonucleotides. Collagen synthesis was initiated by treating growth-arrested cells for 48 hours with DMEM supplemented with fresh oligonucleotides and \(^{1}\)H-proline (1 \(\mu\)Ci/mL) in the presence or absence of various treatments, and \(^{1}\)H-proline incorporation in the acid-insoluble fraction was analyzed as described previously.\(^5\)

**Cell Migration Studies**

For cell migration studies, we used 6.5-mm diameter Transwell plates (Costar) with 8-\(\mu\)m polycarbonate membrane pore size. Endothelial cells were starved overnight in 0.25% FCS and trypsinized, and 30 000 cells were placed on each membrane. The treatments (in a volume of 0.5 mL) were placed in the lower well chamber. After 5 hours of incubation at 37°C, the medium was removed and cells from the upper phase of the membrane were washed away. The membranes were then placed in methanol for fixation for 30 minutes, followed by a 30-minute incubation in Hoescht stain solution (0.5 \(\mu\)g/mL). The membranes were then separated from the wells and mounted on glass slides, and the labeled nuclei of the migrated cells were visualized by fluorescence microscopy. Migrating cells were determined by counting 12 different spots on each slide and taking the average.

**cAMP Synthesis**

Confluent monolayers of endothelial cells were treated every 24 hours for 2 days with or without 0.5 \(\mu\)mol/L antisense, sense, or scrambled oligonucleotides to the \(\beta_\text{2}\) receptor. After 48 hours, monolayers were washed with PBS and endothelial cells were treated for 30 minutes under standard tissue culture conditions with PBS (buffered with HEPES and NaHCO\(_3\), 13 and 25 \(\mu\)mol/L, respectively) containing fresh oligonucleotides and supplemented with 1 \(\mu\)mol/L 3-isobutyl-1-methylxanthine (IBMX) and containing or lacking 1 \(\mu\)mol/L Cl-Ad or MECA, in the presence and absence of DPCPX, KF17837, or DPCPX. Next, the cells along with the supernatants were collected after scraping, and cAMP levels were analyzed using an ELISA kit.\(^{11}\) In endothelial cells 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 triplicate or quadruplicate 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**

Treatment of porcine coronary artery endothelial cells with Cl-Ad enhanced all measures of cell growth, including DNA synthesis, cell proliferation, collagen synthesis, and cell migration (Figure 1). The lowest concentration of Cl-Ad that significantly induced cell growth was 0.1 \(\mu\)mol/L; at a concentration of 1 \(\mu\)mol/L, Cl-Ad induced cell growth by approximately 50%. A maximal mitogenic response of 70% increase in cell proliferation was observed in response to 10 \(\mu\)mol/L Cl-Ad after 4 days of treatment. Compared with Cl-Ad and its analogs, 2.5% FCS and 25 ng/mL platelet-derived growth factor BB were more potent mitogens and induced endothelial proliferation after 4 days of treatment by 7.88-fold and 3.4-fold, respectively. No growth effects were observed in cells treated with 0.1 to 10 \(\mu\)mol/L CPA or CGS21680 (Figure 1). NECA was more potent than AB-

**Figure 1.** Concentration-response relationships for the effects of Cl-Ad, MECA, AB-MECA, NECA, CPA, and CGS21680 (CGS) on DNA synthesis (A), cell number (B), and collagen synthesis (C) in porcine coronary artery endothelial cells. \(D\), the effects of 1 \(\mu\)mol/L of the various adenosine analogs on endothelial cell migration. Values represent mean±SEM from 3 separate experiments, each conducted in quadruplicate. \(^{*}P<0.05\) compared with control. \(\$P<0.05\) versus same concentration of Cl-Ad or MECA.

MECA, CPA, and CGS21680 but less potent than MECA or Cl-Ad in inducing cell growth (Figure 1). The stimulatory effects of Cl-Ad on all measures of cell growth were significantly blocked by KF17837 and DPCPX but not by DPCPX (Figure 2). Similar to Cl-Ad, the stimulatory effects of MECA were blocked by KF17837 and DPCPX but not by DPCPX (Figure 2).

Treatment of porcine coronary artery endothelial cells with EHNA plus IDO augmented DNA synthesis, collagen synthesis, cell proliferation, and cell migration (Figure 3). The stimulatory effects of EHNA plus IDO on cell growth were significantly attenuated by KF17837 and DPCPX 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, DPCPX, or DPCPX.

**Figure 2.** Effects of Cl-Ad (1 \(\mu\)mol/L) and MECA (1 \(\mu\)mol/L) on DNA synthesis (A), cell number (B), collagen synthesis (C), and cell migration (D) in porcine coronary artery endothelial cells in the presence and absence of KF17837 (KF; 10 \(^{-6}\) mol/L), DPCPX (10 \(^{-6}\) mol/L), and DPCPX (10 \(^{-6}\) mol/L). Values represent mean±SEM from 4 separate experiments, each conducted in quadruplicate. \(^{*}P<0.05\) compared with control.
Similar to porcine coronary endothelial cells, Cl-Ad, MECA, and NECA but not CPA, CGS21680, or AB-MECA induced growth of rat aortic endothelial cells (Figure 4). Compared with NECA, Cl-Ad and MECA were more potent in inducing growth, and the stimulatory effects of Cl-Ad and MECA were blocked by DPSPX and KF17837 but not by DPCPX (data not shown). Also, the stimulatory effects of Cl-Ad, MECA, EHNA plus IDO, and cAMP (a precursor of adenosine) on cell proliferation, DNA synthesis, collagen synthesis, and cell migration were completely abolished in rat aortic endothelial cells pretreated with antisense oligonucleotides but not in endothelial cells treated with sense or scrambled oligonucleotides (Figure 4). We have previously shown that at a concentration of 0.5 μmol/L, the antisense oligonucleotides used in this study effectively block A_2B receptor–mediated growth effects in smooth muscle cells and cardiac fibroblasts.5,6 Moreover, trypan blue exclusion and MTT assay showed no cell toxicity at 0.5 μmol/L.

In porcine coronary artery endothelial cells, the stimulatory effects of adenosine were also mimicked by its precursor cAMP, and these effects were blocked by DPSPX and KF17837 but not by DPCPX. The growth effects of cAMP were mimicked by its nonmetabolizable and permeable analog 8-bromo-cAMP; however, in contrast to cAMP, the inhibitory effects of 8-bromo-cAMP were not blocked by DPSPX, KF172837 or DPCPX (Figure 5).

Treatment of rat aortic endothelial cells with Cl-Ad and MECA increased cAMP levels (intracellular plus extracellular), and these stimulatory effects were blocked by DPSPX and KF17837 but not by DPCPX. In rat aortic endothelial cells, the stimulatory effects of Cl-Ad and MECA on cAMP synthesis activity were completely abolished in endothelial cells pretreated with antisense but not with sense or scrambled oligonucleotides (Figure 6).
We have observed similar effects of antisense oligonucleotides against $A_{2B}$ receptor mRNA on cAMP synthesis in vascular smooth muscle cells$^5$ and cardiac fibroblasts.$^6$ These results suggest that the antisense oligonucleotides against $A_{2B}$ receptor mRNA effectively inhibit the expression of functional $A_{2B}$ receptors. However, Western blots or ligand binding studies are needed to confirm this inference.

An important issue is whether adenosine made by endothelial cells affects endothelial cell 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,$^{1,12}$ reduces the levels of endogenous adenosine, and inhibition of adenosine deaminase by EHNA and inhibition of adenosine kinase by IDO increases endogenous levels of adenosine.$^{13}$ The current study demonstrates that treatment of endothelial cells with EHNA plus IDO stimulates endothelial cell growth. Importantly, KF17837 and DPCPX but not DPCPX block the stimulatory effects of EHNA plus IDO on endothelial cell growth, a finding consistent with the involvement of $A_1$ receptors. Moreover, antisense oligonucleotides to $A_{2B}$ receptors but not by sense or scrambled oligonucleotides block the growth-promoting effects of EHNA plus IDO. These results provide strong evidence that the stimulatory effects of endogenous endothelial cell–derived adenosine are $A_{2B}$ receptor mediated.

The present study demonstrates that exogenous cAMP stimulates growth of endothelial cells. Because exogenous cAMP would not penetrate cell membranes to any significant extent, these results suggest that extracellular cAMP stimulates endothelial cell growth. Our previous findings indicate that extracellular cAMP is a precursor of adenosine, and endothelial cells metabolize extracellular cAMP via an extracellular cAMP–adenosine pathway involving ecto-5′-nucleotidases.$^{12}$ Thus, it is possible that adenosine via $A_{2B}$ receptors mediates the effects of extracellular cAMP on endothelial cell growth. In support of this conclusion, the growth-stimulatory effects of extracellular cAMP but not 8-bromo-cAMP (a nonmetabolizable cAMP analog that penetrates cell membranes) are blocked by DPCPX and KF17837, whereas DPCPX does not block the growth-promoting effects of extracellular cAMP. Moreover, antisense but not sense or scrambled oligonucleotides block the growth-stimulatory effects of exogenous cAMP on endothelial cells. Taken together, these findings suggest that $A_{2B}$ receptors play an important role in mediating the growth-promoting effects of extracellular cAMP on endothelial cells. In this context, our recent findings indicate that cAMP-derived adenosine also regulates growth of vascular smooth muscle cells and cardiac fibroblasts via $A_{2B}$ receptors.$^{14,15}$

$A_{2B}$ receptors seem to mediate divergent effects on growth in different cell lines. In vascular smooth muscle cells and cardiac fibroblasts, $A_{2B}$ receptors inhibit cell growth,$^{14,15}$ whereas in endothelial cells, $A_{2B}$ receptors stimulate cell growth. Previous studies indicate that in human retinal endothelial cells, $A_{2B}$ receptors stimulate the production of vascular endothelial growth factor.$^8$ It is likely that the effects of adenosine to stimulate vascular endothelial growth factor...
production via A_2B receptors accounts for the cell line–specific effects of adenosine on cell proliferation. In the vasculature, the net result of this cell line–specific effect would be to facilitate the recovery of blood vessels from injury by inhibiting the inappropriate migration and proliferation of vascular smooth muscle cells into the intima while stimulating the regrowth of endothelial cells over the endothelium-denuded intima. In the heart, inhibition of cardiac fibroblast growth and stimulation of endothelial cell growth would attenuate cardiac fibrosis and increase revascularization after ischemic injury of the myocardium.

Stimulation of adenylyl cyclase is the most widely known signal transduction pathway activated by A_2B receptors, and, as demonstrated in the current study, 8-bromo-cAMP stimulates endothelial cell growth. It is possible, therefore, that the growth-promoting effects of adenosine in endothelial cells is mediated by intracellular cAMP. However, the signal transduction mechanism by which A_2B receptors stimulate endothelial cell growth was not investigated in the present study.

In conclusion, we provide evidence that A_2B receptors, not A_1, A_2A, or A_3 receptors, are responsible for mediating the growth-stimulatory effects of both exogenous and endogenous adenosine on vascular endothelial cells. Thus, A_2B receptors may play a vital role in regulating endothelial cell growth in health and disease. Within the heart/coronary circulation, via A_2B receptors, adenosine may play an important role in inducing collateral vessel development after ischemia or hypoxia. Moreover, abnormal/decreased expression of A_2B receptors may contribute to abnormal angiogenesis and endothelial cell recovery, thereby influencing the vascular remodeling process associated with balloon injury and collateral vessel development after hypoxia, ischemia, and reperfusion injury.

Acknowledgment

Supported by the Swiss National Science Foundation grant 32–64040.00 and National Institutes of Health grant HL55314.

References

A2B Adenosine Receptors Stimulate Growth of Porcine and Rat Arterial Endothelial Cells
Raghvendra K. Dubey, Delbert G. Gillespie and Edwin K. Jackson

Hypertension. 2002;39:530-535
doi: 10.1161/01.Hyp.0000019140.63713.71

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/39/2/530

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/