Adenosine Receptor-mediated Adhesion of Endothelial Progenitors to Cardiac Microvascular Endothelial Cells

On-line Supplement

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Expanded Methods

Reagents

N^6-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.[1] 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-$2K^b$-tsA58 transgenic mice containing a gene encoding the thermolabile SV40 T antigen.[2] 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, Hemdon, 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/cm^2 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_2PO_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 β-actin were provided by Applied Biosystems. RT-PCR reactions utilizing 1 μg 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 IgG_1 (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 NaH_2PO_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 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 drown 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/cm^2) using the formula $\tau = 6Q\mu/a^2b$, where Q is flow rate, μ 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/cm^2 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/CO_2 (19:1) at 37^0C 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.[7] In brief, MCEC-1 cells were incubated in the presence of 10 μmol/L NECA or its vehicle (DMSO) at 37^oC 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.

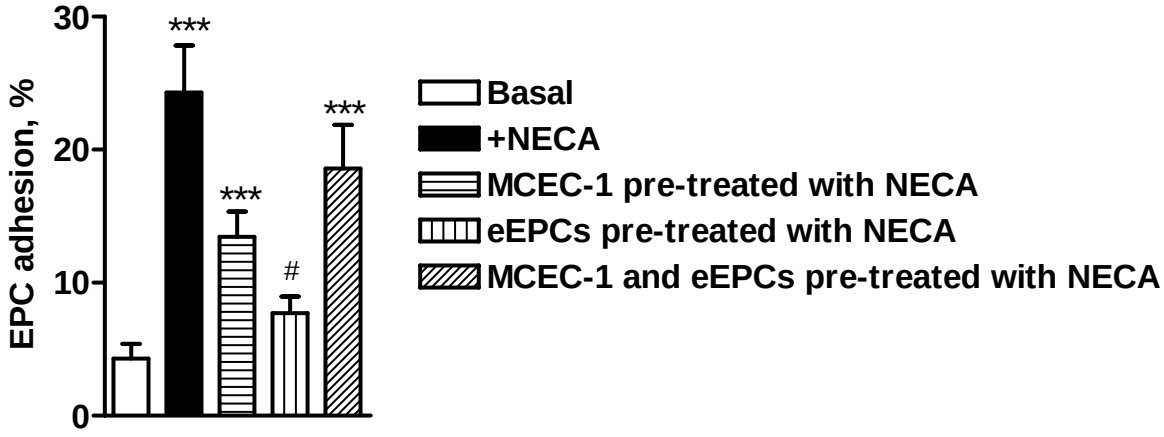
Isolated mouse heart model

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

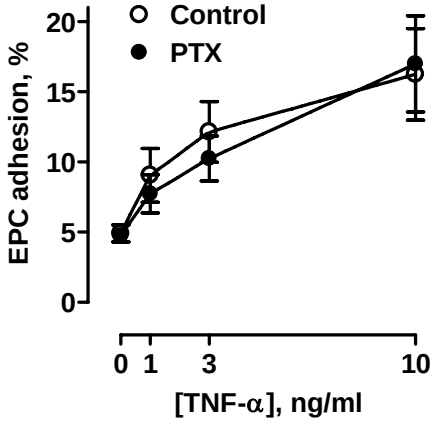
Krebs-Henseleit (KH) buffer (118 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L NaH_2PO_4, 2.5 mmol/L $CaCl_2$, 11 mmol/L glucose, 0.5 mmol/L EDTA, pH 7.4) equilibrated with a gas mixture of 95% O_2 and 5% CO_2 at 37ºC. After a 30 min stabilization period, hearts were 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

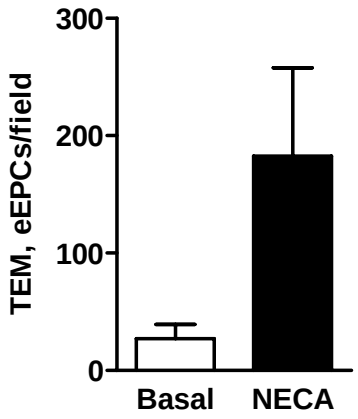
flow using a Harvard infusion pump (Harvard Apparatus) to achieve a desired drug concentration in the perfusate. All agonist concentration-response curves were constructed noncumulatively as previously described[8] and one concentration-response curve was performed on each heart.



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^{o}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.[9] 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 (K_i, K_D, K_B, IC_{50} or EC_{50} values in nmol/L with 95% confidence intervals or ±SEM in parentheses and in log mol/L).

Compounds	Receptor subtypes		
	A_1	A_{2A}	A_{2B}
NECA	h 14 (6.4-29); -7.9 [1]*	h 20 (12-59); -7.7 [1]	h 330 (±60); -6.5 [2]
	r 6.3 (±0.52); -8.2 [4]	r 10 (±0.5); -8 [4]	h 360 (±120); -6.4 [3]
	r 11 (7-17); -8 [5]	r 22 (20-25); -7.7 [5]	**m 449 (291-693); -6.3†**
	r 30 (21-43); -7.5 [6]	r 4.2 (3-5.9); -8.38 [6]	
	m 15 (10-22); -7.8 [6]		
CPA	h 2.3 (1.5-3.4); -8.6 [1]	h 790 (470-1,360); -6.1 [1]	h 34,400 (±11,100); -4.5 [2]
	r 0.59 (±0.02); -9.2 [4]	r 460 (±15); -6.3 [4]	h 21,000(±4,300); -4.7 [3]
	r 0.8 (0.6-1.0); -9.1 [5]	r 2,000 (1,400-2,900); -5.7 [5]	
	r 4 (2.8-5.8); -8.4 [6]	r 148 (42-525); -6.8 [6]	
	m 3.3 (0.9-12); -8.5 [6]		
	m 1.2 (0.6-2.4); -8.9		
CGS21680	h 290 (230-360); -6.5 [1]	h 27 (12-59); -7.6 [1]	h 361,000 (±21,000); -3.4 [2]
	r 3,100 (±470); -5.5 [7]	r 22 (±4.3); -7.7 [7]	
	r 36,300 (20,000-66,100); -4.44 [6]	r 3.6 (1.2-10.5); -8.44 [6]	
	m 14,100 (7,000-28,200); -4.85 [6]		

DPCPX	h 3.9 (3.5-4.2); -8.4 [1]	h 129 (35-260); -6.9 [1]	h 50 (±3.7); -7.3 [2]
	r 0.3; -9.5 [8]	r 340; -6.5 [8]	h 51 (±6.1); -7.3 [3]
	r 2.8 (2.6-3.1); 9.55 [6]	r 151 (141-170); -6.8 [6]	m 86 (±36); -7.1 [9]
	m 1.5 (±0.5); -8.8 [9]	m 598 (±71); -6.2 [9]	
	m 0.4 (0.3-0.5); -9.3 [6]		
	m 1.5 (1.1-2.1); -8.8		
SCH58261	h 290 (210-410); -6.5 [10]	h 0.6 (0.5–0.7); -9.2 [10]	h > 100; > -5 [11]
	r 120 (100-140); -6.9 [12]	r 2.3 (2-2.7); -8.6 [12]	**m 1,868 (1,404-2,486); -5.7**
	m 854 (464-1,570); -6.1	m 1.0 (0.4-1.6); -9.0 [13]	
IPDX	h 24,000 (±8,000); -4.6 [14]	h 36,000 (±8,000); -4.4 [14]	h 625 (±71); -6.2 [14]
	m 20,000 (16,230-24,870); -4.6		**m 603; -6.2**
N-0861	gp 575 (±86); 6.2 [15]		gp 56,200 (±11,400), -4.3 [15]
	m 511 (398-656); -6.3		**m 39,350 (23,600-65,610); -4.4**

* data from references cited within brackets

† data from the current study are presented in boldface.

1. Klotz et al., 1998, transfected CHO cells; radioligands [^{3}H]CCPA (A_1), [^{3}H]NECA (A_{2A}).[10]

2. Linden et al., 1999, transfected HEK 293 cells; radioligand ^{125}I-ABOPX.[11]

3. Ji and Jacobson, 1999, transfected HEK 293 cells; radioligand [^{3}H]ZM241685.[12]

4. Bruns et al., 1986; brain membranes (A_1), striatum (A_{2A}); radioligands [^{3}H]CHA (A_1), [^{3}H]NECA (A_{2A}).[13]

5. Cristalli et al., 1992; brain membranes (A_1), striatum (A_{2A}); radioligands [^{3}H]DPCPX (A_1), [^{3}H]NECA (A_{2A}).[14]

6. Maemoto et al, 1997; brain cortex (A_1), striatum (A_{2A}); radioligands [^{3}H]DPCPX (A_1), [^{3}H]CGS21680 (A_{2A}).[15]

7. Hutchison et al., 1989; rat brain; radioligands [^{3}H]CHA (A_1), [^{3}H]NECA (A_{2A}).[16]

8. Lohse et al., 1987; brain membranes (A_1), striatum (A_{2A}); radioligands [^{3}H]PIA (A_1), [^{3}H]NECA (A_{2A}).[17]

9. Kreckler et al, 2006; transfected HEK 293; radioligands [^{125}I]I-AB-MECA (A_1), [^{125}I]ZM241385 (A_{2A}), [^{3}H]MRS1754 (A_{2B}).[18]

10. Ongini et al., 1999, transfected CHO cells (A_1 and A_{2A}) or HEK 293 cells (A_{2A}); radioligands [^{3}H]DPCPX (A_1), [^{3}H]SCH58261 (A_{2A}).[19]

11. Feoktistov and Biaggioni, 1998, NECA-stimulated HEL cells.[20]

12. Baraldi et al., 1994; brain membranes (A_1), striatum (A_{2A}); radioligand [^{3}H]CHA (A_1), [^{3}H]CGS21680 (A_{2A}).[21]

13. Lopes et al, 2004; striatum, radioligand [^{3}H]SCH58261.[22]

14. Feoktistov et al, 2001; transfected CHO cells (A_1 and A_{2A}), transfected HEK 293 or HEL cells (A_{2B}); radioligands [^{3}H]DPCPX (A_1), [^{3}H]NECA (A_{2A}), [^{3}H]ZM241685 (A_{2B}).[1]

15. Martin et al, 1992; NECA-stimulated atrium (A_1) or aorta (A_{2A}).[23]

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