Effect of VEGF on Retinal Microvascular Endothelial Hydraulic Conductivity: The Role of NO

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PURPOSE. Vascular endothelial growth factor (VEGF) increases microvascular permeability in vivo and has been hypothesized to play a role in plasma leakage in diabetic retinopathy. Few controlled studies have been conducted to determine the mechanism underlying the effect of VEGF on transport properties (e.g., hydraulic conductivity [Lp]). This study was conducted to determine the effect of VEGF on bovine retinal microvascular endothelial Lp and the role of nitric oxide (NO) and the guanylate cyclase/guanosine 3’,5’-cyclic monophosphate/protein kinase G (GC/cGMP/PKG) pathway downstream of NO in mediating the VEGF response.

METHODS. Bovine retinal microvascular endothelial cells (BRECs) were grown on porous polycarbonate filters, and water flux across BREC monolayers in response to a pressure differential was measured to determine endothelial Lp.

RESULTS. VEGF (100 ng/ml) increased endothelial Lp within 30 minutes of addition and by 13.8-fold at the end of 3 hours of exposure. VEGF stimulated endothelial monolayers to release NO and incubation of the BRECs with the nitric oxide synthase inhibitor NG-monomethyl-L-arginine (L-NMMA; 100 μM) significantly attenuated the VEGF-induced Lp increase. It was observed that incubation of the monolayers with the GC inhibitor LY-83583 (10 μM) did not alter the VEGF-mediated Lp response. Addition of the cGMP analogue 8-br-cGMP (1 mM) did not change the baseline Lp over 4 hours. Also, the PKG inhibitor KT5825 (1 μM) did not inhibit the response of BREC Lp to VEGF.

CONCLUSIONS. These experiments indicate that VEGF elevates hydraulic conductivity in BRECs through a signaling mechanism that involves NO but not the GC/cGMP/PKG pathway. (Invest Ophthalmol Vis Sci. 2000;41:4256 - 4261)

Diabetic retinopathy (DR), a major cause of visual impairment in the United States,1 can be divided, for purposes of discussion, into two stages: a proliferative stage and a preceding nonproliferative stage. Nonproliferative DR is characterized by changes in the retina such as increased transport of water and proteins from the retinal microvessels to the surrounding tissue leading to microaneurysms, edema, and hard exudates.2,5 There are two mechanisms by which materials can traverse the endothelial barrier: convection and diffusion. Diffusion of a solute, which is driven by a concentration gradient, is characterized by the permeability coefficient (Pe), and convective transport is characterized by hydraulic conductivity (Lp). Lp is a measure of the ease by which the microvessel wall allows water flow.4 This study focuses on the regulation of endothelial Lp by vascular endothelial growth factor (VEGF).

In nonproliferative DR, platelets clump together to form small stable aggregates that can lead to capillary closure.5 When new blood vessels begin to form in the eye, the disease progresses to the proliferative stage. VEGF is an endothelial cell-specific mitogen that has been hypothesized to play a major role in DR for several reasons: VEGF increases hydraulic conductivity6 as well as solute permeability of microvessels7,8; VEGF production is enhanced by hypoxia9,10; a wide variety of cells in the hypoxic retina such as glial cells, astrocytes, and Müller cells express VEGF; and VEGF produced by these cells may be responsible for the breakdown of the retinal barrier.11 It has been shown that bovine retinal microvascular endothelial cells (BRECs) have a larger number of high-affinity receptors for VEGF than aortic vascular endothelial cells, thereby suggesting that VEGF may be an important mediator of neovascularization induced by hypoxic retinopathies.12 It has also been reported that there is increased VEGF production in both vitreous13 and ocular fluids14 of patients with retinopathy. Murata et al.11 observed in diabetic rats that the rates of retinal endothelial barrier breakdown characterized by albumin extravasation and VEGF activity increase in proportion to the duration of diabetes. The same group showed that there was more albumin leakage in the vessels with high VEGF immunoreactivity than in the vessels without VEGF immunoreactivity.11 Thus, VEGF expression in nonproliferative DR increases extravasation of plasma proteins and may contribute to macular edema.
It has been widely reported that VEGF increases solute permeability in a variety of tissue preparations in vivo including the skin, subcutaneous tissue, peritoneal wall, mesentery, and tumors. However, only a few studies have reported the effect of VEGF on $L_p$. A fivefold increase in $L_p$ of frog mesenteric microvessels has been reported after 24 hours of exposure to 1 mM VEGF. The effect of VEGF on transport properties of cultured endothelial cell lines has also been investigated. Yaccino et al. showed that addition of 50 ng/ml VEGF to BREC monolayers causes a significant increase in $L_p$ within 30 minutes of addition of VEGF, which continues to increase to approximately 18-fold over the next hour. In a second study, Chang et al. determined the effect of VEGF (100 ng/ml) on $L_p$ and albumin permeability in three well-established cell culture models: human umbilical vein endothelial cells (HUVECs), bovine aortic endothelial cells (BAECs), and BREC. They showed that, in addition to affecting BREC $L_p$, VEGF also induces a 9.9-fold increase in albumin $P_e$ in 3 hours. These studies, however, did not explore the biochemical signaling mechanisms mediating the responses of BREC $L_p$ to VEGF.

The upregulation of nitric oxide (NO) production by VEGF has been observed in cultured HUVECs and rabbit vasculature, and the VEGF-induced increase in solute permeability in coronary venules was inhibited by nitric oxide synthase (NOS) inhibitors, as was VEGF-induced angiogenesis in rabbit corneas. Therefore, the hypothesis for this study was that the VEGF-induced increase in BREC monolayer $L_p$ is mediated by NO and its downstream effectors.

**Materials and Methods**

**Chemicals**

The following chemicals were obtained from Sigma (St. Louis, MO): Bovine serum albumin (BSA, fraction V, 30% solution), minimal essential medium with β-valine (MEM β-valine), fetal bovine serum (FBS), gelatin, sodium bicarbonate, fibronectin, penicillin-streptomycin solution, Nω-monomethyl-l-arginine acetate salt (L-NMMA), β-nicotinamide adenine dinucleotide phosphate (β-NADPH), N-(1-naphthyl) ethylenediamine (NAD), flavin adenine dinucleotide (FAD), sulfanilamide, nitrate reductase, and 8-brc-cGMP. LY-83583 and KT5823 were purchased from Calbiochem (La Jolla, CA); recombinant VEGF from R&D systems (Minneapolis, MN); D,L-acetylated low-density lipoprotein from Biomedical Technologies (Stoughton, MA); polycarbonate filters (Transwell chambers, 0.4-μm pore size, 24.5-mm diameter) from Corning Costar (Cambridge, MA); trypsin from Gibco (Gaithersburg, MD); and high-vacuum grease from Dow Corning (Midland, MI).

**Cell Culture**

Primary BREC were harvested from bovine eyeballs and subsequently grown in MEM-β-valine containing 20% FBS, as described by Yaccino et al. Cells were plated at a density of 100,000 cells/cm² on polycarbonate membrane Transwell filters. The filters were pretreated with gelatin (5 mg/ml, type A from porcine skin), exposed to UV light overnight, and precoated with fibronectin (50 μg/ml). Cells between passages 7 and 10 were used in experiments.

**Measurement of Water Flux**

Water flux was measured using an apparatus that was built in our laboratory, the details of which are described by Sill et al. The entire apparatus was housed in a plexiglas box maintained at 37°C. The polycarbonate membrane Transwell filter that contained the BREC monolayer was sealed between a two-piece polycarbonate assembly that separated it into luminal (above the monolayer) and abluminal (below the monolayer) compartments. A 5% CO₂/95% air gas port provided continual positive pressure outgassing and maintained pH at 7.4. The abluminal chamber was attached by Tygon tube to a borosilicate glass tube followed by additional Tygon tubing leading to an abluminal reservoir. The difference in level of the fluid in the luminal compartment and in the abluminal reservoir provided an adjustable hydrostatic pressure differential ($ΔP$) that was the driving force for fluid movement across the monolayer. Because the same medium (MEM β-valine-1% BSA) was used in both the luminal and the abluminal compartments, any osmotic gradient was eliminated from the system. BSA (1%) was needed to seal the monolayers.

For water flux measurements, an air bubble was introduced into the medium in the glass tube, and the abluminal reservoir was lowered so that a $ΔP$ of 10 cm H₂O was applied across the BREC monolayer. The movement of the air bubble, indicative of volume flux across the monolayer, was tracked by a photometer interfaced to a personal computer. The photometer consisted of an emitter-detector unit mounted on a movable carriage. When the borosilicate tubing containing the bubble was clamped in place between the emitter and the detector, the transmittance of the infrared light from the emitter decreased due to scattering by the meniscus of the bubble. The computer controlled the movement of the carriage to maintain the transmittance profile of the light at a constant level. During a typical 4-hour experiment, the photometer detected from 6,000 to 10,000 data points (bubble displacement versus time points).

The volumetric flow rate $J_v$ was calculated using the equation

$$J_v = (Δd/Δt)A_{tubing}$$

where $Δd/Δt$ is the rate of change of the bubble displacement and $A_{tubing}$ is the cross-sectional area of the glass tubing (0.1206 cm²). The hydraulic conductivity was then determined from the definition

$$L_p = J_v/SΔP$$

where $S$ is the surface area of the BREC monolayer (4.714 cm²) and $ΔP$ is the hydrostatic pressure differential across the monolayer (10 cm H₂O).

To study the effect of VEGF on BREC $L_p$, baseline $J_v$ was measured for 1 hour after imposition of $ΔP$, and then 100 ng/ml VEGF was added to the luminal media through a small port on the side of the upper assembly. $L_p$ was measured for a 3-hour period after addition of VEGF.

**Nitrite/Nitrate Determination**

Endothelial cells grown to confluence on polycarbonate filters were rinsed twice with MEM (not containing phenol red)-1%
addition of VEGF. Samples were first reduced with nitrate reductase to convert NO$_3^-$ to NO$_2^-$ and then exposed to Greiss reagent, which reacted with nitrite to yield a diazochromophore. A colorimetric assay (EL311; Bio-tek Instruments, Burlington, VT) was then performed at an optical density of 540 nm.

**Effect of NOS Inhibitor on VEGF-Induced Increase in $L_p$**

In these experiments, the endothelial cells were preincubated for 1 hour with an NOS inhibitor l-NMMA (100 μM) and then exposed to a pressure differential of 10 cm H$_2$O without VEGF for 1 hour to seal the monolayer until baseline $L_p$ was established. Then 100-ng/ml VEGF was added and $L_p$ measured for another 3 hours.

To investigate the biochemical pathway downstream of NO, experiments were conducted in which endothelial cells were exposed to VEGF in the presence of analogues or inhibitors of enzymes affected by NO.

**GC/cGMP/PKG Pathway**

In separate experiments, monolayers were incubated with LY-85583 (10 μM, a specific GC inhibitor) and KT5823 (1 mM, a PKG inhibitor) for 30 minutes before addition of VEGF. In addition, $L_p$ response to the cGMP analogue 8-br-cGMP (1 mM) was also observed. In all these experiments, the usual procedures for hydraulic conductivity measurements were followed.

**Effect of cAMP on VEGF-Induced Increase in $L_p$**

To determine whether increases in $L_p$ could be reversed, a cAMP analogue dibutyryl cAMP (1 mM), which has been effective in reversing $L_p$ increases in other cell types (e.g., BAECs, $^{23}$), was added to the monolayers at the end of 3 hours of VEGF exposure, and $L_p$ was measured for another 30 minutes.

**Data Presentation and Statistical Analysis**

A 1-hour period was allowed for a stable baseline to be established before further intervention. Five-minute $L_p$ averages were calculated, normalized in relation to the established baseline (value at 55 minutes), and presented as mean ± SEM. Significant differences between group means were analyzed by a two-way (time and treatment) repeated-measure analysis of variance (ANOVA) using statistical analysis software (SAS, Cary, NC) incorporating a Bonferroni correction. Time was the repeated factor. $P < 0.05$ was used as the significance level for the statistical analysis. The Bonferroni correction gives a conservative significance level of $P/m$ where $m$ is the number of comparisons to be performed. For example, if two groups were to be compared, $P = 0.05$ would be replaced by $P = 0.05/2 = 0.025$.

**RESULTS**

Figure 1 illustrates a significant increase in $L_p$ of cultured BRECs when exposed to 100 ng/ml of VEGF for 3 hours. For the control samples, normalized $L_p$ decreased with time and was 0.55 ± 0.17 at the end of 3 hours, whereas addition of VEGF increased $L_p$ significantly ($P < 0.025$) to a level of 7.61 ± 0.54 for the same period. The VEGF-induced response relative to the control samples represents a 13.8-fold increase in $L_p$ after 3 hours.

The main objective of this research was to investigate the role of NO in mediating the VEGF-induced increase in $L_p$ shown in Figure 1. First, experiments were conducted to determine whether BRECs altered their production of NO when exposed to VEGF (Fig. 2). The NO concentration in the control samples was 14.03 ± 1.08 nmol/mg protein at time 0 and was 23.37 ± 5.39 nmol/mg protein at the end of the 3-hour experiment. For the VEGF-treated monolayers, the initial concentration of NO was 13.87 ± 1.22 nmol/mg protein, which increased to 22.77 ± 0.92 nmol/mg protein within 5 minutes of VEGF addition and increased to a level of 74.54 ± 10.97 nmol/mg protein at the end of 3 hours. VEGF increased the NO concentration significantly ($P < 0.025$) relative to control samples.

In the next set of experiments, we further examined the role of NO by using an NOS inhibitor l-NMMA (100 μM). Figure 3 shows that the NOS inhibitor greatly attenuated the VEGF response. The normalized $L_p$ of filters that were incubated with l-NMMA increased to 1.96 ± 0.46 at 3 hours after addition of VEGF. This was significantly different from the response of VEGF-treated control samples, which had a normalized $L_p$ of approximately 7.61 ± 0.54 at the end of the same period ($P < 0.025$). Separate experiments (not shown) demonstrated that the NOS inhibitor at the concentration of 100 μM did not significantly affect the baseline $L_p$ in the absence of VEGF (0.71 ± 0.01-fold at the end of 3 hours).

Having established that NO is a key signaling intermediate, we next investigated the role of the GC/cGMP/PKG pathway downstream of NO. In the first set of experiments, BRECs monolayers were incubated with the GC inhibitor LY-85583...
Figure 2. Effect of 100 ng/ml VEGF (n = 4) on cumulative NOx concentration. Exposure to VEGF elevated NO production significantly within 15 minutes compared with the control samples (n = 3). *P < 0.05 is the level of significance. Data are presented as mean ± SEM.

Figure 3. Effect of the NOS inhibitor L-NMMA on VEGF-induced Lp response in BREC monolayers. The monolayers were incubated with 100 μM L-NMMA for 2 hours before addition of 100 ng/ml VEGF. *Compared with the VEGF control samples (n = 6) without the inhibitor, the NOS inhibitor (n = 4) significantly inhibited the Lp response to VEGF (P < 0.05). Data are presented as normalized mean ± SEM.

Figure 4. Response of BREC monolayer Lp to 100 ng/ml VEGF, with and without the GC inhibitor LY-83583. The cells were incubated with 10 μM LY-83583 (n = 5) for 30 minutes before addition of VEGF. The GC inhibitor failed to inhibit the response elicited by 100 ng/ml VEGF. At the end of 3 hours, VEGF in the presence of the inhibitor increased Lp by 6.35 ± 1.44-fold, which was not significantly different from the VEGF control samples (7.61 ± 0.54-fold) at the end of 3 hours of exposure time (P > 0.4).

When a cAMP analogue db cAMP was added to the monolayers at the end of 3 hours of VEGF exposure, normalized Lp decreased from 7.61 ± 0.54 to 2.19 ± 0.31 in just 30 minutes (Fig. 1), indicating that Lp is sensitive to intracellular cAMP levels.

**DISCUSSION**

This study was a continuation of work undertaken to examine the effect and mechanism of VEGF on transport properties of BREC monolayers. Yaccino et al. found that addition of 50 ng/ml VEGF to cultured BREC causes a significant increase in Lp within 30 minutes of addition, which continues to increase dramatically (18-fold) over the next hour. Chang et al. studied the effect of VEGF (100 ng/ml) on three different cell lines, BAECs, BREC, and HUVECs, and observed a fivefold increase in endothelial Lp with BREC monolayers that were exposed to 100 ng/ml VEGF for 3 hours. Bates and Curry used the Landis...
FIGURE 6. Response of endothelial monolayer Lp to 100 ng/ml VEGF, with and without the PKG inhibitor KT5823. Cells were incubated in KT5823 (1 μM; n = 4) for 30 minutes before addition of 100 ng/ml VEGF. KT5823 failed to inhibit the VEGF response. At the end of 3 hours of exposure to VEGF in the presence of the PKG inhibitor, Lp increased to 6.44 ± 0.33-fold, which was not significantly different from the Lp response to VEGF without the inhibitor (P > 1.0). Data are presented as normalized mean ± SEM.

VEGF acts selectively on endothelial cells through the two high-affinity receptor tyrosine kinases, fms-like tyrosine kinase (Flt-1)27,28 and kinase domain region/fetal liver kinase-1 (KDR/Flk-1),29–31 which initiate signaling pathways within the cell. In the present study, we examined the role of NO in mediating the VEGF-induced increase in Lp of BREC monolayers. We found that addition of 100 ng/ml VEGF to cultured BREC stimulated them to increase their NO production levels threefold compared with control samples over a 3-hour period (Fig. 2). Hood et al.19 measured NO release from cultured HUVECs in response to VEGF. NO release increased to 230% of control after 1 hour of incubation and returned to near baseline by 12 hours. By 24 hours, this release was again more than 200% of control, a level maintained through 48 hours of incubation. At both the 1- and 24-hour time points, NO release was increased in a dose-dependent manner by VEGF. It has also been reported that VEGF produced a dose-dependent increase in NO concentration from vascular segments of rabbit thoracic aorta, pulmonary artery, and inferior vena cava, which peaked 8 minutes after addition.20 These studies corroborate our observation that VEGF stimulates increased NO production in endothelial cells.

Another finding of the present study was that the NOS inhibitor L-NMMA blocked the VEGF-elicited increase in albumin permeability in isolated and perfused porcine coronary venules. They observed that topical application of VEGF independently and transiently increased albumin permeability by two- to threefold, and this increase was abolished by L-NMMA. In contrast, Chang et al.18 observed that L-NMMA did not alter BREC Lp response to VEGF. They found that VEGF in the presence of the inhibitor induced a 5.09 ± 0.28-fold Lp increase, which was not significantly different from the Lp response of the VEGF control samples (5.06 ± 0.65-fold) at the end of 3 hours of exposure time. Therefore, although BAECS, similar to BREC, display an increase in Lp in response to VEGF, it is PKC that mediates the Lp response to VEGF in BAECS,18 whereas NO mediates the Lp response in BREC. This demonstrates a divergence of behavior between aortic and microvascular endothelial cells in the same species.

Although we have shown that NO mediates the VEGF-induced increase in BREC Lp, the mechanism is not completely understood. It has been well established in many cell types that NO stimulates soluble GC, which elevates the level of cGMP. Several investigators have examined the role of the GC/cGMP/PKG pathway downstream of VEGF.21,22,32 Wu et al.21 studied the albumin permeability response to VEGF in the presence of the selective GC inhibitor 1H-[1,2,4]oxadiazole[4,3-c]quinoxalin-1-one (ODQ) and the specific PKG inhibitor KT5823 in isolated porcine coronary venules. Both inhibitors reduced basal permeability and prevented the hyperpermeability response to VEGF. Therefore, they suggested that VEGF modulates microvascular permeability through a signaling cascade involving NO synthesis, GC stimulation, and PKG activation. Morbidelli et al.32 observed that exposure of cultured microvascular endothelium isolated from coronary postcapillary venules to VEGF induces a significant increment in cGMP levels and that this effect is abolished by NOS inhibitors. They concluded that VEGF stimulates postcapillary endothelial cells through the production of NO and then cGMP. It was shown that the NOS inhibitor nitro-l-arginine methyl ester (l-NAME) completely blocks angiogenesis induced by VEGF,121 overexpression by MCF-7 breast carcinoma cells.22 Postcapillary endothelial cell migration and growth induced by VEGF were blocked by both the NOS inhibitor l-NMMA and by the GC inhibitor LY-83583, which suggests that VEGF acts through the NOS-GC pathway.

In the present study, to our surprise, when BREC were incubated with the GC inhibitor LY-83583 and VEGF was added to the monolayers after 1 hour, there was no significant inhibition of the VEGF response (Fig. 4). Lp was also measured in the presence of a cGMP analogue 8-br-cGMP, which did not alter the baseline Lp (Fig. 5). In the third set of experiments, use of the specific PKG inhibitor KT5823 did not attenuate the VEGF response (Fig. 6). These results indicate that the VEGF-induced increase in Lp in BREC is mediated by NO but not through the GC/cGMP/PKG pathway.

The mechanism downstream of NO in BREC is thus unclear at the present time. One possibility is that NO inhibits cAMP production. This is plausible, because we have seen that the cAMP analogue db cAMP reverses the increase in Lp induced by VEGF, and others have shown that NO can directly inhibit adenylate cyclase, which catalyzes the formation of cAMP33 or GAPDH, a key glycolytic enzyme, which can decrease the level of ATP.34 We have also shown in other studies...
using BRECs that VEGF reduces the expression and increases the phosphorylation of the tight junction protein occludin.\textsuperscript{35,36} 
This observation and the results of the present study suggest that VEGF induces an increase in water permeability ultimately by modulating tight junction proteins, with NO serving as a key intermediate in the upstream signaling cascade. Further elucidation of these mechanisms may help to identify therapeutic targets for the treatment of retinal brain edema.

References

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