Hypoxia Modulates Adenosine Receptors in Human Endothelial and Smooth Muscle Cells Toward an \( \text{A}_{2\text{B}} \) Angiogenic Phenotype

Igor Feoktistov, Sergey Ryzhov, Hongyan Zhong, Anna E. Goldstein, Anton Matafonov, Dewan Zeng, Italo Biaggioni

Abstract—We previously reported that adenosine \( \text{A}_{2\text{B}} \) receptor activation stimulates angiogenesis. Because hypoxia is a potent stimulus for the release of both adenosine and angiogenic factors, we tested the hypothesis that hypoxia alters the expression of adenosine receptors toward an “angiogenic” phenotype. We used human umbilical vein endothelial cells (HUVECs) and bronchial smooth muscle cells (BSMCs) because, under normoxic conditions, adenosine does not release vascular endothelial growth factor (VEGF). HUVECs expressed a characteristic \( \text{A}_{2\text{A}} \) phenotype (the selective \( \text{A}_{2\text{A}} \) agonist CGS21680 was as potent as the nonselective agonist 5'-N-ethylcarboxamidoenosine [NECA] in generating cAMP). Hypoxia (4.6% \( \text{O}_2 \), 3 hours) decreased \( \text{A}_{2\text{A}} \) mRNA from 1.56±0.3% to 0.16±0.01% of \( \beta \)-actin expression but increased \( \text{A}_{2\text{B}} \) mRNA from 0.8±0.01% to 0.27±0.05%. Consistent with changes in receptor expression, CGS21680 failed to increase cAMP in hypoxic HUVECs, whereas NECA remained active (\( \text{A}_{2\text{B}} \) phenotype), and NECA increased VEGF release from 9.5±1.0 to 14.2±1.2 pg/mL (\( P<0.05 \)), indicating that increased \( \text{A}_{2\text{B}} \) receptors were functionally coupled to upregulation of VEGF. Hypoxia had similar effects on BSMCs, increasing \( \text{A}_{2\text{B}} \) mRNA by 2.4±0.3-fold, from 0.42±0.04% to 1.00±0.13% of \( \beta \)-actin. Whereas NECA had no effect on VEGF release in normoxic BSMCs, it increased VEGF release in hypoxic BSMCs, from 74.6±9.6 to 188.3±16.7 pg/mL (\( P<0.01 \)), and a selective \( \text{A}_{2\text{B}} \) antagonist, CVT-6694, inhibited this increase. \( \text{A}_{3\text{B}} \) receptors activated a VEGF reporter made unresponsive to hypoxia by mutating its hypoxia-inducible factor-1 (HIF-1) binding element, indicating a mechanism independent of HIF-1. In conclusion, hypoxia modulates the expression of adenosine receptors in human endothelial and smooth muscle cells toward an \( \text{A}_{2\text{B}} \) “angiogenic” phenotype. (Hypertension. 2004;44:649-654.)

Key Words: adenosine ■ hypoxia ■ receptors, purinergic ■ endothelium ■ vasculature ■ muscle, smooth ■ endothelium-derived factors

The purine nucleoside adenosine is an intermediate product of adenine nucleotide metabolism. Adenosine serves as a “retaliatory metabolite” in situations where oxygen supply is decreased or energy consumption is increased. Under these conditions, adenosine is released into the extracellular space and signals to restore the balance between energy supply and demand. Four extracellular G protein–coupled receptors, namely, \( \text{A}_1 \), \( \text{A}_{2\text{A}} \), \( \text{A}_{2\text{B}} \), and \( \text{A}_3 \), mediate adenosine actions. \( \text{A}_{2\text{B}} \) receptors have a lower affinity compared with other subtypes and require micromolar concentrations of adenosine for their stimulation.\(^1\) Such high levels of extracellular adenosine can be reached during hypoxia, ischemia, inflammation, and injury.\(^2\) The low affinity of \( \text{A}_3 \) receptors suggests that they are primarily engaged under these pathophysiologic conditions.

\( \text{A}_{2\text{B}} \) receptors regulate various pathological processes, including mast cell activation,\(^3\) vasodilation,\(^4\) inhibition of cardiac fibroblast\(^5\) and vascular smooth muscle growth,\(^6\) stimulation of endothelial cell (EC) growth,\(^7\) and angiogenesis.\(^8\)–\(^10\) Stimulation of angiogenesis appears to be an important function of \( \text{A}_{2\text{B}} \) receptors. We have previously shown that \( \text{A}_{2\text{B}} \) receptors upregulate the production of angiogenic factors in human mast cells, retinal ECs, and human microvascular endothelial cells (HMEC-1s) under normoxic conditions.\(^8\)–\(^10\)

Tissue hypoxia is a powerful stimulus for the expression of genes associated with angiogenesis and, as mentioned previously, it is during hypoxia that adenosine levels increase to concentrations that engage \( \text{A}_{2\text{B}} \) receptors. Therefore, we tested the hypothesis that hypoxia would also modulate expression of adenosine receptor subtypes toward an “angiogenic” \( \text{A}_{2\text{B}} \) phenotype.
Methods

Cell Culture and Treatment Conditions
Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science (Walkersville, Md), HMEC-1s were provided by Dr D.E. Vaughan (Vanderbilt University, Nashville, Tenn), and normal human bronchial smooth muscle cells (BSMCs) were obtained from Clonetics (San Diego, Calif). Cells were maintained as described previously.9,11

Before each experiment, the growing medium was replaced with one containing 1 U/mL adenosine deaminase. Confluent monolayer cultures were exposed to hypoxia in an incubation chamber (Billups-Rothenberg) flushed with a 5% CO2/95% N2 gas mixture until the oxygen concentration inside the chamber reached 4.6%. The hypoxic chamber was then sealed and placed in a 37°C cell culture incubator for the indicated time.

Chemicals
5′-N-ethylcarboxamidoadenosine (NECA) and 2-p-(2-carboxyethyl)phenethylamino-NECA (CGS21680) were purchased from Research Biochemicals, Inc. CVT-6694 was made at CV Therapeutics (Palo Alto, Calif).

Gene Expression Assay and Real-Time RT-PCR
A human adenosine receptor gene-expression array was custom-designed by Super Array (Bethesda, Mass). The assay and real-time RT-PCR were performed as previously described.9

Measurement of cAMP
cAMP concentrations were determined as previously described.12

Determination of VEGF Levels
HUVECs were lysed with phosphate-buffered saline containing 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a 0.01% protease inhibitor cocktail (Roche). After being passed through a 23-gauge needle, cell debris was pelleted by centrifugation, and vascular endothelial growth factor-A (VEGF) was measured in the supernatant by using an ELISA kit (PeproTech). The concentration of VEGF in the cell media of BSMCs was determined with an ELISA kit (Biosource).

Transfections and Luciferase Reporter Assay
HMEC-1 cells were transfected as previously described.9 p11w, a firefly luciferase reporter plasmid, comprising the 5′-flanking 985 base pairs of the human VEGF gene that include a constitutive promoter, and p11m, the mutated version of p11w containing a nonfunctional HIF-1–binding site, and p11m. The assay and real-time RT-PCR were performed as previously described.9

Results

Effect of Hypoxia on mRNA Expression of Adenosine Receptor Subtypes in HUVECs
Gene-expression array showed that HUVECs incubated under normoxic conditions preferentially express A2A receptor mRNA (Figure 1): the levels of A3 and A2B receptors were 1.56±0.3: and 0.08±0.01% of β-actin, respectively, and no mRNA encoding the A1 or A3 receptor was detected. In HUVECs exposed to hypoxia, A3 receptor mRNA expression was decreased from 1.56±0.3 (normoxia) to 0.51±0.12 (1-hour hypoxia) and to 0.16±0.01% (3-hour hypoxia) of β-actin expression. In contrast, A2B receptor mRNA was increased from 0.08±0.01 (normoxia) to 0.30±0.01 (1-hour hypoxia) and to 0.27±0.05% (3-hour hypoxia). The ratio of mRNA expression of A2B to A2A changed from a predominance of A2B (0.05:1 under normoxia) to a predominance of A2A (1.6:1, 3-hour hypoxia).

Effect of Hypoxia on Functional Expression of Adenosine Receptors in HUVECs
Activation of A2A and A2B adenosine receptors stimulates adenylyl cyclase and thus, we used the accumulation of cAMP as a measure of functional phenotypes. Our results confirmed that in normoxic HUVECs, the A2A receptor is the functional, predominant adenosine receptor (Figure 2A). The A2A-selective agonist CGS21680 activated adenylyl cyclase with an EC50 of 849 nmol/L. The nonselective agonist NECA had a potency (EC50 of 948 nmol/L) and efficacy similar to that of CGS21680. This pharmacological profile is consistent with the predominant expression of A2A adenosine receptors.14

In contrast, in hypoxic HUVECs (3-hour hypoxia), NECA stimulated the accumulation of cAMP with an EC50 of 1.4 μmol/L, whereas CGS21680 was virtually ineffective (Figure 2B). This pharmacological profile is consistent with the functional predominance of A2B receptors.12,15

Effect of Hypoxia on Regulation of VEGF by Adenosine in HUVECs
NECA, 100 μmol/L, had no effect on the production of VEGF protein in HUVECs incubated for 3 hours under normoxic conditions (Figure 3), in agreement with our...
previous studies. Under hypoxic conditions, however, NECA increased VEGF protein levels in HUVECs from 9.5 ± 1.0 to 14.2 ± 1.2 pg/mL (P < 0.05).

**Effect of Hypoxia on A2B Receptor mRNA Expression and Regulation of VEGF by Adenosine in SMCs**

We used the human SMC line BSMCs as a model of a nonvascular cell type expressing higher levels of VEGF because we previously demonstrated the presence of A2B receptors in these cells and the lack of effect of adenosine on VEGF secretion under normoxic conditions. Under normoxic conditions, these cells preferentially express A2B receptors (0.42 ± 0.04% of β-actin, Figure 4). Lower levels of A1 and A2A receptors were also detected (0.0023 ± 0.0003 and 0.0097 ± 0.002% of β-actin, respectively), whereas transcripts for A3 receptors were below detection levels. Incubation of BSMCs under hypoxic conditions for 1 hour increased A2B receptor transcript levels by 2.4 ± 0.3-fold (from 0.42 ± 0.04% to 1.00 ± 0.13% of β-actin, P < 0.05) but had no significant effect on the expression of A1 and A2A receptors (0.004 ± 0.0006% and 0.0087 ± 0.0003% of β-actin, respectively).

As shown in Figure 5, NECA (10 μmol/L) had no effect on VEGF release in normoxia but increased VEGF release by 2.5 ± 0.2-fold in hypoxia. The selective A2B antagonist CVT-669411 (1 μmol/L) attenuated the effect of NECA by 64 ± 21%, indicating the involvement of A2B receptors.

**Role of HIF-1 in Regulation of VEGF Transcription by Hypoxia and Adenosine**

HIF-1 is a transcription factor that mediates the effects of hypoxia on VEGF expression by binding to the hypoxia-response element of the VEGF promoter. We have previously shown that NECA, acting via A2B receptors, stimulates the VEGF promoter in HMEC-1s. To examine whether NECA interacts with the HIF-1 pathway to upregulate VEGF transcription, we used 2 previously described luciferase reporters. The p11w reporter is regulated by a fragment of the VEGF promoter that includes an HIF-1–binding site. The p11m reporter is identical except for a 3-bp mutation that prevents HIF-1 binding. We coexpressed these reporters with A2B receptors to minimize the variability in expression levels of A2B receptors because of potential effects of hypoxia.
on native adenosine receptors. As shown in Figure 6A, 10 μmol/L NECA increased luciferase activity of the p11w reporter by 2.3±0.03-fold in HMEC-1s incubated under normoxic conditions for 12 hours. Incubation of cells under hypoxic conditions for 12 hours resulted in a 2.2±0.3-fold and 4.1±0.1-fold increase in p11w reporter activity in the absence and presence of NECA, respectively. NECA also stimulated activity of the p11m reporter by 2.3±0.04-fold and 2.0±0.27-fold under normoxic and hypoxic conditions, respectively, but hypoxia itself had no effect (Figure 6B). These results suggest that in contrast to hypoxia, adenosine-induced VEGF upregulation does not require the HIF-1 pathway.

Discussion

Hypoxia is a feature of many pathophysiologic conditions, including ischemia, inflammation, and tumor growth. Cell hypoxia is a potent stimulus for adenosine release. The physiologic importance of adenosine, therefore, is more relevant during hypoxic than normoxic conditions. Mammalian cells respond to hypoxia by significant genetic reprogramming with selective gene induction and downregulation. Our data show that hypoxia produces dramatic changes in the expression of adenosine receptor subtypes in HUVECs. These cells normally express both A2A and A2B receptors, but the A2A receptor predominates in terms of both mRNA expression and functional coupling. Hypoxia downregulated the expression of high-affinity A2A receptors, but at the same time it increased expression of low-affinity A2B receptors. Hypoxia also altered responses to adenosine agonists, indicating that changes in mRNA expression led to changes in expression of functional receptors. The nonselective A2 agonist NECA stimulated adenylate cyclase in hypoxic HUVECs, whereas the selective A2A agonist CGS21680 was no longer able to do so, indicating that A2B receptors became functionally predominant in HUVECs after hypoxic treatment.

It should be noted, however, that hypoxia does not always downregulate A2A receptors. It has been reported that hypoxia increased expression of A2A receptors in rat PC12 pheochromocytoma cells. It is possible, therefore, that the effects of hypoxia on the expression of A2A adenosine receptors can be cell- and tissue-specific.

In contrast to downregulating high-affinity A2A receptors, hypoxia increased mRNA expression of low-affinity A2B receptors in HUVECs. These results agree with our previous data, which showed that simulation of hypoxia by cobalt ions upregulated the expression of A2B receptors in U87MG cells. Studies from other laboratories have also shown an upregulation of adenosine A2B receptors by hypoxia. Of interest, hypoxia not only upregulates A2B receptors but also increases the levels of endogenous ligand by enhancing hydrolysis of ATP to adenosine through upregulation of surface apyrase (CD39) and 5’-nucleotidase (CD73). Therefore, the functional importance of the low-affinity A2B subtype is probably more relevant under hypoxic conditions, when concentrations of extracellular adenosine are increased. The differential upregulation of adenosine receptors that we report here may provide a positive-feedback mechanism for the engagement of A2B receptors during hypoxia. The observation that adenosine induced upregulation of VEGF protein levels only after treatment with hypoxia, an effect that was absent in HUVECs maintained under normoxic conditions,
indicates that hypoxia-induced modulation of adenosine receptors was functionally important. A switch from expression of mostly A_2A receptors to predominantly the A_2B phenotype could be beneficial if it can promote cell and tissue survival, or it may be detrimental if A_2B receptors participate in inflammatory processes or pathologic angiogenesis. In the cardiovascular system, an upregulation of A_2B receptors in cardiac fibroblasts and SMCs may prevent cardiac remodeling associated with hypertension, myocardial infarction, and myocardial reperfusion injury after ischemia, while promoting EC growth and angiogenesis. 

Although adenosine has long been known to promote angiogenesis, only recently have attempts been made to identify adenosine receptor subtypes that mediate proangiogenic responses. A_2A adenosine receptors can have proangiogenic or antiangiogenic actions depending on the cell type studied. For example, adenosine blunts hypoxia-induced VEGF production in rat pheochromocytoma PC12 cells, a cell line that predominantly expresses A_2A adenosine receptors. Interestingly, selective antagonism of A_2A receptors resulted in increased basal levels of VEGF mRNA in these cells, possibly by unmasking the “angiogenic” effects of A_2B receptors. In contrast, A_2A agonists act synergistically with endotoxin to upregulate VEGF in murine macrophages but had no effect on their own. By comparison, A_2B receptors directly stimulate VEGF production in human retinal ECs, HMEC-1 cells, and human mast cells, even under normoxic conditions. In this study, we have demonstrated that adenosine A_2B receptors stimulated VEGF production only after exposure to hypoxia in 2 different cell types. This gain of function occurs in parallel with the upregulation of A_2B receptors. We have not explored whether enhanced coupling to pathways associated with the regulation of VEGF expression also contributes to this phenomenon.

HIF-1 is a major transcription factor regulating expression of many genes in response to hypoxia, including VEGF. It should be noted that regulation of VEGF transcription is complex and can also be regulated by HIF-1–independent pathways. Indeed, the current study shows that adenosine can regulate VEGF expression independently of HIF-1.

Depending on the cell type studied, adenosine has been shown to upregulate VEGF under normoxic or hypoxic conditions. Some studies have suggested that this effect is more important in normoxia. Others have shown that the effects of hypoxia and adenosine are additive. Our results in HUVECs show that even in cells lacking regulation by adenosine of VEGF under normoxic conditions, adenosine can upregulate VEGF under hypoxic conditions. Even though HUVECs produce relatively small quantities of VEGF in response to hypoxia or to A_2B receptor stimulation, it is possible that modulation of VEGF release through these mechanisms provides an autocrine pathway regulating EC growth. However, ECs are generally not considered a major source of VEGF. Cells located outside the vasculature are thought to release greater amounts of VEGF, establishing a gradient that recruits new blood vessel formation toward the hypoxic focus. Therefore, we extended our studies to BSMCs, a human SMC that expresses greater levels of VEGF compared with HUVECs, both at baseline and during hypoxia. We confirmed that hypoxia increased A_2B receptor expression and that activation of these receptors further increased VEGF release. Thus, A_2B receptors can participate in autocrine and paracrine regulation of EC growth, particularly during hypoxia. It should be noted that our studies were limited to in vitro models of cell function. Additional studies will be required to define the in vivo relevance of these findings. Similarly, it remains to be determined whether hypoxia modulates other functions of adenosine A_2B receptors. For example, both A_2A and A_2B receptors play an important role in the regulation of vascular tone, and it would be interesting to assess whether hypoxia-induced changes in the relative expression of adenosine receptor subtypes translates into changes in the sensitivity of vascular tone to adenosine.

Perspectives

In most cells, both high-affinity A_2A and low-affinity A_2B adenosine receptors are expressed, but only one is functionally predominant. Even though both receptor subtypes are coupled to adenylyl cyclase, other signaling pathways are differentially activated by these receptors. We found that hypoxia modulates expression of adenosine receptors in ECs and SMCs toward the low-affinity “proangiogenic” A_2B adenosine receptor subtype. We propose that during pathophysiologic conditions accompanied by hypoxia, A_2B receptors, otherwise “silent,” become the predominant adenosine receptor subtype and can be engaged in activation of compensatory mechanisms.

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