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Vascular Endothelial Growth Factor Increases Endothelial Nitric Oxide Synthase Transcription In Huvec Cells

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Abstract

Although it is known that VEGF increases eNOS protein, the mechanisms responsible remain unclear. To determine if VEGF alters eNOS transcription, human umbilical vein endothelial cells were transfected with reporters under the control of the eNOS promoter and stimulated with VEGF₁₆₅. VEGF significantly increased eNOS-mRNA after 2 hours exposure. VEGF significantly increased eNOS reporter activity as early as one hour (268±32%), but this increase returned to baseline after 6 hours. Using deletion constructs, the VEGF response region was initially localized to within the -722/-494 region. GMSA indicated that VEGF increased DNA binding to both a cAMP-like and AP1-like response elements. Site-specific mutations and heterologous constructs indicated that the site centered at AP1-like site was both necessary and sufficient to mediate VEGF transcriptional activation. These results indicate that VEGF rapidly activates eNOS transcription prior to a rise eNOS-mRNA, an effect mediated by a cis-trans interaction localized to an AP1-like site within the eNOS promoter.

Keywords

eNOS; VEGF; Endothelial cells; HUVEC; Transcription

Introduction

Nitric oxide (NO) signaling regulates vascular tone, inhibits of components of the atherogenic process, and influences myocardial energy consumption [1-5]. NO synthesis is governed by nitric oxide synthase (NOS). Three isoforms of NOS have been identified which are the products of three separate genes; endothelial NOS (eNOS), inducible NOS

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(iNOS), and neuronal NOS (nNOS). These isoforms share about 50-60% sequence identity and all use L-arginine, O₂, and NADPH to catalyze the synthesis of NADP, citrulline, and NO as well as superoxide. Dimerization is a requirement for catalytic activity of eNOS, although the truly active form is a complex that includes calmodulin, FAD, tetrahydrobiopterin (BH₄), and iron protoporphyrin IX (haem) [6]. The products catalyzed by eNOS are subject to complex regulation that we are just now beginning to understand. Of these isoforms, eNOS predominates in the endothelium.

Vascular endothelial growth factor (VEGF) was first discovered as a protein secreted from tumors but also by smooth muscle and endothelial cells [7]. VEGF is a potent angiogenic factor and has a vasculoprotective role on endothelial function by promoting re-endothelialization and by restoring endothelium-dependent vasomotor responses and thromboresistance. VEGF acts through two tyrosine receptor kinases, Flt-1 and Flk-1/KDR, to increase vascular permeability and stimulate endothelial growth. In part, the actions of VEGF are dependent upon the production of nitric oxide (NO) [8, 9]. Endothelial nitric oxide synthase (eNOS) is the rate-limiting step in the production of NO, and it has been reported that VEGF produces a rapid increase in eNOS activity in part be a shift in its phosphorylation status [10, 11]. More recently, it has been shown that VEGF will also increase eNOS protein level, an effect that peaks after two hours before returning to basal levels [12-14]. However it remains unclear if the pathways that mediate changes in eNOS protein level are operational at the transcriptional or translational levels.

We therefore have undertaken a series of experiments to test the hypothesis that VEGF will increase eNOS-mRNA levels as a function of increased eNOS transcription. We have found that VEGF will transiently increase eNOS transcription via a cis-trans interaction which contributes to the initial increases in NO production in response to change in cardiovascular demand.

Methods

Cell Preparation and culture

HUVEC cells were originally purchased from the ATCC. Cells were grown in T75 flasks using Endothelial Growth Media (Cell Applications Inc. San Diego CA), and passages 3-8 were used in the experiments. For transfection experiments, cells were plated onto 12 or 24 well plates and on the following morning the cells were washed twice with PBS and the cells transfected using NovaFECTORä (VennNova, Pompano Beach, FL). Following transfection the cells were washed and the media replaced with growth media. The cells were permitted to recover for 24 hours before the media was changed to basal media. On the following day, VEGF (20ng/ml) or vehicle was added to individual wells and the cells were harvested after the indicated times.

Reporter Plasmids

The eNOSLuc constructs were derived from the human eNOS 5' flanking region that was the kind gift of W. Sessa and described by Zhang et al., [15]. The -1033/+22 (with respect to the start of transcription) region was subcloned into the pGL3B firefly-luciferase plasmid

(Promega, Madison WI). Site specific mutations were cloned using the sequences as listed in Table 1 and used the eNOS-pGL3B plasmid by a QuickChange protocol (Stratagene, La Jolla, CA) protocol. The heterologous constructs were cloned using the sequence as listed in Table 1 into the pGL3-P reporter under the control of the SV40 promoter. Table 1 lists the sense strand sequences used to make these constructs. . To control for transfection efficiency, cells were cotransfected with a *renilla* luciferase reporter under the control of the SV40 early promoter (Promega, Madison, WI). To validate its use, we determined VEGF responsiveness of two permissive promoter reporter constructs pGL3P and RSV.

Luciferase Assay

At the end of the treatment, the cells were harvested in the Promega passive lysis buffer and assayed immediately or stored at -80°C until analyzed. Luciferase activity was determined using the Promega Dual Luciferase protocol (Promega, Madison, WI). This assay permits the sequential determination of firefly and sea pansy (*renilla*) luciferase based reporters. Chemiluminescence was determined using an OptoComp I luminometer (MGM Instruments, Hamden CT). The relative light units (RLU) values measured were normalized to their respective controls and presented as mean $\pm$ SEM of 6-12 wells.

Gel Mobility Shift Analysis (GMSA)

Nuclear extracts were prepared as a modification of Deryckere and Gannon [16, 17]. Protein concentration was determined using the BioRad Protein Assay and aliquots frozen at -80°C until use. GMSA analysis was carried out using double strand endlabeled DNA oligonucleotides of the eNOS wild type sequence as listed in Table 1. Endlabeling of the double strand oligonucleotides was performed using ^{32}P -gATP and T_4 polynucleotide kinase. The GMSA binding buffer used was modified from that described by Mar and Ordahl [18], and included 50mM Tris-Cl pH 7.9, 50 mM KCl, 2 mM MgCl_2 , 5 mM DTT, 5% glycerol, 0.5 mM EDTA, 400 ng/ml polydIdC. Following incubation of the nuclear extracts with the DNA fragments, the mixture was electrophoresed through a 6% 0.5xTBE polyacrylamide gel, dried, and autoradiographed. Band quantification analysis was performed from a phosphoimage using a Storm 840 Phosphoimager (Molecular Dynamics, Palo Alto, CA).

RNA Analysis

Total RNA was isolated using a TRIzol (Gibco-BRL) protocol [19]. 5ug total RNA was reverse transcribed using the oligo-dT and ImPromII reverse transcriptase (Promega, Madison WI). QPCR was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA). Primers used included eNOS (forward; 5'-ggacggagctggctgc-3', reverse; 5'-GCGTATGCGGCTTGTCAC-3') and HPRT (forward; 5'-CCGTGTGTTAGAAAAGTAAGA-3', reverse; 5'-AACTGCTGACAAAGATTCACT-3'). Crossing point analysis was used to quantify PCR product yield and the data presented is expressed as function of control values.

Statistical analysis

Statistical analyses were performed using NCSS Software (NCSS, Kaysville UT). Where appropriate, student t-test or ANOVA was utilized; post-hoc analysis was done using a Fisher's LSD analysis. Values presented are mean $\pm$ SEM, and statistical significance was set at $p<.05$.

Results

In cultured endothelial cells, exogenous VEGF increases both eNOS protein levels and NO production [10, 13]. We observed that eNOS-mRNA levels were transiently increased 2 hours after the addition of VEGF and had returned to control levels after 6 hours (Figure 1).

Transfection efficiency varies and to control for this, a reporter under the control of a constitutively active promoter was co-transfected. It is critical that this reporter does not react to the experimental conditions. Combinations of luciferase plasmids under the control of different promoters were transfected in conjunction with the SV40-renilla plasmid. Of these, only the RSV-luciferase control was significantly influenced by exogenous VEGF (Figure 2). Neither the pGL3P plasmid which has high levels of activity, nor the pGL3B plasmid which has low levels of activity, were influenced by VEGF. Also the raw activity values for the SV40-driven renilla reporter were not altered by VEGF (data not shown). Collectively, these results indicated that the SV40-driven renilla reporter activity was not influenced by VEGF and validates its use to correct for variations in transfection efficiency.

VEGF significantly increased eNOS reporter ($-1033/+22$ construct) activity that achieved its apogee after one hour before declining to control levels by 6 hours (Figure 3). Separate experiments determined that activity of this construct was not different from control (vehicle) after 24 or 48 hours of VEGF exposure (data not shown). To localize the response to VEGF, different deletion constructs of the eNOS promoter were used. Both the $-1022/+22$ and the $-779/+22$ were significantly increased in response to VEGF, while the $-494/+22$ construct was not (Figure 4). These results localized the responsible elements to the $-779/-494$ region.

Zhang et.al observed that within the $-779/-494$ region there are near consensus sequences for both the cAMP-CREB protein (CRE) and the activator 1 protein (AP1) response elements [15]. Using radio-labeled oligonucleotides in a GMSA analysis, we observed that both regions had increased binding in response to exogenous VEGF (Figure 5). Separate experiments using cold competitors indicated that DNA binding was specific (data not shown). These findings suggest that either or both regions might participate in VEGF induction of eNOS transcription.

To test the relative role for each region, site specific mutations were created in the $-1033/+22$ eNOS-luciferase reporter. We observed that site specific mutation of either region blocked VEGF activation of the reporter activity (Figure 6). Both the cAMP and AP1 regions were separately subcloned into the pGL3P construct to create heterologous reporters regulated by either the CRE-like or AP1 sequences. These constructs were transfected into the HUVEC cells and challenged with VEGF for one hour. Only the construct containing the

AP1-like sequence was responsive to VEGF (Figure 7). These results indicated that while both regions were essential only the AP1 sequence (–667/–654) was both necessary and sufficient to mediate the effects of VEGF.

Discussion

eNOS was originally thought to be constitutively expressed in endothelial cells and unresponsive to the shifting demands on the cardiovascular system [20]. However it is now apparent that many factors may dynamically regulate eNOS. VEGF produces a rapid increase in eNOS activity and these effects are initiated through the KDR receptors [10, 14]. eNOS activity is regulated both at the translational and post-translational levels in part by VEGF-induced increases in eNOS-mRNA [12]. The major findings of this study are that VEGF transiently increased eNOS transcription, an effect that is mediated by an AP1 site within the 5' flanking eNOS promoter region.

We and others have observed that VEGF induces an increase in eNOS-mRNA [12]. Using a semi-quantitative approach, Bouloumie et. al. observed that VEGF transiently elevated eNOS-mRNA levels for up to 12 hours in primary endothelial cells before returning to control levels after 24 hours [12]. In separate studies, using actinomycin D to block transcription, both VEGF-induced and shear stress induced mRNA stabilization which contributed to maintenance of elevated eNOS-mRNA levels [12, 21]. However, mRNA stabilization does not account for the rapid increases in mRNA levels, whereas an increase in transcription does. In the present study, VEGF induced a transient increase in eNOS transcription that returned to controls levels after 6 hours. In contrast, shear stress significantly increased eNOS transcription only after 12 hours, suggesting that a distinctly different signaling pathway mediated this increase [21]. Despite an only transient increase in transcription, eNOS-mRNA levels were elevated in response to increases in VEGF or shear stress.

VEGF-induced activation of eNOS transcription has been shown to be initiated by intracellular signaling pathways beginning with VEGF binding to the Flk1 receptors and ending with a cis-trans interaction localized to the 5' flanking region of the eNOS promoter. VEGF activation influences many transcription factors, including Erg-1, Ets-1, forkhead, NFAT1, Nfkb, and Stat 3 transcription factors and these effects are mediated by a specific cis-trans interaction [22-25]. For example, promoter analysis revealed that the *eNOS* promoter contains a conserved optimal forkhead-responsive element (FHRE: TTGTTTAC) at position –2,753 relative to the start of transcription [26]. In response to shear stress, a Nfkb binding site centered at –987 was shown to mediate activation of eNOS transcription [21]. Using a combination of site specific mutations and construction of heterologous promoters, we identified that an AP1 site was responsible for the transient increase in eNOS transcription. GMSA analysis verified that VEGF significantly increased AP1 DNA binding but also a nearby cAMP response element. Rapid and transient activation of AP1 expression has been known for sometime and appears to be a mechanism allowing the cells to rapidly adapt to stress. In addition to an increase in AP1 protein, a rapid increase in AP1 binding is linked to decreased phosphorylation of c-jun one of the partners in AP1 activation [27]. The shift has been shown to be dependent upon activation of a protein kinase C, another

signaling pathway that VEGF is known to activate [14]. In combination, this permits a rapid increase in eNOS transcription.

Using a combination of site-specific mutations and heterologous constructs, we determined that the AP1 site centered at –659 was both necessary and sufficient to mediate VEGF activation. Although site specific mutation of either the AP1 or the cAMP elements blunted the effects of VEGF, when these consensus sequences were cloned into a heterologous construct, only the AP1 response element was activated by VEGF. It is unclear what role the cAMP element may have with respect to eNOS transcriptional regulation. It is clear that cAMP is elevated by VEGF and cAMP has significant post-translational effects on eNOS activity and stability serving to increase NO production [11, 28]. Kim et.al reported that VEGF increased CREB phosphorylation with the apogee of the response occurring some 3 hours after the addition of VEGF past the time when eNOS transcription had peaked [29]. With respect to the present study, this suggests that any possible cAMP response may also occur after the peak of the VEGF induction of eNOS transcription.

Conclusion(s)

Control of eNOS function is a complex operation that is mediated on several levels including eNOS transcription, mRNA stability, and via several post-translational modifications. We have found that VEGF-induced activation of eNOS transcription is dominated by a cis-trans interaction localized to an AP1 site within the eNOS promoter. These results indicate that VEGF rapidly increases eNOS transcription via the transiently activated AP1 signaling pathway. This finding is consistent with the apparent transient impact VEGF has on the vasculature.

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Figure 1.

RNA analysis of VEGF stimulated HUVEC cells. VEGF (20 ng/ml) was added and the cells harvested at the times indicated. mRNA was analyzed as described in Methods. Values are mean $\pm$ SEM and normalized to control. * $p < .05$ compared to control

Figure 2.

Firefly luciferase reporter plasmids (pGL3B, pGL3P, and RSV) were cotransfected with a renilla luciferase reporter (pRLSV40) plasmid. Values shown are the mean $\pm$ SEM of the ratio of firefly:renilla activity and normalized to control. The pRLSV40 data are mean $\pm$ SEM of raw renilla values and normalized to control. * $p < .05$ compared to control.

Figure 3.

VEGF transiently increases eNOS transcription. HUVEC cells were transfected with eNOS reporters as described in Methods. VEGF (20 ng/ml) was added and the cells harvested at the times indicated. Values are mean $\pm$ SEM and normalized to control. * $p < .05$ compared to control.

Figure 4.

VEGF activates longer but not shorter eNOS promoter constructs. HUVEC cells were transfected with eNOS deletion constructs as described in Methods. VEGF (20 ng/ml) was added and the cells harvested after one hour. Values are mean $\pm$ SEM and normalized to control for each construct pair. Two-way ANOVA using construct and VEGF as main effects found that VEGF significantly increased eNOS reporter activity. * $p < .05$ compared to respective control.

Figure 5.

VEGF alters DNA binding of HUVEC nuclear extracts. Where indicated VEGF (20 ng/ml) was added and the cells were harvested after 30 minutes. Nuclear extracts were prepared from HUVEC cells as described in Methods. 2 μ g of extract was incubated with the indicated probe for 10 minutes before being loaded onto a nondenaturing gel. ne: no extract.

Figure 6. Mutation of the cAMP and Ap1-like regions negated VEGF activation of eNOS transcription. HUVEC cells were transfected with eNOS deletion constructs as described in Methods. VEGF (20 ng/ml) was added and the cells harvested after one hour. Values are mean $\pm$ SEM and normalized to control for each construct pair. * $p < .05$ compared to respective control.

Figure 7.
The -659 AP1 region was sufficient for VEGF induced activation of eNOS transcription.

Table 1

Primers used for eNOS Promoter Analysis

Site Specific Mutation Primers	
AP1 site:	5'-TCCCCCACTTGGTACCTGGGGGTG-3'
cAMP site:	5'-CGGGAAGCGTGGTACCCTGAATGAC-3'
GMSA & Heterologous Primers	
AP1 site:	5'-TCCCCCACTTGAGTCATGGGGGTG-3'
cAMP site:	5'-CGGGAAGCGTGCCTCACTGAATGAC-3'

Sense strand only shown. Antisense primers are complimentary.