Vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen that promotes angiogenesis, vascular hyperpermeability, and vasodilation by autocrine mechanisms involving nitric oxide (NO) and prostacyclin (PGI₂) production. These experiments used immunoprecipitation and immunoblotting procedures to characterize the signaling pathways by which VEGF induces NO and PGI₂ formation in cultured endothelial cells. The data showed that VEGF stimulates complex formation of the flk-1/kinase-insert domain-containing receptor (KDR) VEGF receptor with c-Src and that Src activation is required for VEGF induction of phospholipase C (PLC) γ₁ activation and inositol 1,4,5-trisphosphate formation. Reporter cell assays showed that VEGF promotes a ~50-fold increase in NO formation, which peaks at 5–20 min. This effect is mediated by a signaling cascade initiated by flk-1/KDR activation of c-Src, leading to phospholipase C (PLC) γ₁ activation, inositol 1,4,5-trisphosphate formation, release of [Ca²⁺], and nitric oxide synthase activation. Immunoassays of VEGF-induced 6-keto prostaglandin F₁₂ formation as an indicator of PGI₂ production revealed a 3–4-fold increase that peaked at 45–60 min. The PGI₂ signaling pathway follows the NO pathway through release of [Ca²⁺], but diverges prior to NOS activation and also requires activation of mitogen-activated protein kinase. These results suggest that NO and PGI₂ function in parallel in mediating the effects of VEGF.

Vascular endothelial growth factor (VEGF) is a potent, endothelial cell-specific mitogen that stimulates angiogenesis, vascular hyperpermeability, and vasodilation (1–3). Three tyrosine-kinase signaling receptors for VEGF have been identified: flt-1, flk-1/KDR, and flt-4. The specific actions of VEGF on vascular endothelial cells are regulated by flt-1 and flk-1/KDR. Flt-1, which has higher affinity for VEGF than flk-1/KDR and is required for endothelial cell morphogenesis; flk-1/KDR is involved primarily in mitogenesis (4, 5).

The physiological effects of VEGF on endothelial cells are well established, but the postreceptor signaling pathways are not yet fully understood. VEGF-receptor binding triggers a signaling cascade that results in tyrosine phosphorylation of phospholipase C (PLC) γ₁, leading to increases in intracellular levels of inositol 1,4,5-trisphosphate (1,4,5-IP₃) and elevation of intracellular calcium (6, 7). The increase in intracellular calcium activates nitric oxide synthase (NOS) to produce nitric oxide (NO). NO formation activates guanylate cyclase within vascular smooth muscle cells and endothelial cells, causing cGMP production. This NO/cGMP cascade is thought to have an important role in the vasoactive effects of VEGF. NO production and elevation of cGMP levels have been found to contribute to the effects of VEGF on vascular tone (8). The effects of VEGF in stimulating angiogenesis and increasing vascular permeability also require NOS activity (9–13). In addition, VEGF-induced activation of the mitogen-activated protein kinase (MAPK) cascade has recently been shown to involve NOS-dependent signaling events (14, 15). The NOS/guanylate cyclase-dependent activation of the MAPK cascade is thought to lead to VEGF-induced proliferation of endothelial cells (16). VEGF has recently been found to induce the nuclear translocation of endothelial NOS together with Flk-1/KDR and caveolin-1 (17), suggesting a possible role for NO in transcription factor activation. Another pathway that appears to be involved in mediating the vasoactive effects of VEGF is the prostacyclin (PGI₂) release pathway. VEGF induces PGI₂ production via activation of phospholipase A₂ as a consequence of initiation of the MAPK cascade (18). Although the relationship between NO-mediated signaling events and PGI₂ production in the VEGF signal transduction cascade are not yet known, results of in vivo analysis have suggested that vascular hyperpermeability induced by VEGF results from the synergistic action of both NO and PGI₂ (12). Therefore, the fact that PGI₂, like NO, has vasodilating effects and has been shown to stimulate vascular hyperpermeability and angiogenesis under some conditions (19, 20) suggests that both molecules are likely to be involved in transducing the vascular effects of VEGF.

Finally, another group of signaling molecules that may be involved in the VEGF signaling cascade is the Src family tyrosine kinases. c-Src and Src family proteins have been shown to interact functionally with the transmembrane tyrosine kinase receptors for several growth factors, including platelet-derived...
growth factor (PDGF) (21), epidermal growth factor (EGF) (22), basic fibroblast growth factor (23), and colony-stimulating factor-1 (24). These interactions are thought to modulate growth factor signaling due to the mutual stimulation of catalytic activity and enhanced phosphorylation of downstream targets of each protein tyrosine kinase. Phosphorylation of specific tyrosine residues in receptors promotes their interactions with a variety of proteins containing SH2 domains (the SH2 domain is a conserved sequence of ~100 amino acids with homology for region 2 of Src family proteins) (25). VEGF has been shown to promote tyrosine phosphorylation of several mediators of signal transduction that contain SH2 domains, including PLC-γ1, phosphoinositide 3-kinase, GTPase activating protein, and the oncogenic adapter protein Nck (7, 26, 27). However, although the VEGF receptors have been shown to interact with SH2 domain-containing proteins, their specific interactions with Src family tyrosine kinases remain unclear. Studies with stably transfected cell lines expressing either flt-1 or flk-1/KDR have shown weak association between the Src family proteins Fyn and Yes with flt-1 but not flk-1/KDR (26). Work done using sinusoidal endothelial cells and flk-1/KDR expressing NIH3T3 fibroblasts failed to show activation of the Src family members c-Src, Fyn, Lyn, or Yes upon VEGF stimulation (29).

The goal of the present study was to determine the role of Src family proteins in the VEGF signal transduction process and to characterize the signaling pathways underlying VEGF-activated production of NO and PGL2. Therefore, experiments in primary cultures of bovine aortic endothelial cells (BAECs) were designed to determine the effects of VEGF on NO and PGL2 production in relation to the activity of Src family proteins, tyrosine kinases, PLC-γ1, PKC, NOS, and the MAPK cascade.

**EXPERIMENTAL PROCEDURES**

**Materials**—GF 109203X (GF1) and U73122 were obtained from BIOMOL (Plymouth Meeting, PA). AG-490, PP2, and PD98059 were from Calbiochem (La Jolla, CA). The cGMP radioimmunoassay kit was from PerSeptive Biosystem (Framingham, MA). The 1,4,5-IP3 radioimmunoassay kit was from NEN Life Science Products. The 6-keto-prostaglandin F1α (PGF1α) enzyme immunoassay system was from Amersham Pharmacia Biotech. Recombinant human VEGFα was from R&D systems (Minneapolis, MN). Human VEGFα has been found to be highly potent as an angiogenic and permeability increasing factor in bovine endothelial cell systems (7, 13, 17). The anti-phosphotyrosine monoclonal antibody (PY20) was from Transduction Laboratories (Lexington, KY). Anti-flk-1 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Src and anti-PLC-γ1 antibody were from Upstate Biotechnology (Lake Placid, NY). Other reagents were from Sigma. Rat fetal lung fibroblasts (RFL-6) cells were obtained from American Type Culture Collection.

**Cell Culture**—Primary BAEC cultures were prepared from bovine aortas as described (30) and used at passages 3–6. Unless otherwise indicated, cells were grown in M199 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, penicillin (100 unit/ml), streptomycin (100 μg/ml), and 1-glutamine (2 mM). Media was changed every 3 days and cells were subcultured every 7 days. BAECs were incubated overnight in serum-free M199. The next day, cultures were treated with inhibitors for 30–60 min. Then, VEGF was added to the wells for 10 min, and then 500 μl of conditioned medium aliquots were removed for 6-keto PGF1α analysis. The amount of 6-keto PGF1α in the medium was measured using an enzyme immunoassay kit according to the manufacturer's recommendations.

**Measurement of cGMP—RFL-6 reporter cell assays were done as described (31). Briefly, confluent BAEC cultures were maintained in serum-free medium overnight and then equilibrated in Locke's buffer (154.0 mM NaCl, 5.6 mM KCl, 2.0 mM CaCl2, 1.0 mM MgCl2, 3.6 mM NaHCO3, 5.6 mM glucose, and 10.0 mM Hepes (pH 7.4)). All treatment solutions were prepared with the same buffer. For experiments, BAECs were pretreated with inhibitors or vehicle alone and then with VEGF with or without inhibitors. Immediately before treatment, 0.3 mM 3-isobutyl-1-methylxanthine and 100 units/ml superoxide dismutase were added to the cultures. After treatment, the conditioned media were transferred to RFL-6 cultures. After 3 min at 37 °C, the conditioned medium was replaced by ice-cold 25 mM sodium acetate buffer (pH 3.3). Cells were frozen overnight at −20 °C. The next day, the samples were collected and stored at −70 °C until analyzed for cGMP using a cGMP 125I radioimmunoassay kit.

**Measurement of 6-keto PGF1α**—6-keto PGF1α production in relation to the activity of Src family proteins, tyrosine kinases, PLC-γ1, PKC, NOS, and the MAPK cascade.

**Results and Discussion**—VEGF-stimulated Activation of PLC-γ1 Requires the Activity of Src Family Tyrosine Kinases—We first investigated the involvement of Src family tyrosine kinases in the VEGF signal transduction cascade. Because tyrosine phosphorylation of PLC-γ1 is a well established early event in the VEGF signal transduction cascade (29, 36), the VEGF-induced PLC-γ1 activation in BAECs.

![Fig. 1. Effects of inhibition of Src family kinases on VEGF-induced PLC-γ1 activation in BAECs.](Image)

**Data Analysis**—The data were analyzed by analysis of variance and Dunnett's post hoc comparison test for comparing several treatment groups with a control (35). The results are expressed as the mean ± S.E.

**Figure 1.** Effects of inhibition of Src family kinases on VEGF-induced PLC-γ1 activation in BAECs. BAECs were treated with VEGF (10 ng/ml) for the indicated times in the presence or absence of PP2 (1 μM) or genistein (50 μM). Cells were immunoprecipitated with PY20 and probed with anti-PLC-γ1 antibody. The same results were obtained when the immunoprecipitation was done using anti-phosphotyrosine, and the blots were probed with anti-PLC-γ1. The results shown are representative of three separate experiments.
soluble guanylate cyclase activators and can be used as re-

smooth muscle cells. RFL-6 cells have been shown to respond to

activation through activation of soluble guanylate cyclase in vascular

NO-induced vasodilation occurs due to increased cGMP forma-

The relationship between c-Src activation and the phys-

ical actions of VEGF is not yet understood, but it appears

very likely that c-Src activation has an important role in coor-

dinating the effects of VEGF on growth with its effects on cell

adhesion and cell motility. Tyrosine phosphorylation of recep-

tors for both PDGF and EGF has also been found to promote

formation of receptor complexes with Src family proteins, and

sequent activation of Src family kinases has been impli-

cated in cell proliferation events. For example, Src family ki-

nases have been found to be required for PDGF-induced pro-

gression through the G1 stage of the cell cycle and entrance

into S phase (38). Analyses of interactions between the EGF

receptor and mutated Src family proteins indicate that c-Src is

also required for EGF-induced mitogenesis and that Src may

mediate regulation of a cell cycle checkpoint (39). Src activation

has also been implicated in regulation of cell motility by both

PDGF and EGF due to complex formation between Src and the

focal adhesion tyrosine kinase FAK, together with the focal

adhesion-associated protein paxillin (25). VEGF is also known
to stimulate tyrosine phosphorylation of FAK and paxillin (40).
Thus, c-Src may serve to link the VEGF receptor and adhesion

receptor signaling pathways in vascular endothelial cells, as

has been suggested for PDGF and EGF in other cell types.

VEGF Stimulation of NO Formation Requires the Activity

of Tyrosine Kinases and Src Family Proteins—The next series of

experiments analyzed the relationship between VEGF-induced
c-Src activation and NO production. It is well established that

NO-induced vasodilation occurs due to increased cGMP forma-
tion through activation of soluble guanylate cyclase in vascular

smooth muscle cells. RFL-6 cells have been shown to respond to

soluble guanylate cyclase activators and can be used as re-

porter cells to detect NO release by other cells under various

experiments analyzed the relationship between VEGF-induced 1,4,5-IP3 production in BAECs. BAECs were pretreated with genistein (20 μg/ml) or PP2 (1 μM) for 60 or 30 min, respectively, and then treated with VEGF (10 ng/ml) for the indicated times. Controls were treated with VEGF alone. Cell lysates were analyzed 1,4,5-IP3 formation. Results shown are mean ± S.E. for three experiments: ■, VEGF alone; ○, + genistein; ▲, + PP2.

vein endothelial cells (41) and a

line of coronary venule endothelial cells (9, 10). We have

also obtained similar results using an NO electrode to directly

measure NO accumulation within the media of VEGF-treated

BAECs. The change in the amount of NO in the BAEC condi-
tioned medium must be the net result of formation, release, and
degradation. The decline in NO at later times indicates that

NO is continuously degraded, even in the presence of a high

concentration of superoxide dismutase (100 units/ml). How-

ever, the fact that the VEGF-induced release of NO remains

above the basal levels for up to 60 min after VEGF addition

indicates a sustained effect of the growth factor on NOS activ-

ity. The overall pattern of VEGF release shown by our experi-

ments is quite consistent with the pattern of VEGF-induced in-

creases in intracellular calcium shown by previous work done

in human umbilical vein endothelial cells. Those studies

showed that VEGF induced an increase in intracellular cal-

cium levels that was preceded by a latent period of 15 s, peaked

at ~60 s, and was sustained for up to 90 min (6, 8).

2 H. He and R. B. Caldwell, unpublished observations.
Specificity of the VEGF effects on NO release was demonstrated by experiments comparing the effects of different inhibitors on VEGF-induced formation of NO using the calcium ionophore A23187 as positive control. The NOS inhibitor L-NAME (42), the PKC inhibitor GFX (43), the PLC inhibitor U73122 (44), the specific MAPK kinase (MEK) inhibitor PD98059 (45), or the JAK2 inhibitor AG490 (46) was used to pretreat the cells before stimulation with VEGF. As expected, inhibition of NOS by L-NAME completely blocked NO formation (9, 10, 41, 47). Furthermore, inhibition of PKC or PLC activity also completely blocked VEGF-stimulated NO release (Fig. 6). In contrast, the inhibition of MEK and JAK2 had no effect. These results suggest that PKC and PLC are involved in VEGF activation of the NOS/guanylate cyclase pathway, whereas MEK and JAK2 are not.

To determine which VEGF receptor is involved in NOS activation and NO production, we tested the effects of placental growth factor (PlGF). PlGF is a member of the VEGF family of proteins that binds flt-1 with high affinity but fails to bind flk-1/KDR (48). The results showed that PlGF did not increase cGMP production (Fig. 7). Thus, activation of the flt-1/KDR receptor is responsible for VEGF-induced NO production, whereas flt-1 is not involved.

*Increased Intracellular Calcium Is Required for VEGF-induced cGMP Production*—In endothelial cells, VEGF-induced activation of PLCγ1 leads to formation of 1,4,5-IP₃, which induces increases in intracellular calcium levels and activation of calcium/calmodulin-dependent protein kinases (27, 49). The interdependence of tyrosine phosphorylation and calcium signaling in endothelial cells is well established (50, 51). Stimulation of NOS activation and NO formation by increased intracellular Ca²⁺ is also well known (52). To determine the source of the Ca²⁺ increase responsible for VEGF-induced NO production, we studied the effects of the following inhibitors on VEGF-induced cGMP production by reporter cells: TMB-8 (intracellular Ca²⁺ channel blocker) (53), BAPTA/AM (intracellular Ca²⁺ chelator), and verapamil (extracellular Ca²⁺ channel blocker) (55). As shown in
VEGF Signals NO and Prostacyclin Production

FIG. 8. Role of different sources of calcium in VEGF-induced NO production. Confluent BAECs were pretreated with TMB-8 (200 μM), BAPTA/AM (50 μM), EGTA (5 mM), and verapamil (10 μM) and then treated with VEGF (10 ng/ml) for 5 min. Controls were treated with VEGF alone. Treatment effects on NO formation was analyzed by determining cGMP formation in reporter cells. The results shown (mean ± S.E.) are representative of three separate experiments.

FIG. 9. The effect of VEGF on PGI2 production. The accumulation of 6-keto PGF1α in the supernatant of cells stimulated in six-well plates was used as an index of PGI2 release. BAECs were pretreated with PP2, genistein, GFX, BAPTA/AM, PD98059, and L-NAME for 30–60 min. Then VEGF (10 ng/ml) was added to the wells for 10 min. PGI2 formation was assayed by enzyme immunoassay of 6-keto PGF1α. The results shown (mean ± S.E.) are representative of three separate experiments.

Fig. 8, TMB-8 and BAPTA/AM attenuated VEGF-induced NO production, whereas EGTA and verapamil had no effect. These data indicate that Ca2+ release from internal stores is required for VEGF-induced activation of NOS. This conclusion is supported by results of previous experiments analyzing VEGF effects on cGMP formation in human umbilical vein endothelial cells, which showed that BAPTA/AM totally blocked the VEGF-induced increase in cGMP (41). Previous analysis of VEGF effects on intracellular Ca2+ accumulation have shown that the initial VEGF-induced increase in intracellular Ca2+ results from a combination of influx of external Ca2+ and Ca2+ release from internal stores and that sustained elevation of intracellular Ca2+ levels requires the influx of external Ca2+ (6). The lack of inhibitory effects of verapamil and EGTA shown in our experiments indicates that influx of extracellular Ca2+ into the cell is not required for NOS activation under acute conditions (5 min of VEGF treatment). However, influx of external Ca2+ would probably be needed to replenish the intracellular Ca2+ if treatment were continued longer.

VEGF-induced PGI2 Production Is Independent of NOS Activity—PGI2, like VEGF, is known to be involved in the regulation of vascular permeability and angiogenesis (19, 20). VEGF has been shown to stimulate PGI2 production in human umbilical vein endothelial cells via activation of flk-1/KDR (18). Previous analysis of VEGF stimulation of the MAPK pathway is well established (14), but the relationship between the MAPK cascade and NOS activation is not yet clear. Results of work done using late passage coronary vein endothelial cells indicated that the NOS/guanylate cyclase pathway lies upstream of the MAPK cascade (16). However, others have also shown that activation of the MAPK cascade by shear stress parallels NO production. Our observation that VEGF-induced production of PGI2 was blocked by the MEK inhibitor but not by the NOS inhibitor indicates that this effect is mediated by the MAPK pathway independent of NOS.

Based on the above results, we propose a working model for VEGF signaling leading to increased NO and PGI2 formation: VEGF binding to flk-1/KDR results in formation of receptor complexes with c-Src and c-Src activation. Activated c-Src activates PLCγ1, which leads to formation of diacylglycerol and 1,4,5-IP3. Diacylglycerol activates PKC, and 1,4,5-IP3 stimulates Ca2+ release from internal stores, which activates NOS, leading to NO production. The signaling pathway for VEGF stimulation of PGI2 production follows the above pathway though the level of intracellular Ca2+ release but diverges prior...
to initiation of the NOS/guanylate cyclase and MAPK cascades (Fig. 10). Our model, therefore, suggests that VEGF-induced NO and PGI2 formation occur in parallel. This model, however, does not necessarily imply that direct phosphorylation or interactions occur between each of the indicated components in either pathway.

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