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Hypoxia Regulates Vascular Endothelial Growth Factor Receptor KDR/Flk Gene Expression Through Adenosine A2 Receptors in Retinal Capillary Endothelial Cells

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Purpose. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific angiogenic factor that serves an important role in numerous ischemic retinopathies. The authors studied the hypoxic gene regulation of two known VEGF receptors (KDR and Flt) and its mechanism in cultured bovine retinal endothelial cells (BREC).

Methods. Confluent monolayers of BREC were exposed to various oxygen concentrations using a computer-controlled, infrared, water-jacketed CO2 incubator with reduced oxygen control. Northern blot analysis and 125I-VEGF binding analysis were used to identify hypoxia-induced alterations of VEGF receptor at mRNA and protein levels.

Results. KDR was detectable by Northern blot analysis in BREC, whereas Flt was not. Hypoxia decreased KDR gene expression in a dose- and time-dependent manner with maximal inhibition to 0.5 ± 0.2% (P = 0.019) of normoxic control observed after 24 hours exposure to 0% oxygen and with significant inhibition at oxygen concentrations below 5%. Blockade of oxygen respiration decreased KDR mRNA expression to 58% ± 7.1% of control (P = 0.001) after 3 hours. CPA, a stable adenosine A1 receptor (A1R) agonist, did not affect KDR mRNA expression at A1R stimulatory concentrations, but it decreased KDR mRNA levels to 30% ± 4.9% (P = 0.002) of control at higher concentrations that react with A1R. DPMA, an adenosine A2 receptor (A2R) agonist, decreased KDR mRNA in a dose-dependent manner with an EC50 of 5 to 10 nM. A2R antagonists, 8-phenyltheophylline and 8-phenyltheophylline, did not inhibit the hypoxic response of KDR mRNA at A1R inhibitory concentrations but did inhibit the response at A2R effective doses (P = 0.001). The A2R antagonist, CSC, inhibited the KDR hypoxic response by 42% ± 7.8% (P = 0.008) at 10 μM. Specific VEGF binding to BREC was decreased from 15.1% ± 0.3% to 12.7% ± 0.4% per milligram protein (P < 0.001) after exposure to 1% oxygen for 24 hours. In contrast, long-term exposure to 1% oxygen (72 hours) resulted in an increase of VEGF binding from 13.5% ± 1.1% to 18.3% ± 0.8% per milligram protein (P < 0.001). Scatchard analysis detected a decrease of receptor binding sites without change in binding affinity after 30 hours of exposure to hypoxia but demonstrated an increase in specific binding sites (4.2 ± 0.6 × 10^4 sites/cell to 6.7 ± 1.0 × 10^4 sites/cell, P = 0.049) with unaltered receptor affinity after 72 hours of hypoxic exposure.

Conclusions. These data suggest that hypoxia induces an initial decline in KDR mRNA levels and VEGF binding sites as mediated through adenosine binding to the A2R. Exposure to prolonged periods of hypoxia, however, results in an increase in VEGF binding sites by an as yet unidentified mechanism.


Ischemic retinal diseases such as diabetic retinopathy, retinopathy of prematurity, and central retinal vein occlusion are major causes of blindness in the United States. A common characteristic of these diseases is the formation of retinal nonperfusion, often followed by pathologic angiogenesis leading to visual loss. This neovascularization presumably results from elevated expression of intraocular growth factors induced by inner retinal ischemia.

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and angiogenesis factor. It is an inducer of tumor angiogenesis, and its expression is increased in ischemic areas surrounding regions of tumor necrosis. Vascular endothelial
VEGF levels are elevated in patients with active ocular diseases, similarities to the platelet-derived growth factor expression is dramatically increased by VEGF stimulation activates MAP kinase and cell proliferation in vitro. These characteristics of VEGF make it an ideal candidate to mediate the retinal vasoproliferative response to hypoxic stimuli observed in a variety of ischemic ocular diseases.

Vascular endothelial growth factor receptors are high-affinity, membrane-bound proteins located primarily on endothelial cells. Vascular endothelial growth factor binds to two high-affinity tyrosine kinase receptors—kinase insert domain-containing receptor (KDR) and the fms-like tyrosine kinase receptor, Flt. The murine homologue of KDR is fetal-liver kinase receptor (Flk). These receptors are autophosphorylating tyrosine kinase receptors with similarities to the platelet-derived growth factor receptor family. Recent reports have demonstrated that the two VEGF receptors differ both in function and intracellular signal transduction.

In this study, we have investigated the regulation of VEGF receptors (KDR/Flk and Flt) under hypoxic conditions in retinal endothelial cells, and we have studied the role of adenosine as a mediator of these changes in gene expression.

**MATERIALS AND METHODS**

**Materials**

Carbonyl cyanide p-(trifluromthoxy)phenyl-hydrazone (FCCP) was obtained from Sigma (St. Louis, MO). Fetal bovine serum, plasma-derived horse serum, and calf serum were obtained from HyClone (Logan, UT), respectively. The BBL GasPakPlus and BBL Disposable Anaerobic Indicators were obtained from Becton Dickson (Cockeysville, MD). N6-cyclopentyladenosine (CPA), N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl] adenosine (DPMA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-phenyltheophylline, and 8-(3-Chlorostyryl)catechol (GSC) were purchased from Research International Biochemicals (Natick, MA). 32P-dATP was obtained from Amersham (Arlington Heights, IL). Human VEGF and KDR cDNA were kindly provided by Genentech, Inc. (San Francisco, CA). Human and mouse Flt cDNA was a generous gift from Dr. Masahumi Shibuya (The Institute of Medical Science, Tokyo, Japan) and Kevin Claflay (Beth Israel Hospital, Boston, MA), respectively.

**Cell Cultures**

Primary cultures of bovine retinal endothelial cells (BREC) were isolated from slaughterhouse eyes by homogenization and by a series of filtration steps as previously described. Primary BREC cells were grown on fibronectin (NY Ben Reagents; New York Blood Center, New York, NY)-coated dishes (Costar, Cambridge, MA) containing Dulbecco’s modified Eagle’s medium with 5.5 mM glucose, 10% plasma-derived...
Hypoxic Regulation of VEGF Receptors

Hypoxic Studies

Confluent cell monolayers were exposed to various oxygen concentrations using an 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 cultured under these conditions showed no morphologic changes by light microscopy after exposures exceeding 72 hours, excluded trypan blue dye (>98%) and subsequently could be passaged normally. In some experiments, cells were exposed to 0% oxygen using the BBL GasPakPlus system (Becton Dickinson). Cells incubated under standard normoxic conditions from the same batch and passage were used as controls (95% air, 5% CO₂). Although this “normoxia” may represent higher oxygen concentrations than normally found in the retina, the hypoxic conditions studied are certainly “relatively” hypoxic compared to control conditions and are truly hypoxic compared with the in vivo situation at the lowest oxygen levels. Retinal endothelial cells demonstrate a hypoxic response below 5% oxygen with maximal response observed below 3%.3

Northern Blot Analysis

Total RNA was isolated from individual P-100 tissue culture plates using guanidium thiocyanate.40 Northern blot analysis was performed on 20 to 25 µg total RNA after 1% agarose−2 M formaldehyde gel electrophoresis and subsequent capillary transfer to ICN Biochemicals (Irvine, CA) Biotrans nylon membranes and ultraviolet cross-linking using a Stratagene UV Stratallinker 2400 (La Jolla, CA). Radioactive probes were generated using Amersham Multiprime labeling kits and [32P]-dATP. Blots were prehybridized, hybridized, and washed in 0.5 × SSC, 5% sodium dodecyl sulfate (SDS), at 65°C with four changes over 1 hour in a rotating hybridization oven (model 400; Robbins Scientific, Sunnyvale, CA). Fli gene was detected using 2 × SSC, 5% SDS, at 50°C as the washing condition. All signals were analyzed using the Molecular Dynamics (Sunnyvale, CA) Computing Densitometer and Pho-photomager, and lane loading differences were normalized using 36B4 cDNA probe.41,42

Vascular Endothelial Growth Factor Binding Analysis

Monolayers of confluent endothelial cells grown in 12-well cluster dishes (Costar) were placed on ice and washed three times with cold phosphate-buffered saline containing calcium and magnesium. 125I-labeled VEGF was added along with increasing amounts of unlabeled VEGF, and binding was carried out on a rocking platform for 4 hours at 4°C. Binding was terminated by washing each well three times with cold phosphate-buffered saline containing 0.1% bovine serum albumin. The cells subsequently were lysed in 1 ml of 0.1% SDS and counted in a gamma counter (model 1825; Tracor Analytic, Elk Grove Village, IL).

Statistical Analysis

Determinations were performed in triplicate, and experiments were repeated at least three times. Results are expressed as mean ± standard error unless otherwise indicated. Statistical analysis with the paired Student’s t-test was used 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. P <0.05 was considered statistically significant.

RESULTS

Characterization of KDR/Flk and Flt mRNA in Various Cultured Cells

Expression of VEGF receptors KDR/Flk and Flt mRNA were assessed by Northern blot analysis in BREC, bovine aortic endothelial cells, bovine retinal pigment epithelial cells, and human umbilical vein endothelial cells. As demonstrated in Figure 1, KDR/Flk mRNA was observed readily in all three endothelial cell types but not, as expected, in bovine retinal pigment epithelial cells. Flt, however, could not be detected by Northern blot analysis in BREC or bovine aortic endothelial cells even when using polyadenylated RNA and low stringency washing conditions. Flt was detectable in human umbilical vein endothelial cells. Thus, subsequent studies evaluated only KDR/Flk expression in BREC.

Hypoxia Suppresses KDR/Flk mRNA Expression in a Time- and Dose-Dependent Manner

Because VEGF expression is regulated by hypoxia in many ocular cell types, such as retinal endothelial cells, retinal pericytes, retinal pigment epithelial cells,
KDR

Flt

36B4

HUVEC BREC BAEC BRPE

FIGURE 1. RNA expression of KDR and Flt in bovine retinal endothelial cells, bovine aortic endothelial cells, bovine retinal pigment epithelial cells, and human umbilical vein endothelial cells. Fifteen micrograms of total RNA from each cell type was evaluated by Northern blot analysis. Hybridization was performed using human KDR and Flt cDNA probe. Low-stringency washing was performed for the Flt probe (2 X SSC at 50°C). Similar findings were observed with murine Flt probe and the use of polyadenylated RNA. 36B4 was used as a lane loading control.

and Müller cells, we investigated whether hypoxia affected VEGF receptor mRNA expression in retinal endothelial cells. Cells were exposed to 0% oxygen, 5% CO₂, and 95% N₂ for various periods, and Northern blot analysis was performed. KDR/Flk mRNA expression was decreased in a time-dependent manner to 16% ± 2.3% of normoxic levels (P = 0.006) after 12 hours and to 0.5% ± 0.2% (P = 0.019) after 24 hours (Fig. 2A). KDR mRNA expression was inhibited in an oxygen dose-dependent manner, with half-maximal inhibition observed at 5% to 10% O₂ (Fig. 2B). Exposure to 5% oxygen, 5% CO₂, and 90% N₂ decreased mRNA expression to 35% ± 14% of 21% oxygen levels (P = 0.031).

Hypoxia-Induced Suppression of KDR/Flk mRNA Expression Is Reversed by Reinstitution of Normoxia

We evaluated the ability of KDR/Flk mRNA expression to recover after hypoxia by returning cells to normal oxygen conditions after an 18-hour exposure to 0% O₂ (Fig. 3), which decreased KDR/Flk mRNA to 17% ± 5.6% of control (P = 0.001). After reinstitution of normoxia for 18 and 24 hours, mRNA levels increased to 72% ± 25% and 84% ± 16% of control, respectively. Neither of these levels was significantly different from control cells.

Inhibition of Electron Transport Decreases KDR/Flk mRNA Expression

We next investigated whether inhibition of oxidative phosphorylation in a normoxic environment could induce a similar response as observed under hypoxic conditions. We used FCCP, a cyanide-containing compound that inhibits mitochondrial oxidative phosphorylation at its most distal site, preventing the electron transport from cytochrome aa₃ to oxygen.
Thus, this compound more closely mimics an oxygen deficit than do proximal transport inhibitors, such as rotenone and antimycin A. When BREC cells were exposed to 10 μM FCCP, KDR/Flk mRNA expression decreased to 58% ± 7.1% of control (P = 0.001) after 3 hours (Fig. 4).

**Adenosine Receptor Agonists Reduce KDR/Flk mRNA Expression**

It is well documented that hypoxia increases intracellular adenosine levels by reducing adenosine kinase-mediated recycling of adenosine to adenosine monophosphate. We investigated whether adenosine analogues could mimic the hypoxia-induced reduction of KDR/Flk mRNA. N\(^6\)-cyclopentyl adenosine (CPA) is a stable adenosine A\(_1\) receptor (A\(_1\)R) agonist, whose IC\(_{50}\) for A\(_1\)R and for A\(_2\) receptor (A\(_2\)R) binding is approximately 1 nM and 600 nM, respectively. CPA did not affect KDR/Flk mRNA expression at concentrations up to 100 nM for 24 hours but did reduce the mRNA level at higher concentrations (Fig. 5A). Stimulation of cells with 500 nM, 50 nM, and 500 nM CPA decreased the mRNA level to 59% ± 1.4% (P = 0.001), 56.7% ± 19% (P = 0.011), and 30% ± 4.9% (P = 0.002) of control, respectively. CPA (50 μM) reduced KDR/Flk mRNA level in a time-dependent manner with significant reductions noted after exposure for 12 or more hours (Fig. 5B).

Because CPA at concentrations less than 500 nM—which specifically stimulate A\(_1\)R—did not reduce KDR/Flk mRNA levels, and because higher doses—which can stimulate both A\(_1\)R and A\(_2\)R—did reduce KDR/Flk mRNA levels, we investigated whether adenosine could be reducing KDR/Flk mRNA expression through specific effects on A\(_2\)R. Cells were stimulated with the A\(_2\)R specific agonist, N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl] adenosine (DPMA), whose IC\(_{50}\) for A\(_2\)R binding is 4.4 nM as opposed to 142 nM for A\(_1\)R. DPMA decreased KDR/Flk mRNA levels in a dose-dependent manner with an EC\(_{50}\) of 5 to 10 nM (Fig. 6A) and maximal inhibition of more than 50% observed at 10 to 100 nM (P = 0.026). Stimulation of the cells with 100 nM DPMA reduced the KDR/Flk mRNA level within 3 hours (P < 0.001), which lasted for more than 24 hours.
Adenosine Receptor Antagonists Inhibit the Hypoxia-Induced Reduction of KDR/Flk mRNA

To determine whether the hypoxia-induced reduction of KDR/Flk mRNA could be prevented by blocking adenosine receptor activation, we studied the effects of A_1R antagonists, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-phenyltheophylline, and the A_2R antagonist 8-(3-chlorostyryl)caffeine (CSC), on hypoxic BREC. DPCPX did not inhibit the hypoxic response of KDR/Flk mRNA at 10 nM but did inhibit the response at 50 μM (P = 0.001) (Fig. 7A). Because the IC_{50} of this compound is 0.1 nM and 465 nM for A_1R and A_2R binding, respectively, the observed response suggests that the hypoxic regulation of KDR/Flk mRNA is mediated through A_2R. Similarly, 8PT, whose IC_{50} is 22 nM for A_1R binding and 850 nM for A_2R binding, inhibited the hypoxic response at 1 to 10 μM (P < 0.001) (Fig. 7B), again suggesting the involvement of A_2R. The A_3R antagonist CSC, whose IC_{50} is 25 μM for A_1R binding and 100 nM for A_2R binding, inhibited the hypoxic response by 42% ± 7.8% (P = 0.008) at 10 μM, a concentration specific for blocking A_3R (Fig. 7C).

Vascular Endothelial Growth Factor Receptor Binding

To determine whether the number or affinity of VEGF binding sites was changed in BREC under hypoxic conditions, we performed ^{125}I-VEGF binding analysis. When exposed to 1% oxygen, specific VEGF binding decreased within 30 hours but increased after that time period. Hypoxia exposure for 24 hours decreased specific VEGF binding from 15.1% ± 0.3% to 12.7% ± 0.4% per milligram protein (P < 0.001), whereas
specific binding was increased from 13.5% ± 1.1% to 18.3% ± 0.8% per milligram protein (P < 0.001) after 72 hours (Fig. 8A). Scatchard analysis detected a non-significant decrease of receptor binding sites without changes of binding affinity after 30 hours of exposure (Fig. 8B) to hypoxia (6.1 ± 0.9 sites/cell to 5.4 ± 0.4 sites/cell, P = 0.185; 58 ± 9.3 nM to 60 ± 10 nM, P = 0.184). However, after 72 hours of hypoxia, an increased number of VEGF binding sites (4.2 ± 0.6 × 10^4 sites/cell to 6.7 ± 1.0 × 10^4 sites/cell, P = 0.049) with unchanged receptor affinity (50 ± 17 nM to 54 ± 14 nM, P = 0.207) were observed (Fig. 8C). Flt mRNA was still not detectable in BREC after this period of hypoxia, and KDR mRNA levels did not demonstrate a significant increase (data not shown).

**DISCUSSION**

Ocular diseases associated with retinal nonperfusion often result in the formation of retinal neovascularization leading to visual loss. This response is thought to be mediated by the hypoxic induction of angiogenic factors that subsequently stimulate pathologic angiogenesis.\(^7\) Studies have demonstrated that expression of the angiogenic factor, VEGF, is increased greatly in response to hypoxia in retinal cells.\(^7\) In addition, the level of VEGF in the eyes of patients with diabetes mellitus are associated closely with the presence of active proliferative diabetic retinopathy.\(^2,5\) Indeed, inhibition of VEGF suppresses ischemia-induced retinal neovascularization in animal models.\(^45\) Thus, VEGF could mediate much of the intraocular neovascularization arising from ischemic retinal diseases.

In this study, we demonstrated that the VEGF receptor mRNA of KDR/Flk, rather than Flt, is predominantly expressed in bovine retinal endothelial cells. We could easily detect KDR/Flk in retinal endothelial cells; however, Flt mRNA expression was not observed in these cells by Northern blot analysis, despite low stringency conditions, use of two different Flt cDNA probes, and polyadenylated mRNA analysis. Interestingly, a similar observation was reported by Barleon et al.\(^46\) In that study, Flt was detected in human umbilical-vein endothelial cells but not in bovine aortic endothelial cells. This does not appear to be an artifact of chronic culture conditions because Flt mRNA could not be detected in cultured BREC from either early or late passage. However, one cannot exclude the possibility of culture conditions initially selecting a BREC subtype that expresses only KDR while suppressing BREC subtypes that express Flt.

We also demonstrated that KDR/Flk mRNA levels were reduced by short-term hypoxic conditions. Adenosine receptor stimulation decreased KDR/Flk mRNA under normoxic conditions, and adenosine receptor
inhibition prevented the reduction of KDR/Flk under hypoxic conditions. These data suggest that the increased adenosine concentrations present under hypoxic conditions might mediate the short-term hypoxic downregulation of KDR/Flk mRNA. In contrast to short-term hypoxia (<36 hours), chronic hypoxia induced an increase of VEGF binding sites. This biphasic response was not observed after adenosine stimulation, suggesting that other mechanisms may mediate the chronic upregulation of VEGF receptors.

Although the in vivo significance of increased VEGF expression, combined with initial decreased KDR expression observed in vitro, is not certain, it is interesting to speculate that the physiologic significance of the biphasic VEGF receptor response may be to regulate hypoxia-induced neovascularization more tightly. Initial, possibly transient, decreases in oxygen concentrations, where VEGF levels can be dramatically elevated but where angiogenesis may not be urgently required, lead to a reduction of KDR and thus an amelioration of VEGF's angiogenic stimuli. However, under conditions of chronic oxygen deficits, in which angiogenesis is a more appropriate response, VEGF receptors are increased and thus potentially facilitate VEGF action. Indeed, hypoxia increases VEGF binding sites in cultured bovine retinal endothelial cells, and a recent study showed increased mRNA levels of both KDR/Flk and Flt in the lung of rats exposed to hypoxia.

It can be seen that decreased KDR/Flk mRNA levels do not result from a toxic effect because the cells show no morphologic changes by light microscopy, exclude trypan blue dye (>98%), reverse mRNA changes when they are returned to normoxia, and express genes that are not downregulated by hypoxic exposure. In addition, VEGF itself does not reduce KDR/Flk mRNA at doses that fully stimulate retinal endothelial cell mitogenic activity (25 ng/ml, data not shown). The mRNA levels of KDR/Flk begin to decrease after 6 hours of hypoxia and at 5% to 10% O2. This time and dose response is similar to that of VEGF stimulation by hypoxia in retinal pericytes and endothelial cells. The relatively modest VEGF binding decline observed during this period, compared with mRNA reduction, may be a consequence of relative message and/or protein stability and blunting caused by the subsequent induction of binding capacity discussed below.

Hypoxia induces large increases of adenosine, and adenosine can mediate multicellular functions through cell surface adenosine receptors. For example, erythropoietin, a molecule whose expression is increased by hypoxia and whose regulation is analogous to that of VEGF, is stimulated by adenosine, as is VEGF. Our investigation demonstrated similar adenosine effects on the hypoxic downregulation of KDR/Flk mRNA. CPA, an adenosine agonist, showed little effect at concentrations up to 100 nM but decreased KDR/Flk mRNA expression at higher concentrations (maximal decrease at 50 μM). Because the
IC_{50} for A_{1}R binding is 1 nM for A_{1}R binding and 680 nM for A_{2}R binding. The observed response suggests that the KDR/Flk mRNA level is most likely mediated through A_{2}R. DPMA decreased KDR/Flk mRNA levels at concentrations as low as 10 nM. Because the IC_{50} of this ligand for A_{2}R binding is 4.4 nM and for A_{1}R it is 142 nM, these data again suggest that the VEGF response is mediated by A_{2}R.

If adenosine and its receptor mediate the hypoxic response of the KDR/Flk mRNA, inhibition of adenosine receptor activation should prevent the hypoxic decreases in KDR mRNA. Both A_{1}R antagonists, 8PT and DPCPX, inhibited hypoxia-induced reduction of the KDR/Flk mRNA only at high concentrations that block both A_{1}R and A_{2}R stimulation. CSC, however, inhibited the hypoxic response of the mRNA at doses as low as 100 nM, at which only A_{2}R is inhibited. These data further support the hypothesis that A_{2}R mediation might play a role in the hypoxic regulation of the KDR/Flk mRNA and suggest that the A_{2}R subtype may be predominantly involved.

Increased VEGF binding sites have been reported in bovine endothelial cells exposed to anoxic conditions. However, the retinal ischemia responsible for inducing retinal neovascularization in numerous pathologic clinical conditions is hypoxic rather than anoxic. Therefore, we investigated the retinal endothelial cell binding capacity under hypoxic conditions (1% to 5% O_{2}). Hypoxia for periods up to 30 hours decreased specific binding as a result of fewer binding sites rather than from the decreased affinity of the receptors when assessed by Scatchard analysis. This change, however, did not reach statistical significance. Although confirmation by direct analysis of KDR protein levels is desirable, this has not been achieved because appropriate KDR antibodies do not yet exist.

Long-term hypoxic exposure, however, induced an increase in specific binding as a result of increased receptor affinities as determined by Scatchard analysis. Because KDR/Flk mRNA is still decreased after 72 hours of incubation in 1% oxygen, the observed binding response probably is not regulated transcriptionally. Instead, posttranscriptional regulation of the receptor protein, including increased translocation of intracellular receptors to the plasma membrane or receptor stabilization, may be involved. Increased activity of another VEGF receptor could produce this phenomenon; however, we did not detect Flt mRNA expression in bovine retinal endothelial cells in normoxic or hypoxic cells, and Scatchard analysis suggests only a single affinity receptor class. Further studies are necessary to elucidate the mechanism of biphasic regulation of the VEGF receptors by hypoxia.

Angiogenesis not only plays a role in pathologic conditions, such as tumor growth and neovascularization from ischemic retinal diseases, it also contributes to normal developmental physiology. In these situations, a tightly coordinated regulation of angiogenic factor response is important, and feedback mechanisms probably exist. Overall, we find that VEGF receptor KDR/Flt mRNA expression in BREC is tightly regulated by hypoxic conditions in a complex manner, further suggesting that VEGF plays a critical role in the modulation of ischemia-induced angiogenesis.

**Key Words**
endothelial cell, KDR, Flk, receptor, retina, vascular endothelial growth factor (VEGF), vascular permeability factor (VPF)

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