Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells

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Adenosine Mediates Hypoxic Induction of Vascular Endothelial Growth Factor in Retinal Pericytes and Endothelial Cells

Hitoshi Takagi,* George L. King,*++ Gregory S. Robinson,§ Napoleone Ferrara,|| and Lloyd Paul Aiello*††

Purpose. To determine the mechanistic role for adenosine and adenosine receptors in the hypoxic induction of vascular endothelial growth factor (VEGF) in retinal microvascular cells.

Methods. Bovine retinal capillary endothelial cells and microvascular pericytes were studied under normoxic (95% air, 5% CO₂) or hypoxic conditions (0% to 2% O₂, 5% CO₂, 93% to 95% N₂) using a variety of well-characterized adenosine and adenosine receptor agonists and antagonists. Vascular endothelial growth factor mRNA expression was evaluated by Northern blot analysis, VEGF protein levels were determined by Western blot analysis, and cyclic adenosine monophosphate (cAMP) accumulation was measured by radioimmunoassay.

Results. Inhibitors of oxidative respiration increased VEGF mRNA 5 ± 3 times (P < 0.001) after 3 hours. Adenosine A₁ receptor (A₁R) agonist N-cyclopentyl-adenosine did not increase VEGF mRNA at A₁R stimulatory concentrations; however, adenosine A₂ receptor (A₂R) agonists DPMA, NECA, and CGS21680 increased VEGF mRNA in a dose-dependent manner with elevations of 2 ± 0.3 (P < 0.001), 2.3 ± 0.5 (P = 0.016), and 2 ± 0.2 (P = 0.002) times, respectively. A₁R antagonist CSC and adenosine degradation by adenosine deaminase reduced hypoxic stimulation of VEGF mRNA 68% ± 18% (P = 0.038) and 37% ± 6% (P = 0.025), respectively, in a dose-dependent manner. A₂R antagonists DPCPX and 8-PT had no significant effect. Hypoxia and NECA increased VEGF protein secretion 4.7 times, whereas CSC inhibited hypoxia-induced VEGF protein secretion by 96%. NECA and CGS21680 increased cAMP production within 10 minutes, and cAMP stimulation increased VEGF mRNA 4.8 ± 2.6 times (P = 0.034). CSC suppressed the hypoxic elevation of cAMP (P < 0.05). Inhibition of protein kinase A using H-89 reduced hypoxia-induced VEGF expression 61% ± 6.3% (P = 0.043) in a dose-dependent manner.

Conclusions. These data suggest that the hypoxia-induced accumulation of adenosine stimulates VEGF gene expression through stimulation of adenosine A₂ receptors and subsequent activation of the cAMP-dependent protein kinase A pathway in retinal vascular cells. Invest Ophthalmol Vis Sci. 1996; 37:2165–2176. Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and vasopermeability factor whose expression is increased dramatically in vivo and in many types of cultured cells, including retinal vascular cells. Vascular endothelial growth factor exerts its action on endothelial cells through high-affinity, endothelial-specific, autophosphorylating tyrosine kinase receptors. Elevated levels of VEGF have been associated with tumor neovascularization,7 developmental angiogenesis,10 and ischemia-induced proliferative ocular diseases, such as diabetic retinopathy and central retinal vein occlusion.11 Recently accumulating evidence suggests that the hypoxic induction of VEGF gene expression appears to be regulated by transcriptional activation and increased mRNA stability.12–15 cis elements and trans factors are reported to affect the hypoxic regulation of VEGF. A second-messenger pathway, such as the protein kinase C pathway,16 the cyclic adenosine monophosphate (cAMP)-dependent pathway,16 and
the Ca\(^{2+}\) ion channel\(^{17}\) can regulate VEGF gene expression. Activation of c-Src has been reported to be a regulator of hypoxic VEGF induction.\(^{18}\) However, the mechanism by which hypoxia induces cells to increase VEGF expression is not well understood.

Adenosine is released by hypoxic tissues, and concentrations of this naturally occurring nucleoside are elevated (>30 times) in hypoxic hepatocytes, primarily as a result of decreased recycling by adenosine kinase.\(^{19}\) In addition, adenosine is released from, and induces the proliferation and migration of, chick astrocytes, human astrocytoma cells,\(^{20}\) and human umbilical vein endothelial cells.\(^{21}\) Erthropoietin, a molecule whose expression is increased by hypoxia,\(^{22,23}\) and whose regulation is analogous to that of VEGF,\(^{24}\) is similarly stimulated by adenosine through \(A_2R\).\(^{25}\)

Adenosine is a metabolic precursor for nucleic acid biosynthesis and a regulator of numerous mammalian systems, including neurologic, cardiovascular, renal, respiratory, immunologic, gastrointestinal, and metabolic.\(^{26}\) Physiologic responses include coronary vasodilatation, renal vasoconstriction, inhibition of platelet aggregation, sedation, and reduced cardiac rate and contractile force.\(^{27}\) The majority of adenosine effects are mediated through cell surface receptors that characteristically bind adenosine more than nucleotides, are selectively blocked by methylxanthines, couple to adenyly cyclase, and do not alter prostaglandin synthesis.\(^{28}\) High concentrations of adenosine, however, can inhibit adenyly cyclase directly through an intracellular "P-site." Adenosine receptors act through guanosine triphosphate binding proteins, coupling not to adenyly cyclase but also to ion channels and phospholipases.\(^{29}\) Adenosine receptors have seven transmembrane helical structures\(^{30}\) and are categorized as \(A_1\), \(A_2a\), \(A_2b\), \(A_3\), and possibly other \(A_1\) subtypes by pharmacologic interaction with a variety of agonists and antagonists.\(^{31,32}\) Adenosine \(A_2a\) and \(A_3\) receptor binding decreases cAMP levels, whereas \(A_2\) receptor binding increases these same levels.\(^{33,34}\) Four adenosine receptor subtypes have been cloned,\(^{35}\) and numerous species homologues have been identified. Recently, adenosine has been shown to stimulate VEGF expression in U-937 mononuclear cells\(^{36}\) and pig cerebral endothelial cells,\(^{37}\) although the mechanism of stimulation and mediating receptors appear to differ.

Because adenosine is released from hypoxic tissue and can stimulate endothelial cell proliferation, we investigated the possibility that the hypoxia-induced increase in VEGF expression is mediated by adenosine interaction with its receptors on retinal vascular cells. Our results demonstrate that hypoxia-induced adenosine release results in adenosine \(A_2\) receptor stimulation and activation of the cAMP-dependent protein kinase A (PKA) pathway, which together account for much of the hypoxic induction of VEGF in retinal microvascular endothelial cells and pericytes.

**MATERIALS AND METHODS**

**Chemicals and Antibodies**

Carbonyl cyanide \(p\)-(trifluoromethoxy) phenyl-hydrazone (FCCP) and N\(^6\)-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)-ethyl] adenosine (DPMA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-(3-chlorostyryl)-caffeine (CSC), 8-phenylthioethylamine (B-PT), 5'-[(N-ethylcarboxamido)-adenosine (NECA), 2-\(p\)-(2-carboxyethyl)-phenethyl-amino-5'-N-ethylcarboxyamidoadenosine (CGS21680), and N\(^6\)-cyclopentyl-adenosine (CPA) were obtained from Research Biochemicals International (Natick, MA). N-[2-((3-bromophenyl)-2-propenyl)-amino]-ethyl]-5-isooquinolinesulfonamide (HS89) and RO-20-1724 were purchased from Calbiochem (San Diego, CA). Cyclic adenosine monophosphate radioimmunoassay kits were obtained from DuPont-New England Nuclear (Boston, MA), \(^{38}P\)-dATP was acquired from Amersham (Arlington Heights, IL), and adenosine deaminase was obtained from Worthington Biochemicals (Freehold, NJ). Recombinant human vascular endothelial growth factor and human VEGF cDNA was provided by Genentech (San Francisco, CA).

**Cell Cultures**

Bovine retinal endothelial cells and retinal pericytes were isolated from slaughterhouse eyes by homogenization and a series of filtration steps as previously described.\(^{39}\) Primary endothelial cell cultures similarly were obtained and grown on fibronectin (NYBen Reagents; New York Blood Center, New York, NY)-coated dishes. Homogeneity of retinal pericytes was confirmed by reactivity with anti-factor VIII antibodies. Retinal pericytes were isolated as described above and cultured in DMEM with 5.5 mM glucose, 10% plasma-derived horse serum (Wehaton, Pipersville, PA), 50 mg/l heparin, and 50 U/l endothelial cell growth factor. The cells were cultured in 5% CO\(_2\) at 37°C, and media were changed every 3 days. Endothelial cell homogeneity was confirmed by reactivity with anti-factor VIII antibodies. Retinal pericytes were isolated as described above and cultured in DMEM with 5.5 mM glucose and 20% fetal bovine serum (Hyclone, South, UT) on fibronectin-coated dishes. Homogeneity of retinal pericytes was confirmed by reactivity with monoclonal antibody 3G5.\(^{40}\) Retinal endothelial cells and retinal pericytes were evaluated in these studies, each yielding similar results. Data from experiments with a single cell type, as described in the text, are presented.
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1 μM FCCP
100 μM FCCP

VEGF
36B4

FIGURE 1. Inhibition of electron transport stimulates vascular endothelial growth factor (VEGF) mRNA expression. Confluent monolayers of retinal endothelial cells were treated with 1 μM or 100 μM FCCP under normoxic conditions for the duration indicated. Total RNA was isolated and Northern blot analysis performed as described in Materials and Methods. Representative Northern blots using VEGF cDNA and control 36B4 probe (top) and quantitation of multiple experiments after normalization to the control signal are shown (bottom). Results are expressed as percent of control VEGF mRNA expression ± standard error.

Hypoxia Studies

Confluent cell monolayers were exposed to oxygen concentrations noted in the text using a Lab-Line Advanced Computer Controlled Infrared Water-Jacketed CO2 Incubator with reduced oxygen control (model 480; Lab-Line, Melrose Park, IL). All cells were maintained at 37°C in a constant 5% carbon dioxide atmosphere, with oxygen deficit induced by nitrogen replacement. Cells under these conditions showed no morphologic changes by light microscopy after exposure periods exceeding 72 hours, excluded Trypan Blue dye (>98%), and subsequently could be passaged normally. Cells incubated under standard culture conditions from the same batch and passage were used as controls (95% air, 5% CO2). Throughout this article, normoxic refers to standard cell culture conditions (95% air, 5% CO2), whereas hypoxia refers to lower oxygen concentrations (2% and 0.5% in these experiments) than present under standard conditions (21% oxygen). It should be noted that “normoxic” culture conditions are hyperoxic compared to physiologic conditions. However, the 2% and 0.5% oxygen concentrations used in these studies are relatively hypoxic compared to control conditions, cultured retinal cells respond to hypoxia similarly below 5% oxygen, and 0.5% oxygen corresponds to <20 mm Hg, which is physiologically hypoxic.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from individual P-100 tissue culture plates using guanidium thiocyanate. Radioactive probes were generated using Amersham Multiprime labeling kits and 32P-dATP. Northern blot analysis was performed using ICN Biochemicals (Irvine, CA) Biotrans nylon membranes, ultraviolet crosslinking (UV Stratalinker 2400; Stratagene, La Jolla, CA), and a rotating hybridization oven (model 400; Robbins Scientific, Sunnyvale, CA). Analysis was performed with a Molecular Dynamics Computing PhosphorImager (Mountain View, CA). Lane-loading differences were normalized using 36B4 control cDNA. Graphs of Northern blot quantitation represent results from at least three experiments, with each point within each experiment performed in triplicate. Each lane was normalized to its own 36B4 control signal.

Cyclic Adenosine Monophosphate Accumulation

Cells grown in six-well cluster dishes were washed twice with phosphate-buffered saline and were incubated with adenosine receptor subtype-specific ligands at the indicated concentration in 1 ml of serum-free DMEM in the presence of 300 μM RO-20-1724 for 10 minutes. The incubation was terminated by aspirating the media and the addition of 1 ml ice-cold 6% trichloroacetic acid. After a 20-minute incubation on ice, the cells were scraped off the plate, sonicated, and stored at —20°C. The sample was thawed and centrifuged at 2000g for 15 minutes at 4°C, the supernatant was extracted five times with 2 vol water-saturated ethyl ether, and the remaining ether was evaporated to dryness. The dried samples were resuspended in 50 mM sodium acetate (pH 6.2) and assayed for cAMP by radioimmunoassay using a cAMP assay kit from New England Nuclear.

Vascular Endothelial Growth Factor Protein Secretion

Either CSC (10 μM), NECA (1 μM), or phosphate-buffered saline was added to 90% confluent cultures
FIGURE 2. Stimulation of adenosine A1 receptor does not induce vascular endothelial growth factor (VEGF) mRNA expression. Normoxic bovine retinal pericytes were exposed to increasing concentrations of CPA and VEGF mRNA levels evaluated by Northern blot analysis after 4.5 hours (A). A1R are significantly more sensitive to CPA than are A2R (Ki = 1 nM and 680 nM, respectively). The time course of VEGF expression in retinal pericytes exposed to 50 µM CPA is presented in B. Representative Northern blots and quantitation of multiple experiments after normalization to the control signal are shown at the top and bottom of each panel, respectively.

of bovine retinal pericytes for 1 hour. The culture media then were replaced with 5 ml of labeling media (3.75 ml DMEM minus methionine and cysteine, 1.25 ml DMEM with 20% fetal bovine serum, 200 µCi 35S-Translabel [ICN, Costa Mesa, CA]) supplemented with CSC or NECA as described above. CSC-treated and -untreated cells were exposed to hypoxic conditions, whereas NECA-treated and -untreated cells were exposed to normoxic conditions for 17 hours before collection and centrifugation of the media. Equal media protein (as determined by equal trichloroacetic acid precipitable counts) were immunoprecipitated overnight at 4°C using a polyclonal rabbit anti-human VEGF antibody (a generous gift from Kevin Claffey, Department of Pathology, Beth Israel Hospital, Boston, MA). The antibody–VEGF complexes were pelleted using protein A sepharose (Pharmacia, Uppsala, Sweden), washed 3X (10 mM Tris, pH 8, 140 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.01% sodium azide), and resuspended in 2X sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer containing 7 mM dithiothreitol. Samples were separated on a 5.5%–12.5% SDS polyacrylamide gel, enhanced using Entensify solution (New England Nuclear), and dried. Results were quantitated using a phosphoimager (Molecular Dynamics, Mountainview, CA).

Statistical Analysis
All determinations were performed in triplicate, and experiments were repeated at least three times. Results are expressed as the mean ± standard deviation. Statistical analysis used the paired Student’s t-test to compare quantitative data populations with normal distributions and equal variance. Data were analyzed using the Mann–Whitney Rank Sum Test for populations with nonnormal distributions or unequal variance. Data within multiple sets were compared to a
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**RESULTS**

**Inhibition of Electron Transport Stimulates Vascular Endothelial Growth Factor mRNA Expression**

To determine whether the mechanism of hypoxia-induced VEGF mRNA induction was dependent on the absence of oxygen itself, we examined whether artificial inhibition of oxidative phosphorylation in a normoxic environment could mimic the VEGF mRNA regulation observed under hypoxic conditions. FCCP inhibits mitochondrial oxidative phosphorylation at its most distal site, preventing the electron transport from cytochrome aa3 to oxygen.35 Thus, this compound more closely mimics an oxygen deficit than do proximal transport inhibitors such as rotenone and antimycin A. When confluent normoxic retinal endothelial cells were exposed to either 1 μM or 100 μM FCCP, VEGF mRNA levels increased 2.4 ± 0.8 times (P = 0.024) and 5 ± 0.3 times (P < 0.001) within 3 hours, respectively (Fig. 1). These data suggest that the mechanism of hypoxia-induced VEGF mRNA induction is not dependent on the absence of oxygen itself but might involve byproducts of an inactive mitochondrial respiratory system or effects from depleted energy stores within the cell.

**Adenosine Agonists Stimulate Vascular Endothelial Growth Factor mRNA Expression**

It has been reported that hypoxia reduces adenosine kinase-mediated recycling of adenosine to adenosine monophosphate, resulting in dramatic elevations of intracellular adenosine.10 These levels of adenosine are capable of mediating physiologic effects through binding to various adenosine receptors. Because hypoxic tissues release adenosine and adenosine induces proliferation and migration of endothelial cells,21 we investigated whether adenosine stimulation could increase the expression of VEGF mRNA. Stable adenosine receptor agonists were used to test this hypothesis because adenosine itself degrades rapidly in culture, and a steady media concentration cannot be assured. CPA is a stable adenosine A1 receptor (A1R) agonist at low concentrations (Ki = 1 nM) and both an A1R and an A2R (Ki = 680 nM) agonist at high concentrations.36,37 Stimulation of normoxic retinal pericytes with CPA did not affect VEGF mRNA expression at levels up to 100 nM (Fig. 2A) but did increase VEGF mRNA expression at higher concentrations (P = 0.005). High-dose CPA (50 μM) increased VEGF mRNA levels 2 ± 0.1 times (P < 0.001), with...
Consistent induction of expression noted within 3 hours (Fig. 2B).

To determine whether adenosine induced VEGF expression through specific effects on A$_1$R, we stimulated normoxic retinal endothelial cells with the A$_1$R agonists DPMA, NECA, and CGS21680. Each compound increased VEGF mRNA expression in a dose-dependent manner at concentrations of 10 nM and higher (Fig. 3). Vascular endothelial growth factor mRNA expression was increased 2 ± 0.3 ($P < 0.001$), 2.3 ± 1.1 ($P = 0.016$), and 2 ± 0.2 times ($P = 0.002$) after stimulation with DPMA (100 nM), NECA (1 µM), and CGS21680 (1 µM) respectively. Vascular endothelial growth factor stimulation by the A$_1$R specific agonist CGS21680 was not statistically different from the stimulation observed with the nonsubtype specific A$_1$R agonist NECA.

**Adenosine Antagonists Inhibit Hypoxia-Induced Vascular Endothelial Growth Factor mRNA Expression**

To determine whether the hypoxic VEGF response could be reduced by blocking adenosine receptor activation, we studied the effects of A$_2$R antagonists DPCPX and 8-phenyltheophylline (8-PT), the A$_2$R antagonist CSC, and the adenosine-degrading enzyme adenosine deaminase (AdD), which is expressed in U/ml.

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Adenosine deaminase. Both DPCPX and 8-PT had little effect on hypoxia-induced (2% O₂, 5% CO₂, 93% N₂) expression of VEGF at AₐR inhibitory concentrations (Fig. 4). Adenosine deaminase reduced VEGF expression 37% ± 6% (P = 0.025). Blocking AₐR (IC₅₀ = 54 nM) with CSC inhibited hypoxia-induced VEGF expression by 68% ± 18% (P = 0.038) in a dose-dependent manner at levels not expected to affect AₐR (IC₅₀ = 28 μM).35-36

Adenosine Agonists Stimulate Cyclic Adenosine Monophosphate Synthesis

If adenosine mediates hypoxia-induced VEGF production, then retinal pericytes and endothelial cells should express adenosine receptors. As previously mentioned, AₐR stimulation decreases cAMP whereas A₂R stimulation increases it.26-27 We measured cAMP accumulation in normoxic bovine retinal pericytes after the application of adenosine agonists for 10 minutes in the presence of the cAMP phosphodiesterase inhibitor RO-20-1724. Both AₐR agonists NECA and CGS21680 increased cAMP accumulation in a dose-dependent manner, with consistent increases noted at concentrations greater than 1 to 10 nM (Fig. 5). NECA and CGS 21680 increased cAMP levels by 2.4 ± 0.9 (P = 0.008) and 2.9 ± 0.5 times (P < 0.001) at a concentration of 100 nM. At low (AₐR effective) concentrations, stimulation of AₐR with CPA did not reduce cAMP accumulation after cAMP stimulation with forskolin (data not shown).

Cyclic Adenosine Monophosphate Analogs Stimulate Vascular Endothelial Growth Factor mRNA Expression

Because adenosine receptor agonists increased cAMP levels and VEGF mRNA expression in retinal cells, we determined whether cAMP could induce VEGF expression. Confluent normoxic retinal pericytes were exposed to 50 μM of the cAMP analog dibutyryl cAMP, and VEGF mRNA expression was analyzed by Northern blot analysis. VEGF mRNA levels increased 4.8 ± 2.6 times (P = 0.034) after 6 hours (Fig. 6).

Hypoxia-Induced Cyclic Adenosine Monophosphate Synthesis Is Suppressed by Adenosine A₂ Receptor Inhibition

Because AₐR stimulation increases cAMP, we studied whether hypoxia could stimulate cAMP synthesis in retinal cells. In addition, to determine whether the observed hypoxia-induced cAMP increase resulted from the stimulation of AₐR, we studied the effect of an A₂R antagonist. Bovine retinal endothelial cells were incubated in 0.5% or 21% oxygen for 4 hours with or without 10 μM of the A₂R antagonist CSC. Cyclic adenosine monophosphate levels were increased nearly 50% (P < 0.01) in cells exposed to hypoxic conditions compared to those exposed to normoxic conditions (Fig. 7). This hypoxia-induced cAMP elevation was inhibited 69% by CSC (P < 0.05); CSC had no significant effect on cells incubated under normoxic conditions.

VEGF Protein Secretion Is Stimulated by Adenosine Receptor Agonists and Hypoxia-Induced VEGF Protein Expression Is Inhibited by A₂R Antagonists

To determine whether VEGF protein levels responded to adenosine in a manner similar to VEGF mRNA, VEGF protein was immunoprecipitated from media conditioned by retinal pericytes exposed to 35S-methionine under normoxic or hypoxic (0.5% O₂, 5% CO₂, 94.5% N₂) conditions for 17 hours, and SDS-PAGE was performed (Fig. 8). In the media, VEGF protein was increased 4.6 times by 17 hours of hypoxia. Exposure to 1 μM NECA under normoxic conditions also increased VEGF protein concentrations 4.7 times. However, pretreatment with the A₂R antagonist CSC for 1 hour before 17 hours of hypoxia inhibited 96% of the hypoxia-induced increase in VEGF protein.

Protein Kinase A Inhibition Suppresses Hypoxic Induction of VEGF mRNA

Because cAMP has been well documented to stimulate PKA and because we have demonstrated that cAMP...
FIGURE 8. Adenosine agonists stimulate vascular endothelial growth factor (VEGF) protein expression, and A$_1$R antagonists prevent hypoxia-induced VEGF protein expression. Compounds were added to confluent cultures of retinal pericytes in the presence of $[^35]$S-methionine for 17 hours as indicated. Cells receiving CSC were pretreated with the drug for 1 hour before induction of hypoxia. The media were then immunoprecipitated using anti-VEGF antibody, run on SDS–PAGE, and analyzed by phosphoimager. Equal protein loading was confirmed using trichloroacetic acid precipitable counts. Relative trichloroacetic acid-normalized VEGF expression was 1, 4.6, 4.7, and 1.1 for left to right lanes, respectively. Similar results have been obtained from three experiments.

analogs stimulate VEGF mRNA expression, we investigated whether PKA is involved in the hypoxic response of VEGF. Bovine retinal pericytes and endothelial cells were exposed to 21% or 0.5% oxygen for 12 hours in the presence of various concentrations of PKA inhibitor H-89. H-89 inhibited the hypoxic stimulation of VEGF mRNA in a dose-dependent manner, achieving a 61% ± 6.3% ($P = 0.043$) suppression at 20 µM (Fig. 9).

**DISCUSSION**

Recent studies have demonstrated that the expression of the angiogenic protein VEGF is increased greatly in ocular cells on exposure to hypoxic conditions in vitro and in vivo. Retinal microvascular endothelial cells and pericytes are among the cell types with strong VEGF induction that would experience hypoxia in ischemic retinal disorders. In addition, the intraocular concentration of VEGF in patients with diabetes mellitus is correlated closely with the presence of active proliferative diabetic retinopathy, and the inhibition of VEGF reduces ischemia-induced retinal neovascularization in animals. The mechanism by which hypoxia induces VEGF expression, however, remains incompletely understood.

We have investigated the mechanism of hypoxic VEGF induction in retinal microvascular endothelial cells and pericytes because of their known responsiveness and their key roles in intraocular neovascularization. Although it is not known which ocular cell types contribute most to physiologically relevant VEGF expression, endothelial cells and the intimately endothelial cell-associated pericyte could have a substantial impact on endothelial responses caused by either autocrine mechanisms or high local concentrations. Our investigations demonstrate that retinal vascular cells increase VEGF mRNA and protein levels after adenosine receptor stimulation under normoxic conditions. Conversely, adenosine receptor inhibition reduces the induction of VEGF mRNA and protein expression when cells are exposed to hypoxic conditions. Indeed, the magnitude of VEGF protein stimulation produced by hypoxia can be achieved by physiologically relevant concentrations of adenosine, and adenosine receptor inhibition prevents nearly all the hypoxia-induced increase.

Hypoxia >

H89 (µM) > 0 20 0 0.1 5 20

**FIGURE 9.** Protein kinase A inhibition suppresses hypoxic induction of vascular endothelial growth factor (VEGF) mRNA. The PKA inhibitor H-89 was added to confluent monolayers of retinal bovine pericytes at the indicated concentrations, and its effect on hypoxic stimulation (0.5% oxygen for 12 hours) of VEGF mRNA expression was evaluated by Northern blot analysis. Representative Northern blots using VEGF cDNA and control 36B4 probe (top) and quantitation of multiple experiments after normalization to the control signal are shown (bottom). Values are expressed as the percent of observed stimulation compared to untreated cells, and they represent the mean ± SD of three triplicate experiments.
crease in VEGF protein. These data suggest that the majority of hypoxia-induced VEGF expression may be mediated by the increased concentrations of adenosine present under hypoxic conditions.19

In the current study, we have demonstrated that VEGF gene expression in retinal pericytes and endothelial cells is stimulated by the inhibition of electron transport at its terminal step and does not require the absence of oxygen itself. Similar findings in cardiac myocytes were reported by Levy et al17 using amobarbital and rotenone, which act at complex I of the electron transport chain. Surprisingly, in Levy’s study, FCCP, which blocks more distally in the electron transport chain, did not induce VEGF gene expression. The differences in the FCCP response may result from the differences in cell types, dose, or duration used in these experiments. Further studies are necessary to determine whether a specific portion of the electron transport chain is particularly involved in hypoxic VEGF stimulation.

Comparing the effectiveness of the various adenosine receptor agonists and antagonists suggests that a particular adenosine receptor subtype may mediate the hypoxic VEGF response. CPA demonstrated little effect below 100 nM, whereas maximal VEGF mRNA stimulation occurred at 10 μM. Because the Ki of CPA is approximately 1 nM for A1R and 680 nM for A2R,36,37 the observed dose response suggests that VEGF induction is most likely mediated through A1R. However, DPMA increased VEGF expression at concentrations as low as 5 nM. Because the Ki of DPMA is 142 nM for A1R and 4.4 nM for A2R,42 these data again suggest that the VEGF response is mediated through the A1R. Similarly, NECA and CGS21680 increased VEGF mRNA at adenosine A2R-stimulating concentrations.43 Because VEGF stimulation by the A2bR specific agonist CGS21680 was not statistically different from the stimulation observed with the nonsubtype-specific A2R agonist NECA, a predominant role for A2bR is suggested. However, because VEGF stimulation by NECA was slightly (but nonsignificantly) greater than that observed for CGS21680, a contributing role for A2bR cannot be ruled out. The hypothesis of adenosine mediation through A2bR is supported further by the effectiveness of low-dose A2bR antagonists, but not A1R antagonists, at inhibiting the hypoxia-induced elevation of VEGF mRNA. A2bR-specific antagonists are not currently available.

Although theoretically the Ki derived in vitro for each drug can be significantly different from that observed in cellular systems, the close correlation between expected and observed values using multiple agents strongly suggests that the majority of the adenosine response is mediated through the A2R and probably specifically through the A2bR. Indeed, Hashimoto et al39 have reported that hypoxic induction of VEGF expression in mononuclear U-937 cells is mediated through adenosine A2 receptor. However, they did not differentiate A2b from A1 receptor involvement. In contrast with these studies, Fischer et al41 reported that A1 rather than A2 receptor is involved in the hypoxic VEGF induction in pig cerebral capillary endothelial cells. A1 receptor-mediated expression, however, appears not to be predominant in retinal vascular cells because we could not see A1 receptor stimulation by the inhibition of adenylyl cyclase in these cells. Again, different species and cell types might account for these differences.

Adenosine A1 receptor binding decreases cAMP, whereas A2R receptor binding increases cAMP.20,27 Our finding that A1R agonists increase cAMP in retinal cells and that A1R stimulation does not reduce forskolin-stimulated levels (data not shown) suggests that functional A1R exists on retinal pericytes and endothelial cells, whereas the A1 subclass is either absent, present in low numbers, or relatively inactive biologically. Our finding that hypoxia-induced cAMP accumulation is blocked by A2R antagonists and that cAMP can increase VEGF mRNA levels further supports the role of A2R in mediating the hypoxic response. Similar increases in cAMP production in response to hypoxia have been observed in isolated guinea pig heart,44 although bovine aortic and pulmonary artery endothelial cells have been reported to decrease cAMP levels under hypoxic conditions in vitro.45

Although the manner in which cAMP increases VEGF mRNA remains unknown, our data suggest that cAMP stimulation of PKA may be involved because PKA-specific blocker H89 inhibited hypoxic VEGF induction in a dose-dependent manner. Indeed, the VEGF promoter contains two potential AP-2 binding sites at nucleotides -135 to -128 and -1875 to -1868, which would indicate that VEGF expression could be increased by cAMP stimulation of PKA.46 In fact, the 5' flanking region, which includes the latter AP-2 binding site, has been reported to contain an enhancer element for hypoxic VEGF induction.12 In contrast, other reports suggest no involvement of this 5' flanking region,14 and indeed this region is not conserved in rat genomic sequences.15 It is still controversial whether the PKA pathway is involved in hypoxic VEGF induction. Blocking PKA in cardiac myocytes using KT5720 failed to inhibit hypoxia-induced VEGF expression.17 Forskolin and rolipram, which are known to stimulate the cAMP–PKA pathway, did not increase VEGF expression in one study,51 whereas stimulation of the PKA pathway has increased VEGF expression in others.52,53 Some reports have suggested a predominant role for the PKC pathway because hypoxia increases membrane-associated PKC57 and the transcriptional factors, fos and jun.58 AP-1 binding sites, however, are reported not to be regulated by
vascularization in diabetic retinopathy and other ischemic diseases.

Hypoxic regulation of VEGF shares several similarities with other hypoxia-inducible proteins, such as erythropoietin (Epo) and GLUT-1. The steady state mRNA expression of Epo and VEGF are increased by hypoxia and cobalt chloride, whereas it is inhibited by carbon monoxide. This suggests that the sensor regulating the expression of these molecules might be a heme protein. Epo has cis elements and trans factors involved in its hypoxic regulation. Indeed, VEGF has been reported to have a 5'-flanking enhancer element homologous to the erythropoietin hypoxia-responsive enhancer. A homologous 3' flanking enhancer also is reported to be a cis-acting element for hypoxic induction, although this region is reported to be a 3' untranslated region by another group. The other 5' flanking element (a potential binding site for the transcriptional factor, Sfp1) is reported to be a cis-acting element for hypoxic VEGF induction. Thus, it is possible that hypoxic expression of VEGF in retinal cells may be regulated by factors that interact at several locations in response to the adenosine-mediated stimulation of A2R. In addition, the hypoxic induction of VEGF expression appears to be regulated by mRNA stability, as is GLUT-1. The 3' untranslated region of these mRNA contains the destabilizing AUUUA motif in the context of an AU rich region. It also is possible that binding of regulatory proteins to these 3'UTR regions may occur in response to adenosine A2R stimulation and may result in stabilization of the VEGF mRNA.

Taken together, currently available data suggest the following mechanism for the development of neovascularization in diabetic retinopathy and other ischemic retinal disorders. The retinal capillary loss or nonperfusion characteristic of all these diseases results in relative hypoxia that causes an elevation of adenosine levels primarily caused by decreased recycling by adenosine kinase. Adenosine binds to adenosine receptors located on hypoxia-responsive VEGF-producing cells, such as retinal pericytes, endothelial cells, Müller cells, and pigment epithelial cells. Binding of adenosine, probably to the A2R, induces adenyl cyclase-mediated cAMP elevation by G protein coupling. Cyclic AMP may then activate PKA, which induces VEGF expression through an as yet uncharacterized signal transduction pathway. The soluble isoforms of VEGF are then free to diffuse within the eye and bind to retinal endothelial cells through autophosphorylating VEGF receptors. Vasculogenic endothelial growth factor receptor stimulation leads to autophosphorylation and possible phosphorylation of intermediate signaling molecules, eventually resulting in increased endothelial cell mitogenesis and permeability.

In conclusion, prior evaluation of the adenosine induction of VEGF expression has been performed in mononuclear and pig endothelial cells in which the involved mechanisms appear to differ. Our findings provide the first mechanistic information regarding the hypoxic stimulation of VEGF in retinal vascular cells. In addition, because the inhibition of VEGF has been shown to suppress VEGF-mediated retinal and iris angiogenesis in animals, these data suggest that adenosine A2 receptor antagonists might prove effective inhibitors of ischemia-induced intraocular neovascularization.

Key Words
adenosine, adenosine receptor, cyclic adenosine monophosphate (cAMP)-dependent protein kinase A, hypoxia, ischemia, neovascularization, vascular permeability factor (VPF)

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