Adenosine Receptor Activation Induces Vascular Endothelial Growth Factor in Human Retinal Endothelial Cells

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Abstract—Adenosine, released in increased amounts by hypoxic tissues, is thought to be an angiogenic factor that links altered cellular metabolism caused by oxygen deprivation to compensatory angiogenesis. Adenosine interacts with 4 subtypes of G protein–coupled receptors, termed A₁, A₂A, A₂B, and A₃. We investigated whether adenosine causes proliferation of human retinal endothelial cells (HRECs) and synthesis of vascular endothelial growth factor (VEGF) and, if so, which adenosine receptor subtype mediates these effects. The nonselective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA), in a concentration-dependent manner, increased both VEGF mRNA and protein expression by HRECs, as well as proliferation. This proliferative effect of NECA was inhibited by the addition of anti-human VEGF antibody. NECA also increased insulin-like growth factor-I and basic fibroblast growth factor mRNA expression in a time-dependent manner and cAMP accumulation in these cells. In contrast, neither the A₁ agonist N⁶-cyclopentyladenosine nor the A₂₃ agonist 2-p-(2-carboxyethyl) phenethylamino-NECA caused any of the above effects of NECA. The effects of NECA were not significantly attenuated by either the A₂₃ antagonist SCH58261 or the A₁ antagonist 8-cyclopentyl-1,3-dipropylxanthine. However, the nonselective adenosine receptor antagonist xanthine amine congener completely inhibited the effects of NECA. Addition of antisense oligonucleotide complementary to A₂B adenosine receptor mRNA inhibited VEGF protein production by HRECs after NECA stimulation. Thus, the A₂B adenosine receptor subtype appears to mediate the actions of adenosine to increase growth factor production, cAMP content, and cell proliferation of HRECs. Adenosine activates the A₂B adenosine receptor in HRECs, which may lead to neovascularization by a mechanism involving increased angiogenic growth factor expression. (Circ Res. 1999;85:699-706.)

Key Words: adenosine receptor ▪ angiogenesis ▪ ischemia ▪ hypoxia ▪ diabetes

Tissue hypoxia and ischemia are known to initiate a series of events that lead to the development of collateral blood vessels in a process referred to as compensatory angiogenesis. However, the cellular and molecular mechanisms underlying compensatory angiogenesis have been only partially elucidated. Potential mediators of compensatory angiogenesis include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and nucleosides such as adenosine.

Adenosine, the subject of the present study, has been proposed to be a factor that links altered cellular metabolism caused by oxygen deprivation to the formation of new capillaries. This proposed role of adenosine is based on the observation that this nucleoside is released in increased amounts by hypoxic and/or ischemic cells and promotes proliferation of endothelial cells. Consistent with this hypothesis, adenosine and adenosine analogs have been reported to affect a number of steps involved in angiogenesis, including endothelial cell proliferation, migration, and blood vessel formation in various vascular beds. Adenosine can interact with at least 4 subtypes of G protein–coupled receptors, designated A₁, A₂A, A₂B, and A₃. These receptor subtypes are encoded by distinct genes and can, for the most part, be differentiated on the basis of their affinities for selected agonists and antagonists. Adenosine receptors are coupled to pertussis toxin–sensitive inhibitory G proteins that inhibit adenyl cyclase activity, whereas A₂₃ (high-affinity) and A₂B (low-affinity) adenosine receptors are coupled to chola toxin–sensitive G proteins that stimulate adenyl cyclase activity. In most cell types and organ systems, activation of A₁ adenosine receptors results in decreased work, and therefore, reduced O₂ consumption. Activation of A₂₃ adenosine receptors, on the other hand, increases O₂ supply by causing vasodilation. Thus, adenosine is an ideal metabolite to respond to imbalances between O₂ supply and demand. In the retina, hypoxia is followed by...
VEGF is a potent endothelial mitogen, induced by hypoxia and hyperglycemia, and has been shown to be an important factor in ischemic ocular neovascularization. VEGF causes hyperpermeability of blood vessels, which is observed in both nonproliferative and proliferative diabetic retinopathy. VEGF acts through 2 receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), both of which are tyrosine kinases. VEGF signaling occurs through tyrosine phosphorylation of phospholipase C (PLC) and phosphatidylinositol 3′-kinase.

The effects of VEGF are also mediated by activation of protein kinase C (PKC) to induce membrane translocation of PKC isofoms, especially the β-isofom of the enzyme. VEGF-R1 mediates the permeability effects associated with VEGF, whereas VEGFR-2 mediates the proliferative effects of VEGF. Elevated levels of VEGF have been detected in vitreous humor of diabetic patients with proliferative retinopathy. More importantly, in animal models of retinal neovascularization, inhibition of VEGF blocks neovascularization.

Other growth factors have been implicated in ocular angiogenesis, including bFGF and IGF-1.

The experiments described were performed to test the hypothesis that adenosine regulates expression of the angiogenic growth factor VEGF and to determine the adenosine receptor subtype that mediates the effect of the nucleoside in retinal endothelial cells of human origin.

### Materials and Methods

5′-N-ethylcarboxamidoadenosine (NECA), 2-p-(2-carboxyethyl)phenethylamino-NECA (CGS21680), N6-cyclopentyladenosine (CPA), xanthine amine congener (XAC), and 8-cyclopentyl-1,3-dipropylxanthine (CPX) were from Research Biochemicals, Inc. 9-(2-carboxyethyl)-6-cyclopentyladenosine (NECA), 2-p-(2-furyl)-pyrazolo(4,3-E)-1,2,4-triazolo(1,5-c) pyrimidine (SCH58261) was a gift from Schering-Plough Research Institute. Rabbit anti-human VEGF was from Peprotech. Antibodies and conjugates for immunofluorescence were as follows. Chicken anti-human A2B adenosine receptor, FITC-conjugated rabbit anti-chicken IgG, and nonimmune sera were from Sigma-Aldrich. Sense and antisense oligonucleotides corresponding to either VEGF or A2B adenosine receptor sequences were synthesized by Life Technologies. The sequences were chosen from the region proximal to and including the start codon using published sequence information for these genes. The antisense sequences are as follows: VEGF, AGACAGACAGAAAGTTCATGG, and A2B adenosine receptor, CAGCCGGTCTTCGTCCTCAGAAGCATGG. Sense sequences are the complement of the antisense sequences shown.

Human retinal endothelial cells (HRECs) were prepared and maintained as previously described. Cells in passages 3 to 6 were used for the studies. The identity of HRECs was validated by demonstrating endothelial cell incorporation of fluorescently labeled acetylated LDL and by fluorescence-activated cell sorting analysis as previously described. For all experiments, cells were starved of serum overnight and then incubated with adenase deaminase type III (2 U/mL, Sigma-Aldrich) for 20 minutes before test agents were added. Adenosine receptor agonists and antagonists were added at concentrations ranging from 5 μmol/L to 100 μmol/L in serum-free medium containing adenase deaminase type III and then incubated for additional times as indicated in specific results.

cAMP was measured in response to adenosine receptor agonists and/or antagonists as described. Conditioned medium was used to measure changes in VEGF protein in response to adenosine receptor agonists and/or antagonists using an ELISA kit (R&D Systems, Inc).

HREC proliferation was determined by measuring DNA synthesis via colorimetric detection of bromodeoxyuridine (Brdu) incorporation using a kit (Roche Molecular Biochemicals), and also by changes in cell number. Brdu incorporation was also used to measure the effect of anti-VEGF antibody on adenosine receptor agonist–induced HREC proliferation. The potential reduction of VEGF synthesis induced by adenosine receptor agonist was tested by ELISA after inclusion of antisense oligonucleotides directed against mRNA for either A2B adenosine receptor or VEGF. Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), using a competitive synthetic multiplex template as described previously, was performed to measure changes in mRNA after treatment with adenosine receptor agonists and/or antagonists. Immunofluorescent confocal microscopy was used as described to demonstrate both the presence of A2B adenosine receptors and uptake of fluorescence-labeled acetylated LDL in HRECs grown on multichamber glass slides (Nalge Nunc International).

### Statistical Analysis

Comparisons between treatment groups (as described in the figure legends) were analyzed by 1-way ANOVA followed by the Bonferroni t test. Data are expressed as mean±SEM. Values of P<0.05 were considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

### Results

The nonselective adenosine receptor agonist NECA, after 48 hours of exposure, induced a concentration-dependent increase in DNA synthesis by HRECs, as indicated by Brdu incorporation (Figure 1A). In contrast, neither the A2A adenosine receptor agonist CGS21680 (10 nmol/L to 10 μmol/L) nor the A1 adenosine receptor agonist CPA (10 nmol/L to 10 μmol/L) increased Brdu incorporation by HRECs (Figure 1A). The addition of the adenosine receptor antagonist XAC completely prevented NECA-stimulated Brdu incorporation (Figure 1B). In contrast, neither the selective A1 adenosine receptor antagonist CPX (20 nmol/L) nor the selective A2A adenosine receptor antagonist SCH58261 (60 μmol/L) attenuated the stimulatory effect of NECA on Brdu incorporation by HRECs (Figure 1B).

The data for cell counts were consistent with those for Brdu incorporation. Treatment with NECA for 48 hours resulted in a concentration-dependent increase in HREC number, whereas neither CGS21680 nor CPA caused an increase in cell number (Figure 2A). Of the 3 adenosine receptor antagonists tested, only XAC (10 μmol/L) significantly inhibited the increase in cell number induced by 10 μmol/L NECA (Figure 2B).

### cAMP Accumulation

To obtain evidence for the presence of the A2B adenosine receptors in HRECs, we performed assays for cAMP content in intact HRECs after treatment of cells with adenosine receptor agonists and antagonists. The nonselective adenosine receptor agonist NECA increased the cAMP content of HRECs in a concentration-dependent manner (Figure 3A), with an EC50 value of 24 μmol/L. In contrast, the selective high-affinity A2B adenosine receptor agonist CGS21680 (at concentrations up to 100 μmol/L) had no significant effect on cAMP content of HRECs (Figure 3A). The effect of selective A1 and A2B adenosine receptor antagonists on NECA-induced accumulation of cAMP was also examined. NECA (10 μmol/L)-
induced increase in cAMP content in HRECs was not significantly inhibited either by the selective A2A adenosine receptor antagonist SCH58261 (60 nmol/L) or by the selective A1 adenosine receptor antagonist CPX (20 nmol/L) (Figure 3B). On the other hand, the nonselective adenosine receptor antagonist XAC (10 μmol/L) completely blocked the effect of NECA on cAMP accumulation.

**Quantification of VEGF, IGF-I, and bFGF mRNA in HRECs**

To determine whether NECA stimulates angiogenic growth factor mRNA expression in HRECs, total mRNA from HRECs exposed to NECA was subjected to quantitative RT-PCR. Treatment of HRECs with NECA (10 nmol/L to 10 μmol/L) for 2 hours induced a concentration-dependent increase in expression of mRNA for VEGF by up to 4.6-fold (from 0.43 × 10⁶ to 1.85 × 10⁶ copies/μg RNA), compared with untreated control cells. After 8 hours of exposure to NECA, cell mRNA levels for VEGF in cells treated with NECA had returned to baseline. HRECs were also treated with the A2A adenosine receptor agonist, CGS21680 (10 nmol/L to 10 μmol/L), and the A1 adenosine receptor agonist, CPA (10 nmol/L to 10 μmol/L). In contrast to NECA, neither CGS21680 nor CPA caused a significant change in VEGF mRNA expression (data not shown).

The increase in mRNA for VEGF caused by 10 μmol/L NECA was not attenuated significantly either by the selective A2A adenosine receptor antagonist SCH58261 (60 nmol/L) or by the selective A1 adenosine receptor antagonist CPX (20 nmol/L) (Figure 4). The nonselective antagonist XAC (10 μmol/L) completely attenuated NECA-induced increases in mRNA for VEGF (Figure 4). NECA (10 μmol/L) also induced a time-dependent increase in mRNA for both IGF-I and bFGF. IGF-I mRNA levels increased by 2.2-fold after 2
hours of exposure (from $30 \times 10^3$ copies/µg RNA to $65 \times 10^3$ copies/µg RNA) and 11.7-fold ($350 \times 10^3$ copies/µg RNA)

after 8 hours of exposure to NECA. Similarly, bFGF mRNA increased 3.7-fold after 2 hours of exposure (from $2 \times 10^3$ copies/µg RNA to $7.4 \times 10^3$ copies/µg RNA) and 11.4-fold ($22.8 \times 10^3$ copies/µg RNA) after 8 hours of exposure to NECA.

Quantification of VEGF Protein in Conditioned Medium

To determine whether the increase in VEGF mRNA expression resulted in increased protein levels, VEGF was measured in conditioned medium after 8 hours of exposure to NECA, in the presence or absence of adenosine receptor antagonists (Figure 5). NECA increased VEGF protein, but neither the A1 adenosine receptor antagonist CPA (not shown) nor the A2A adenosine receptor agonist CGS21680 caused an increase in VEGF protein (Figure 5). The increase in VEGF protein caused by NECA was not attenuated by either the selective $A_{2A}$ adenosine receptor antagonist SCH58261 (60 nmol/L) or by the selective $A_1$ adenosine receptor antagonist CPA (20 nmol/L). Only the nonselective adenosine receptor antagonist XAC (10 µmol/L) completely inhibited the action of NECA to increase VEGF protein expression (Figure 5).

Effect of Anti-VEGF Antibody on NECA-Induced HREC Proliferation

Incubation with 10 ng/mL VEGF resulted in BrdU incorporation to a level approximating that induced by normal growth medium. The anti-VEGF antibody at 100 ng/mL significantly reduced DNA synthesis induced by VEGF (Figure 6). Incubation with NECA (10 µmol/L) increased
DNA synthesis to levels comparable with that induced by normal growth medium. The addition of anti-VEGF antibody resulted in a decrease in NECA-induced BrdU incorporation, which was statistically significant at the highest concentration of antibody used (Figure 6). Similar results were observed at either 24 or 48 hours of exposure to the test agents.

Effect of Antisense Oligonucleotides on VEGF Induction by NECA

Both A2B adenosine receptor and VEGF antisense oligonucleotides caused a significant decrease of VEGF in the conditioned medium after NECA exposure (Figure 7). This effect was most pronounced for the receptor antisense oligonucleotide with 10 nmol/L NECA, but it was evident for all concentrations of NECA tested. The VEGF antisense oligonucleotide also caused a decrease in secreted VEGF in response to NECA, although not to the same magnitude as that observed with the A2B adenosine receptor antisense.

Immunofluorescence

Analysis of acetylated LDL uptake indicates that the cells are indeed of endothelial origin (Figure 8A). These results were confirmed by immunofluorescent labeling with antibody to coagulation factor VIII (data not shown). Labeling with A2B adenosine receptor antibody clearly demonstrated that the tested cells express the A2B receptor subtype (Figure 8B and 8C).

Discussion

In this report, we demonstrate that the nonselective adenosine receptor agonist NECA, but neither the A2A adenosine receptor agonist CGS21680 nor the A1 adenosine receptor agonist CPA, stimulates HREC DNA synthesis, proliferation, and cAMP accumulation. Furthermore, neither the selective A2A adenosine receptor antagonist SCH58261 nor the A1 adenosine receptor selective antagonist CPX attenuated significantly the effects of NECA. Only the nonselective adenosine receptor antagonist XAC reduced significantly the NECA-mediated increase of DNA synthesis, cell proliferation, cAMP content, and VEGF synthesis. The A2B adenosine receptor, which we localized in HRECs using a specific antibody, is the predominant adenosine receptor subtype responsible for mediating the actions of NECA. Equally or more importantly, the addition of VEGF antibody decreased significantly NECA-induced BrdU incorporation. This finding provides strong evidence that VEGF plays a major role in mediating the mitogenic effect of NECA and, presumably, the natural ligand adenosine. We also demonstrated a significant increase in expression of mRNA for both IGF-I and bFGF after 8 hours of exposure to NECA. Thus, our data also raise the possibility that, in addition to VEGF, IGF-I and
bFGF may contribute in part to the proliferative effect of NECA.

In HRECs, NECA caused a concentration-dependent increase in VEGF mRNA as well as an increase in secreted VEGF protein that was blocked by an antisense oligonucleotide complementary to A2B adenosine receptor mRNA. In contrast, neither the A2A agonist CGS21680 nor the A1 agonist CPA affected the expression either of VEGF mRNA or protein, ruling out a role for either A2A or A1 adenosine receptors in mediating increased VEGF expression, increased BrdU incorporation, and cell proliferation. NECA-induced increases in expression of both VEGF mRNA and protein by HRECs were blocked by the nonselective adenosine receptor antagonist XAC, whereas the A1- and A2A-selective adenosine receptor antagonists CPX (20 nmol/L) and SCH58261 (60 nmol/L), respectively, did not attenuate these increases. The antagonists CPX and SCH58261 were used at concentrations at which their selectivity for A1 and A2A receptors has been demonstrated in cardiovascular preparations.36,37,38 On the other hand, XAC was used at a concentration (10 μmol/L) that should be sufficient to antagonize effectively A1, A2A, A2B, and possibly the A1 receptor-mediated responses. Hence, the evidence supporting the role of A2B adenosine receptors as the adenosine receptor subtype that mediates the effects of NECA reported here can be summarized as follows: (1) A2B receptors were localized in HRECs using immunofluorescence microscopy with the A2B antibody; (2) antisense oligonucleotides homologous to the A2B receptor blocked NECA-stimulated VEGF production; (3) neither the A1 nor the A2A receptor agonists had any effect on BrdU incorporation, cell proliferation, or cAMP production; (4) neither the A1 nor the A2A antagonists, used at the concentration at which they are selective for their receptor subtype, antagonized the effects of NECA; and (5) the nonselective but potent A2B antagonist XAC used at high concentrations significantly attenuated the effects of NECA.

Taken together, the data support the hypothesis that the A2B adenosine receptor, but neither the A1 nor the A2A receptor, is responsible for mediating the actions of NECA on cAMP accumulation and VEGF synthesis in cultured HRECs. The results of our studies do not rule out a possible role of A1 receptor in mediating the effects of NECA. However, this is unlikely, because the affinity (Kd) of XAC for the A1 adenosine receptor is 29 μmol/L, which is higher than the concentration (10 μmol/L) used in our studies.37,39 Our conclusion that the A2B receptor is the most likely adenosine receptor subtype that mediates the effects of NECA—and presumably adenosine—on HRECs differs from that reported by Takagi et al.40,41 These investigators, using retinal endothelial cells of bovine origin, concluded that the proliferative action of adenosine is mediated by A2A receptor. Takagi et al.40 also reported that acute hypoxia causes a decline in KDR/Flk mRNA levels as well as VEGF binding sites on the cell surface. On the other hand, chronic hypoxia was associated with increased KDR/Flk message levels.40 More importantly, Takagi et al.41 also reported that the endogenous adenosine released by hypoxic bovine retinal endothelial cells was sufficient to stimulate VEGF message expression. Species differences and passage number may account for differences in the observed adenosine receptor subtype in retinal endothelial cells. Furthermore, distinct adenosine receptor subtypes may mediate the proliferative effects of adenosine in endothelial cells from different vascular beds, even within the same species.

Protein kinase A and members of the mitogen-activated protein kinase, family such as extracellular signal–regulated kinases 1 and 2 (ERK1/ERK2) are potential mediators of adenosine–mediated cell proliferation.42–44 Activation of the A2B adenosine receptor results in cAMP generation via Gs. A2 receptor activation and stimulation of adenylyl cyclase/protein kinase A pathways can either activate45 or inhibit46–48 growth factor–stimulated ERK activity.

A2B adenosine receptor signaling through Gαi also results in increased levels of ERK.42 Therefore, A2B receptor stimulation of Gαi, PLC, and PKC may synergize with or potentiate the effects of traditional tyrosine kinase–
coupled growth factors, either through c-src–dependent activation of ERK or through PKC-dependent, src-independent, pathways. Because forskolin-mediated adenylyl cyclase activity does not activate VEGF expression in endothelial cells, Gq/11- and PKC-mediated activation of ERK may contribute to activation of transcription factors and lead to the induction of message for VEGF. Thus, A2B receptor activation can mediate proliferation by inducing growth factor synthesis and through stimulation of Gq/11, PLC, and PKC pathways.

Angiogenesis is a compensatory mechanism in response to insufficient tissue oxygenation. In the retina of diabetic individuals, homeostatic abnormalities lead to retinal nonperfusion and subsequent ischemia. Ischemia leads to new vessel formation and disruption of the normal retinal vasculature, the hallmarks of proliferative diabetic retinopathy. Our findings raise the possibility that selective A2B adenosine receptor antagonists could be used as a novel therapeutic approach to block the inciting events leading to aberrant angiogenesis in proliferative diabetic retinopathy. Pharmacological modulation of the neovascular response in a nondestructive manner should have significant advantages over current therapeutic approaches.

By blocking the A2B adenosine receptor, the action of adenosine to induce the growth factor cascade may be inhibited, and blocking the A2B adenosine receptor may attenuate aberrant cellular proliferation. In summary, our results provide strong evidence that the proliferative effect of adenosine on HRECs is caused by increased expression of VEGF and probably other growth factors, and this effect is mediated by the A2B adenosine receptor.

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References

Figure 8. A, Fluoromicrograph of subconfluent HRECs that were incubated for 6 hours with 5 µg/mL fluorescence-labeled acetylated LDL. Magnification, ×120. Cells were visualized using a standard FITC long-pass excitation:emission filter set. Note the abundance of punctate perinuclear fluorescence in the HRECs. Cultured human coronary artery smooth muscle cells were used as a negative control and showed no evidence of acetylated LDL uptake (data not shown). B, Confocal image of HRECs at ~50% confluence reacted with FITC-conjugated rabbit anti-chicken Ab showing nonspecific binding of this antibody. Magnification, ×60. C, Confocal image of HRECs at ~50% confluence reacted first with AF5 chicken anti-A2B adenosine receptor Ab, followed by FITC-conjugated rabbit anti-chicken Ab, demonstrating specific reactivity of the cells to the anti-A2B receptor antibody. Magnification, ×60.


