Adenosine upregulates VEGF expression in cultured myocardial vascular smooth muscle cells

JIAN-WEI GU, ANN L. BRADY, VIVEK ANAND, MICHAEL C. MOORE, WHITNEY C. KELLY, AND THOMAS H. ADAIR.

Department of Physiology and Biophysics, and Center for Excellence in Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi 39216-4505

Gu, Jian-Wei, Ann L. Brady, Vivek Anand, Michael C. Moore, Whitney C. Kelly, and Thomas H. Adair. Adenosine upregulates VEGF expression in cultured myocardial vascular smooth muscle cells. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H595–H602, 1999.—We tested whether adenosine has differential effects on vascular endothelial growth factor (VEGF) expression under normoxic and hypoxic conditions, and whether A1 or A2 receptors (A1R; A2R) mediate these effects. Myocardial vascular smooth muscle cells (MVSMCs) from dog coronary artery were exposed to hypoxia (1% O2) or normoxia (20% O2) in the absence and presence of adenosine agonists or antagonists for 18 h. VEGF protein levels were measured in media with ELISA. VEGF mRNA expression was determined by Northern blot analysis. Under normoxic conditions, the adenosine A1R agonists, N6-cyclopentyladenosine and R-N'H2-(2-phenylisopropyl)adenosine did not increase VEGF protein levels at A1R stimulatory concentrations. However, adenosine (5 μM) and the adenosine A2R agonist N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)]ethyl adenosine (DPMA; 100 nM) increased VEGF protein levels by 51 and 132% and increased VEGF mRNA expression by 44 and 90%, respectively, in cultured MVSMCs under normoxic conditions. Hypoxia caused an approximately fourfold increase in VEGF protein and mRNA expression, which could not be augmented with exogenous adenosine, A1R agonist (DPMA), or A2R agonist [1,3-diethyl-8-phenylxanthine (DPX)]. The A2R antagonist 8-(3-chlorostyryl)caffeine completely blocked adenosine-induced VEGF protein and mRNA expression and decreased baseline VEGF protein levels by up to ~60% under normoxic conditions but only by ~20% under hypoxic conditions. The A1R antagonist DPX had no effect. These results are consistent with the hypothesis that 1) adenosine increases VEGF protein and mRNA expression by way of A1R. 2) Adenosine plays a major role as an autocrine factor regulating VEGF expression during normoxic conditions but has a relatively minor role during hypoxic conditions. 3) Endogenous adenosine can account for the majority of basal VEGF secretion by MVSMCs under normoxic conditions and could therefore be a maintenance factor for the vasculature. 

vascular endothelial growth factor; hypoxia; normoxia; vascular smooth muscle cells; adenosine receptor; vascular maintenance factor

ADENOSINE is a purine nucleoside composed of adenine and ribose joined by a glycosidic bond. It is a metabolic precursor for nucleic acid biosynthesis. All cells are possible sources of adenosine because all cells can use free energy derived from catabolism of ATP to perform their various functions. Many of the cells that “produce” adenosine also have receptors for adenosine. Adenosine receptors on the cell surface membrane are members of the G protein-coupled receptor family and are categorized as A1, A2a, A2b, and A3 (and possibly A1 subtypes) by pharmacological interaction with a variety of agonists and antagonists (10, 39). Adenosine is a regulator of numerous bodily functions associated with the cardiovascular, renal, respiratory, neurological, immunologic, gastrointestinal, and metabolic systems (39).

Physiological effects of adenosine on the cardiovascular system include vasodilation throughout the body except in the kidney where vasoconstriction occurs, bradycardia, decreased arteriovenous nodal conduction velocity, reduction of atrial contractility, inhibition of cardiac pacemaker activity, inhibition of platelet aggregation, anti-β-adrenergic effects, inhibition of renin release, and renal sodium retention (36). Evidence for novel cardiovascular actions mediated by the different adenosine receptors is accumulating. The importance of adenosine as a negative feedback modulator of cell and organ energy demand and consumption is a basic tenet of adenosine biology (6, 8, 30).

It is now well known that angiogenesis is often a compensatory response to prolonged imbalances between the metabolic requirements of the tissues and the perfusion capabilities of the vasculature (2). Although angiogenesis is associated with tissue hypoxia and increased metabolic rate, the mechanisms that regulate angiogenesis under these conditions is less well understood. Adenosine is attractive as a negative feedback modulator of angiogenesis (1, 2, 11, 38) because adenosine levels in tissues are elevated during hypoxic conditions and when metabolic rate increases (7, 19, 21, 35), and adenosine can stimulate endothelial proliferation in vitro (12, 13, 28, 29) as well as growth of blood vessels in a variety of in vivo models (1–3).

Mounting evidence suggests that hypoxia-induced vascular endothelial growth factor (VEGF) plays an important regulatory role in angiogenesis in both physiological and pathological conditions. Recent evidence has shown that adenosine can enhance VEGF expression in a variety of cultured cells (15, 16, 23, 34, 37). Hashimoto et al. (23) reported that 5′-(N-ethylcarboxamide)adenosine, an adenosine analog, upregulated VEGF mRNA expression in cultured U-937 cells under normoxic conditions. Takagi et al. (37) found that 8-(3-chlorostyryl)caffeine (CSC), an adenosine A2 receptor antagonist, reduced hypoxic stimulation of VEGF mRNA by 68% in cultured bovine retinal capillary endothelial cells. However, the mechanism by which adenosine upregulates VEGF expression in cultured myocardial vascular smooth muscle cells remains to be elucidated.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
which adenosine induces blood vessel growth and the relation between adenosine and VEGF in the regulation of angiogenesis under normoxic and hypoxic conditions are still poorly understood.

The present study seeks to determine whether adenosine has a differential effect on VEGF expression under normoxic and hypoxic conditions and whether the effects of adenosine are mediated by adenosine A1 or A2 receptors in cultured myocardial vascular smooth muscle cells (MVSMCs) from dog coronary artery. The results suggest that adenosine, acting by way of A2 receptors, plays a dominant role in establishing basal levels of VEGF during normoxic conditions and a relatively minor role during hypoxic conditions.

MATERIALS AND METHODS

Chemicals. Adenosine was purchased from Fujisawa USA (Deerfield, IL). N6-cyclopentyladenosine (CPA), R(-)-N6-(2-phosphorylpropyl)adenosine (R-PIA), N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine (DPMA), 1,3-dithioly-8-phenylxanthine (DPX), and CSC were obtained from Research Biochemicals International (Natwick, MA). [32P]dCTP was obtained from DuPont New England Nuclear (Boston, MA). Multiprime DNA-labeling system was obtained from Amersham International (Amersham, UK). VEGF ELISA kits were provided by R & D Systems (Minneapolis, MN). Cell cultures. MVSMCs were isolated from adult male mongrel dogs with a method previously described (20). Briefly, the left anterior descending coronary artery was removed from the heart and cut into small pieces. The tissue pieces were digested with 0.1% collagenase (Sigma), 0.02% elastase (Sigma), and 0.02 trypsin inhibitor (Sigma) in DMEM (Sigma) from the heart and cut into small pieces. The tissue pieces were digested with 0.1% collagenase (Sigma), 0.02% elastase (Sigma), and 0.02 trypsin inhibitor (Sigma) in DMEM (Sigma) for 2 h at 37°C. The cells were seeded into sterile culture flasks at ~5 x 10^5 cells/cm^2 and incubated at 37°C in a humidified atmosphere of 5% CO2-20% O2-75% N2. The culture medium was 50% DMEM plus 50% medium 199 (GIBCO) supplemented with 10% fetal bovine serum (HyClone), 100 µM penicillin, 100 µg/ml streptomycin, and 0.25 µM amphotericin B. The primary culture reached confluence in 5–7 days. The purity of the culture was confirmed by immunohistochemical staining with mouse anti-α-smooth muscle actin-fluorescein isothiocyanate conjugate (Sigma). The cell lines were used between passages 4 and 8 in the experiments. When monolayers of MVSMCs reached ~80% confluence, standard medium was replaced with media having 4% heat-inactivated fetal bovine serum in the absence and presence of adenosine and a variety of A1- or A2-receptor agonists and antagonists. Samples were taken at different periods of incubation in normoxic (20% O2-5% CO2-75% N2) and hypoxic (1% O2-5% CO2-94% N2) environments.

Exposure to hypoxia. The experiments were performed in a water-jacketed triple gas incubator (Queue System, Parkersburg, WV) that controls the O2 and CO2 to within ±0.1% of the set-point value. After 24 h of incubation in a normoxic environment, the medium was replenished with similar complete media and the ~80% confluent MVSMC monolayers were exposed to a hypoxic environment (1% O2-5% CO2-94% N2) in the absence and presence of various adenosine agonists and antagonists. Cells exposed to hypoxia still excluded trypan blue dye (~95%) and showed no morphological changes by light microscopy, and the levels of lactate dehydrogenase (LD-L 20 assay kit, Sigma) were not increased in the media. MVSMCs incubated under normoxic conditions (20% O2-5% CO2-75% N2) from the same batch and passage were used as controls.

Measurement of VEGF protein. The amount of VEGF was measured in the normoxic and hypoxic conditioned media of cultured MVSMCs with sandwich ELISA (R & D Systems). In these assays, the angiogenic growth factor in the test sample is sandwiched between a monoclonal antibody against human recombinant VEGF. A second polyclonal antibody against the growth factor is conjugated to horseradish peroxidase and then added to the mixture. Color develops by addition of hydrogen peroxide and chromogen tetramethylbenzidine, and the intensity is measured at 450 nm. VEGF protein levels were normalized to the total amount of cellular protein and expressed as picograms per milligrams of total cell protein. Cell protein content was determined in duplicate with BSA as the standard (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories).

Northern blot analysis. Total RNA was prepared with a total RNA isolation kit (catalog no. 1910; Ambion, Austin, TX). Total RNA (20 µg) was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde in 1× MOPS buffer. The gel was stained with ethidium bromide to visualize rRNA, and the RNA was transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH) overnight by capillary action in 20× saline sodium citrate (SSC; 3 M NaCl, 0.3 M Na2-citrate-2H2O, pH 7.0). After transfer, the nitrocellulose paper was baked at 80°C under vacuum for 2 h and then prehybridized for 3 h at 42°C in a buffer containing 25 mM KPO4 (pH 7.4), 6× SSC, 5 × Denhardt’s solution, 50% formamide, and 50 µg/ml salmon sperm DNA. Dextran (10%, final concentration) was then added to the prehybridization solution. cDNA probes were denatured by boiling and added to the hybridization solution. After hybridization overnight at 42°C, the blots were washed at 42°C in 0.2× SSC/0.1% sodium dodecyl sulfate until the background radioactivity was removed.

The VEGF cDNA probe is a 580-bp EcoRI-BamHI fragment of the murine VEGF cDNA cloned into pBluescript plasmid (kindly provided by Dr. Werner Risau (33) at Max-Planck Institute). The cDNA probe was labeled with [α-32P]dCTP (New England Nuclear, Boston, MA) with a multiprime random-promoted DNA labeling kit (Amersham, UK). Quantification of VEGF mRNA expression was performed on phosphor images of blots collected with a Phospho-Imager (Molecular Dynamic; software: ImageQuant, version 3.3, Molecular Dynamics). To verify the relative amount of total RNA, filters were hybridized with a 32P-labeled 28S rRNA antisense oligonucleotide probe (Ambion). The VEGF mRNA was normalized against 28S rRNA in each sample.

Statistical analysis. All determinations were performed in duplicate, and each experiment was repeated at least three times. Where indicated, data are presented as means ± SD. Differences were considered statistically significant when P < 0.01 by paired t-test. All statistical calculations were performed with StatView software (BrainPower, Calabasa, CA).

RESULTS

VEGF protein expression in normoxia. VEGF protein levels in media were measured with sandwich ELISA (R & D Systems) after MVSMCs were cultured under normoxic conditions in the absence and presence of adenosine and adenosine A1− and A2− receptor agonists and antagonists. Adenosine administered into culture media caused a dose-related increase in VEGF protein expression (Fig. 1A). VEGF protein levels, expressed as
Adenosine (Ado) and the adenosine A<sub>2</sub>-receptor agonist DPMA (100 nM) significantly increased VEGF protein levels in media compared with the 2-h control group (P < 0.01). The changes in VEGF expression caused by adenosine and A<sub>2</sub>-receptor agonist and antagonist that occurred after 18 h of treatment (Fig. 1) were also present after 2, 6, and 12 h of treatment (Fig. 2). In these experiments, small samples of media (200 µl) were taken from T-75 flasks at various times after cells were exposed to fresh media in the absence (control) and presence of adenosine (5 µM) and A<sub>2</sub>-receptor agonist DPMA (100 nM) or antagonist CSC (5 µM). VEGF protein levels in media increased progressively in control cultures throughout the 12-h experiment (Fig. 2). Adenosine (5 µM) and A<sub>2</sub>-receptor agonist DPMA (100 nM) significantly increased VEGF protein levels by 27 and 60%, respectively, after only 2 h of treatment compared with the 2-h control group (P < 0.05), and the effects of adenosine and DPMA to increase VEGF levels in media persisted for the next 10 h (Fig. 2). The A<sub>2</sub>-receptor antagonist CSC (5 µM) decreased VEGF protein levels by 16% (P < 0.05) after only 2 h of treatment, and the inhibitory effects of CSC continued throughout the experiment.

VEGF protein expression in hypoxia. Hypoxia (hypoxic control) caused a 4.3-fold increase in VEGF protein levels in media after 18 h of treatment (Fig. 3), compared with the normoxic control group (normoxic control, 0.77 ± 0.09 vs. hypoxic control, 3.28 ± 0.63). When MVSMCs were cultured under normoxic conditions (Fig. 1B). However, the adenosine A<sub>1</sub>-receptor agonists, CPA and R-PIA, did not increase VEGF protein levels at A<sub>1</sub>-receptor stimulatory concentrations (Fig. 1B). The A<sub>2</sub>-receptor antagonist CSC (5 µM) completely blocked adenosine-induced increases in VEGF protein expression and decreased baseline VEGF protein levels by 44% under normoxic conditions (Fig. 1C: 0.77 ± 0.09 vs. 0.43 ± 0.05, P < 0.01). The A<sub>1</sub>-receptor antagonist DPX (5 µM) had no effect on VEGF protein levels (Fig. 1C).

The changes in VEGF expression caused by adenosine and A<sub>2</sub>-receptor agonist and antagonist that occurred after 18 h of treatment (Fig. 1) were also present after 2, 6, and 12 h of treatment (Fig. 2). In these experiments, small samples of media (200 µl) were taken from T-75 flasks at various times after cells were exposed to fresh media in the absence (control) and presence of adenosine (5 µM) and A<sub>2</sub>-receptor agonist DPMA (100 nM) or antagonist CSC (5 µM). VEGF protein levels in media increased progressively in control cultures throughout the 12-h experiment (Fig. 2). Adenosine (5 µM) and A<sub>2</sub>-receptor agonist DPMA (100 nM) significantly increased VEGF protein levels by 27 and 60%, respectively, after only 2 h of treatment compared with the 2-h control group (P < 0.05), and the effects of adenosine and DPMA to increase VEGF levels in media persisted for the next 10 h (Fig. 2). The A<sub>2</sub>-receptor antagonist CSC (5 µM) decreased VEGF protein levels by 16% (P < 0.05) after only 2 h of treatment, and the inhibitory effects of CSC continued throughout the experiment.

VEGF protein expression in hypoxia. Hypoxia (hypoxic control) caused a 4.3-fold increase in VEGF protein levels in media after 18 h of treatment (Fig. 3), compared with the normoxic control group (normoxic control, 0.77 ± 0.09 vs. hypoxic control, 3.28 ± 0.63).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 1. A: dose-related effect of adenosine (Ado) on vascular endothelial growth factor (VEGF) protein expression in myocardial vascular smooth muscle cells (MVSMCs) cultured under normoxic conditions. Cells were cultured in absence (control as 0) and presence of various concentrations of Ado for 18 h under normoxic conditions (5% CO<sub>2</sub>-20% O<sub>2</sub>-75% N<sub>2</sub>). B: effects of Ado and Ado A<sub>1</sub>- and A<sub>2</sub>-receptor agonists on VEGF protein expression in MVSMCs cultured under normoxic conditions. When monolayers reached ~80% confluence, cells were cultured in absence (control) and presence of Ado (5 µM), A<sub>1</sub>-receptor agonists [R(-)-N<sup>6</sup>(2-phenylisopropyl)adenosine (R-PIA), 100 nM and N<sup>6</sup>-cyclopentyladenosine (CPA), 100 nM], and A<sub>2</sub>-receptor agonist [N<sup>6</sup>-2-(3,5-diethoxyphenyl)-2-(2-methylphenyl)ethyl adenosine (DPMA), 100 nM] for 18 h under normoxic conditions. C: effects of Ado and Ado A<sub>1</sub>- and A<sub>2</sub>-receptor antagonists on VEGF protein expression in MVSMCs cultured under normoxic conditions. Cells were cultured in absence (control) and presence of Ado (5 µM), A<sub>2</sub>-receptor antagonist [8-(3-chlorostyryl)caffeine (CSC), 5 µM], and A<sub>1</sub>-receptor antagonist [1,3-diethyl-8-phenylxanthine (DPX), 5 µM] for 18 h under normoxic conditions. VEGF protein levels in media were measured by ELISA and expressed as ng/mg total cell protein. Data are means ± SD from 2 independent series of experiments (n = 8). *P < 0.01 vs. control. **P < 0.01 vs. Ado.

Adenosine (5 µM) and the adenosine A<sub>2</sub>-receptor agonist DPMA (100 nM) increased VEGF protein levels by 51% (0.77 ± 0.09 vs. 1.16 ± 0.04, P < 0.01) and 132% (0.77 ± 0.09 vs. 1.79 ± 0.21, P < 0.01), respectively, when MVSMCs were cultured under normoxic conditions (Fig. 1B). However, the adenosine A<sub>1</sub>-receptor agonists, CPA and R-PIA, did not increase VEGF protein levels at A<sub>1</sub>-receptor stimulatory concentrations (Fig. 1B). The A<sub>2</sub>-receptor antagonist CSC (5 µM) completely blocked adenosine-induced increases in VEGF protein expression and decreased baseline VEGF protein levels by 44% under normoxic conditions (Fig. 1C: 0.77 ± 0.09 vs. 0.43 ± 0.05, P < 0.01). The A<sub>1</sub>-receptor antagonist DPX (5 µM) had no effect on VEGF protein levels (Fig. 1C).
Neither adenosine (5 µM), A2-receptor agonist DPMA (100 nM), nor A1-receptor agonist DPX (5 µM) could increase VEGF protein expression beyond that of the hypoxic control group (Fig. 3). Interestingly, A2-receptor antagonist CSC (5 µM) reduced VEGF protein levels by only 20% during 18 h under hypoxic (H) or normoxic (N) conditions. VEGF protein levels in media were measured by ELISA and expressed as ng/mg total cell protein. Data are means ± SD from 2 independent series of experiments (n = 8). *P < 0.01 vs. hypoxic control.

Dose-related inhibition of VEGF expression. Dose-related inhibitory effects of A2-receptor antagonist (CSC) on VEGF protein expression were examined in MVSMCs incubated in hypoxic and normoxic environments (Fig. 4). Cells were cultured in the absence and presence of various concentrations of CSC (0.5 µM, 5.0 µM, and 50 µM) for 18 h. VEGF protein levels in media were measured by ELISA and expressed as percentage of control. CSC caused a greater percent decrease in VEGF protein expression during normoxic conditions compared with hypoxic conditions at each concentration (Fig. 4). At the highest dosage of CSC (50 µM), VEGF protein levels in media were decreased by ~60% under normoxic conditions and by ~25% under hypoxic conditions. This suggests that adenosine may be more important for establishing basal levels of VEGF during normoxic conditions, compared with hypoxic conditions.

VEGF mRNA expression in normoxic and hypoxic conditions. Northern blot analyses were used to determine the effect of adenosine and adenosine-receptor agonists and antagonists on VEGF mRNA expression in MVSMCs cultured under normoxic (Fig. 5) and hypoxic (Fig. 6) conditions. Adenosine (5 µM) and the A2-receptor agonist DPMA (100 nM) increased VEGF mRNA by 44 and 90%, respectively, after 18-h treatment, compared with the normoxic control group (Fig. 5; control, 1.03 ± 0.16; adenosine, 1.48 ± 0.38; DPMA, 1.96 ± 0.49; P < 0.01 for both). The A2-receptor antagonist CSC (5 µM) decreased VEGF mRNA by 41% under normoxic conditions but had no effect (Fig. 5). Hypoxia alone caused a 4.1-fold increase in VEGF mRNA expression compared with the normoxic control group (Fig. 6: normoxic control, 1.03 ± 0.16 vs. hypoxic control, 4.27 ± 1.01). A2-receptor antagonist CSC (5 µM) caused a 17% decrease in VEGF mRNA expression (hypoxic CSC, 3.54 ± 0.76) compared to the hypoxic control group (P < 0.05). There were no significant differences in VEGF mRNA expression in...
understood. The results shown on Fig. 3 indicate that induced upregulation of VEGF expression is less well 
the role of adenosine in the mediation of hypoxia 
retinal pericytes under normoxic conditions. However, 
creased VEGF mRNA expression in cultured bovine 
mRNA expression in a dose- and time-dependent man-

protein and mRNA under normoxic conditions. Previ-
caused a significant increase in the expression of VEGF 
containing adenosine or the A2-receptor agonist DPMA 
expression in MVSMCs cultured under hypoxic conditions. Cells 
expression in MVSMCs cultured under hypoxic conditions. Cells 

DISCUSSION

The results show that exposing MVSMCs to media 
containing adenosine or the A2-receptor agonist DPMA 
caused a significant increase in the expression of VEGF 
protein and mRNA under normoxic conditions. Previous 
studies suggested this might be so. Fischer et al. 
(16) demonstrated that adenosine upregulated VEGF 
mRNA expression in a dose- and time-dependent manner in porcine brain-derived microvascular endothelial cells under normoxic conditions. Takagi et al. (37) reported that the adenosine A2 agonist DPMA increased VEGF mRNA expression in cultured bovine retinal pericytes under normoxic conditions. However, the role of adenosine in the mediation of hypoxia 
induced upregulation of VEGF expression is less well 
understood. The results shown on Fig. 3 indicate that neither adenosine nor the adenosine A2-receptor ago-
nist DPMA could upregulate VEGF expression in 

MVSMCs under hypoxic conditions. The results also indicate that the A2-receptor antagonist CSC (5 µM) reduced the expression of VEGF protein and mRNA by only ∼20% under hypoxic conditions, whereas a ∼40% reduction was observed in normoxic cultures. Therefore, these findings support the hypothesis that adeno-
sine, acting by way of A2 receptors, plays a major role in the regulation VEGF expression during normoxic conditions and has a relatively minor role during hypoxic conditions.

Adenosine is thought to have a physiological role as an angiogenic factor (1–3, 8, 38). This hypothesis can be attributed to the following findings: 1) adenosine can stimulate the proliferation of endothelial cells in vitro (12, 13, 28, 29) but not the proliferation of other types of cells (12). 2) Blood vessel growth in chick embryos is

stimulated by dipyridamole, an adenosine reuptake inhibitor, and greatly attenuated by aminophylline, a nonselective adenosine-receptor antagonist (2, 3). 3) Adenosine production is increased in hypoxic tissues as well as in tissues with increased metabolic rate (7, 19, 35); in fact, the interstitial concentration of adenosine can increase from a normal value on the order of ∼100 nM (32) to as high as 40 µM (21) during hypoxic conditions. 4) Exogenous adenosine can stimulate blood vessel growth in the intact animal, and both hypoxia-
induced angiogenesis and endothelial proliferation can be partly attributed to adenosine accumulation in the hypoxic environment (for review, see Refs. 1 and 2).

The mechanism by which adenosine stimulates blood vessel growth is still poorly understood. However, the results of the present study as well as studies from other laboratories suggest that adenosine can enhance VEGF expression in a variety of cultured cells (15, 16, 23, 34). VEGF is a key mediator of angiogenesis in both physiological and pathological conditions (14, 17, 25) and can be upregulated by various cytokines, growth factors, steroid hormones, and mutated oncogenes in tumor cells (14, 31); however, the induction of VEGF expression by hypoxia is considered to be most signif-
ciant from a physiological perspective. VEGF regulation by hypoxia occurs at both the transcriptional and posttranscriptional levels. Transcriptional regulation of VEGF is mediated by hypoxia-inducible factor 1, which accumulates under hypoxic conditions and activates VEGF transcription by binding to specific pro-
moter sequences (18). In addition, hypoxia leads to stabilization of VEGF mRNA (24, 26), which acts to increase steady-state levels of VEGF mRNA. Recent evidence suggests that the protein kinase C pathway (9), the Ca2+ channel (27), and the AMP-dependent pathway (9) can also regulate VEGF gene expression. Fischer et al. (16) reported that rolipram, an inhibitor of AMP phosphodiesterase, completely blocked the upregulation of VEGF mRNA induced by adenosine in cultured microvascular endothelial cells. Pueyo et al. (34) found that a 2.2-fold stimulation of VEGF mRNA expression by adenosine was enhanced after cotreatment with cobalt chloride, a hypoxia-mimicking agent.

The results of the present study indicate that the adenosine A2 antagonist CSC could not block more than ∼20–25% of the VEGF expression induced by hypoxia. These results together with those from studies discussed previously support the hypothesis that aden-
osine has a relatively minor role in the upregulation of VEGF expression caused by exposure to a hypoxic environment. However, this contention is based en-
tirely on in vitro experimentation in which tissue hypoxia was simulated by exposing cells to a 1% oxygen atmosphere for a relatively short period of time. The quantitative role of adenosine in the mediation of hypoxia-induced VEGF expression in vivo is likely to depend on the degree of hypoxia to which the tissues are exposed as well as the duration and frequency of the hypoxic exposure. Additional studies will be required to confirm or refute our present hypothesis that adenosine

![Fig. 6. Effects of Ado and A2-receptor antagonist on VEGF mRNA expression in MVSMCs cultured under hypoxic conditions.](http://ajpheart.physiology.org/)
Adenosine stimulates VEGF expression

Adenosine has a relatively minor role in the hypoxic induction of VEGF.

An interesting finding in the present study was that the adenosine A_{2} antagonist CSC reduced the expression of VEGF protein and mRNA by >60% under normoxic conditions. It is unlikely that the suppression of VEGF resulted from a toxic and/or nonspecific effect of the molecule for the following reason: a 10-fold increase in the concentration of CSC (from 5 to 50 µM) did not cause a further lowering of VEGF protein levels in the hypoxic group, as shown in Fig. 4. Additional studies showed that basal levels of VEGF protein in the media of rat cardiomyoblasts cultured under normoxic conditions could be decreased from a control value of 1.84 ± 0.05 to a value of 0.74 ± 0.06 ng/mg total cell protein by subjecting the cells to 10 µM adenosine deaminase in the media for 18 h (n = 6, P < 0.01). Because adenosine deaminase converts adenosine to inosine, an inactive metabolite, it is clear that adenosine could account for at least 60% of the basal levels of VEGF protein in the media under normoxic conditions. These findings support the hypothesis that adenosine plays an autocrine role to stimulate VEGF expression.

On the basis of these findings, we can speculate that adenosine, acting by way of VEGF protein, is a maintenance factor for the vasculature. What is a vascular maintenance factor? The concept of a “vascular maintenance factor” is based on the knowledge that the size of a capillary network is dependent on the metabolic activity of the tissues that it serves. Factors that increase metabolic activity can stimulate angiogenesis in skeletal muscles; whereas decreases in metabolic activity caused by immobilizing a limb in a plaster cast, cutting the Achilles tendon, or unloading hindlimb muscles by tail suspension in rats can decrease capillarity in the affected skeletal muscles by as much as 50% within a few weeks (for review, see Ref. 2). Therefore, a normal level of muscular activity seems to be required to maintain the structural integrity of the capillary network. We are speculating that adenosine is a vascular maintenance factor not only because adenosine may have an autocrine role to maintain basal levels of VEGF in the muscles, but also because the levels of adenosine in a muscle are proportional to metabolic rate of the muscle (5), as discussed further below.

Inasmuch as the concentration of vascular maintenance factor in a muscle should be proportional to the capillarity of the muscle, we would predict that oxidative muscles with their relatively high capillarity should have higher levels of vascular maintenance factor under normoxic conditions compared with glycolytic muscles that have relatively low capillarity. The following findings are consistent with this prediction: 1) the concentration of 5'-nucleotidase, which converts AMP to adenosine, is higher in oxidative muscles with glycolytic muscles in many different species of animals, suggesting that adenosine levels are higher in oxidative muscles (5). 2) Rabbit oxidative muscles (semitendinosis and soleus) have higher levels of VEGF protein compared with glycolytic muscles (plantaris and tibialis anterior) under basal, resting conditions (4). 3) When a glycolytic muscle is converted to an oxidative muscle by prolonged electrical stimulation of a motor nerve, an approximately twofold increase in capillarity is associated with an approximately twofold increase in basal levels of VEGF mRNA in the muscle tissues (22).

Therefore, the available data from intact animals show correlations between muscle capillarity and basal levels of adenosine and VEGF protein in the muscles under normoxic conditions. These correlations, along with our new finding that endogenous adenosine has an autocrine role to establish basal levels of VEGF protein under normoxic conditions, are all consistent with the supposition that adenosine, acting by way of VEGF, is a maintenance factor for the vasculature. It will be interesting to determine whether increasing endogenous levels of adenosine in the muscles of intact animals will increase basal levels of VEGF in the tissues and therefore increase the capillarity under normoxic conditions or whether adenosine antagonists will decrease the basal levels of VEGF and cause rarefaction of the vasculature under normoxic conditions.

The present study indicates that adenosine has differential effects on the regulation of VEGF expression in MVSMCs cultured under normoxic compared with hypoxic conditions. This conclusion is based on the following findings: 1) exogenous adenosine or the adenosine A_{2} agonist DPMA did not increase VEGF expression in hypoxic cultures of MVSMCs; however, both factors upregulated VEGF protein and mRNA expression under normoxic conditions. 2) The adenosine A_{2} antagonist CSC (5 µM) reduced the expression VEGF protein as well as mRNA by ~40% in normoxic cultures but only by ~20% under hypoxic conditions. 3) A dose-response experiment (Fig. 4) showed that the maximal effects of the adenosine A_{2} antagonist CSC could block only 25% of the VEGF protein expression induced by hypoxia but could decrease basal levels of VEGF protein by >60% under hypoxic conditions. Why does exogenous adenosine or adenosine A_{2} agonist induce VEGF expression in normoxic cultures but not in hypoxic cultures? A likely possibility is that hypoxia greatly increased the adenosine levels in the cultures so that maximum adenosine effects were achieved in the absence of exogenous adenosine or adenosine A_{2} agonist. This differential response to adenosine in regulating VEGF expression under normoxic and hypoxic conditions could have a significant clinical impact in terms of developing angiogenic therapy with adenosine and adenosine agonists or developing antiangiogenic therapy with adenosine antagonists.

Adenosine caused a dose-related increase in VEGF protein expression with a maximal approximately threefold increase in VEGF protein levels at an adenosine concentration of 500 µM, as shown in Fig. 1A. However, 1,000 µM adenosine decreased VEGF protein levels by 23%, compared with the control group. These results are consistent with a study by Ethier et al. (12), which showed that adenosine caused a dose-related increase...
in the proliferation of human umbilical vein endothelial cells over a wide range of adenosine concentrations but caused a reduction in cell number at an adenosine concentration of 1,000 µM. We can speculate that the inhibitory effects of adenosine on VEGF expression and endothelial cell growth are caused by toxic effects of adenosine at high concentrations.

In comparing the effectiveness of the various adenosine A1- or A2-receptor agonists and antagonists, our data demonstrate that the effects of adenosine on VEGF expression are mediated by adenosine A2 receptors but not A1 receptors in cultured MVSMCs. These findings are consistent with previous reports from Hashimoto et al. (23) and Takagi et al. (37). Hashimoto et al. (23) reported that exogenous adenosine upregulated VEGF mRNA via adenosine A2 receptors in cultured U-937 cells under normoxic conditions. Takagi and associates (37) found that adenosine stimulated VEGF gene expression through the stimulation of adenosine A2a receptors in cultured retinal vascular cells. However, Fischer and associates (16) reported that the effect of adenosine on VEGF mRNA expression was mediated via A2 rather than A1 receptors in cultured pig cerebral capillary endothelial cells. This disparity may reflect species differences or cell or organ specific differences in the distribution of A1 and A2 receptors. For example, A1 receptors are found in greatest numbers in the brain, spinal cord, testis, and adipose tissue, and they are found in lesser numbers in many other tissues, including kidney, spleen, and heart (39).

In conclusion, we have demonstrated that exposing MVSMCs to media containing adenosine or the adenosine A2 agonist DPMA caused a significant increase in the expression of VEGF protein and mRNA under normoxic conditions but not under hypoxic conditions. The adenosine A2 antagonist CSC greatly decreased the expression of VEGF protein and mRNA under normoxic conditions but had a relatively small effect in hypoxic cultures. These findings suggest that 1) adenosine, acting by way of A2 receptors, plays a major role in regulating VEGF expression during normoxic conditions and minor role during hypoxic conditions; 2) adenosine has an autocrine role to stimulate VEGF secretion and can account for the majority of basal VEGF secretion by MVSMCs under normoxic conditions; and 3) most of the hypoxic induction of VEGF expression is independent of adenosine accumulation in the microenvironment of the hypoxic cells.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-51971 and a grant from the American Heart Association Mississippi Affiliate (9810183MS). J.-W. Gu was a recipient of a Grant-in-Aid award of the American Heart Association Mississippi Affiliate.

Address for reprint requests and other correspondence: T. H. Adair, Dept. of Physiology & Biophysics, Univ. of Mississippi Medical Center, 2500 North State St., Jackson, Mississippi 39216-4505 (E-mail: tadair@physiology.umsmmed.edu).

Received 23 December 1998; accepted in final form 23 March 1999.

REFERENCES


