VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells

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VEGF is an endothelium-specific peptide that potently stimulates angiogenesis, vasodilation, and microvascular hyperpermeability. Hypoxia upregulates VEGF production and release in a wide variety of cells and organs (16). This relationship provides an elegant feedback loop by which decreased nutrient supply elicits the angiogenic signal and directs it to the target vascular endothelium (15). Recent reports indicate that NO may play an intimate role in VEGF signaling (9, 13, 18).

Therefore, we hypothesized that ecNOS expression and activity may be regulated by VEGF. This study examines that hypothesis using human endothelial cells as an in vitro model.

METHODS

Cell line and culture conditions. Unless otherwise noted, all supplies were purchased from Sigma (St. Louis, MO). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA). These were cultured at 37°C on gelatin-coated plates in the basal nutrient media MCDB-131, supplemented with 5% fetal bovine serum (FBS; Hydnone, Logan, UT), 20 U/ml heparin, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. Cells were passaged by trypsinization in Dulbecco’s phosphate-buffered saline (DPBS) containing 0.25% trypsin and 0.02% EDTA. The cells used in this study were between passages 2 and 4.

Measurement of NOS activity by quantification of NO release. NO production was evaluated by measurement of nitrite (NO−) and nitrate (NO−) [nitrogen oxides (NOx)], the stable degradation products of NO, using a modification of previously described techniques (11). Briefly, HUVECs were grown to confluence in six-well plates and then incubated in MCDB-131 with or without VEGF for the indicated time periods. After the 12-, 24-, and 48-h incubation periods, the media was replaced with fresh MCDB-131 without VEGF for 1 h and then sampled. Protein was precipitated from the sampled media, and 100 µl of supernate was injected into a reflux chamber containing vanadium(III) in 3 N HCl heated to >85°C. The released NO was purged with a stream of nitrogen gas directed by vacuum into the reaction chamber of a chemiluminescence analyzer (model 270B, Sievers, Boulder, CO). The analyzer was calibrated on the day of the experiment with NO3− standards, and the results were normalized to the cell number in the plate.

Northern blot analysis. Confluent 100-mm dishes of HUVECs were incubated in MCDB-131 plus 1% FBS with or without VEGF for 20 h. Total cellular RNA was isolated with
an acid guanidinium thiocyanate-phenol-chloroform protocol according to previously described methods (4). The isolated RNA (10 µg) was size-fractionated on a 1.1% agarose-3% formaldehyde gel, transferred to a nylon membrane, and covalently linked with ultraviolet (UV) irradiation using a UV cross-linker system (Stratagene Cloning Systems, La Jolla, CA). Hybridizations were performed at 42°C for 18 h with a [32P]-ATP-end-labeled probe. The probe for this assay was generated using the Oligo analysis software program (National Biosciences, Plymouth, MN) to locate an appropriate 40-mer sequence matching the ecNOS cDNA sequence from GeneBank. After the sequence was chosen, it was compared with the other known human sequences using the Blast section of GeneBank to ensure a unique sequence. The following sequence, complementary to base pairs 2,986–3,025 of a published mRNA strand (8), was then commercially synthesized (Genosys, Houston, TX) for use as a ecNOS probe in northern blotting: 5′-AAGCGAGCTCAAGCCCCGAGACCTGTGCCCTGCTTCATC-3′. After hybridization, membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) at 55°C for 30 min and then with 0.2× SSC, 0.1% SDS at 55°C for 30 min. Autoradiography was performed overnight with an intensifying screen at −70°C. Laser densitometry and digital analysis of scanned images were used for quantification of autoradiograms. Variation in RNA loading was internally controlled by stripping and rehybridizing the membranes with a commercially available glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Calbiochem, San Diego, CA).

Western blot analysis. After 24-h treatment with VEGF (Peprotech, Rocky Hill, NJ), confluent 100-mm dishes of treated (or untreated control) HUVECs were rinsed with DPBS and scraped to remove the cells. Cells were spun into a pellet at 100 g. The pellet was resuspended in lysis buffer containing 1% sodium deoxycholate, 0.1% SDS, 10 mm tris(hydroxymethyl)aminoethane (Tris, pH 8.0), 0.14 M NaCl, 1 µg/ml aprotinin, 0.5 mg/ml Pefabloc, 1 µg/ml pepstatin, and 1 µg/ml leupeptin and subjected to two to three freeze-thaw cycles. Protein content was quantified using the bicinchoninic acid protocol (Pierce, Rockford, IL) with bovine serum albumin as a standard. Total protein (2 µg/lane) was subjected to SDS-polyacrylamide gel electrophoresis. The membrane was blocked for 12 h at 4°C in Tris-buffered saline (25 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20 and 5% skim milk and incubated with 1 µg/ml mouse monoclonal anti-ecNOS antibody (Transduction Laboratories, Lexington, KY) for 12 h at 4°C. After washing, the membrane was incubated with 0.1 µg/ml donkey anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase; peroxidase activity was visualized using an enhanced chemiluminescence substrate system (Amer sham, Arlington Heights, IL).

Statistical analysis. Data are expressed as means ± SE. Comparisons of data between different groups were made by analysis of variance followed by a Fisher's test of least significant difference. For comparison of two variables where paired data were available, paired t-tests were used. Probability values <0.05 were considered significant.

RESULTS

Effect of VEGF on endothelial cell release of NOx. Incubation of HUVECs with VEGF resulted in a biphasic increase in the release of the stable degradation products of NO, NOx. NOx release increased to 230% of control after 1 h of incubation and returned to near baseline by 12 h. By 24 h, this release was again >200% of control, a level maintained through 48 h of incubation (Fig. 1A). At both the 1- and 24-h time points, NOx release was increased in a dose-dependent manner by VEGF (Fig. 1B).

![Fig. 1. A: effect of vascular endothelial growth factor (VEGF) on rate of release of nitrogen oxides (NO2 and NO3; NOx) by human umbilical vein endothelial cells (HUVECs) over time. HUVECs were incubated in MCDB-131 with or without 10 ng/ml VEGF for indicated time-periods. After 12-, 16-, 24-, and 48-h incubation periods, medium was replaced with fresh MCDB-131 without VEGF for 1 h and then sampled. Release of NOx was measured using chemiluminescence after protein precipitation and reduction of the supernate with vanadate(III) chloride and hydrochloric acid. B: dose response of VEGF-induced release of NOx from HUVECs. HUVECs were incubated with indicated doses of VEGF for 1 or 24 h and assayed for NOx release as described in A. Data represent means ± SE from 3 experiments. *P < 0.05 vs. control; †P < 0.05 vs. 1-, 24-, and 48-h incubation.](http://ajpheart.physiology.org/)
Induction of ecNOS mRNA in response to VEGF.
Exposure of HUVECs to VEGF markedly increased ecNOS expression. The ecNOS message of HUVECs exposed to 100 ng/ml VEGF increased slightly by 6 h and by 9 h had reached a peak response equivalent to 310 ± 43% of control (Fig. 2A). In contrast, VEGF had minimal effects on steady-state levels of mRNA for GAPDH, a constitutively expressed housekeeping gene. Therefore, in later experiments the 9-h incubation period was used to obtain maximal effect on ecNOS mRNA expression. The message for ecNOS increased in a dose-dependent fashion to VEGF concentrations between 0.1 and 100 ng/ml. At 9 h, steady-state mRNA for ecNOS increased to 121 ± 34, 156 ± 27, 247 ± 32, and 303 ± 33% at 0.1, 1, 10, and 100 ng/ml, respectively (Fig. 2B).

Induction of ecNOS protein in response to VEGF.
Western blotting revealed a dose-dependent increase in ecNOS content in endothelial cells after a 24-h incubation with VEGF. Peak response occurred at 100 ng/ml and was 271 ± 32% of control (Fig. 3).

VEGF pretreatment augments basal and stimulated release of NOx. Exposure of HUVECs to 10 ng/ml VEGF for 24 h increased both baseline and A-23187-induced production of NOx by approximately twofold (Fig. 4).

DISCUSSION

These experiments demonstrate that VEGF causes a dose-dependent, biphasic release of NO from human endothelial cells, with a transient phase at 1 h and a second increase that occurs by 24 h. Furthermore, the results demonstrate that expression of mRNA and protein for ecNOS is significantly increased in endothelial cells exposed to VEGF. It should be noted that all HUVEC cultures were studied under confluent conditions. This allowed contact inhibition of proliferation, eliminating a variable that is known to alter ecNOS expression (1). Because several investigators have observed that VEGF induces an immediate spiking in cytosolic calcium levels, the acute response is assumed to be caused by calcium activation of NOS enzyme (3). In contrast, the chronic increase appears to be the result of dose-dependent increases in NOS enzyme concentration. The calcium dependence of this upregulated response is supported by the increased NO production elicited by A-23187 after 24-h treatment with VEGF.

Since ecNOS was first cloned five years ago, studies have shown that ecNOS is a calcium/calmodulin-dependent "constitutive" enzyme present in all mammalian endothelial cells and responsible for the formation of endothelium-derived relaxing factor. Despite its initial designation as a constitutive enzyme, recent studies have shown that ecNOS is regulated to some degree by several factors (6). Although the degree of upregulation seen in this study and others is modest (2- to 3-fold), it is important to recognize that the dose-response curve for NO effects can be quite steep. For example, after a threshold level is reached, a two- to threefold increase in NO levels can increase vascular relaxation by nearly 100% (12). Therefore, small changes...
in basal enzyme levels of NOS can have a profound effect on vascular function.

In conjunction with alterations in vascular function, alterations in NOS activity can have dramatic effects on angiogenic processes. Angiogenesis is known to occur coincident with vasodilation and hyperemia of preexisting microvessels (14). Clearly, these are functions that can be tightly regulated by NO. Furthermore, recent studies indicate that NO stimulates endothelial cell proliferation, migration, protease release, and permeability increases (22, 23, 21). Especially relevant to this study are reports indicating that the NO pathway is a core component of VEGF signaling mechanisms. NO blockers prevent both VEGF-induced proliferation and VEGF-mediated activation of mitogen-activating protein kinase in venular endothelial cells (Ref. 13 and M. Ziche, unpublished observation). Other investigators have observed that VEGF-induced hyperpermeability in venules is an NO-dependent event (18).

Our findings are also consistent with recent studies indicating biphasic changes in hydraulic conductivity ($L_p$) elicited by VEGF. One study found VEGF-mediated alterations in $L_p$ with a time course that closely parallels the changes in NO release found in this study. The investigators found that VEGF increased $L_p$ initially within 2 min, followed by a return to baseline and a secondary increase of approximately fivefold by 24 h (2). Other studies have observed that NO mediates VEGF-induced increases in permeability. These studies, in conjunction with our own findings, suggest that NOS upregulation might explain the biphasic changes in permeability associated with VEGF and provide a mechanism by which permeability is chronically increased in VEGF-mediated angiogenesis (18).

The present findings may have important implications as VEGF is garnering ever-increasing attention by investigators interested in using it, or its inhibitors, to modulate angiogenesis for therapeutic reasons. Already, studies attempting to use bolus injections of VEGF to stimulate angiogenesis have encountered potentially lethal levels of hypotension as a side effect (20). This is ameliorated by the use of lower doses of VEGF intravenously, corroborating our findings that VEGF-induced NO release is dose dependent (19). Similarly, it is possible that anti-VEGF treatment could reduce ecNOS expression, compromising the capacity of the endothelium to produce NO in response to physiological stimuli. Better understanding of the regulation of ecNOS by VEGF may be critical to safely take advantage of the full therapeutic potential of VEGF.

In conclusion, VEGF stimulates a biphasic, dose- and time-dependent release of NO. The initial phase of this release is likely regulated by calcium spiking, whereas the secondary phase appears to be caused by increased intracellular enzyme levels. This is corroborated by the finding that VEGF upregulates both ecNOS mRNA and protein levels in a dose-dependent manner.

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