Vascular Endothelial Growth Factor/Vascular Permeability Factor Enhances Vascular Permeability Via Nitric Oxide and Prostacyclin

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Background—Vascular endothelial growth factor (VEGF), an endothelial cell mitogen that promotes angiogenesis, was initially identified as a vascular permeability factor (VPF). Abundant evidence suggests that angiogenesis is preceded and/or accompanied by enhanced microvascular permeability. The mechanism by which VEGF/VPF increases vascular permeability (VP), however, has remained enigmatic. Accordingly, we used an in vivo assay of VP (Miles assay) to study the putative mediators of VEGF/VPF-induced permeability.

Methods and Results—VEGF/VPF and positive controls (platelet-activating factor [PAF], histamine, and bradykinin) all increased vascular permeability. Prior administration of the tyrosine kinase inhibitors genistein or herbimycin A prevented VEGF/VPF-induced permeability. Placenta growth factor, which binds to Flt-1/VEGF-R1 but not Flk-1/KDR/VEGF-R2 receptor tyrosine kinase, failed to increase permeability. Other growth factors such as basic fibroblast growth factor (FGF), acidic FGF, platelet-derived growth factor-BB, transforming growth factor-β, scatter factor, and granulocyte macrophage-colony stimulating factor (8 to 128 ng) failed to increase permeability. VEGF/VPF-induced permeability was significantly attenuated by the nitric oxide (NO) synthase inhibitors $\text{N}^-\text{nitro-L-arginine}$ (10 mg/kg) or $\text{N}^-\text{nitro-L-arginine methyl ester}$ (20 mg/kg) and the cyclooxygenase inhibitor indomethacin (5 mg/kg). The inactive enantiomer $\text{N}^-\text{nitro-D-arginine methyl ester}$ (20 mg/kg) did not inhibit VEGF/VPF-induced permeability. In vitro studies confirmed that VEGF/VPF stimulates synthesis of NO and prostaglandin metabolites in microvascular endothelial cells. Finally, NO donors and the prostacyclin analogue taprostene administered together but not alone reproduced the increase in permeability observed with VEGF/VPF.

Conclusions—These results implicate NO and prostacyclin produced by the interaction of VEGF/VPF with its Flk-1/KDR/VEGF-R2 receptor as mediators of VEGF/VPF-induced vascular permeability. Moreover, this property appears unique to VEGF/VPF among angiogenic cytokines. (*Circulation*. 1998;97:99-107.)

Key Words: endothelium ■ growth substances ■ nitric oxide ■ prostaglandins ■ permeability
Protocol 4: Role of Receptor Tyrosine Kinase in VEGF/VPF-Induced VP

The receptor tyrosine kinases Flk-1/KDR/VEGF-R2 and Flt-1/VEGF-R1 bind VEGF/VPF with high affinity.28–31 Accordingly, we tested the effect of the tyrosine kinase inhibitors genistein and herbimycin A on VEGF/VPF-stimulated VP. Ten minutes after administration of EBD, saline (control), genistein (100 μM/L), or herbimycin A (1 μM/L) was intradermally injected as 0.1 mL volume. Ten minutes later (ie, 20 minutes after EBD), the maximum dose of VEGF/VPF used here to stimulate permeability (128 ng) was injected in 0.1 mL volume into the identical dermal area.

Although VEGF/VPF binds to both Flt-1/VEGF-R1 and Flk-1/KDR/VEGF-R2 receptor tyrosine kinases, PAF has been shown to bind principally to Flk-1/VEGF-R1.28–31 To further determine which receptor tyrosine kinase is responsible for VEGF/VPF-induced VP, we tested PIGF (R&D Systems) (8 to 128 ng) on VP as well.

Protocol 5: Role of NO in VEGF/VPF-Induced Vascular Permeability

Two NO synthase inhibitors, L-NNA (10 mg/kg) or L-NAME (20 mg/kg) were used to investigate the role of NO on VEGF/VPF-induced VP. These were injected through the penile or femoral vein immediately before administration of EBD. Twenty minutes later, (a) saline alone (negative control), (b) VEGF/VPF (8, 16, 32, and 64 ng), or (c) PAF (0.01, 0.1, 1, 10, and 100 μM/L) was injected intradermally and VP assessed.

To exclude a nonspecific action of L-NAME, the effect of the inactive enantiomer D-NNA (20 mg/kg) on VEGF/VPF-induced VP was also tested. D-NNAME was injected through the penile vein before administration of EBD. At 20 minutes after EBD, (a) saline alone or (b) VEGF/VPF (8, 16, 32, 64, and 128 ng) was injected intradermally.

To assess whether NO donors can mimic VEGF/VPF-induced VP, we administered NO donors, SNP (10, 50, 100, and 500 μM/L) or S-nitroso-N-acetyl penicillamine (SNAP) (10, 100, and 500 μM/L in 0.1 mL solution). At 20 minutes after EBD administration, (a) saline alone, (b) SNP or SNAP, or (c) PAF was injected intradermally.

Protocol 6: Role of Prostaglandin(s) in VEGF/VPF-Induced VP

To examine a role for prostaglandins in VEGF/VPF-induced VP, indomethacin (5 mg/kg), a cyclooxygenase inhibitor, was injected intraperitoneally,26 before EBD administration. At 20 minutes after EBD injection, (a) saline, (b) VEGF/VPF (8, 16, 32, and 64 ng), or (c) PAF was injected intradermally.

To test whether prostacyclin, a major metabolite of arachidonic acid, can mimic VEGF/VPF-induced VP, we investigated the direct effect of intradermal injection of prostacyclin or its stable analog taprostene.35 At 20 minutes after EBD, (a) saline, (b) prostacyclin or taprostene (0.01, 0.1, 1, and 10 μM/L), or (c) PAF was injected intradermally.

Finally, SNP and taprostene were mixed together and injected intradermally to assess the combined activity of NO and prostacyclin in enhancing VP.

Protocol 7: Effect of NO-Independent Vasocostriction on VP

To exclude the possibility that inhibition of VEGF/VPF-induced VP by L-NAME was simply the result of L-NAME–induced vasocostriction, we examined the effects of phenylephrine on VEGF/VPF-induced VP. Phenylephrine was administered intravenously to achieve a comparable increase in systemic blood pressure (BP) to that resulting from L-NAME (20 mg/kg). After administration of sodium pentobarbital (60 mg/kg IP), a 22-gauge cannula was inserted into the left common carotid artery for BP monitoring. After similar increases in
BP were recorded in response to either L-NAME or phenylephrine, EBD was administered to evaluate VEGF/VPF-induced VP.

Protocol 8: Effect of Locally Administered L-NAME or Phenylephrine on VEGF/VPF-Induced VP
To analyze the effects of L-NAME and phenylephrine administered locally rather than systemically on VP, VEGF/VPF was dissolved (8 to 128 ng in 0.1 mL) in saline containing either L-NAME (1 mmol/L) or phenylephrine (1 μmol/L). We also examined the impact of either L-NAME (0.001 to 1 mmol/L) or phenylephrine (0.01 to 10 μmol/L) administered alone.

Bovine Microvascular Endothelial Cell Culture
Because modulation of VP involves the microcirculation, we used BMEC (American Type Culture Collection) to study the effect of VEGF/VPF on stimulating release of NO and prostacyclin in vitro. BMEC express mRNA and protein for both constitutive endothelial NO synthase and prostaglandin H synthase-1 and thus may secrete NO and prostaglandins. Cells were grown at 37°C in a humidified atmosphere of 5% CO2 +95% air in phenol red-free MEM containing 20% FBS, 0.6 mmol/L L-arginine, ampicillin, and kanamycin. Cells were plated at confluence at 10^6 cells/mL (1 mL per well). After attachment to plates, medium was replaced by 1 mL of sterile Krebs/Henseleit (K-H) buffer.

Measurement of Nitrite Production in BMEC
After release, NO reacts with O2 to yield stable nitrite metabolite. Nitrite concentrations were measured by the Griess reaction to estimate total amounts of NO released from the BMEC. At different time points after addition of VEGF/VPF, 300 μL of K-H buffer was removed and mixed with 30 μL of Griess reagent (1% sulfanilic acid, 0.1% naphthalene-ethylene diamine in 5% phosphoric acid; Sigma), incubated for 10 minutes at room temperature, and absorbance measured at 548 nm. For a blank, 300 μL of fresh K-H buffer was mixed with 30 μL of Griess reagent. Concentrations were determined by comparison with sodium nitrite standard. Lower limit of detection was 0.1 mmol/L of nitrite.

Measurement of VEGF/VPF-Induced Prostacyclin Production in BMEC
The stable metabolite of prostacyclin, 6-keto PGF1α in K-H buffer conditioned by BMEC treated with or without VEGF/VPF, was measured by enzyme immunoassay kit (Amersharn) at serial time points in response to VEGF/VPF. The lower limit of detection was 6 pg/mL.

Histological Examination After Intradermal Injection of VEGF/VPF
Five-micron-thick sections of skin were cut, stained with hematoxylin-eosin and/or Giemsa, and examined by light microscopy after treatment with BMEC conditioned by saline or with VEGF/VPF. Histological examination was obtained from the skin injected with saline (0.1 mL).

Results

Analysis of VP for Angiogenic Cytokines and Putative Mediators

VEGF/VPF Increases VP
Repeated intradermal injection of the vehicle control saline (0.1 mL) did not increase VP. In contrast, intradermal injection of VEGF/VPF (8, 16, 32, 64, and 128 ng) significantly increased VP in a dose-dependent manner (Fig 1A). VEGF/VPF started to render injected area blue at 149 ± 5 seconds (n=5) after intradermal injection. As positive controls, we used PAF (0.01, 0.1, 1, 10, and 100 μmol/L), bradykinin (1, 10, 100, and 1000 nmol/L), and histamine (1, 10, 100, and 1000 nmol/L), all of which increased VP (Fig 1, B, C, and D).

Effects of PAF Receptor Antagonist and Histamine H1 Receptor Antagonist on VEGF/VPF-Mediated Increase in VP
Because PAF and histamine are well-known endogenous mediators of VP, we tested the effect of PAF receptor antagonist CV-6209 and histamine H1 receptor antagonist...
diphenhydramine on VEGF/VPF-mediated VP. CV-6209 (2 mg/kg) and diphenhydramine (10 mg/kg) inhibited PAF-mediated (0.1, 1, and 10 μmol/L) and histamine-mediated (1, 10, 100, and 1000 nmol/L) VP, respectively. However, both CV-6209 and diphenhydramine failed to inhibit VEGF/VPF-induced VP (Fig 1, E and F). Thus PAF and histamine do not mediate VEGF/VPF-induced VP.

**Other Angiogenic Cytokines Do Not Increase VP**

Because increased VP has been considered to be associated with in vivo angiogenesis,13 we investigated the effects of bFGF (8, 16, 32, 64, and 128 ng), aFGF (8, 16, 32, 64, and 128 ng), GM-CSF (8, 16, 32, 64, and 128 ng), scatter factor (8, 16, 32, 64, and 128 ng), PDGF-BB (8, 16, 32, 64, and 128 ng), and TGF-β (8, 16, 32, 64, and 128 ng) on VP. None enhanced VP (Fig 2, A through F), consistent with previous reports.16

**Role of Receptor Tyrosine Kinase in VEGF/VPF-Induced VP: PlGF Does Not Increase VP**

Although VEGF/VPF stimulates at least two class III receptor tyrosine kinases in the endothelial cells, Flt-1/VEGF-R1 and Flk-1/KDR/VEGF-R2, it is unknown which (or if both) receptor mediates VP in response to VEGF/VPF.

We first assessed the role of functional receptor tyrosine kinase in VEGF/VPF-induced VP by using the tyrosine kinase inhibitors genistein (100 μmol/L) and herbimycin A (1 μmol/L) followed by an intradermal injection of VEGF/VPF (128 ng). Genistein or herbimycin A alone did not change VP (Fig 3). When VEGF/VPF was injected into an untreated skin area, VEGF/VPF increased VP as before. When VEGF/VPF was injected at the area previously treated with genistein or herbimycin A, however, VP was attenuated (Fig 3). These results document that tyrosine phosphorylation is required for VEGF/VPF-mediated VP.

We then tested the effects of PlGF on VP. PlGF binds to only Flk-1/KDR/VEGF-R2 receptor tyrosine kinase.32,37 PlGF (8, 16, 32, 64, and 128 ng) did not increase VP (Fig 3), suggesting that Flk-1/KDR/VEGF-R2 is the relevant receptor tyrosine kinase for VEGF/VPF-induced VP.

**Effects of the NO Synthase Inhibitors on VEGF/VPF-Induced VP**

We next examined the role of NO in VEGF/VPF-mediated VP by using the NO synthase inhibitors L-NNA (10 mg/kg)
or L-NAME (20 mg/kg). Both attenuated VEGF/VPF-induced VP. The inhibitory effect of L-NAME on VEGF/VPF-induced VP is shown in Fig 4B. In contrast, pretreatment with NO synthase inhibitors did not alter VP induced by PAF (Fig 4B), indicating that PAF does not induce VP through NO. The inactive stereoisomer D-NAME (20 mg/kg), which does not inhibit endothelial NO synthesis, failed to inhibit VEGF/VPF-induced VP (Fig 4C). VEGF/VPF-mediated increase in VP was thus dependent on local NO production.

**Effects of the Cyclooxygenase Inhibitor Indomethacin on VEGF/VPF-Induced VP**

Pretreatment with indomethacin (5 mg/kg) inhibited VP stimulated by VEGF/VPF (Fig 4D). In contrast, indomethacin pretreatment had no effect on PAF-induced VP. Histological examination disclosed no evidence of VEGF/VPF-associated inflammatory cell infiltrate and/or mast cell degranulation at the site of intradermal injection with or without indomethacin pretreatment. Thus VP due to VEGF/VPF, in contrast to PAF, was also dependent on local prostaglandin(s).

**Effect of NO-Independent Vasoconstriction on VEGF/VPF-Induced Increase in VP**

In vivo injection of L-NAME (20 mg/kg) increased systemic BP from 96/70 to 132/96 mm[Hg] and inhibited VEGF/VPF-induced VP (Fig 5A). Phenylephrine (0.05 to 0.2 mg/kg) was injected in a cumulative fashion, and 0.2 mg/kg of phenylephrine increased BP from 91/69 (baseline) to 140/92 mm Hg, similar to the increase in BP recorded after administration of L-NAME. In contrast to L-NAME, however, phenylephrine did not inhibit VEGF/VPF-induced VP despite similar increases in systemic BP (Fig 5B). These results support the notion that VEGF/VPF-induced VP is mediated by NO but is unaffected by NO-independent vasoconstriction.

**Effect of Locally Administered L-NAME and Phenylephrine on VEGF/VPF-Induced Increase in VP**

Neither L-NAME (0.001, 0.01, 0.1, and 1 mmol/L) nor phenylephrine (0.01 to 10 µmol/L) alone increased VP (Fig 5C, D). VP was reduced after administration of local VEGF/VPF with L-NAME (1 mmol/L) (Fig 5C). Local administration of VEGF/V PF with phenylephrine (1 µmol/L) resulted in increased VP similar to that seen with VEGF/VPF alone (Fig 5D).
prostacyclin) is required for increased VP. That interaction between NO and prostaglandin (possibly no evidence of mast-cell degranulation.) These results indicate (Histological examination of Giemsa-stained sections disclosed however, VP increased in a dose-dependent manner (Fig 6C).

When SNP and taprostene were mixed and injected intradermally together, administered alone, to increase VP (Fig 6B). When SNP and taprostene were mixed and injected intradermally together, however, VP increased in a dose-dependent manner (Fig 6C). (Histological examination of Giemsa-stained sections disclosed no evidence of mast-cell degranulation.) These results indicate that interaction between NO and prostaglandin (possibly prostacyclin) is required for increased VP.

Measurement of Nitric Oxide and Prostaglandin Metabolites in Cultured Microvascular Endothelial Cells

VEGF/VPF Stimulates NO Release From Microvascular Endothelial Cells

Because increased VP implies functional alteration in the microcirculation, we investigated the effects of VEGF/VPF on NO production in BMEC. Cross-reactivity of rhVEGF/VPF with BMEC was previously confirmed in our laboratory.38

As shown in Fig 7A, VEGF/VPF (50 ng/mL) stimulated NO synthesis, with peak NO production 5 to 15 minutes after the addition of VEGF/VPF.

VEGF/VPF Stimulates Prostaglandin Release From BMEC

To assess the effects of VEGF/VPF on prostacyclin production, we measured 6-keto-PGF1α, a stable metabolite of prostacyclin. VEGF/VPF (50 ng/mL) significantly stimulated 6-keto-PGF1α production in cultured BMEC in a time-dependent manner (Fig 7B).

Effect of Inhibitors on VEGF/VPF-Mediated Nitrite/Nitrate and 6-keto-PGF1α Production

We tested the effects of the NO synthase inhibitor L-NAME (100 μmol/L), the cyclooxygenase inhibitor indomethacin (10 μmol/L), and the tyrosine kinase inhibitor genistein (100 μmol/L) on VEGF/VPF-mediated NO and 6-keto-PGF1α production. L-NAME significantly inhibited both NO and 6-keto-PGF1α production (Fig 8). Indomethacin significantly inhibited 6-keto-PGF1α production but not NO release. Tyrosine kinase inhibitor genistein significantly attenuated both 6-keto-PGF1α and NO production significantly, consistent with the results of the Miles assay.

Role of Flt-1/VEGF-R1 Receptor Tyrosine Kinase in VEGF/VPF-Induced NO Synthesis

Because PGF failed to increase VP in Miles assay, we tested whether PGF stimulates NO synthesis in vitro. Consistent with the results of the Miles assay, PGF (100 ng/mL) could not be shown to increase NO production from cultured BMEC (Fig 9). Because PGF ligates only Flt-1/VEGF-R1, these findings suggest that Flt-1/VEGF-R2 but not Flt-1/VEGF-R1 receptor tyrosine kinase mediates VEGF/VPF-induced NO synthesis.

Discussion

The present study demonstrates that (a) VEGF/VPF-induced VP is attenuated by NO synthase inhibitors, the cyclooxygenase inhibitor indomethacin, and tyrosine kinase inhibitor genistein; (b) PGF, known to bind Flt-1/VEGF-R1 but not Flk-1/KDR/VEGF-R2,32–34 did not increase VP; (c) other angiogenic cytokines including aFGF, bFGF, scatter factor (HGF), PDGF-BB, GM-CSF, and TGF-β all failed to increase VP; (d) VEGF/VPF stimulated NO and prostacyclin production in cultured microvascular endothelial cells, whereas PGF failed to release NO; (e) neither NO donor (SNP or SNAP) nor prostacyclin administered alone increased VP, but combined administration of SNP and taprostene, a stable prostacyclin analogue, did lead to an increase in VP. These findings thus provide evidence that NO and prostacyclin production in microvascular endothelial cells mediate VEGF/VPF-induced VP. Although histamine and PAF are known endogenous mediators of VP, our studies using receptor antagonists indicate that these molecules are not mediators of VEGF/VPF-induced increase in VP. Furthermore, histological examination revealed neither inflammatory cell infiltrates nor degranulated mast cells on Giemsa-stained sections (data not
shown) after intradermal injection of VEGF/VPF, consistent with previous data from Collins et al.39

Our studies using genistein and herbimycin A confirmed essential role of receptor tyrosine kinase(s) in VEGF/VPF-mediated VP. The demonstration that PlGF fails to augment VP confirms recent findings by Park et al32 and implies that the Flt-1/VEGF-R1 tyrosine kinase receptor alone cannot mediate VEGF/VPF-induced VP32–34; this effect instead appears to be mediated wholly or in part by Flk-1/KDR/VEGF-R2. Furthermore, this feature is not shared by other growth factors that nevertheless stimulate angiogenesis in vivo.

VEGF/VPF stimulates transient accumulation of cytoplasmic calcium in cultured endothelial cells.41 VEGF/VPF-induced increase in endothelial cytosolic Ca2+ probably activates calcium-calmodulin–dependent enzymes such as endothelial constitutive NO synthase. In fact, Ku et al41 previously showed that VEGF/VPF stimulates NO production in isolated canine coronary arteries. The present study extends these previous observations by demonstrating that VEGF/VPF also stimulates NO release from cells regulating VP at the microvascular level.

Previous studies have implicated NO as a factor regulating VP.19–21 Nguyen et al21 demonstrated that substance P, a potent endothelium-dependent vasodilator, increases micro-VP through NO-dependent pathways. More recent findings suggested that NO-mediated alteration of VP may depend on its local concentration.20

VEGF/VPF-induced VP appears dependent on not only NO but also prostaglandin production. It has been suggested that prostaglandins are involved in the regulation of both angiogenesis and VP.25,26 Yamamoto et al25 recently reported that a stable prostacyclin analogue, SM-10902, accelerated wound healing in a murine model of diabetic skin ulcers. Fujii et al26 have demonstrated that lipopolysaccharide-induced VP is attenuated by either an NO synthase inhibitor or indomethacin. These results collectively support the notion that prostaglandins produced from arachidonic acid by cyclooxygenase can regulate VP.

Our data indicate that NO synthase inhibitor L-NAME inhibited not only nitrite production but also 6-keto PGF1α production from microvascular endothelial cells. This finding suggests a possible interaction between NO and prostaglandin. In this context, recent studies demonstrated that either endogenous or exogenous NO can stimulate prostacyclin production through cyclooxygenase activation.36,42 Thus increased production of prostacyclin in response to VEGF/VPF observed in our study might derive in part from NO stimulated by VEGF/VPF.

Figure 8. A, VEGF/VPF-induced (50 ng/mL) nitrite production as a marker of NO synthesis and B, VEGF/VPF-induced (50 ng/mL) 6-keto PGF1α production as a marker of prostacyclin synthesis. L-NAME (100 μmol/L), an NO synthase inhibitor, and genistein (100 μmol/L), a tyrosine kinase inhibitor, significantly inhibited VEGF/VPF-induced nitrite production, whereas indomethacin (10 μmol/L) did not alter nitrite release. L-NAME, indomethacin, and genistein significantly inhibited VEGF/VPF-induced 6-keto PGF1α production (n=6 in each bar). *P<.05, **P<.01.

Figure 9. VEGF/VPF (100 ng/mL) significantly increased nitrite production, whereas PlGF (100 ng/mL), compared with controls (nontreated microvascular endothelial cells), did not (n=4 in each group). *P<.05, **P<.01.
but instead involved transendothelial transport through a novel cytoplasmic organelle that they termed the vesicular–vacular organelle. Others have reported VEGF/VPF-induced ultrastructural features consistent with endothelial fenestration. The downstream details by which NO/prostacyclin-mEDIATE VP may involve these routes remains to be fully elucidated.

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