Review

Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family

Ian Zachary*, Georgia Gliki

Centre for Cardiovascular Biology and Medicine, Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, UK

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Abstract

The central role of vascular endothelial growth factor (VEGF) in angiogenesis in health and disease makes it attractive both as a therapeutic target for anti-angiogenic drugs and as a pro-angiogenic cytokine for the treatment of ischaemic heart disease. While VEGF binds to two receptor protein tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (KDR), most biological functions of VEGF are mediated via VEGFR2, and the role of VEGFR1 is currently unknown. Neuropilin-1, a non-tyrosine kinase transmembrane molecule, may function as a co-receptor for VEGFR2. Considerable progress has recently been made towards delineating the signal transduction pathways distal to activation of VEGFR2. Activation of the mitogen-activated protein kinase, protein kinase C and Akt pathways are all strongly implicated in mediating diverse cellular biological functions of VEGF, including cell survival, proliferation, the generation of nitric oxide and prostacyclin and angiogenesis. Upregulation of metalloproteinases, activation of focal adhesion kinase and interactions between VEGF receptors and integrins are strongly implicated in VEGF-induced endothelial cell migration. Recent findings suggest important roles for the vasodilators nitric oxide and prostacyclin, in linking post-receptor signaling networks to downstream biological effects and in mediating some in vivo endothelial functions of VEGF. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Angiogenesis; Nitric oxide; KDR; Apoptosis; Endothelial receptors; Prostaglandins; Signal transduction

1. Introduction

Vascular endothelial growth factor (VEGF) is essential for endothelial cell differentiation (vasculogenesis) and for the sprouting of new capillaries from pre-existing vessels (angiogenesis) during development [1,2]. VEGF is also thought to play a key role in postnatal angiogenesis during pregnancy, wound healing and in human pathophysiological conditions including cancer, rheumatoid arthritis, ocular neovascular disorders, and cardiovascular disease [3–5].

VEGF was initially recognized as a factor which increased vascular permeability, and it is now apparent that this cytokine regulates multiple biological functions in endothelial cells, among which are enhanced production of vasoactive mediators, increased expression of components of the thrombolytic and coagulation pathways, suppression of neointimal vascular smooth muscle cell (VSMC) hyperplasia, inhibition of thrombosis, hypotension and vasorelaxation [5,6]. VEGF is also a pleiotropic factor with effects on diverse cell types and tissues [7–11].

The complexity of VEGF biology is paralleled by the emerging complexity of interactions between VEGF ligands and their receptors, and the downstream signaling pathways they mediate. This review considers the signal transduction mechanisms underlying the biological actions of VEGF. An overview of VEGF ligands and receptors will be followed by a thematic discussion of biologically relevant VEGF signaling.

Abbreviations: bFGF, basic fibroblast growth factor (FGF-2); eNOS, endothelial constitutive nitric oxide synthase; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HUVEC, human umbilical vein endothelial cell; PGI2, prostacyclin; PKC, protein kinase C; VEGF, vascular endothelial growth factor A; VSMC, vascular smooth muscle cell; vWF, von Willebrand factor

*Corresponding author. Tel.: +44-20-7679-6620; fax: +44-20-7679-6212.
E-mail address: i.zachary@ucl.ac.uk (I. Zachary).

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2. VEGF ligands

VEGF (VEGF-A) is a member of a family of closely-related growth factors, which now includes VEGFs B, C, D, E and placenta growth factor (PIGF) [1,12,13]. All of these polypeptides are endogenously expressed in mammals except VEGF-E which is encoded by the double stranded DNA virus, orf. Alternative splicing of human VEGF mRNA from a single gene containing eight exons gives rise to at least five different isoforms of 121, 145, 165, 189 and 206 amino acid residues [14,15]. Exon 6 is absent in VEGF_{121} and VEGF_{165} and exon 7 is absent from VEGF_{121} and VEGF_{145}. VEGF_{121}, VEGF_{145} and VEGF_{165} are secreted and form dimeric proteins whereas VEGF_{189} and VEGF_{206} are thought to remain sequestered in the pericellular matrix upon their secretion. VEGF_{189} was reported to undergo proteolytic cleavage by urokinase to generate a smaller biologically active isoform which could bind to VEGFR2 and stimulate proliferation [16]. All isoforms, excepting VEGF_{121}, bind heparin to differing degrees via a region rich in charged basic amino acid residues encoded by exon 6 and an exon 7-encoded domain [14]. Human VEGF_{165} is glycosylated at Asn75 [14] and is typically expressed as a 46-kDa homodimer of 23-kDa monomers and is the most abundant and, in vitro studies, most biologically active form [13,14]. VEGF_{121} and VEGF_{145} are also biologically active in endothelial cells.

The central role of VEGF in embryonic blood vessel development has been highlighted by the finding that targeted inactivation of only a single allele of the VEGF gene in mice causes a lethal impairment of angiogenesis [17]. Transgenic homozygous mice expressing only VEGF_{120} and lacking the longer heparin-binding VEGF_{164} and VEGF_{189} isoforms coded by exons 6 and 7 [18] die shortly after birth due to bleeding and ischaemic cardiomyopathy.

VEGF expression is regulated by hypoxia, which induces binding of the transcription factor complex, hypoxia inducible factor 1, to the hypoxia response element in the VEGF gene promoter region [19–22]. VEGF mRNA expression is also regulated in VSMC, keratinocytes and other cultured cell types by diverse stimuli including basic fibroblast growth factor (bFGF), TGF-β and interleukin-1β [23–28].

VEGF-B and PIGF are both expressed as two isoforms produced from alternative splicing [29,30]. VEGF-C and -D are produced as large precursor forms which subsequently undergo processing to variety of partially processed and mature forms with different receptor-binding specificities [31]. The role of these VEGFs is not well understood at present. A major function of VEGF-C may be as a growth factor for lymphatic vessels [32,33]. Transgenic mice overexpressing VEGF-C in keratinocytes of the skin epidermis develop enlarged lymphatic vessels in their skin but mice overexpressing VEGF_{164} in the same location showed only blood vessel hyperplasia [32]. VEGF-B knockout mice are healthy and fertile but have smaller hearts and exhibited vascular dysfunction after coronary occlusion and impaired recovery following experimental cardiac ischaemia [34,35]. VEGF-B has been reported to induce expression and activity of urokinase type plasminogen activator [36]. A role for PIGF in placental angiogenesis has been proposed [37], but PIGF homodimers appear to have little effect on angiogenesis in vivo. However, VEGF/PIGF heterodimers do bind to VEGFR2, are mitogenic for endothelial cells and stimulate angiogenesis in vivo [38].

3. VEGF receptors: structure, ligand affinity, expression and function

3.1. VEGFR1, VEGFR2 and VEGFR3

Two receptor tyrosine kinases have been identified for VEGF, VEGF receptor(R)1 (known as Flt-1) and VEGFR2 (KDR/Flk-1) which share ~44% amino acid homology [12,13]. VEGF-C and -D bind to a third receptor, VEGFR3 (Flt-4), which does not recognize VEGF-A. VEGF-C and -D also bind VEGFR2 with lower affinity than they bind VEGFR3 [39,40]. PI GF and VEGF-B bind with high-affinity only to VEGFR1 [36,41], while VEGF-E binds specifically to VEGFR2 [42]. The specificities of members of the VEGF family for their receptors are illustrated in Fig. 1.

The three VEGF receptor tyrosine kinases are structurally related to the PDGF family of receptors (class III) and have a similar domain structure characterized by cytoplasmic regions with an insert sequence within the catalytic domain, a single hydrophobic transmembrane domain and seven immunoglobulin-like domains in the extracellular regions (Fig. 1). The reported affinities of VEGF for VEGFR1 and VEGFR2 are, respectively, 16–114 pM and 0.4–1 nM [12,13,43–46]. VEGFR3 is synthesized as a glycosylated 195-kDa molecule which undergoes cleavage in its extracellular domain to generate 120- and 75-kDa chains which are linked by disulphide bridges [12].

Elucidation of the crystal structure and mutational analysis of VEGF_{9–108} has identified residues critical for binding to VEGFR1 and R2 [47,48]. Analysis of deletion and point mutants and chimeric forms of VEGFR1 and R2 indicate that Ig-like extracellular domains 2 and 3 are the most important for ligand binding [48–50]. Monomeric receptors have 100-fold less affinity for VEGF, which preferentially binds predimerized receptors [49]. Heterodimeric receptors with one functional and one non-functional unit are unable to transduce signals and antagonize VEGF activity.

Targeted disruption of VEGFR1 and R2 in mice prevents normal vascularization and embryonic development, but the two knockouts have distinctive phenotypes [51,52].
Fig. 1. VEGF ligands and receptors. VEGF tyrosine kinase receptors possess an extracellular domain (ECD) containing seven immunoglobulin-like loops (red ovals), a single hydrophobic membrane-spanning domain (TM, white), and a large cytoplasmic domain comprising a single catalytic domain (pink) which is interrupted by a non-catalytic region, called the kinase insert (KI, yellow). The extracellular domain of VEGFR1 is also independently expressed as a soluble protein. VEGFR3 undergoes proteolytic processing to yield disulphide-linked 120- and 75-kDa polypeptides. VEGFRs 1 and 2 are present in vascular endothelium, VEGFR1 is also uniquely expressed on monocytes, while VEGFR3 is preferentially expressed on lymphatic endothelium. NP-1 is a transmembrane receptor with a short cytoplasmic domain, and five extracellular domains comprising a C domain which shares homology with MAM domains found in diverse proteins thought to mediate homophilic protein–protein interactions, b1 and b2 domains related to the C1 and C2 domains of coagulation factors VIII and V, and a1 and a2 domains related to the non-catalytic regions of complement components C1r and C1s.

Whereas VEGFR2-deficient mice produce neither differentiated endothelial cells nor organized blood vessels, the VEGFR1 knock-out mice possess mature, differentiated endothelial cells, but have large, disorganized vessels. VEGFR3 expression starts during E8 in developing blood vessels but subsequently is largely confined to the lymphatic vasculature, consistent with a major specific role of this receptor in lymphangiogenesis. Disruption of the VEGFR3 gene in mice did not prevent vasculogenesis or angiogenesis but caused defects in normal vascular development leading to fluid accumulation from leaky vessels by E9.5 [53].

3.2. Neuropilin-1

Neuropilin-1 (NP-1) was recently identified as a receptor for VEGF165 (Kd 0.3 nM) and also recognizes VEGF-B and the PIGF-2 splice form but not VEGF121 [54–56] (Fig. 1). NP-1 is a non-tyrosine kinase transmembrane protein originally identified as a receptor for members of the semaphorin/collapsin family of secreted polypeptides implicated in axonal guidance and neuronal patterning [57]. Overexpression of NP-1 in mice causes diverse abnormalities in the embryonic cardiovascular system, including excess capillaries and blood vessels, dilated blood vessels, and malformation of the heart [58]. NP-1 knock-out mice displayed impaired neural vascularization, defects in development of the aorta and other large blood vessels, and aberrant yolk sac vascularization [59]. In the human fetal heart, NP-1 is expressed in the endocardium, coronary vessels, myocardial capillaries and epicardial blood vessels and is co-expressed with VEGFR1 and VEGFR2 in the endocardium and myocardial capillaries, but only with VEGFR1 in coronary vessels [60].

Co-expression of NP-1 with VEGFR2 increased binding of VEGF165 4-fold and enhanced chemotaxis and mitogenicity of VEGF165 [54]. Since NP-1 has a short cytoplasmic tail with no known signaling function, it is likely that NP-1 by itself is not a functional receptor but acts as a ‘docking’ co-receptor. A major NP-1 binding site for VEGF165 has been mapped to the domain encoded by exon 7 which is contained in VEGF165 and is absent from VEGF121 [54].

3.3. Heparan sulphate proteoglycans

Heparan sulphate proteoglycans may act as an additional low-affinity binding site for VEGF able to modulate binding of VEGF to its receptors. Low concentrations of heparin have been reported to augment the binding of VEGF to human umbilical vein endothelial cell (HUVECs), while higher concentrations reduced binding [61]. The glycosylphosphatidylinositol anchored HSPG, glypican-1, has also been reported to restore VEGF binding to heparinase-treated cells [62].

4. VEGF receptor signaling

4.1. VEGFR1

It remains enigmatic whether VEGFR1 is able to transmit a biologically meaningful signal in endothelial cells. Studies of VEGFR1 signaling have yielded contrasting results. Whereas tyrosine 1169 in the VEGFR1 kinase
domain has been reported to provide a binding site for PLC-γ [63] and VEGF caused activation of phospholipase C-γ but weak activation of extracellular signal-regulated kinase (ERK) in VEGFR1-expressing NIH3T3 cells [64]. VEGFR1-expressing porcine aortic endothelial (PAE) cells showed ERK activation but no effect on PLC-γ activity in response to PlGF [65]. VEGFR1 was found to support Ca^{2+} fluxes in Xenopus laevis oocytes [44] and VEGFR1 has been reported to mediate Ca^{2+} mobilization in trophoblast cells [66], but PlGF and VEGFR1 did not mediate Ca^{2+} mobilization in HUVECs [67]. VEGFR1 was also reported to interact with the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) in a yeast two-hybrid system [68], but so far this has not been associated with a biological activity in VEGFR1-expressing cells.

The VEGFR1-specific ligand, PlGF does not stimulate either migration or proliferation in HUVECs, and VEGF has no reported biological effects in PAE/VEGFR1 cells. A recent study suggests that PlGF, but not VEGF, can stimulate ERK activation and mitogenesis, but not migration in PAE/VEGFR1 cells [65]. The best-characterized biological responses mediated by VEGFR1 are the stimulation of migration and increased tissue factor expression in monocyes [8,43], though their biological role in vivo is unclear. VEGFR1 is also implicated in VEGF stimulation of metalloproteinase expression in SMC [69]. Remarkably, mice expressing only the extracellular domain of VEGFR1 and lacking its cytoplasmic kinase domain are fertile and develop normally [70]. Suppression of VEGF-induced migration of macrophages in vitro was the only observed phenotype in these mice [70].

There is increasing evidence that VEGFR1 functions as a negative regulator of VEGFR2. A soluble form of the extracellular VEGFR1 domain occurs naturally and over-expression of this form, but not of an artificial soluble VEGFR2, inhibits VEGF-induced migration and proliferation of human microvascular endothelial cells and HUVECs by forming an inactive complex with VEGF and the full-length VEGFR2 [71,72]. A study of chimeras made between the extracellular domain of the receptor for colony-stimulating factor 1 and the kinase domains of either VEGFR1 or R2., found that activation of the R1 kinase domain chimera with CSF-1 suppressed ERK activation and proliferation mediated via the R2 kinase chimera [73]. Analysis of domain swapping between VEGFR1 and VEGFR2 indicates that a short motif (ANGG) unique to the intracellular juxtamembrane VEGFR1 domain can suppress VEGFR2-mediated signaling and cell migration [74].

4.2. VEGFR2

VEGFR2 is activated through ligand-stimulated receptor dimerization and transphosphorylation (autophosphorylation) of tyrosine residues in the cytoplasmic kinase do-
cellular machinery. VEGF induces expression of the antiapoptotic proteins Bcl-2 and A1 [89], which inhibit activation of upstream caspases, and upregulates two members of the IAP (inhibitors of apoptosis) family, survivin and X-chromosome-linked IAP (XIAP) which inhibit terminal effector small pro-domain caspases [90].

The non-receptor protein tyrosine kinase, focal adhesion kinase (FAK), plays a key role in integrin-dependent signaling [91] and is strongly implicated in the maintenance of survival signals in several adherent cell types including endothelial cells [92,93]. Consistent with a role for FAK in VEGF survival signaling, VEGF increases tyrosine phosphorylation and focal adhesion association of FAK and the FAK-associated protein paxillin in HUVECs [94–96] (Fig. 2), and stimulates tyrosine phosphorylation of the FAK-related tyrosine kinase, Pyk2, in a bone marrow endothelial cell line [97].

Integrin $\alpha_\beta_3$ is an important survival system for nascent blood vessels during angiogenesis [98]. Cross-talk between this system and VEGF receptors is indicated by the findings that VEGFR2 has been reported to associate selectively with $\alpha_\beta_3$ and VEGF mitogenicity and receptor activity were enhanced by endothelial adhesion to the $\alpha_\beta_3$ ligand, vitronectin [99]. Although a VEGFR2–$\alpha_\beta_3$ network is an attractive mechanism for integrating cell survival and angiogenic functions of VEGF, a caveat to this is that $\beta_3$-null mice exhibited no defects in retinal neovascularization, a physiological angiogenic context in which $\alpha_\beta_3$ has been strongly implicated [100]. This latter finding suggests that at least in the case of some angiogenic functions of VEGF, interactions between VEGFR2 and $\alpha_\beta_3$ may play a redundant role. This is perhaps unsurprising given that other integrins such as $\alpha_\beta_1$ and $\alpha_\beta_4$ have also been implicated in mediating VEGF-dependent angiogenesis [101].

Other signaling pathways may also play a role in endothelial cell survival functions of VEGF. For example, the protein kinase C (PKC) activator phorbol myristate acetate, was shown to promote survival of HUVECs and endothelial cell tube formation in 3-D collagen gels [102].

5.2. Proliferation

VEGF stimulates VEGFR2-mediated DNA synthesis and proliferation in a variety of endothelial cell types (Table 1). VEGF strongly induces the activity of extracellular signal-regulated kinases (ERKs) 1 and 2 and activation of this pathway presumably plays a central role in the stimulation of endothelial cell proliferation [79,94] (Table 1, Fig. 3). VEGF also induced the MAP kinase pathway leading to activation of c-Jun N-terminal protein kinase (JNK). VEGF-induced mitogenesis, cyclin D1 synthesis and cyclin-dependent kinase 4 activation were inhibited by both a dominant negative JNK-1 mutant and PD98059, a selective inhibitor of ERK activation [103]. Interestingly, expression of the dominant negative Y185F ERK2 mutant also blocked JNK activation by VEGF indicating that the mitogenic response to VEGF might involve cross-talk between the ERK and JNK pathways [103].

VEGF also stimulates PLC-$\gamma$ tyrosine phosphorylation
Table 1
**VEGF-induced cell signalling and related biological effects in endothelial cells**

<table>
<thead>
<tr>
<th>Reference(s)</th>
<th>Cell type</th>
<th>Signaling response</th>
<th>Related biological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>[88]</td>
<td>HUVECs</td>
<td>PI3K activation, ERK activation</td>
<td>Transcription from c-fos promoter, entry into S-phase</td>
</tr>
<tr>
<td>[83]</td>
<td>HUVECs</td>
<td>KDR-mediated PI3K and Akt activation</td>
<td>Survival, inhibition of apoptosis</td>
</tr>
<tr>
<td>[89]</td>
<td>HUVECs</td>
<td>Upregulation of Bcl-2 and A1</td>
<td>Gene expression, cell survival</td>
</tr>
<tr>
<td>[90]</td>
<td>HUVECs</td>
<td>Upregulation of XIAP and survivin</td>
<td>Cell survival</td>
</tr>
<tr>
<td>[105]</td>
<td>HUVECs</td>
<td>PKCα, PKCζ translocation and specific inhibition using antisense</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>[94]</td>
<td>HUVECs</td>
<td>ERK 1/2 activation, tyrosine phosphorylation of PLC-γ, FAK, paxillin</td>
<td>Migration, focal adhesion formation</td>
</tr>
<tr>
<td>[96]</td>
<td>HUVECs</td>
<td>Phosphorylation of PLC-γ, FAK, AKT, ERK and PKCζ translocation</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>[99]</td>
<td>HUVECs</td>
<td>β3 Integrin and KDR-mediated PI3 kinase stimulation</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>[130,111]</td>
<td>HUVECs</td>
<td>Upregulation of eNOS</td>
<td>NO production, gene expression</td>
</tr>
<tr>
<td>[113]</td>
<td>Post-capillary endothelial cells</td>
<td>No production, ERK activation</td>
<td>NO-mediated ERK activation</td>
</tr>
<tr>
<td>[114]</td>
<td>HUVECs</td>
<td>Raf-1 and PKG activation</td>
<td>Cell proliferation, permeability</td>
</tr>
<tr>
<td>[43]</td>
<td>HUVECs</td>
<td>Fh1 activation</td>
<td>Tissue factor production</td>
</tr>
<tr>
<td>[121]</td>
<td>HUVECs</td>
<td>Ca²⁺ and PI3K-mediated cGMP increase and eNOS upregulation</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>[95]</td>
<td>HUVECs</td>
<td>Recruitment of vinculin to focal adhesions, ERK</td>
<td>Cell migration, actin filament reorganization</td>
</tr>
<tr>
<td>[108]</td>
<td>HUVECs, EC</td>
<td>NO-mediated depression of PKCβ activity</td>
<td>Cell migration, proliferation</td>
</tr>
<tr>
<td>[153]</td>
<td>HUVECs</td>
<td>Phosphorylation of VE-cadherin, β-catenin, plakoglobin, p120, PECAM-1</td>
<td>Cell migration, permeability</td>
</tr>
<tr>
<td>[78]</td>
<td>HUVECs</td>
<td>Increased expression of α,β₁ and α,β₂ integrins</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>[101]</td>
<td>Human dermal microvascular EC</td>
<td>Activation of Raf-1, Mek, ERK, PKC β translocation</td>
<td>DNA synthesis, PKC-dependent ERK activation</td>
</tr>
<tr>
<td>[107]</td>
<td>Sinusoidal EC</td>
<td>Activation of Raf-1, Mek, ERK, PKC α translocation</td>
<td>Ras-independent, PKC-dependent ERK activation</td>
</tr>
<tr>
<td>[111]</td>
<td>HUVECs</td>
<td>ERK activation</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>[103]</td>
<td>BAEC</td>
<td>ERK-mediated JNK activation; cyclin D1 synthesis, Cdk4 activation</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>[104]</td>
<td>BAEC</td>
<td>PKCζ, PKCβ translocation, PLC-γ phosphorylation, PI3K</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>[79]</td>
<td>HUVECs</td>
<td>eNOS ser1177 phosphorylation</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>[122]</td>
<td>HUVECs</td>
<td>Akt-dependent eNOS phosphorylation</td>
<td>Migration</td>
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<tr>
<td>[123,133]</td>
<td>HUVECs</td>
<td>Occludin and ZO-1 tyrosine phosphorylation</td>
<td>Ca²⁺-independent NO production</td>
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<tr>
<td>[155]</td>
<td>Bovine retinal EC</td>
<td>ERK, cPLA₂ activation</td>
<td>Permeability</td>
</tr>
<tr>
<td>[78]</td>
<td>BAEC</td>
<td>Association of src with KDR, PLC-γ activation, intracellular Ca²⁺ mobilization</td>
<td>NO production, PGI₂ production</td>
</tr>
<tr>
<td>[128]</td>
<td>Bovine microvascular EC</td>
<td>NO and PGI₂ production</td>
<td>Permeability</td>
</tr>
<tr>
<td>[161]</td>
<td>Bovine retinal EC</td>
<td>ERK and PKC-mediated upregulation of Ang2</td>
<td>Gene expression</td>
</tr>
<tr>
<td>[148]</td>
<td>Bovine retinal EC</td>
<td>KDR, c appearing 1 and eNOS translocation to the nucleus</td>
<td>Intracellular trafficking</td>
</tr>
<tr>
<td>[156]</td>
<td>Bovine pulmonary artery EC</td>
<td>Loss of β-catenin staining from adherens junctions, PLC-γ, paxillin and β-catenin phosphorylation</td>
<td>Cell proliferation, permeability</td>
</tr>
<tr>
<td>[81]</td>
<td>Bovine adrenal cortex EC</td>
<td>PKC-mediated eNOS upregulation</td>
<td>Gene expression</td>
</tr>
<tr>
<td>[146]</td>
<td>Porcine brain microvascular EC</td>
<td>cGMP production</td>
<td>Permeability</td>
</tr>
<tr>
<td>[151]</td>
<td>Porcine isolated coronary venules</td>
<td>PLC-γ phosphorylation, PKC-mediated eNOS phosphorylation</td>
<td>Permeability</td>
</tr>
<tr>
<td>[77]</td>
<td>PVE/VEGFR</td>
<td>Shc-Gh2 complex formation, Gh2 and Nck association with KDR, SHP1 and SHP2 association with KDR</td>
<td>DNA synthesis, VEGFR2 specific signaling and proliferation</td>
</tr>
<tr>
<td>[76]</td>
<td>BAEC</td>
<td>PLC-γ, Ras GTPase-activation, Nck tyrosine phosphorylation</td>
<td>Migration</td>
</tr>
<tr>
<td>[123]</td>
<td>HUVECs</td>
<td>PLD activation</td>
<td>VEGFR2 specific signaling</td>
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<tr>
<td>[67]</td>
<td>HUVECs</td>
<td>Ca²⁺ mobilization</td>
<td>VEGFR2 specific signaling</td>
</tr>
<tr>
<td>[80]</td>
<td>HUVECs, PVE/VEGFR</td>
<td>NO, cGMP production</td>
<td>VEGFR2 specific signaling</td>
</tr>
</tbody>
</table>

* BAEC, bovine aortic endothelial cells; PI3K, phosphatidylinositol 3'-kinase; PKG, protein kinase G. Other abbreviations are defined in the text.

and activation leading to generation of diacylglycerol and inositol 1,4,5-trisphosphate and subsequent activation of PKC and Ca²⁺ mobilization. Studies using PKC inhibitors and isoform-specific anti-sense approaches indicate that activation of PKC and particularly PKCα and ζ isoforms, plays a crucial role in VEGF mitogenic signaling [104–107] (Fig. 3). Intriguingly, VEGF-induced proliferation was reported to be dependent on a NO-mediated reduction in PKCβ activity [108]. Consistent with this finding, overexpression of PKCβ retards cell-cycle progression of microvascular endothelial cells [109]. These findings suggest that the mitogenic effects of VEGF may be mediated...
phorylation and receptor association of the adapter protein, GRB-2, subsequent stimulation of the guanine nucleotide exchange protein SOS and activation of ras which in turn activates Raf-1 and the distal ERK cascade [110]. Though the role of this pathway in VEGF-stimulated activation of Ras or ERKs has not been demonstrated, it was reported that VEGF induces ras-independent and PKC-mediated induction of the Raf–MEK–ERK pathway [107,111,112], findings which suggest that VEGFR2 may be unique among receptor tyrosine kinases in activating the ERK cascade via a Ras-independent pathway mediated via PKC. Two reports have suggested another novel pathway for VEGF-induced ERK activation involving NO-mediated Raf-1 activation, but the mechanism involved is unclear [113,114].

5.3. Migration

Degradation of the basement membrane is essential for endothelial cell migration and is a key early step in the initiation of pathophysiological angiogenesis [2]. VEGF induces the expression of matrix-degrading metalloproteinases [115] and these enzymes are likely to play an essential permissive role in VEGF-induced migration in vivo.

A large body of evidence indicates that FAK-associated signaling is critical for regulating focal adhesion turnover, actin filament organization, and cell migration [116]. VEGF induces tyrosine phosphorylation of FAK and paxillin and promotes recruitment of FAK to new focal adhesions in HUVECs [94] (Fig. 4). It was reported that the heat shock protein, Hsp90, facilitated FAK phosphorylation and inhibition of Hsp90 with geldanamycin arrested via a coordinated increase in activity of some PKC isoforms and decrease in PKCδ activity.

The major pathway through which protein tyrosine kinase receptors activate ERKs involves tyrosine phos-

![Figure 3](image1.png)

Fig. 3. VEGF mitogenic signaling. VEGF is a strong activator of ERKs 1/2 and this pathway is a major mediator of VEGF-induced cell proliferation. Another pathway which mediates VEGF-induced mitogenesis involves PKC isoforms and possibly PKC-dependent ERK activation. PLC-γ-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate leads to generation of diacylglycerol (DAG) and activation of PKC. VEGFR2 associates with several SH2 domain adapter proteins including Shc and Grb2 which may also lead to ERK activation via an as yet undefined pathway.

![Figure 4](image2.png)

Fig. 4. VEGF chemotactic signaling. VEGF has been shown to activate FAK via an unknown mechanism. FAK activity may also be induced via engagement of integrin α,β3. VEGF-induced activation of the p38 kinase has also been implicated in reorganization of the actin cytoskeleton. Recent findings indicate that Akt-dependent phosphorylation of eNOS at Ser 1177 is required for VEGF-induced migration.
VEGF-induced migration [95]. Via an independent pathway, VEGF activated p38 MAP kinase in HUVECs and the p38 kinase inhibitor SB203580 inhibited actin reorganization and cell migration whereas the MEK inhibitor PD98059 had no effect on these biological effects [95,117].

There is growing evidence that NO production may play a role in VEGF-induced endothelial cell migration (Fig. 4). VEGF induces NO production and NO is implicated in non-chemotactic scalar movement (podokinesis) of endothelial cells and as a permissive factor in VEGF-induced endothelial cell migration [118,119] and angiogenesis [120,121]. NO has been reported to regulate focal adhesion integrity and FAK tyrosine phosphorylation in endothelial cells [119], suggesting cross-talk between FAK and NO pathways in migration signaling (Fig. 3). Direct evidence for a role of NO has come from a recent study in which Akt-dependent phosphorylation of eNOS at serine 1177 was shown to be required for VEGF-induced cell migration [122].

PLC-γ activation is implicated in mediating cellular responses linked to VEGF-induced cell migration [65,116] and VEGF-induced phospholipase D activation via a pathway sensitive to the PKC inhibitor Ro 31-8220, has been more tentatively implicated in the cell migratory response to VEGF [123].

5.4. Signaling mechanisms mediating VEGF-induced NO and PG12 production

It is well-established that VEGF stimulates endothelial production of NO and prostacyclin (PG12) [79,124–128], and these intercellular mediators have been implicated in mediating diverse biological effects of VEGF including angiogenesis, increased vasopermeability and inhibition of neointimal VSMC hyperplasia [5]. In common with other activators of eNOS, short-term NO production induced by VEGF probably involves activation of the constitutive eNOS isoform in part through VEGF-induced Ca2+ mobilization (Fig. 5). Another mechanism for VEGF-dependent NOS activation may be through activation of the heat shock protein Hsp 90 or an Hsp 90-associated protein [129]. Activation of c-Src was reported to mediate VEGF-stimulated NO and PG12 production through PLC-γ activation leading to inositol 1,4,5-trisphosphate formation and Ca2+ mobilization [84]. In this study it was unclear, however, how c-Src couples with and activates PLC-γ.

VEGF-induced PG12 production results from PKC-mediated ERK1/2 activation and ERK-mediated phosphorylation and activation of cPLA2 [79] (Fig. 5). The Ca2+-chelating agent BAPTA-AM also prevents production of PG12 [112]. These findings suggest that stimulation of PG12 biosynthesis by VEGF occurs via ERK-dependent cPLA2 activation, while release of PG12 is mediated via a Ca2+-triggered pathway (Fig. 5). VEGF-induced PG12 production and ERK activation in HUVECs were unaffected by inhibitors of eNOS, suggesting that VEGF signaling leading to NO and PG12 generation bifurcates upstream of ERK [112].

VEGF upregulates eNOS mRNA and protein expression [81,130,131] providing a mechanism for prolonged VEGF-induced NO production. VEGF-stimulated eNOS expres-

![Fig. 5. VEGF signaling mediating NO and PG12 synthesis. Short-term NO production is mediated via increased cytosolic Ca2+, resulting from activation of PLC-γ and generation of inositol 1,4,5-trisphosphate (IP3). c-Src has been implicated in signaling upstream of PLC-γ. Akt activation leads to phosphorylation and activation of eNOS (eNOS-P), providing a mechanism for sustained Ca2+-independent NO synthesis. PKC activation is strongly implicated in VEGF-induced ERK activation. ERK activation mediates cPLA2 activation, mobilization of arachidonic acid (AA) and PG12 synthesis. Increased cytosolic Ca2+ stimulates the cellular release of PG12. PKC is also reported to mediate VEGF-induced upregulation of eNOS, another mechanism leading to long-term NO generation.](image-url)
sion occurs via activation of VEGFR2 and was inhibited by selective PKC inhibitors suggestive of a role for PKC in VEGF-dependent gene expression [81]. VEGF-induced Akt activation mediates phosphorylation of eNOS at serine 1179 to cause Ca++-independent NO generation [132,133]. Combined with VEGF-induced upregulation of eNOS mRNA, this could be an important signaling mechanism underlying the stimulation of long-term NO production by VEGF, and may contribute to other biological functions of Akt signaling (Figs. 4 and 5).

NO and PGI2 are best known as vasodilators, but they have several vascular protective effects, including inhibition of VSMC proliferation, anti-platelet actions, and in the case of NO, inhibition of leukocyte adhesion [5]. VEGF may have similar vascular protective effects through enhanced NO and PGI2 production [5]. VEGF protein or gene transfer accelerates reendothelialization and reduces intimal thickening and thrombus formation following balloon endothelial denudation in the rat carotid artery [5,134,135]. Evidence that VEGF has NO-dependent vascular protective effects independent of angiogenesis or endothelial cell proliferation, comes from the finding that periadventitial VEGF gene transfer in the rabbit carotid artery suppressed VSMC neo-intimal hyperplasia induced by a perivascular silastic collar without endothelial injury [125]. The endothelial nitric oxide synthase (eNOS) inhibitor L-NAME prevented VEGF-mediated inhibition of neo-intima formation in this model [125]. The paradoxical ability of VEGF to inhibit VSMC proliferation while stimulating endothelial cell mitogenesis may reflect differential effects of NO and PGI2 on growth of these two cell types, reflecting either differential expression of PGI2 receptors or a divergence in growth regulatory mechanisms in the two cell types.

An anti-thrombotic effect of VEGF may result both from inhibition of platelet aggregation mediated by NO and PGI2 (Fig. 2), and from VEGF-induced expression and activation of the serine proteases, urokinase and tissue type plasminogen activator [136], which cleave plasminogen to generate the key thrombolytic enzyme plasmin. Paradoxically, VEGF also has potentially pro-thrombogenic effects, including the induction of endothelial von Willebrand factor (vWF) secretion [79,137], essential for platelet adhesion to subendothelial collagen, and upregulation of tissue factor [54], an essential step in the extrinsic pathway of coagulation and clot formation. However, VEGF only appears to increase surface expression of active tissue factor on endothelial cells in cooperation with tumor necrosis factor-α [138]. Furthermore, other findings point towards roles for von Willebrand factor and tissue factor in angiogenic functions of VEGF. Mice embryos deficient in tissue factor have an impaired pattern of extraembryonic angiogenesis [139], and von Willebrand factor increases endothelial cell adhesion, suggestive of a role in the maintenance of endothelial integrity [140].

The stimulation of angiogenesis and the enhancement of vascular protective effects via NO and PGI2 production are both consonant with the view that VEGF may be useful for promoting therapeutic angiogenesis in the ischaemic heart [4], and for reducing neo-intima formation in situations such as coronary and peripheral bypass graft stenosis [5]. This favourable assessment of the beneficial effects of VEGF for cardiovascular disease should, however, be tempered in the light of recent findings. Increasing evidence suggests that VEGF is insufficient for viable collateralization of the ischaemic heart, a process which involves arteriogenesis and VSMC proliferation rather than angiogenesis. Moreover, intra-plaque neovascularization has long been recognized as a feature of atherosclerosis and recent findings suggest that antiangiogenic agents can reduce atherosclerosis in the apo E-deficient mouse. These findings suggest that while VEGF may not be an effective agent for stimulating myocardial collateral artery formation, VEGF-driven neovascularization could contribute to atherogenesis. These issues are discussed in more detail in our recent review [5].

5.5. VEGF signaling in the regulation of vascular permeability

VEGF was originally identified as a factor which increased vascular permeability [7,141]. The signaling mechanisms which underly this effect remain largely obscure, however.

The formation of fenestrae, specialized regions of the plasma membrane that are highly permeable to small solutes, may be an important mechanism by which VEGF modulates vascular permeability. Fenestrated endothelia are restricted to the kidney glomeruli, gastrointestinal tract, endocrine organs and particular areas of the brain, and are absent from most of the brain, skeletal muscle, skin and lung [142,143]. A correlation between tissues maintaining high levels of VEGF expression in the adult and the presence of fenestrated endothelia has been noted [143]. The neovasculature induced by VEGF in glioblastoma is also highly fenestrated, in contrast to the non-fenestrated endothelia of the blood–brain barrier [144,145]. In cultures of bovine adrenal cortex endothelial cells and porcine brain microvessel endothelial cells, VEGF induced the formation of abundant fenestrae [143,146]. The signaling pathways mediating VEGF-induced fenestration are unclear, though a permissive environment involving changes in the composition of the extracellular matrix may be required [143,147]. VEGF-induced formation of fenestrations is associated with caveolae, small invaginations of the plasma membrane implicated in endocytotic and transcytotic transport [143,148]. VEGF also induces the appearance of vesicular-vacuolar organelles [149,150] which interconnect upon stimulation to form channels that connect the blood vessel lumen to the interstitium and facilitate passage of molecules.

A role for NO and PGI2 in mediating VEGF-induced
vascular permeability is supported by the observations that the cyclooxygenase inhibitor indomethacin and the eNOS inhibitor L-NAME both inhibited permeability changes induced by VEGF in vivo as measured by the Miles assay [128]. Several studies also implicate PLC-γ tyrosine phosphorylation, mobilization of intracellular Ca²⁺ and PKC activation as effectors of VEGF-induced venular permeability [151].

Phosphorylation of components of intercellular endothelial adherens and tight junctions may mediate disruption of cell–cell adhesions, leading to increased vascular permeability [152]. VEGF increased phosphorylation of the adherens junction components VE-cadherin [153], and β-catenin [154], though these effects were not associated in HUVECs with readily detectable changes in the morphology or organization of adherens junctions. VEGF also stimulated tyrosine phosphorylation of the tight junction proteins, occludin and zona occluden 1 [155].

Some evidence exists against the involvement of VEGFR2 in the ability of VEGF to trigger vascular permeability. Mutant VEGF that has lost the ability to activate VEGFR2 and induce mitogenesis, retained the ability to induce vascular permeability as evaluated in the Miles assay [156]. In addition, VEGF-D, which binds VEGFR2 but not VEGFR1, has no effect on vascular permeability [156].

5.6. VEGF regulation of gene expression

Regulation of several genes by VEGF has already been mentioned (Table 1). VEGF has been shown to increase expression of several transcription factors. Upregulation of Ets1 has been implicated in VEGF-mediated cell migration [157], while Egr-1 and nuclear factor of activated T-cells (NFAT) have been reported to regulate VEGF-induced tissue factor expression [158,159]. Recent findings also suggest that VEGF receptors may activate Stat 3 and 5 [160], members of the family of signal transducers and activators of transcription (Stats). Whether these transcriptional activators are essential for developmental or angiogenic effects of VEGF is unclear, however.

6. Perspectives and concluding remarks

Despite considerable progress in understanding the intracellular mechanisms mediating the actions of VEGF in the endothelium, important areas of VEGF receptor signal transduction remain to be elucidated. A striking instance of this is the role of VEGFR1. Another major challenge will be to elucidate the endothelial function of neuropilin-1. The mechanisms which mediate VEGF regulation of vasopermeability are still largely undefined. Another future goal will be to delineate the pathways through which signals are relayed from VEGF receptors to the nucleus in order to generate information required for endothelial cell differentiation, and the complex cellular changes involved in branching angiogenesis. These responses presumably involve the generation of long-range signals mediated through a programme of gene expression. VEGF has been shown to induce the expression of several transcription factors (Table 1) [21], but if or how these are linked to developmental or angiogenic effects of VEGF remains to be fully elucidated.

The potential for using VEGF and anti-VEGF therapy for human disease is an exciting one. In order to effectively harness this potential, an improved understanding of the signal transduction mechanisms which mediate the complex biological functions of VEGF will be essential, and may itself generate new approaches for treating human disease.

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