Differential Expression of Adenosine Receptors in Human Endothelial Cells

Role of A2B Receptors in Angiogenic Factor Regulation

Igor Feoktistov, Anna E. Goldstein, Sergey Ryzhov, Dewan Zeng, Luiz Belardinelli, Tatyana Voyno-Yasenetskaya, Italo Biaggioni

Abstract—Adenosine has been reported to stimulate or inhibit the release of angiogenic factors depending on the cell type examined. To test the hypothesis that differential expression of adenosine receptor subtypes contributes to endothelial cell heterogeneity, we studied microvascular (HMEC-1) and umbilical vein (HUVEC) human endothelial cells. Based on mRNA level and stimulation of adenylate cyclase, we found that HUVECs preferentially express A2A adenosine receptors and HMEC-1 preferentially express A2B receptors. Neither cells expressed A1 or A3 receptors. The nonselective adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) increased expression of interleukin-8 (IL-8), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) in HMEC-1, but had no effect in HUVECs. In contrast, the selective A2A agonist 2-p-(2-carboxyethyl)phenylethylamino-NECA (CGS 21680) had no effect on expression of these angiogenic factors. Cotransfection of each type of adenosine receptors with a luciferase reporter in HMEC-1 showed that A2B receptors, but not A1, A2A, or A3, activated IL-8 and VEGF promoters. These effects were mimicked by constitutively active α12, α132, and α133, but not α1, or αG13-3. Furthermore, stimulation of phospholipase C indicated coupling of A2B receptors to Gq proteins in HMEC-1. Thus, differential expression of adenosine receptor subtypes contributes to functional heterogeneity of human endothelial cells. A2B receptors, predominantly expressed in human microvascular cells, modulate expression of angiogenic factors via coupling to Gq, and possibly via G12/13. (Circ Res. 2002;90:531-538.)

Key Words: adenosine receptors ■ vascular endothelium ■ angiogenesis ■ vascular endothelial growth factor ■ interleukin-8
has been attributed, at least in part, to activation of endothelial-derived factors in some vascular beds.\textsuperscript{10,11} Adenosine is thought to promote endothelial barrier function and to maintain vascular integrity.\textsuperscript{4,7} Adenosine has also been found to stimulate the proliferation of capillary endothelial cells\textsuperscript{12-14} and to promote neovascularization.\textsuperscript{15}

Regulation of neovascularization depends on a delicate balance of proangiogenic and antiangiogenic factors, proteases and their inhibitors, and adhesion molecules. Vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) occupy a particular place among positive modulators of angiogenesis due to their potency and the reported essential role each of them plays in promoting angiogenesis.\textsuperscript{16-18} Extracellular adenosine, generated in high concentrations in hypoxic tissues, has been found to modulate secretion of VEGF.\textsuperscript{12-14,19-22} However, depending on the cell type studied, adenosine has been shown to either stimulate or inhibit secretion of proangiogenic factors. Recent studies demonstrated that adenosine stimulates VEGF production in human retinal endothelial cells.\textsuperscript{23,24} This effect was attributed to activation of $A_2A$ adenosine receptors.\textsuperscript{13,14} In contrast, adenosine inhibited hypoxia-induced VEGF production in rat pheochromocytoma P12 cells, and this effect was attributed to activation of $A_2B$ adenosine receptors.\textsuperscript{21,22}

In this study, we characterized the adenosine receptor subtypes present in 2 different human endothelial cell types: one obtained from large venous conduit vessels, HUVECs, and the other derived from skin microvasculature, HMEC-1. Our purpose was to test the hypothesis that diverse endothelial cells would show differential expression of adenosine receptor subtypes and that this will translate into distinct phenotypes in regards to expression of angiogenic factors.

**Materials and Methods**

**Cell Culture**
HUVECs and HMEC-1 were kindly provided by Dr D.E. Yanghan (Vanderbilt University, Nashville, Tenn). Chinese hamster ovary CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, Va).

**Chemicals**
5′-N-ethylcarboxamidoadenosine (NECA) and 2-p-(2-carboxyethyl)phenylethylamino-NECA (CGS21680) were purchased from Research Biochemicals, Inc. 3-Isobutyl-1-methylxanthine (IPDX) was synthesized as previously described.\textsuperscript{25}

**Gene Expression Assay**
Total RNA was isolated using Quiagen RNeasy Mini Kit. Expression of angiogenic factors was evaluated using gene expression arrays (Super Array, Inc). Human adenosine receptors gene expression array was custom designed by Super Array, Inc. The assay was performed according to manufacturer’s instructions. In brief, gene-specific [$^{32}$P]-labeled cDNA probes were generated from 5 to 10 $\mu$g of total RNA using gene-specific set of primers for reverse transcription. The cDNA probes were then hybridized with gene-specific cDNA fragments spotted on nylon membrane. The relative expression level of each gene was analyzed using a PhosphorImager and Image Quant software (Molecular Dynamics).

**Reverse Transcription–Polymerase Chain Reaction**
Two micrograms of total RNA from each sample were subjected to reverse transcription followed by 40 cycles of amplification using Promega Access reverse transcription–polymerase chain reaction (RT-PCR) system in accordance with manufacturer’s instructions. PCR primers were synthesized to target mRNA sequence representing parts of different exons in human $A_1$, $A_{2A}$, and $A_2B$ adenosine receptors genes to yield PCR products of 500 to 800 base pairs. The following primer pairs were used for $A_1$: 5′-TCTGGGGCGTGAAGGTGAAC-3′ (sense) and 5′-AGTTGGCGCGGTCGAAGAAG-3′ (antisense); $A_{2A}$: 5′-TGCTTCGTCGTCCTACAC-3′ (sense) and 5′-GGTCCGGTCGTCACTGGCAT-3′ (antisense); and $A_{2B}$: 5′-CCCTTTGGCATTACCATCAG-3′ (sense) and 5′-CCTGACACCTTCACGTGTA-3′ (antisense). PCR primers for human $A_1$ adenosine receptor were used as described by Mitchell et al.\textsuperscript{24} Purified PCR fragments were analyzed by restrictive digestion.

Real-time RT-PCR was performed on ABI Prism Sequence Detection System 5700 (PE Applied Biosystems) in accordance with manufacturer’s recommendations.

**Transfections and Luciferase Reporter Assay**
HMEC-1 and CHO-K1 cells were transfected using Fugene 6 transfection reagent (Roche) with cDNA described in the Results section and luciferase reporters at a ratio of 10:1. The same ratio, 10:1, was used for experimental firefly luciferase reporter:control Renilla luciferase reporter combination. VEGF promoter–driven luciferase reporter, a firefly luciferase reporter plasmid, comprising 5′-flanking −1005 to +379 base pairs of the human VEGF gene\textsuperscript{25} was kindly provided by Dr G.L. Semenza, Johns Hopkins Hospital, Baltimore, Md. IL-8 promoter-driven luciferase reporter −133-loc, a firefly luciferase reporter plasmid, comprising 5′-flanking −133 to +44 base pairs of the human IL-8 gene\textsuperscript{26} was generously gifted by Dr Naofumi Mukaida, Kanazawa University, Ishikawa, Japan. The cDNAs encoding the human adenosine receptors in the pRc/CMV expression vector (Invitrogen) were a generous gift from Drs P.R. Schofield and A. Townsend-Nicholson (Garvan Institute of Medical Research, Australia). Plasmids $\alpha$RCpCDNA, $\alpha$LQpCDNA, $\alpha$QLpCDNA, $\alpha$LPpCDNA, $\alpha$LPpCDNA, $\alpha$LCPpCDNA, $\alpha$CRpCDNA, and $\alpha$QpCDNA, encoding constitutively active $\alpha$ subunits of G-proteins, were constructed as described previously.\textsuperscript{27-30} Reporter activity was measured 30 hours after transfection using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activities were normalized against Renilla luciferase activities from the coexpressed pRL-TK (Promega) and expressed as relative luciferase activities over basal (set as 1).

**Measurement of Second Messengers and Angiogenic Factors**

**cAMP concentrations and formation of [H]inositol phosphates** were determined as previously described.\textsuperscript{31} IL-8 and VEGF concentrations were measured using ELISA kits (R&D Systems) as previously described.\textsuperscript{31}

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

**Expression of Adenosine Receptor mRNA**
Gene expression array results indicate that HUVECs preferentially express mRNA encoding $A_2A$ receptors (Figure 1A). As a percentage of $\beta$-actin expression, HUVECs expressed levels of $A_{2A}$ and $A_{2B}$ receptors of 3.2±0.09% and 0.38±0.04%, respectively (Figure 1B). Therefore, the ratio of expression of $A_{2A}$:$A_{2B}$ was approximately 10 to 1. In contrast, HMEC-1 preferentially express mRNA encoding $A_{2A}$ receptors (Figure 1A). HMEC-1 expressed mRNA levels for $A_{2B}$ and $A_{2A}$ receptors of 0.55±0.07% and 0.13±0.01% of $\beta$-actin mRNA, respectively (Figure 1B). Therefore, the ratio of
expression of A_{2B}/A_{2A} was approximately 4 to 1. No mRNA encoding A_1 or A_3 receptors was detected by gene expression array in either HUVECs or HMEC-1. In control experiments, all 4 subtypes of adenosine receptors were detected in RNA isolated from human brain using the same technique (4.5\pm0.3\% for A_1, 5.8\pm0.4\% for A_{2A}, 0.48\pm0.02\% for A_{2B}, and 1.5\pm0.1\% for A_3 adenosine receptors as a percentage of \beta-actin expression).

To further verify the absence of A_1 and A_3 mRNA in HUVECs and HMEC-1, we used RT-PCR techniques. Only A_{2A} and A_{2B}, but not A_1 or A_3, receptor mRNA was detected by RT-PCR in both cells (Figure 1C). All 4 subtypes of adenosine receptor mRNA were detected in control experiments using total RNA isolated either from human brain or from CHO cells expressing each of the human adenosine receptor subtypes. Differential expression of adenosine receptors was also confirmed using real-time RT-PCR, which showed preferential expression of A_{2A} receptors in HUVECs and A_{2B} in HMEC-1 (online Figure 1 in the online data supplement available at http://www.circresaha.org).

**Regulation of Adenylate Cyclase: Functional Expression of Adenosine Receptor Subtypes**

Both A_{2A} and A_{2B} adenosine receptors are known to stimulate adenylate cyclase. Therefore, we measured accumulation of cAMP as a way to determine if differential expression of mRNA translates into differential pharmacological profile of adenosine receptors expressed in HUVECs and HMEC-1. Our results confirmed the predominance of A_{2A} receptors in HUVECs (Figure 2A). The A_{2A}-selective agonist CGS 21680 activated adenylate cyclase with an EC_{50} of 469 nmol/L. The nonselective agonist NECA was slightly less potent (EC_{50} of 950 nmol/L), but was more efficacious than CGS 21680. This pharmacological profile is consistent with predominant expression of A_{2A} adenosine receptors.\textsuperscript{32}

In contrast, we observed no evidence of functional coupling of A_{2B} adenosine receptors to adenylate cyclase in HMEC-1. NECA stimulated accumulation of cAMP with an EC_{50} of 14 \mu mol/L, whereas CGS 21680 was virtually ineffective (Figure 2B). This pharmacological profile is consistent with the functional presence of A_{2B} receptors only.

**Adenosine-Induced Expression of Angiogenic Factors**

Incubation of HMEC-1 in the presence of 100 \mu mol/L NECA and 1 U/mL adenosine deaminase for 6 hours increased mRNA expression of the angiogenic factors IL-8, bFGF, and VEGF by 27.1\pm6.2-, 3.9\pm0.2-, and 3.9\pm0.8-fold, respectively.
Figure 3. Modulation of angiogenic factors expression by adenosine. Increase in the levels of IL-8, bFGF, and VEGF mRNA expression (normalized to β-actin mRNA expression) in HMEC-1 and HUVECs induced by incubating with the stable adenosine agonist 100 μmol/L NECA for 6 hours. Data were calculated from gene expression array analysis and expressed as mean±standard error of 3 experiments.

Effect of adenosine receptors on VEGF and IL-8 levels, compared with cells incubated with vehicle and 1 μmol/L adenosine deaminase. In contrast, NECA did not produce any significant increase in expression of these factors in HUVECs (Figure 3). Expression levels of mRNA for other members of the VEGF family factors VEGF-B, VEGF-C, and VEGF-D were not significantly changed after incubation with NECA in either HMEC-1 or HUVECs (online Figure 2).

These results were confirmed with measurements of IL-8 and VEGF protein levels in conditioned media. NECA produced a dose-dependent increase in levels of VEGF (Figure 4A) and IL-8 (Figure 4B) in conditioned media after 6 hours of incubation with HMEC-1. The selective A2B antagonist IPDX23 competitively inhibited NECA-induced IL-8 secretion with a Ki of 1.5 μmol/L (online Figure 3). In contrast, the A2A selective agonist CGS 21680 at a concentration of 10 μmol/L, at which it produces maximal activation of A2A adenosine receptors, had no significant effect on basal levels of VEGF and IL-8. Incubation of HUVECs with NECA and CGS 21680 up to 24 hours did not change basal levels of VEGF and IL-8 in conditioned media, whereas 1 μmol/L thrombin, used as a positive control, increased IL-8 concentrations from 90±11 pg/mL to 1.1±0.08 ng/mL (online Figure 4).

Effect of Adenosine Receptors on Activity of IL-8 and VEGF Promoters
To examine the role of adenosine receptor subtypes in transcriptional regulation of IL-8 and VEGF mRNA expression, plasmids encoding human A1, A2A, A2B, and A3 adenosine receptors, or an empty expression vector (mock transfection), were cotransfected either with IL-8 promoter–driven luciferase reporter or with VEGF promoter–driven luciferase reporter plasmids in HMEC-1. Twenty-four hours after transfection, cells were incubated in the presence or absence of 100 μmol/L NECA for 6 additional hours. Under the conditions of our experiments, NECA increased activity of VEGF and IL-8 promoters in mock-transfected cells by 1.2- and 2-fold, respectively, compared with vehicle-treated cells. Cotransfection of VEGF or IL-8 reporters with A1 or A3 adenosine receptors had no significant effect, and cotransfection with A2A adenosine receptors attenuated by 84±17% and 73±8% NECA-induced stimulation of VEGF and IL-8 promoters, respectively. In contrast, cotransfection of VEGF or IL-8 reporters with adenosine A2B receptors in HMEC-1 resulted in a 2.6±0.1- and 11.7±0.4-fold NECA-induced stimulation of their activity, respectively (Figure 5). The observed effects were not due to variation of transfection efficiency because such differences were overcome by normalization of data with the cotransfected control constitutively active Renilla luciferase plasmid pRL-TK. Similar results were obtained also in CHO-K1 cells lacking endogenous adenosine receptors, where cotransfection of IL-8 reporter with adenosine A2B receptors resulted in 5.1±0.7-fold NECA-induced stimulation, whereas cotransfection with A1, A2A, or A3 had no effect on reporter activity (online Figure 5).

In ancillary experiments, the expression of recombinant A2A and A2B receptors in CHO-K1 cells was verified by NECA-induced increase in intracellular cAMP levels. The expression of recombinant A1 and A3 adenosine receptors was confirmed using previously described radioligand binding technique.23

Effect of Constitutively Active α Subunits of G Proteins on IL-8 and VEGF Promoters
To examine requirements of IL-8 and VEGF gene expression for specific G-protein activation, we transfected HMEC-1 with vectors encoding mutationally activated α subunits of G proteins together with either a IL-8 promoter–driven luciferase reporter or with a VEGF promoter–driven luciferase reporter. Cells assayed 30 hours after transfection with constitutively active mutants of αq, α12, or α13 G protein subunits produced 7.6±0.1-, 6.1±0.2-, and 5.7±0.1-fold increase in VEGF reporter luciferase activity and 23.2±1.6-, 19.6±0.9-, and 14.1±0.6-fold increase in IL-8 reporter luciferase activity, respectively, when compared with mock-transfected cells. Luciferase activity in cells transfected with
constitutively active $\alpha_\alpha$, $\alpha_i$, $\alpha_\beta$, or $\alpha_\gamma$ was virtually the same as in mock-transfected cells (Figure 6).

**Adenosine-Induced Inositol Phosphate Accumulation in HMEC-1**

G proteins of the $G_q$ family stimulate phosphoinositide hydrolysis by activation of phospholipase C-$\beta$. To determine if this pathway is stimulated by adenosine in HMEC-1, we measured the accumulation of total inositol phosphates in the presence of 20 mmol/L LiCl. As seen in Figure 7, 100 $\mu$mol/L NECA produced a small but significant increase in accumulation of inositol phosphates compared with the basal levels (from 704±35 to 861±22 cpm/tube, n=6, $P<0.01$, Figure 7), and this effect was completely blocked by the selective $A_{2B}$ antagonist, IPDX (10 $\mu$mol/L). In contrast, the selective $A_{2A}$ agonist, CGS 21680, had no effect on basal levels of inositol phosphates.

**Discussion**

Our work demonstrates that human endothelial cells of disparate origin are characterized by differential expression of adenosine receptor subtypes. HUVECs express mRNA for $A_{2A}$ and $A_{2B}$ receptors at a ratio of 10:1, and this preferential gene expression agrees well with the typical pharmacological phenotype of $A_{2A}$ receptor–mediated simulation of adenylate cyclase by adenosine analogs. Using complementary techniques, RT-PCR, and gene expression array, we found that $A_1$ and $A_3$ adenosine receptors are not expressed in HUVECs. Previous studies in HUVECs have suggested a potential role of $A_1$ receptor in maintaining endothelial barrier function and of $A_1$ and $A_3$ receptors in modulation of tissue factors expression. The apparent contradiction between these results and ours can be explained by the use of nonselective concentrations of adenosine receptor ligands in previous studies.

HMEC-1 also express only $A_{2A}$ and $A_{2B}$ mRNA, but in contrast to HUVECs, they express predominantly $A_{2B}$ receptor mRNA, with a ratio $A_{2B}/A_{2A}$ of 4:1. The difference in expression of adenosine receptor subtypes between these cells is even more dramatic when the effects of adenosine receptor activation on inositol phosphate formation. Accumulation of total inositol phosphates in unstimulated HMEC-1 (basal), and in cells stimulated with 10 $\mu$mol/L CGS 21680 (CGS), or with 100 $\mu$mol/L NECA in the absence (NECA), or in the presence of 10 $\mu$mol/L IPDX (NECA+IPDX). Values are expressed as mean±standard error of 6 experiments.

**Figure 5.** Effect of expression of adenosine receptors on VEGF and IL-8 promoters. Activation of VEGF (A) and IL-8 (B) gene promoters was studied by cotransfection of corresponding luciferase reporters together with vectors encoding adenosine receptor subtypes (indicated on graph as $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$) or with an empty vector (mock) in HMEC-1. Twenty-four hours after transfections, cells were incubated in the presence or absence of 100 $\mu$mol/L NECA for 6 additional hours. The results from 3 experiments are expressed as mean±standard error of NECA-induced stimulation of luciferase activity.

**Figure 6.** Regulation of VEGF and IL-8 promoters by $G$ protein $\alpha$-subunits. Activation of VEGF (A) and IL-8 (B) gene promoters was studied by cotransfection of corresponding luciferase reporters together with expression vectors encoding the constitutively active mutants $\alpha_\alpha$, $\alpha_i$, $\alpha_\beta$, $\alpha_\gamma$ and $\alpha_\Omega$ (indicated on graph as s, i, $\Omega_1$, $\Omega_2$, $\Omega_3$, q, 12, and 13, respectively) or with an empty vector (mock) in HMEC-1. Relative luciferase activity was measured 30 hours after transfections, and the results from 3 experiments are expressed as mean±standard error.

**Figure 7.** Effect of $A_{2B}$ adenosine receptor activation on inositol phosphate formation. Accumulation of total inositol phosphates in unstimulated HMEC-1 (basal), and in cells stimulated with 10 $\mu$mol/L CGS 21680 (CGS), or with 100 $\mu$mol/L NECA in the absence (NECA), or in the presence of 10 $\mu$mol/L IPDX (NECA+IPDX). Values are expressed as mean±standard error of 6 experiments.
analog on cAMP accumulation are compared. In contrast to stimulation of adenylate cyclase in HUVECs, the selective A<sub>2A</sub> agonist CGS 21680 had virtually no effect on adenylate cyclase in HMEC-1. Thus, we found no evidence of functional coupling of A<sub>2A</sub> receptors in HMEC-1.

Interestingly, previously published data on expression of adenosine receptors in human and porcine endothelial cells derived from large coronary arteries also demonstrated the predominance of A<sub>2A</sub> receptor-mediated stimulation of adenylate cyclase,<sup>9</sup> similar to that observed in this study in HUVECs derived from human umbilical vein. In contrast, adenosine actions were thought to be mediated by A<sub>2B</sub> receptors in human retinal microvascular endothelial cells,<sup>13,14</sup> a finding similar to that observed in this study in HMEC-1 derived from human skin microvasculature. It is tempting to suggest that endothelial cells lining large conduit vessels express predominantly A<sub>2B</sub> receptors and endothelial cells lining small vessels or capillaries express predominantly A<sub>2B</sub> receptors. However, an expanded comparative study of various endothelial cells of different origin is needed to reach a definite conclusion.

Adenosine is known to upregulate VEGF expression in endothelial cells of microvascular origin. Adenosine was shown to play a role in the hypoxic induction of VEGF in porcine brain-derived microvascular endothelial cells.<sup>9</sup> Adenosine A<sub>2B</sub> is the predominant receptor subtype in human retinal microvascular endothelial cells. In these cells, similarly to HMEC-1, only A<sub>2B</sub> adenosine receptors are functionally linked to activation of adenylate cyclase and stimulate expression of VEGF.<sup>13,14</sup> In the present study, we demonstrated that adenosine stimulates mRNA expression of several proangiogenic factors, namely VEGF, IL-8, and bFGF, in HMEC-1 expressing predominantly A<sub>2B</sub> receptors, but not in HUVECs expressing predominantly A<sub>2A</sub> adenosine receptors. We found that adenosine receptor activation specifically increased levels of VEGF mRNA in HMEC-1, and had no significant effect on mRNA levels for other members of VEGF family angiogenic factors including VEGF-B, VEGF-C, and VEGF-D. We have also confirmed that increases in VEGF and IL-8 mRNA expression in HMEC-1 resulted in increased proteins levels, and these effects were mediated via A<sub>2B</sub> adenosine receptors because the nonselective A<sub>2A</sub>/A<sub>2B</sub> agonist NECA, but not the selective A<sub>2A</sub> agonist CGS 21680, elevated VEGF and IL-8 levels in conditioned media.

We have previously reported that stimulation of A<sub>2B</sub> adenosine receptors increased synthesis and secretion of IL-8 in human mast cells.<sup>31</sup> Adenosine-induced stimulation of IL-8 production in endothelial cells is a novel finding with direct relevance to angiogenesis. There is growing evidence that IL-8 plays an important and specific role in promoting angiogenesis. IL-8 is elevated in wounds and enhances wound healing.<sup>38</sup> IL-8, secreted by inflammatory and neoplastic cells, stimulates angiogenesis by paracrine mechanism in various solid tumors.<sup>36–38,36</sup> Hypoxia has been shown to induce expression of both IL-8<sup>39</sup> and VEGF<sup>40</sup> in endothelial cells, implying the existence of autocrine pathways regulating their growth. Hypoxia can induce angiogenesis by various mechanisms, including but not limited to oxygen-dependent regulation of hypoxia-inducible factor 1, the activator of VEGF transcription,<sup>25</sup> or stabilization of VEGF mRNA.<sup>41</sup> Our results raise the possibility that adenosine, elevated during hypoxia, can also contribute to hypoxia-induced angiogenesis via stimulation of A<sub>2B</sub> receptors.

In experimental models using cotransfection of IL-8 and VEGF reporters with adenosine receptors, we confirmed that only A<sub>2B</sub> receptors mediate adenosine-induced transcription of IL-8 and VEGF genes. In contrast, overexpression of A<sub>2A</sub> adenosine receptors attenuated the stimulation of IL-8 and VEGF promoters mediated by native A<sub>2B</sub> receptors in HMEC-1. These data agree with the previously reported downregulation of IL-8 in HUVECs<sup>32</sup> and VEGF in rat pheochromocytoma PC12 cells<sup>32</sup> mediated via A<sub>2A</sub> adenosine receptors. The exact mechanisms underlying the A<sub>2A</sub>-mediated downregulation of VEGF and IL-8 are currently unknown and will require further investigation. It is of interest, that selective blockade of A<sub>2A</sub> adenosine receptors in PC12 cells, which also express a small number of A<sub>2B</sub> adenosine receptors,<sup>33</sup> resulted in an increase in basal levels of VEGF,<sup>32</sup> presumably due to stimulation of A<sub>2B</sub> adenosine receptors.

It is obvious that the opposing effects of A<sub>2A</sub> and A<sub>2B</sub> adenosine receptors on the synthesis of angiogenic factors imply their coupling to different G-proteins. In this study, we demonstrate that constitutively active G<sub>q</sub>, G<sub>12</sub>, and G<sub>13</sub> α subunits stimulated VEGF and IL-8 promoters. These G proteins are potential candidates for coupling of A<sub>2B</sub> receptors to stimulation of proangiogenic factors. Remarkably, the constitutively active α subunit of G<sub>q</sub>, which is coupled to both A<sub>2A</sub> and A<sub>2B</sub> adenosine receptors, had virtually no effect on VEGF and IL-8 reporters. Activation of G proteins of the G<sub>q</sub> subfamily is known to stimulate phosphoinositide-specific phospholipase C-β. We have previously demonstrated that A<sub>2B</sub> adenosine receptors not only stimulate adenylate cyclase via coupling to G<sub>q</sub>, but can also stimulate phospholipase C-β in human mast cells by cholera toxin– and pertussis toxin–resistant mechanism, presumably via activation of G<sub>q</sub> protein.<sup>31</sup> Here, we report the activation of phospholipase C via A<sub>2B</sub> adenosine receptors in HMEC-1, suggesting that G proteins of the G<sub>q</sub> subfamily may be involved in the signal transduction leading to increase of IL-8 and VEGF production in these cells. In contrast to the G<sub>q</sub> subfamily, the signaling through G<sub>12/13</sub> proteins is still poorly understood. Recent evidence suggests that these G proteins are involved in regulation of cell growth,<sup>44</sup> differentiation,<sup>45</sup> and apoptosis.<sup>46</sup> They have been implicated in regulation of Na<sup>+</sup>/H<sup>+</sup> exchange,<sup>27</sup> MAPK pathways,<sup>37</sup> and actin cytoskeleton rearrangement.<sup>48</sup> It should be noted, that many G<sub>q</sub> coupled receptors have been shown to couple also to G<sub>12</sub> and/or G<sub>13</sub> proteins (for review see Fields and Casey<sup>49</sup>). Of interest, while this article was in preparation, Shepard et al<sup>50</sup> reported stimulation of IL-8 secretion by the receptor of Kaposi’s sarcoma-associated herpesvirus via coupling to G<sub>13</sub> protein in HeLa cells. Potential coupling of G<sub>q</sub> and G<sub>13</sub> proteins to A<sub>2B</sub> adenosine receptors and their involvement in the regulation of angiogenic factors in endothelial cells deserves further study.
In summary, several novel findings are derived from our results. We report differential expression of adenosine receptor subtypes in endothelial cells of disparate origin; A$_{2A}$ receptors are the predominant subtype in HUVECs and A$_{2B}$ receptors are the predominant subtype in the human microvascular cell line HMVEC-1. Adenosine A$_{2B}$ Receptors mediate the expression of the angiogenic factors IL-8, VEGF, and bFGF. These effects are not linked to stimulation of G proteins, but appear to be mediated by G proteins of the G$_{i}$ and possibly of the G$_{12/13}$ subfamilies. These results confirm and expand those previously reported in other human microvascular endothelial cells. Our findings add to a growing evidence that angiogenesis is an important function of adenosine A$_{2B}$ receptors.

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Materials and Methods

Cell culture.

Human umbilical vein endothelial cells (HUVEC) propagated from pooled primary cultures of human umbilical veins were kindly provided by Dr. D.E. Vaughan, Vanderbilt University. The cells exhibited typical endothelial cell morphology, growing as confluent monolayers of polygonal, closely apposed cells with a "cobblestone" appearance, and as evidenced by immunofluorescence staining with antibodies against von Willebrand factor and by uptake of Dil-acetyl-LDL. Only early passage (passage 2 or 1) cultures were used in these studies. HUVEC were maintained in M-199 medium supplemented with 15% (v/v) FBS, 1X Antibiotic-Antimycotic mixture (Gibco BRL catalog # 15240-062), and 0.3 µg/mL bovine hypothalamus endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA). Human microvascular endothelial cells (HMEC-1) were derived from SV40-transformed human skin microvascular endothelial cells and were maintained in the same medium.

Chinese hamster ovary CHO-K1 cells were obtained from the American Type Culture Collection (CRL-9618; Rockville, MD) and maintained in Ham’s F12 medium supplemented with 10% (v/v) FBS and 1X Antibiotic-Antimycotic mixture. All cells were kept under humidified atmosphere of air/CO$_2$ (19:1) at 37°C.
**Chemicals.**

5'-N-ethylcarboxamidoadenosine (NECA) and 2-p-(2-carboxyethyl)phenethylamino-NECA (CGS21680) were purchased from Research Biochemicals, Inc. (Natick, MA). 3-isobutyl-8-pyrrolidinoxanthine (IPDX) was synthesized as previously described².

**Gene expression assay.**

Total RNA was isolated using RNeasy Mini Kit (Quiagen Inc, Valencia, CA). Expression of angiogenic factors was evaluated using gene expression arrays (Super Array, Inc., Bethesda, MA). Human adenosine receptors gene expression array was custom designed by Super Array, Inc. The assay was performed according to manufacturer’s instructions. In brief, gene-specific [³²P]-labeled cDNA probes were generated from 5 to 10 µg of total RNA using gene-specific set of primers for reverse transcription. The cDNA probes were then hybridized with gene-specific cDNA fragments spotted on nylon membrane. The relative expression level of each gene was analyzed using a phosphoimager and Image Quant™ software (Molecular Dynamics). Total RNA isolated from human brain (Clontech, Palo Alto, CA) containing mRNA for all four adenosine receptor subtypes was used as positive control.

**Reverse transcription polymerase chain reaction (RT-PCR).**

Two microgram of total RNA from each sample were subjected to RT-PCR using Access RT-PCR system (Promega Corporation, Madison, WI) in accordance with manufacturer’s instructions. PCR primers targeting mRNA sequences that represent parts of different exones in human A₁, A₂A and A₂B adenosine receptors genes were synthesized to yield amplicons of 500-800 base pairs. PCR primers for human A₃ adenosine receptor were used as described by Mitchell et al³. The following primer pairs were used for A₁: 5'
TCTGGGCGGTGAAGGTGAAC-3' (sense) and 5'-AGTTGCCGTGCGTGAGGAAG-3' (antisense); A2A: 5'-TGCTTCGTCCTGGTCCTCAC-3' (sense) and 5'-GCTCTCCGTCACTGCCAT-3' (antisense); A2B: 5'-CCCTTTGCCATCACCATCAG-3' (sense) and 5'-CCTGACCATTCCCACCTCTTGA-3' (antisense); A3: 5'-GCGCCATCTATCTTGACATCTTTT-3' (sense) and 5'CTTGGCCCAGGCATAACAGG-3' (antisense). Reverse transcription at 48°C for 45 min was followed by 40 cycles of amplification with denaturing at 94°C for 30 sec, annealing at incrementally lowering temperature by 0.2°C per cycle from 70°C to 62°C for 1 min, and extension at 72°C for 2 min. Total RNA isolated from CHO cells expressing each of human adenosine receptor subtype and total RNA isolated from human brain were used as positive controls. Purified PCR fragments were analyzed by restrictive digestion.

Real-time RT-PCR was performed on ABI Prism Sequence Detection System 5700 (PE Applied Biosystems, Foster City, CA). A set of primers was designed for each gene using Primer Express (PE Applied Biosystems), and amplicons of 100-200 base pairs with Tm between 68°C and 85°C were selected. RT-PCR reactions utilizing 4 µg of DNase-treated total RNA were performed under conditions recommended by the manufacturer. A dissociation curve was generated at the end of the polymerase chain reaction cycle to verify that a single product was amplified. A standard curve for each amplicon was obtained using serial dilutions of total RNA prepared from CHO-A1 cells (A1 primers), HEK-A2A (A2A primers), HEK-A2B (A2B primers), CHO-A3 (A3 primers) and HEK-293 cells (S9 and cyclophilins). 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 either cyclophilin or ribosomal protein S9 to correct for fluctuations in input RNA levels and varying efficiencies of reverse transcription reactions. The differential expression curves obtained using either of these genes as the correction factor consistently showed the same pattern. The normalization was done assuming mRNA levels of cyclophilin in each cell line is 100.

**Transfections and luciferase reporter assay.**

HMEC-1 and CHO-K1 cells were transfected using Fugene 6 transfection reagent (Roche Molecular Biochemicals). One microgram of plasmid DNA was mixed with 100 µl serum-free M-199 medium (HMEC-1), or Ham’s F12 medium (CHO-K1) containing 3 µl Fugene 6. After 15 min incubation at room temperature, the transfection mixture was added to cells growing on six-well plates at 40-60% confluency. The volume of transfection mixture was adjusted accordingly if cells were grown on plates of a different size.

Cells were co-transfected with cDNA described in Results section and luciferase reporters at a ratio of 10 : 1. The same ratio, 10 : 1, was used for experimental firefly luciferase reporter : control *Renilla* luciferase reporter combination. VEGF promoter-driven luciferase reporter, a firefly luciferase reporter plasmid, comprising 5' flanking -1005 to +379 base pairs of the human VEGF gene was kindly provided by Dr. G.L. Semenza, Johns Hopkins Hospital, Baltimore, MD. IL-8 promoter-driven luciferase reporter -133-luc, a firefly luciferase reporter plasmid, comprising 5' flanking -133 to +44 base pairs of the human IL-8 gene was generously gifted by Dr. Naofumi Mukaida (Kanazawa University, Ishikawa, Japan). The cDNAs encoding the human adenosine receptors in the pRc/CMV expression vector (Invitrogen) were a generous
gift from Drs. P.R. Schofield and A. Townsend-Nicholson (Garvan Institute of Medical Research, Australia). Plasmids, $\alpha_s$ RC pcDNA I; $\alpha_{i1}$ QL pcDNA I; $\alpha_{i2}$ QL pcDNA I; $\alpha_{i3}$ QL pcDNA I; $\alpha_q$ RC pcDNA I; $\alpha_{12}$ QL pcDNA I neo; $\alpha_{13}$ QL pcDNA I, encoding constitutively active alpha subunits of G-proteins were constructed as described previously. A control constitutively active Renilla luciferase plasmid pRL-TK was purchased from Promega (Madison, WI). Reporter activity was measured 30 hours after transfection using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activities were normalized against Renilla luciferase activities from the co-expressed pRL-TK, and expressed as relative luciferase activities over basal (set as 1).

**Measurement of cAMP accumulation.**

HUVEC or HMEC-1 cells growing in 12-well plates were preincubated in 150 mmol/L NaCl, 2.7 mmol/L KCl, 0.37 mmol/L NaH$_2$P0$_4$, 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 5 min at 37°C. The reaction was stopped by the addition of 200 µl of 5% trichloroacetic acid. The extracts were washed five times with 10 volumes of water-saturated ether. Cyclic AMP concentrations were determined using cAMP assay kit (TRK.432; Amersham Corp., Arlington Heights, IL).

**Determination of IL-8 and VEGF levels in conditioned media.**

Cells were incubated in serum-free media containing 1 U/mL adenosine deaminase for 6 hours under humidified atmosphere of air/CO$_2$ (19:1) at 37°C with the reagents indicated in the
Results. At the end of this incubation period the culture media were collected by centrifugation at 12,000 x g for 1 min at 4°C. IL-8 and VEGF concentrations were measured using ELISA kits (R&D Systems, Minneapolis, MN).

Measurement of $[^3H]$ inositol phosphates formation.

Formation of inositol phosphates was determined using modification of the procedure described by K. Seuwen et al. HMEC-1 cells grown in 12-well plates were labeled to equilibrium with myo-$[^3H]$ inositol (2 µCi/mL, DuPont-NEN, Boston, MA) for 18 h in inositol-free MEM medium. The HMEC-1 cells were then washed twice and pre-incubated in 150 mmol/L NaCl, 2.7 mmol/L KCl, 0.37 mmol/L NaH$_2$P0$_4$, 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 20 mmol/L LiCl$_2$ for 15 min at 37°C. Adenosine agonists and antagonists were added to cells, and the incubation was allowed to proceed for 30 min at 37°C. Reaction was terminated by replacing the incubation buffer with 200 µl ice-cold 10 mmol/L formic acid (pH 3). After 30 min, this solution, containing the extracted inositol phosphates and inositol was collected and diluted with 800 µl of 5 mmol/L NH$_3$ solution (final pH 8 - 9). The resulting mixture was then applied to a column containing 0.2 mL anion exchange resin (AG 1-X8, formate form, 200 - 400 mesh; Bio-Rad Laboratories, Richmond, CA). Free inositol and glycerophosphoinositol were eluted with 1.25 mL of H$_2$O and 1 mL of 40 mmol/L ammonium formate/formic acid, pH 5. Total inositol phosphates were eluted in the single step with 1 mL of 2 mol/L ammonium formate/formic acid, pH 5, and radioactivity was measured by liquid scintillation counting.
References


Online figure 1. Expression of adenosine receptors.

Levels of mRNA expression in HMEC-1 (panel A) and HUVEC (panel B) measured by real-time RT-PCR are compared to those determined by gene expression array. Both techniques show that HMEC-1 cells preferentially express A2B, and HUVEC preferentially express A2A adenosine receptor mRNA.
Online figure 2. Effect of NECA on mRNA expression of the VEGF family angiogenic factors.

Changes in the levels of mRNA expression (normalized to β-actin mRNA expression) in HMEC-1 (panel A) and HUVEC (panel B) induced by incubating with the stable adenosine agonist 100 µmol/L NECA for 6 hours. Data were calculated from two independent gene expression array experiments performed in duplicates.
Online figure 3. Effect of the selective A\textsubscript{2B} antagonist IPDX on NECA-induced IL-8 secretion in HMEC-1.

IL-8 secretion was induced in HMEC-1 by incubating cells with NECA for 6 hours. Concentration-response curves were repeated in the absence (control) and in the presence of increasing concentrations of IPDX, which produced a progressive shift to the right. Results are presented as means±SEM of three experiments. Inset: Schild plot analysis of these data revealed slope close to unity (0.93±0.13), indicating that IPDX acts as a simple competitive antagonist of A\textsubscript{2B} receptors. The intercept of this linear regression, which is used to estimate the dissociation constant of antagonist-receptor complex (K\textsubscript{B}) was -5.8, and the K\textsubscript{B} was 1.5 µmol/L. R represents the ratio of agonist EC\textsubscript{50} in the presence of antagonist to its EC\textsubscript{50} in the absence of antagonist.
Online figure 4. Stimulation of adenosine receptors in HUVEC does not induce IL-8 secretion.

Levels of IL-8 measured by ELISA in conditioned media of HUVEC incubated for 24 hours in the absence (basal), or in the presence of 10 μmol/L NECA (NECA), 10μmol/L CGS 21680 (CGS) or 1 U/mL thrombin (Thrombin). Values are expressed as mean±SEM of three experiments.
Online figure 5. Effect of expression of adenosine receptors in CHO cells on IL-8 promoter.

Activation of IL-8 promoter was studied by cotransfection of IL-8 luciferase reporter with vectors encoding adenosine receptor subtypes (indicated on graph as $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$) or with an empty vector (mock) in CHO-K1 cells. Twenty-four hours after transfections, cells were incubated in the presence of 100 µmol/L NECA for additional 6 hours. The results from three experiments are expressed as mean±SEM.