Adenosine Receptor–Mediated Adhesion of Endothelial Progenitors to Cardiac Microvascular Endothelial Cells

Sergey Ryzhov, Nataliya V. Solenkova, Anna E. Goldstein, Mathias Lamparter, Todd Fleenor, Pampee P. Young, James P. Greelish, John G. Byrne, Douglas E. Vaughan, Italo Biaggioni, Antonis K. Hatzopoulos, Igor Feoktistov

Abstract—Intracoronary delivery of endothelial progenitor cells (EPCs) is an emerging concept for the treatment of cardiovascular disease. Enhancement of EPC adhesion to vascular endothelium could improve cell retention within targeted organs. Because extracellular adenosine is elevated at sites of ischemia and stimulates neovascularization, we examined the potential role of adenosine in augmenting EPC retention to cardiac microvascular endothelium. Stimulation of adenosine receptors in murine embryonic EPCs (eEPCs) and cardiac endothelial cells (cECs) rapidly, within minutes, increased eEPC adhesion to cECs under static and flow conditions. Similarly, adhesion of human adult culture-expanded EPCs to human cECs was increased by stimulation of adenosine receptors. Furthermore, adenosine increased eEPC retention in isolated mouse hearts perfused with eEPCs. We determined that eEPCs and cECs preferentially express functional A₁ and A₂B adenosine receptor subtypes, respectively, and that both subtypes are involved in the regulation of EPC adhesion to cECs. We documented that the interaction between P-selectin and its ligand (P-selectin glycoprotein ligand-1) plays a role in adenosine-dependent EPC adhesion to cECs and that stimulation of adenosine receptors in cECs induces rapid cell surface expression of P-selectin. Our results suggest a role for adenosine in vasculogenesis and its potential use to stimulate engraftment in cell-based therapies. (Circ Res. 2008;102:356-363.)

Key Words: adenosine • adenosine receptors • endothelium • adhesion molecules

Intracoronary injection of bone marrow-derived or culture-expanded endothelial progenitor cells (EPCs) is currently tested for the treatment of patients after acute myocardial infarction. Recent double-blinded, placebo-controlled, multicenter clinical trials have shown that this type of therapy is relatively safe without serious adverse effects and may lead to moderate improvement of cardiac output.¹ However, the number of donor cells retained in the heart is low, in the range of 3% to 5%,² limiting the effectiveness of therapy. To overcome this problem, it would be highly desirable to develop methods to improve adhesion and retention of EPCs to cardiac endothelium.

We have previously shown that homing of EPCs to sites of tumor-induced angiogenesis or cardiac ischemia is mediated by active interaction with the vascular wall,³,⁴ suggesting that preactivation of adhesion molecules in host endothelium and donor-transplanted cells might augment cell retention in target tissues. However, activation of cell adhesion molecules in endothelial cells after ischemic injury or inflammation is likely to be transient and absent by the time of therapeutic intervention. Therefore, there is a need to develop safe ways to activate, locally and acutely, the adhesiveness of vascular beds during cell delivery.

In the present study, we examined the role of adenosine in EPC homing. Adenosine is generated when ATP is catabolized as energy demands increase or oxygen supply decreases in sites of tissue stress, injury, and local hypoxia. Adenosine exerts its actions through interaction with cell surface G protein–coupled adenosine receptors, of which there are 4 subtypes: A₁, A₂A, A₂B and A₃. Once released into the extracellular space, adenosine signals to restore the balance between energy supply and demand.

The concept of adenosine as a retaliatory autacoid, originally proposed by Berne et al, has focused mostly on its acute actions, including vasodilation and negative chronotropic and inotropic effects in the heart.⁵ Accumulating evidence suggests that adenosine is also important for the long-term restoration of oxygen supply by contributing to neovascularization. Adenosine stimulates blood vessel formation in embryos⁶ and promotes capillary proliferation in the adult heart and skeletal muscles.⁷,⁸ These effects are mediated at least in part by adenosine-stimulated production of growth factors that facilitate new blood vessel formation from pre-existing fully differentiated endothelial cells, a process known as angiogenesis.⁹,¹⁰

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Neovascularization also occurs in a process known as vasculogenesis. Bone marrow–derived EPCs are critical to this process by differentiating into mature endothelial cells at the site of development of vascular networks. There is evidence that application of an adenosine receptor agonist to experimental excisional wounds stimulates vasculogenesis in the early phase of wound healing. However, the role of adenosine in EPC homing to the sites of tissue injury or ischemia has not been studied.

In the present study, we tested the hypothesis that adenosine is involved in the recruitment of EPCs to ischemic or damaged tissues by modulating their interaction with vascular endothelium. We focused our study on regulation of EPC adhesion to cardiac endothelium in view of the potential of cell-based therapies for cardiovascular disease and the possibility that adenosine could be developed as a novel adjunct agent for this purpose.

Materials and Methods

Reagents and Cells

N′-Cyclopylenadenosine (CPA), 5′-N-ethylcarboxamidoadenosine (NECA), 4-[(N-ethyl-5′-carboxamyladenos-2-yl)-aminoethyl]-phenyl-propionic acid (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and adenosine were purchased from Sigma (St Louis, Mo), Endonorbornan-2-yl-9-methyladenine (N9061) was a gift from Whitby Research Inc (Richmond, Va), and 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-[1,2,4]triazolo[1,5-c]-pyrimidine (SCH58261) was a gift from Schering Plough (Milan, Italy), 3-Isobutyl-8-pyrrolidinoxantain (IPX) was synthesized as previously described.

Mouse cardiac microvascular endothelial (MCEC-1) cells were generously provided by Dr J. Mason (National Heart and Lung Institute, London, UK). These cells were isolated from mice containing a gene encoding the theromable SV40 T antigen and maintained in the presence of interferon-y at 33°C. Six days before experiments, cells were replated and cultured in the absence of interferon-y at 37°C. Primary cultures of human cardiac microvascular endothelial (HMVEC-c) cells (Cambrex, Walkersville, Md) were maintained according to the recommendations of the supplier up to passages 2 to 5.

Mouse EPCs isolated from E7.5 embryos (eEPCs) have been described previously. Human adult culture-expanded EPCs were generated from mononuclear cells obtained from normal peripheral blood leukocytes by culturing in EBM-2 (Cambrex) with supplements according to previously published protocols. EPCs were harvested on day 7 and were identified by uptake of 1,1′-diodoteadecyl-3,3,3′,3′-tetramethylindocarcyanine (DiI)-labeled acetylated LDL and co-staining with Ulex europaeus agglutinin-1 and vascular endothelial cadherin.

Measurement of cAMP Accumulation

cAMP concentrations were determined using a cAMP assay kit (GE Healthcare, Little Chalfont, UK).

Real-Time RT-PCR

RT-PCR was performed as previously described. Primer pairs and 6-carboxy-fluorescein–labeled probes were provided by Applied Biosystems (Foster City, Calif).

Analysis of Cell Adhesion Under Static Conditions

We incubated progenitor cells (5×10⁶ cells per well) prelabeled with calcine–acetoxymethyl ester (Molecular Probes, Eugene, Ore) in 96-well plates precoated with 1% porcine gelatin type A (Sigma) and covered with confluent endothelial monolayers in DMEM at 37°C. At the end of incubation periods indicated under Results, wells were gently washed twice with DMEM and twice with Tyrode’s buffer. Fluorescence of adhered cells was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, and cell adhesion was calculated using a calibration curve constructed for each experiment by measuring fluorescence of predetermined numbers of labeled cells.

Analysis of Cell Adhesion Under Flow Conditions

Adhesion assays under flow conditions were performed using a parallel plate flow chamber (Glycotech, Rockville, Md) following the instructions of the manufacturer. Endothelial confluent monolayers were perfused for 10 minutes with DMEM containing 10 μmol/L NECA or its vehicle, followed by an EPC suspension (10⁶ cells/mL) in the same medium for another 10 minutes at a constant rate to generate a desired wall shear stress (τ, dynes per centimeter squared) using the formula τ=6μLμs²b, where Q is flow rate, μ is medium viscosity, b is channel width, and a is channel height. EPC adhesion was determined by analysis of digitized video recordings using NIH Image software.

Cell-Based P-Selectin Enzyme-Linked Immunoassay

Cell surface P-selectin expression on MCEC-1 cells was analyzed as previously described using rat anti-mouse CD62P (Fitzgerald Industries, Concord, Mass) or isotype-matched control antibodies (BD Biosciences, San Jose, Calif) and a secondary goat anti-rat horseradish peroxidase–conjugated antibody (Jackson ImmunoResearch, West Grove, Pa).

Isolated Mouse Heart Model

Twenty eight male 6- to 8-week-old C57Bl/6 mice were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Hearts were rapidly removed from mice anesthetized with inhalation of isoflurane. The aorta was cannulated and connected to a Langendorff apparatus. The Langendorff perfusion was performed at a constant rate of 4 mL/min with Krebs–Henseleit buffer equilibrated with a gas mixture of 95% O₂ and 5% CO₂ at 37°C. Drug effects on coronary flow were measured at a constant pressure of 80 mm Hg. After a 30-minute stabilization period, hearts were perfused with 1.5 mg/L fluorescein isothiocyanate (FITC)–conjugated Helix pomatia lectin (Sigma) for 10 minutes to label endothelial cells followed by a 10 minutes washing period. Hearts were then perfused with eEPCs prelabeled with DiI-C16 (Invitrogen, Carlsbad, Calif) and resuspended in Krebs–Henseleit buffer containing 2% FBS (2500 cells/mL) in the presence or absence of 10 μmol/L adenosine, 3 nmol/L CGS21680, or 100 μmol/L, inosine for 10 minutes. After washing for 10 minutes to remove unbound eEPCs, hearts were dissected, and retention of eEPCs was analyzed by taking 10 random images of the left ventricle using epifluorescence microscopy. Area of EPC-emitted fluorescence was measured using NIH ImageJ software and normalized to the area of vascular endothelium stained with FITC–lectin.

Statistical Analysis

All data are presented as means±SEM. The data were analyzed using unpaired 2-tail t test or 1-way ANOVA with Dunnett’s post test. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Adenosine Receptors in Mouse Embryonic EPCs

Real-time RT-PCR showed that eEPCs preferentially express mRNA encoding A₁ receptors (0.248±0.004% of β-actin; Figure 1A). Very low levels of A₃ receptors were also detected (0.009±0.002% of β-actin), whereas transcripts for A₂a and A₃ receptors were below detection levels.
We measured cAMP accumulation as a way to determine whether expression of mRNA translates into functional presence of adenosine receptors in eEPCs; A1A and A2B receptors stimulate adenylate cyclase via coupling to Gs proteins, whereas A3 receptors inhibit this enzyme via coupling to Gi proteins.5 The affinity to adenosine receptor subtypes of the agonists and antagonists used are summarized in the Table.

Forskolin increased cAMP levels in eEPCs from 4.3±0.7 to 35±2 pmol per well, with an EC50 of 1.1 μmol/L, whereas the nonselective adenosine receptor agonist NECA did not elevate cAMP (Figure 1B). This is contrary to what would be expected for activation of A2B receptors. However, the selective A2B agonist CPA inhibited forskolin-stimulated cAMP accumulation with an EC50 of 1.3 nmol/L (Figure 1C), corresponding to its reported affinity at A2B receptors.5 Furthermore, DPCPX and N-0861 antagonized the action of 10 nmol/L CPA on forskolin-stimulated cAMP accumulation (inset, Schild analysis indicated simple competitive antagonism at A2B receptors (slope of 1.1) with a Kd value of 603 nmol/L. D. Effect of the selective A1 receptor agonist CPA on cAMP accumulation induced by 1 μmol/L forskolin. The data are means±SEM (n=3).

Adenosine Receptors in Cardiac Microvascular Endothelial Cells

Real-time RT-PCR analysis of MCEC-1 cells revealed preferential expression of mRNA encoding A2B receptors (0.284±0.012% of β-actin), with lower expression of A1 and A2A receptors (0.016±0.002 and 0.091±0.005% of β-actin, respectively) and no detectable levels of A3 receptor transcripts (Figure 2A).

NECA stimulated cAMP accumulation with an EC50 of 449 nmol/L, corresponding to its affinity at A2B receptors, whereas the A2A agonist CGS21680 had no effect when used at selective concentrations (Figure 2C). The selective A2B antagonist IPDX progressively shifted concentration–response curves of NECA-stimulated cAMP accumulation to the right (Figure 2C). Schild plot analysis (inset) determined

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**Table. Affinity of Agonists and Antagonists to Adenosine Receptor Subtypes**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>A1</th>
<th>A2A</th>
<th>A2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NECA</td>
<td>6.3–30</td>
<td>4.2–20</td>
<td>330–449</td>
</tr>
<tr>
<td>CPA</td>
<td>0.59–4</td>
<td>148–2000</td>
<td>21 000–34 000</td>
</tr>
<tr>
<td>CGS21680</td>
<td>290–36 300</td>
<td>3.6–27</td>
<td>361 000</td>
</tr>
<tr>
<td>DPCPX</td>
<td>0.3–3.9</td>
<td>129–598</td>
<td>50–86</td>
</tr>
<tr>
<td>SCH58261</td>
<td>120–854</td>
<td>0.6–2.3</td>
<td>&gt;100–1868</td>
</tr>
<tr>
<td>IPDX</td>
<td>20 000–24 000</td>
<td>36 000</td>
<td>603–625</td>
</tr>
<tr>
<td>N-0861</td>
<td>511–575</td>
<td>39 350–56 200</td>
<td></td>
</tr>
</tbody>
</table>

*The range of estimated binding or inhibition constants is given in nmol/L. An expanded table with detailed information and references is available in the online data supplement.*
that IPDX inhibits this A2B-mediated process with a dissociation constant of 603 nmol/L, a value similar to that found in human cells. Functional, albeit low, expression of A1 receptors in MCEC-1 cells was also detected; the A1 agonist CPA inhibited forskolin-stimulated adenylyl cyclase at selective (low nanomolar) concentrations (Table). Inhibition was reversed with increasing concentrations of CPA (>100 nmol/L) presumably because of stimulation of A2B receptors (Figure 2D). Taken together, our data suggest that A2B is the predominant receptor subtype regulating adenylyl cyclase in MCEC-1 cells.

HMVEC-c cells preferentially expressed mRNA encoding A2B receptors (0.168±0.003% of β-actin), lower levels of A2A receptor transcripts (0.082±0.015% of β-actin), and no detectable levels of A1 or A3 receptor mRNA (Figure 3A). Similarly to MCEC-1 cells, A2B receptor was the predominant subtype regulating adenylyl cyclase in HMVEC-c cells; the nonselective agonist NECA stimulated cAMP accumulation 5.1±0.1-fold, whereas the selective A2A agonist CGS21680 had no significant effect (Figure 3B).

**Role of Adenosine Receptors in EPC Adhesion to Cardiac Microvascular Endothelial Cells**

Adhesion of fluorescently labeled mouse eEPCs to MCEC-1 cells was rapidly stimulated by 1 μmol/L NECA (Figure 4A and 4B), with a half-maximal effect observed at 5 minutes. Adhesion in the continuous presence of NECA was greater compared with adhesion in the absence of NECA after individual pretreatment of MCEC-1 cells and/or eEPCs with NECA (Figure 1 in the online data supplement). From data in Figure 4A, we selected 30 minutes as the incubation time that produced maximal increase in adhesion, and performed a pharmacological analysis of the adenosine receptor subtypes involved in this action. NECA increased eEPC adhesion from 4.5±0.3% to 21.3±1.4% in a concentration-dependent manner with an EC50 of 139 nmol/L. Selective stimulation of A1 receptors with 10 nmol/L CPA only slightly increased eEPC adhesion to 7.4±0.6%, whereas stimulation of A2A receptors with CGS21680 had virtually no effect (Figure 4C). Based on these results, we selected 1 μmol/L NECA, a concentration...
producing submaximal increase in eEPC adhesion to MCEC-1 cells, to analyze the effects of adenosine receptor-specific antagonists. As seen in Figure 4D, DPCPX, N-0861, and IPDX inhibited NECA-induced eEPC adhesion with IC50 values of 4 nmol/L, 1.5 μmol/L, and 1.6 μmol/L, consistent with their respective potency at A1 and A2B receptors (Table). Of note, the selective A2B antagonist SCH58261 inhibited NECA-induced eEPC adhesion with an IC50 value of 1.7 μmol/L that was consistent with its potency at A1 and A2B receptors, whereas it had no effect at lower concentrations that selectively block A2A receptors (Table). Taken together, these data suggest that A1 and A2B, but not A2A, receptors are involved in stimulation of eEPC adhesion to MCEC-1 cells by adenosine.

We also used a complementary approach to evaluate the contribution of A1 receptors by preincubating eEPCs with 100 nmol/L pertussis toxin for 12 hours to uncouple the receptor to Gi proteins.5 In ancillary studies, we documented that this treatment completely abrogated the ability of CPA to inhibit forskolin-stimulated adenylate cyclase, thus confirming the functional uncoupling of A1 receptors. Pertussis toxin treatment significantly attenuated but did not completely block the stimulation of adhesion induced by 10 μmol/L NECA (Figure 4E). In contrast, this treatment had no effect on TNF-α–induced eEPC adhesion (supplemental Figure II). Thus, we conclude that stimulation of A2B receptors on MCEC-1 cells is essential for EPC adhesion to endothelium but that stimulation of A1 receptors on eEPCs can additionally increase their adherence. An increase in eEPC adhesion induced by stimulation of adenosine receptors can eventually lead to increased numbers of cells transmigrating endothelial layer, and our ancillary studies indicate this possibility (supplemental Figure III).

Next, we evaluated the adhesion of these cells under laminar flow conditions by perfusing eEPCs over MCEC-1 monolayers at 2 different levels at the low end of physiologically relevant range of wall shear stress values,19 0.75 and 1 dyne/cm2 for 10 minutes. As expected, an increase in shear stress reduced adhesion of eEPCs to endothelial cells. However, stimulation of adenosine receptors with 10 μmol/L NECA significantly increased eEPC adhesion at both levels of shear stress (Figure 4F). On stopping and resuming flow, the adhered eEPCs withstood further increase in laminar flow applied in increments of 1 dyne/cm2 and started to detach only when shear stress exceeded 10 dyne/cm2.

We then measured the effect of adenosine receptor stimulation on the adhesion of human adult culture–expanded endothelial progenitor cells (CE-EPCs) to HMVEC-c cells. As seen in Figure 5, NECA stimulated CE-EPC adhesion to HMVEC-c cells in a concentration-dependent manner. These results indicate that adenosine receptors can regulate not only adhesion of mouse embryonic EPCs but also homing of adult human progenitor cells to cardiac microvascular endothelial cells.

**Adenosine Promotes EPC Retention in Isolated Mouse Hearts**

To determine whether the observed adenosine-dependent increase in EPC adhesion to cardiac microvascular endothelial cells translates into increased retention of circulating EPCs in the coronary vasculature, we used a conventional Langendorff retrograde perfusion system. Endothelial cells in coronary vessels were marked with FITC-conjugated Helix pomatia lectin (green; Figure 6A and 6B). Mouse eEPCs were labeled with Dil-C16 to allow their detection at the surface of the left ventricle using epifluorescence microscopy (red; Figure 6C and 6D). Figure 6 shows representative images obtained from hearts perfused with eEPC suspension in the absence (A, C, and E) or presence of 10 μmol/L adenosine (B, D, and F). We found that adenosine significantly increased the relative area of vascular network occupied by eEPCs (Figure 6G).

A2B receptors are known to participate in adenosine-induced coronary vasodilation.5 Perfusion of hearts with the selective A2B agonist CGS21680 (3 nmol/L) produced comparable vasodilation as 10 μmol/L adenosine (Figure 6H) but had a considerably less effect on eEPC retention (Figure 6I), indicating that vasodilation per se cannot explain this phenomenon. In rodents, adenosine can also trigger the release of vasoactive compounds from mast cells via A1 receptors.20 However, stimulation of A1 receptors with 100 μmol/L inosine28 had no effect on eEPC retention in perfused hearts (Figure 6J).

**Role of P-Selectin Glycoprotein Ligand-1 and P-Selectin in the Mechanism of Adenosine-Dependent EPC Adhesion to Cardiac Microvascular Endothelium**

Because the P-selectin glycoprotein ligand (PSGL)-1 has been previously implicated in eEPC adhesion to the vascular wall,7 we investigated its potential role in adenosine receptor-stimulated eEPC adhesion to MCEC-1 cells. Fucoidan, a polysaccharide known to block PSGL-1 interaction with P-selectin,21 inhibited NECA-dependent stimulation of eEPC adhesion (Figure 7A). Furthermore, NECA-induced eEPC adhesion to MCEC-1 cells was partially blocked if mouse eEPCs (106 cells/mL) were preincubated with 10 μg/mL a blocking monoclonal anti-PSGL-1 antibody (clone 2PH1, Fitzgerald Industries) but was not affected by preincubation with a control isotype-matched antibody (Figure 7B). These data suggest that interaction between PSGL-1 and P-selectin plays a role in adenosine-induced eEPC adhesion. Therefore, we next tested whether stimulation of adenosine receptors on
Figure 6. Adenosine promotes retention of eEPCs in isolated hearts. Retention of eEPCs in mouse hearts was studied using a conventional Langendorff retrograde perfusion system. A through F, Representative fluorescent micrographs of perfused vessels (green) (A and B), retained eEPCs (red) (C and D), and their overlay (E and F) were obtained from hearts perfused with eEPC suspension in the absence (A, C, and E) or presence (B, D, and F) of 10 μmol/L adenosine. (Scale bar =50 μm.) G, I, and J, Retention of eEPCs in hearts perfused in the absence (Control) or presence of 10 μmol/L adenosine, 3 nmol/L CGS21680, and 100 μmol/L inosine was estimated by measuring the area of EPC-emitted fluorescence and by normalizing to the area of endothelial staining in 10 random images of the left ventricle taken for each heart. The data are means±SEM (n=3). *P<0.05 (t test). H, Concentration-response curves of CGS21680 and adenosine effects on coronary flow (mL/min per gram). The data are expressed as percentages from baseline and represent means±SEM (n=5).

Figure 7. Interactions between PSGL-1 and P-selectin contribute to NECA-induced adhesion of eEPCs to MCEC-1 cells. A, Effect of fucoidan on eEPC adhesion to MCEC-1 cells in the absence (open bars) or in the presence of 10 μmol/L NECA (closed bars). The data are means±SEM (n=12). **P<0.01 (t test) compared with corresponding control values. B, Effect of blocking anti–PSGL-1 monoclonal antibody on eEPC adhesion to MCEC-1 cells in the absence (open bars) or presence (closed bars) of 10 μmol/L NECA. EPCs were preincubated for 15 minutes with a PSGL-1 blocking or control (rat IgG1) antibodies and then assayed for adhesion to MCEC-1 cells. The data are means±SEM (n=18). **P<0.01 (t test) compared with corresponding control values. C, Effect of NECA on cell surface P-selectin expression in MCEC-1 cells. Cells were incubated in the absence (open bars) or presence (closed bars) of 10 μmol/L NECA for 15 or 30 minutes at 37°C. Cell surface P-selectin expression was measured by an enzyme-linked immunosassay and presented in arbitrary units calculated from optical density of samples by subtracting corresponding values for nonspecific binding. The data are means±SEM (n=6) **P<0.01 (t test) compared with values obtained in the absence of NECA.

MCEC-1 cells could acutely increase P-selectin expression on the cell surface. Indeed, our results show that stimulation of MCCEC-1 cells with 10 μmol/L NECA for 15 or 30 minutes significantly increased P-selectin expression on the surface of endothelial cells (Figure 7C).

Discussion
Because extracellular adenosine is elevated at sites of ischemia and stimulates neovascularization, we examined the potential role of adenosine in augmenting EPC retention to cardiac microvascular endothelium. We chose mouse embryonic EPCs and cardiac microvascular endothelial cells MCCEC-1 cells as a model to study the role of adenosine receptors in promoting adhesion. Our choice of these cells was determined by their robust growth properties in culture, and hence the availability of considerable quantities required for a systematic pharmacological analysis of adenosine receptors and their functions. EPCs express early endothelial receptors and their functions. EPCs express early endothelial type virtually identical to that of primary cardiac microvascular endothelial cells when cultured at 37°C.
Our study demonstrated that mouse eEPCs preferentially express functional high-affinity A<sub>1</sub> adenosine receptors, whereas MCEC-1 cells preferentially express functional low-affinity A<sub>2B</sub> receptors. We verified that primary cultured HMVEC-c cells also preferentially express functional A<sub>2B</sub> receptors, thus validating the use of MCEC-1 cells as a relevant cell model to study adenosine actions on cardiac microvascular endothelium.

Adenosine has been shown previously to modulate adhesion of other cells to vascular endothelium. Studies in neutrophils suggested differential roles of adenosine receptor subtypes in regulating their adhesion to endothelial cells. Stimulation of A<sub>1</sub> receptors promoted neutrophil adhesion to endothelial cells, whereas stimulation of A<sub>2B</sub> receptors inhibited their adhesion<sup>22</sup>; opposite roles of A<sub>1</sub> and A<sub>2A</sub> receptors in neutrophil adhesion to cardiac vascular endothelium were also demonstrated in the guinea pig isolated heart.<sup>23</sup> Endothelial A<sub>2A</sub> receptors have been shown to inhibit the expression of E-selectin and VCAM-1 stimulated by proinflammatory cytokines and endotoxin in human umbilical cord vein endothelial cells.<sup>24</sup> However, we found no functional presence of A<sub>2A</sub> receptors in MCEC-1 or HMVEC-c cells. Based on these observations, we reasoned that in contrast to the inhibitory action of A<sub>2A</sub> receptors in neutrophil adhesion, A<sub>1</sub> and A<sub>2B</sub> receptors might promote EPC adhesion to microvascular endothelium.

In the present study, we found that mouse eEPCs expressing A<sub>1</sub> receptors increased their adherence to MCEC-1 cells in the presence of the nonselective adenosine receptor agonist NECA under static and flow conditions. However, activation of A<sub>1</sub> receptors on eEPCs per se was not sufficient for efficient stimulation of cell adhesion. NECA was as efficacious as adenosine (4.7±0.3 versus 4.3±0.3-fold stimulation) in promoting EPC adhesion to MCEC-1 cells, but the effect of the selective A<sub>1</sub> agonist CPA was considerably lower than the effect of NECA. Furthermore, uncoupling of A<sub>1</sub> receptors from intracellular signaling pathways with pertussis toxin in injected cells may pass through the targeted organ (eg, heart) and accumulate in other organs such as spleen, liver, and kidney.<sup>2</sup> In this study, we demonstrated that adenosine promotes EPC retention in vasculature of isolated hearts, suggesting its potential use for improvement of cell delivery. Adenosine can be given directly into the coronary circulation, and its extremely short half-life in the bloodstream provides the unique advantage of increasing EPC retention locally. Intracoronary adenosine has been administered in humans without significant adverse events.<sup>28,29</sup> Our data suggest that adenosine could further improve delivery of progenitor cells by increasing their adhesion to cardiac endothelium, a particularly appealing prospect because of the clinical availability of adenosine. Future research may validate the utility of this approach.

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Disclosures

Dr. Hatzopoulos received an honorarium from Momenta Pharmaceuticals for a lecture on EPCs.

References


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http://circres.ahajournals.org/content/suppl/2007/11/21/CIRCRESAHA.107.158147.DC1
Adenosine Receptor-mediated Adhesion of Endothelial Progenitors to Cardiac Microvascular Endothelial Cells

On-line Supplement

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Expanded Methods

Reagents

N⁶-cyclopentyladenosine (CPA), 5'-N-ethylcarboxamidoadenosine (NECA), 4-((N-ethyl-5'-carbamoyladenos-2-yl)-aminoethyl)-phenyl-propionic acid (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and adenosine were purchased from Sigma (St. Louis, MO). Endonorbornan-2-yl-9-methyladenine (N-0861) was a gift from Whitby Research, Inc. (Richmond, VA) and 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine (SCH58261) was a gift from Drs C. Zocchi and E. Ongini (Schering Plough Research Institute, Milan, Italy). 3-isobutyl-8-pyrrolidinoxanthine (IPDX) was synthesized as previously described. Dimethyl sulfoxide (DMSO) was purchased from Sigma. When used as a solvent, final DMSO concentrations in all assays did not exceed 1% and the same DMSO concentrations were used in vehicle controls.

Cell isolation and culture

Mouse cardiac microvascular endothelial cells

MCEC-1, conditionally immortalized mouse cardiac microvascular endothelial cells were generously provided by Dr. J. Mason (National Heart and Lung Institute, UK). The cells were isolated from H-2Kᵇ-tsA58 transgenic mice containing a gene encoding the thermolabile SV40 T antigen. Cell cultures were propagated in the presence of 20 U/mL recombinant mouse IFN-γ (PeproTech, Rocky Hill, NJ) at 33°C on 1% gelatin-coated tissue culture plates containing DMEM supplemented with 10% FBS, 1X Antibiotic-Antimycotic mixture (Sigma), 2 mmol/L L-glutamine, 10 U/mL heparin, and 30 µg/mL ECGF. Six days before experiments, cells were replated and cultured in the
absence of IFN-γ at 37°C. Under these conditions MCEC-1 cells assume the phenotype of primary cardiac microvascular endothelial cells.2

**Human cardiac microvascular endothelial cells**

Primary cultures of human cardiac microvascular endothelial cells (HMVEC-c) were obtained from Cambrex (Walkersville, MD), and cultured using EGM™-2 MV growth medium (Cambrex). HMVEC-c from passages 2 to 5 were used.

**Mouse embryonic endothelial progenitor cells**

Mouse endothelial progenitor cells isolated from E7.5 embryos (eEPCs) have been previously described.3 Cells were maintained in DMEM medium supplemented with 20% FBS, 2 mmol/L L-glutamine, 1 mmol/L pyruvic acid, 1X MEM nonessential Amino Acids (Mediatech Inc, Herndon, VA), 1X Antibiotic Antimycotic mixture (Sigma) and 0.1 mmol/L β-mercaptoethanol.

**Human adult culture-expanded EPCs**

Normal human peripheral blood leukocytes were obtained from human blood donor leukocyte reduction filters (LeukotrapRC, Pall Corporation, East Hills, NY) otherwise discarded by the American Red Cross (Nashville, TN) as previously described;4 three to four filters were pooled per prep to reduce donor variability. Mononuclear cells from leukocytes were obtained by centrifugation on Histopaque 1077 (Sigma) gradients according to manufacturer instructions. Mononuclear cells were directly plated at 10^8 cells/cm² culture dishes and maintained in EBM-2 (Clonetics) with supplements according to previously published protocols.4 EPCs were harvested on day 7 and were identified by uptake of DiI-acLDL and co-staining with UEA-1 lectin as well as
anti-VEGFR2 and anti-VE-cadherin by indirect immunofluorescence as described previously.\(^4\)

**Measurement of cAMP accumulation**

Cyclic AMP accumulation was measured as previously described.\(^5\) Cells growing in 12-well plates were pre-incubated in 150 mmol/L NaCl, 2.7 mmol/L KCl, 0.37 mmol/L NaH\(_2\)PO\(_4\), 1 mmol/L MgSO\(_4\), 1 mmol/L CaCl\(_2\), 5 g/L D-glucose, 10 mmol/L HEPES-NaOH, pH 7.4 and 1 U/mL adenosine deaminase containing the cAMP phosphodiesterase inhibitor papaverine (1 mmol/L) for 15 min at 37°C. Adenosine agonists and antagonists were added to cells, and the incubation was allowed to proceed for 3 min at 37°C. The reaction was stopped by the addition of 1/5 volume of 25% trichloroacetic acid. The extracts were washed five times with 10 volumes of water-saturated ether. Cyclic AMP concentrations were determined using a cAMP assay kit (GE Healthcare, Little Chalfont, UK).

**Real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Real-time RT-PCR analysis was performed as previously described.\(^6\) Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Real-time RT-PCR was carried out on ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primer pairs and FAM-labeled probes for murine and human adenosine receptors and \(\beta\)-actin were provided by Applied Biosystems. RT-PCR reactions utilizing 1 \(\mu\)g of DNase-treated total RNA were performed under conditions recommended by the manufacturer. A standard curve for each amplicon was obtained using serial dilutions of total RNA. The results from triplicate polymerase chain reactions for a given gene at each time point were used to determine mRNA quantity.
relative to the corresponding standard curve. The relative mRNA quantity for a given gene measured from a single reverse transcription reaction was divided by the value obtained for β-actin to correct for fluctuations in input RNA levels and varying efficiencies of reverse transcription reactions.

**Analysis of cell adhesion under static conditions**

Endothelial cells were grown to confluency in 96-well plates pre-coated with 1% porcine gelatin type A (Sigma). One hour before experiments, the growth medium in each well was replaced with 70 μl of DMEM. Progenitor cells were fluorescently labeled by incubating with 5 μmol/L calcein-AM (Molecular Probes, Eugene, OR) in DMEM (10^7 cells/mL) for 30 min at 37°C. Labeled cells were washed three times by centrifugation and resuspended in DMEM (10^6 cells/mL). In some experiments, eEPCs were pre-incubated with 10 μg/mL rat monoclonal anti-mouse PSGL-1 antibody (clone 2PH1, Fitzgerald Industries, Concord, MA) or control rat IgG1 (BD Biosciences, San Jose, CA) for 15 min at room temperature. The assay was started by transferring 50 μl of labeled cell suspension to each well covered with endothelial monolayer followed immediately by addition of 30 μl of DMEM containing 5X concentrations of test compounds or controls. Plates were placed in a cell culture incubator at 37°C. At the end of incubation periods indicated in the Results section, 96-well plates were gently washed twice with DMEM and twice with Tyrode’s buffer (150 mmol/L NaCl, 2.7 mmol/L KCl, 0.37 mmol/L NaH2PO4, 1 mmol/L MgSO4, 1 mmol/L CaCl2, 5 g/L D-glucose, 10 mmol/L HEPES-NaOH, pH 7.4) and finally 150 μl of Tyrode’s buffer was added to each well. Cell adhesion was measured using a fluorescence plate reader at excitation and emission wavelengths of 485 and 535 nm, respectively. The percentage of adhered
fluorescent cells was calculated using a calibration curve constructed for each experiment by measuring fluorescence of predetermined numbers of labeled cells.

**Analysis of cell adhesion under flow conditions**

Adhesion assays under flow conditions were performed using a parallel plate flow chamber (Glycotech, Rockville, MD) following the manufacturer’s instructions. Cell suspension or cell-free medium were drawn into chambers by a syringe pump (Model 44, Harvard Apparatus, Inc., Holliston, MA) at a constant rate to generate a desired wall shear stress (τ, dynes/cm²) using the formula \( \tau = \frac{6Q\mu}{a^2b} \), where \( Q \) is flow rate, \( \mu \) is medium viscosity, \( b \) is channel width, and \( a \) is channel height. After flow chamber assembly, the endothelial monolayer was perfused for 10 min with DMEM containing 10 μmol/L NECA or its vehicle, and then with an EPC suspension in the same medium for another 10 min. For cell detachment experiments, the flow was stopped for 1 min and then resumed at increments of 1 dynes/cm² at 30-s intervals. Cells were observed with a Nikon model TMS inverted phase contrast microscope (Nikon USA, Melville, NY) and videotaped with a Sony DCR-TRV480 color video camera (Sony Corporation, Tokyo, Japan). Cell adhesion was determined by analysis of digitized video recordings using NIH Image software.

**Transendothelial migration assay**

MCEC-1 cells were plated on polycarbonate membrane (3-μm pore filters; Corning Costar, Acton, MA) pre-coated with 10 μg/mL fibronectin to obtain confluent endothelial monolayers. Confluency was confirmed by measuring permeability for FITC-dextran 3,000 (Molecular Probes, Eugene, OR). Mouse eEPCs were fluorescently labeled with calcein-AM as described above, and \( 10^5 \) cells in 500 μL of DMEM containing 10
µmol/L NECA or its vehicle were placed in the upper chamber on top of the MCEC-1 monolayer. The chambers were placed in a 24-well culture dish filled with 500 µL of DMEM. After incubation under humidified atmosphere of air/CO₂ (19:1) at 37°C for 4 hours, the lower side of the filter was washed with PBS and fixed with 1% paraformaldehyde. Fluorescently labeled EPCs migrating into the lower chamber were counted manually in 3 random microscopic fields.

**Cell-based P-selectin enzyme-linked immunoassay**

To analyze cell-surface P-selectin expression, we used a previously published method. In brief, MCEC-1 cells were incubated in the presence of 10 µmol/L NECA or its vehicle (DMSO) at 37°C for periods indicated and then fixed for 5 min with 0.5% paraformaldehyde solution. After washing and blocking, cells were incubated with 5 µg/mL rat anti-mouse CD62P antibodies (Fitzgerald Industries) or rat isotype-matched control antibody (BD Biosciences) for 1 hour. After washing, a secondary goat anti-rat horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) was added for 1 hour followed by washing and then analyzed at 450 nM after addition of substrate.

**Isolated mouse heart model**

Twenty eight male C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME) at age of 6-8 weeks were used. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Hearts were rapidly removed from mice anesthetized with inhalation of isoflurane. The aorta was cannulated and connected to a Langendorff apparatus. The Langendorff perfusion was carried out at a constant flow rate of 4 mL/min with modified
Krebs-Henseleit (KH) buffer (118 mmol/L NaCl, 25 mmol/L NaHCO₃, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L NaH₂PO₄, 2.5 mmol/L CaCl₂, 11 mmol/L glucose, 0.5 mmol/L EDTA, pH 7.4) equilibrated with a gas mixture of 95% O₂ and 5% CO₂ at 37°C. After a 30 min stabilization period, hearts were perfused with 1.5 mg/L FITC-conjugated Helix pomatia lectin (Sigma) for 10 min to label endothelial cells of perfused vessels followed by a 10 min washing period with KH buffer. Hearts were then perfused with eEPCs pre-labeled with DiI C16 (Invitrogen, Carlsbad, CA) and resuspended in KH buffer containing 2% FBS (2,500 cells/mL) in the presence or absence of 10 μmol/L adenosine for 10 min. After washing with KH buffer for 10 min to remove unbound eEPCs, hearts were dissected, and placed on a microscopic stage. Retention of eEPCs in hearts was analyzed by taking 10 random images of the left ventricle using epifluorescence microscopy (20X objective). Area of EPC-emitted fluorescence was measured using NIH ImageJ software and normalized to the area of vascular endothelium stained with FITC-lectin.

To compare the vasodilatory effects of adenosine and CGS21680, isolated hearts were perfused with KH buffer at a constant hydrostatic pressure of 80 mmHg. The left ventricle was vented with a small polyethylene apical drain. Total coronary artery flow was measured by timed collection of effluent dripping from the heart into a graduated cylinder. After 20-min stabilization period, hearts were switched to pacing using a Grass S9 stimulator (Grass, Quincy, MA, U.S.A.). Hearts were paced at 400 beats min⁻¹ via silver electrodes (5 ms, 5 V) and stabilized for additional 10 min. Upon reaching a steady-state coronary flow, increasing concentrations of adenosine receptor agonists were infused into the aortic cannula immediately above the heart at a rate of 1% of the basal
flow using a Harvard infusion pump (Harvard Apparatus) to achieve a desired drug concentration in the perfusate. All agonist concentration-response curves were constructed noncumulatively as previously described and one concentration-response curve was performed on each heart.
Online Figure 1. Stimulation of eEPC adhesion to MCEC-1 with NECA. EPC adhesion was measured under static conditions in the absence (Basal) or presence of 10 μmol/L NECA (+NECA) as described in the Methods section. In parallel experiments, we used the following modifications: MCEC-1 monolayers were pre-incubated with 10 μmol/L NECA for 30 minutes at 37°C. After washing MCEC-1 three times with DMEM, untreated eEPCs were added to MCEC-1 monolayers for adhesion assay in the absence of NECA (MCEC-1 pre-treated with NECA); labeled eEPCs were pre-incubated with NECA, washed, and then eEPCs were added to untreated MCEC-1 monolayers for adhesion assay in the absence of NECA (eEPCs pre-treated with NECA); both MCEC-1 and eEPCs were individually pre-incubated with NECA, washed, and then eEPCs were added to MCEC-1 monolayers for adhesion assay in the absence of NECA (MCEC-1 and eEPCs pre-treated with NECA). Data are mean±SEM, n=12, ***p<0.001, # p=0.053; t-test, compared to basal adhesion.
Online Figure 2. Effect of pre-treatment of eEPCs with Pertussis Toxin on their adhesion to MCEC-1 in the presence of TNF-α. eEPCs were pre-incubated in the absence (Control) or presence of 100 nmol/L pertussis toxin (PTX) for 12 hours. After washing with DMEM, eEPCs were added to MCEC-1 monolayers for adhesion assay under static conditions in the presence of indicated concentrations of TNF-α. Cells were co-incubated for 30 minutes. This incubation time in the presence of TNF-α has been previously shown to be adequate for inducing early-onset endothelial adhesivity. Data are mean±SEM, n=7.
Online Figure 3. Transendothelial migration (TEM) of eEPCs. Migration of eEPCs through MCEC-1 monolayers was measured in the absence (Basal) or presence of 10 μmol/L NECA as described in the Expanded Methods section. Data are mean±SEM, n=7, p=0.068.
Expanded Table. Affinity or potency of agonists and antagonists at human (h), rat (r), guinea pig (gp) and mouse (m) adenosine receptor subtypes (Kᵢ, Kₒ, Kᵦ, IC₅₀ or EC₅₀ values in nmol/L with 95% confidence intervals or ±SEM in parentheses and in log mol/L).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Receptor subtypes</th>
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<tr>
<td></td>
<td>A₁</td>
</tr>
<tr>
<td>NECA</td>
<td></td>
</tr>
<tr>
<td>h 14 (6.4-29); -7.9 [1]*</td>
<td>h 20 (12-59); -7.7 [1]</td>
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<tr>
<td>r 6.3 (±0.52); -8.2 [4]</td>
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<td>r 11 (7-17); -8 [5]</td>
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<td>r 30 (21-43); -7.5 [6]</td>
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<tr>
<td>CPA</td>
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<td>h 790 (470-1,360); -6.1 [1]</td>
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<td>r 0.59 (±0.02); -9.2 [4]</td>
<td>r 460 (±15); -6.3 [4]</td>
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<td>r 0.8 (0.6-1.0); -9.1 [5]</td>
<td>r 2,000 (1,400-2,900); -5.7 [5]</td>
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<td>r 4 (2.8-5.8); -8.4 [6]</td>
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<td>m 3.3 (0.9-12); -8.5 [6]</td>
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<td><strong>m 1.2 (0.6-2.4); -8.9</strong></td>
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<td>CGS21680</td>
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<td>h 290 (230-360); -6.5 [1]</td>
<td>h 27 (12-59); -7.6 [1]</td>
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<td>r 3,100 (±470); -5.5 [7]</td>
<td>r 22 (±4.3); -7.7 [7]</td>
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<td>r 36,300 (20,000-66,100); -4.44 [6]</td>
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<td></td>
<td>m 1.5 (1.1-2.1); -8.8</td>
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<td><strong>SCH58261</strong></td>
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<td>m 854 (464-1,570); -6.1</td>
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<td><strong>N-0861</strong></td>
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<td></td>
<td>m 511 (398-656); -6.3</td>
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</table>

* data from references cited within brackets
† data from the current study are presented in boldface.

1. Klotz et al., 1998, transfected CHO cells; radioligands [³H]CCPA (A₁), [³H]NECA (A₂A)⁹
2. Linden et al., 1999, transfected HEK 293 cells; radioligand ¹²⁵I-ABOPX.¹¹
4. Bruns et al., 1986; brain membranes (A1), striatum (A2A); radioligands \[^{3}\text{H}]\text{CHA} (A1), \[^{3}\text{H}]\text{NECA} (A2A)\).

5. Cristalli et al., 1992; brain membranes (A1), striatum (A2A); radioligands \[^{3}\text{H}]\text{DPCPX} (A1), \[^{3}\text{H}]\text{NECA} (A2A)\).

6. Maemoto et al, 1997; brain cortex (A1), striatum (A2A); radioligands \[^{3}\text{H}]\text{DPCPX} (A1), \[^{3}\text{H}]\text{CGS21680} (A2A)\).

7. Hutchison et al., 1989; rat brain; radioligands \[^{3}\text{H}]\text{CHA} (A1), \[^{3}\text{H}]\text{NECA} (A2A)\).

8. Lohse et al., 1987; brain membranes (A1), striatum (A2A); radioligands \[^{3}\text{H}]\text{PIA} (A1), \[^{3}\text{H}]\text{NECA} (A2A)\).

9. Kreckler et al, 2006; transfected HEK 293; radioligands \[^{125}\text{I}]\text{I-AB-MECA} (A1), \[^{125}\text{I}]\text{ZM241385} (A2A), \[^{3}\text{H}]\text{MRS1754} (A2B)\).

10. Ongini et al., 1999, transfected CHO cells (A1 and A2A) or HEK 293 cells (A2A); radioligands \[^{3}\text{H}]\text{DPCPX} (A1), \[^{3}\text{H}]\text{SCH58261} (A2A)\).


12. Baraldi et al., 1994; brain membranes (A1), striatum (A2A); radioligand \[^{3}\text{H}]\text{CHA} (A1), \[^{3}\text{H}]\text{CGS21680} (A2A)\).

13. Lopes et al, 2004; striatum, radioligand \[^{3}\text{H}]\text{SCH58261}\).

14. Feoktistov et al, 2001; transfected CHO cells (A1 and A2A), transfected HEK 293 or HEL cells (A2B); radioligands \[^{3}\text{H}]\text{DPCPX} (A1), \[^{3}\text{H}]\text{NECA} (A2A), \[^{3}\text{H}]\text{ZM241685} (A2B)\).

REFERENCES


