Ligand-Independent Activation of Vascular Endothelial Growth Factor Receptor 2 by Fluid Shear Stress Regulates Activation of Endothelial Nitric Oxide Synthase

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Abstract—Fluid shear stress generated by blood flowing over the endothelium is a major determinant of arterial tone, vascular remodeling, and atherogenesis. Nitric oxide (NO) produced by endothelial NO synthase (eNOS) plays an essential role in regulation of vascular function and structure by blood flow, but the molecular mechanisms that transduce mechanical force to eNOS activation are not well understood. In this study, we found that laminar flow (shear stress=12 dyne/cm²) rapidly activates vascular endothelial growth factor receptor 2 (VEGFR2) in a ligand-independent manner and leads to eNOS activation in cultured endothelial cells. Flow-stimulated VEGFR2 recruits phosphoinositide 3-kinase and mediates activation of Akt and eNOS. Inhibiting VEGFR2 kinase with selective inhibitors blocks flow-induced activation of Akt and eNOS and production of NO. Decreasing VEGFR2 expression with antisense VEGFR2 oligonucleotides significantly attenuates activation of Akt and eNOS. Furthermore, Src kinases are involved in flow-stimulated VEGFR2 because inhibiting Src kinases by PP2, a selective inhibitor for Src kinases, abolishes flow-induced VEGFR2 tyrosine phosphorylation and downstream signaling. Finally, we show that inhibiting VEGFR2 kinase significantly reduces flow-mediated NO-dependent arteriolar dilation in vivo. These data identify VEGFR2 as a key mechanotransducer that activates eNOS in response to blood flow. (Circ Res. 2003;93:354-363.)

Key Words: vascular endothelial growth factor receptor  3B shear stress  3B mechanotransduction  3B endothelial nitric oxide synthase  3B vasodilation

Vascular endothelial cells (ECs), which form the inner lining of the blood vessel wall, are exposed to fluid shear stress, the dragging force generated by the flowing blood. Fluid shear stress modulates endothelial structure and function and is a major determinant of vascular remodeling, arterial tone, and atherogenesis.1,2 It has been shown that atherosclerotic lesions preferentially develop in regions of low shear stress, whereas laminar flow generating high shear stress is atheroprotective.1,2 Although the exact mechanisms by which flow prevents atherosclerosis are not known, nitric oxide (NO) plays an essential role in mediating many effects of flow, including vessel relaxation,3 inhibition of apoptosis,4 inhibition of platelet coagulation,5 and antiinflammation.6,7 Physiologically, fluid shear stress is the most important stimulus for the continuous formation of NO in vessels.8,9 Endothelial-derived NO has a critical role in the local regulation of vascular homeostasis. A decrease in the bioavailability of NO is a characteristic feature in patients with coronary artery disease80 and promotes the development of atherosclerotic lesions.11 In addition, blood flow and NO appear to play important roles in angiogenesis.12-14

Flow stimulates production of NO via endothelial nitric-oxide synthase (eNOS) both in cultured ECs and in intact vessels.8,9,15-18 We and others have previously reported flow-stimulated phosphorylation of eNOS regulates its enzyme activity,16,19,20 and phosphoinositide 3-kinase (PI3K) and its downstream serine/threonine protein kinase Akt (protein kinase B) mediate phosphorylation of eNOS at Ser1179 (based on the bovine eNOS sequence and equivalent to human eNOS-Ser1177).17,21,22 However, the molecular mechanisms by which mechanical force activates the PI3K-Akt-eNOS signaling pathway are not well understood. Results from our laboratory and others suggest a critical role for protein tyrosine kinases in mechanotransduction.7,23 Flow rapidly activates several tyrosine kinases, including Src family kinases,24-26 focal adhesion kinase (FAK),24,27 proline-rich tyrosine kinase (PyK2),28 and vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2, also known as Flk-1 for murine homologue or KDR for human homologue).29 Several studies also suggest that tyrosine kinases are involved in flow-stimulated NO production and vasodilation.16-20 Thus, we hypothesized that tyrosine kinases mediate flow-induced PI3K and Akt activation, leading to eNOS activation and NO production in ECs.

In this study, we report that VEGFR2 associates with PI3K and is rapidly tyrosine phosphorylated by flow in ECs. We...
also demonstrate that activation of VEGFR2 occurs in a ligand-independent manner via Src kinase-dependent phosphorylation of VEGFR2. Based on these findings, we conclude that VEGFR2 is a mechanotransducer for flow-mediated PI3 K-Akt-eNOS activation and NO-dependent vasodilation in vivo, suggesting an important role for VEGFR2 in flow-mediated effects on angiogenesis and atherosclerosis.

Materials and Methods

Materials
VEGF receptor tyrosine kinase inhibitor (VTI, 4-[4-‘chloro-2-‘fluoro phenylamino]-6,7-dimethoxyquinazoline), SU1498, 4-amino-5-(4-chlorophenyl)-7-((t-butyl) pyrazolo [3,4-d] pyrimidine (PP2), genistein, herbimycin A, wortmannin, and LY294002 were purchased from Calbiochem. VEGF165 was purchased from R&D Systems. Anti–phospho-eNOS antibody (phospho-serine-1179 in bovine eNOS, p-eNOS), anti–phospho-Akt antibody (Ser-473, p-Akt), anti–phospho-p44/42MAPK (p-EKR1/2), and anti-Akt antibody were from Cell Signaling Technologies. Anti-eNOS monoclonal antibody was from Transduction Laboratories. Anti-phosphotyrosine 4G10 (pY-4G10) was from Upstate Biotechnology. Anti-VEGFR2 polyclonal antibody was purchased from Santa Cruz Biotechnology.

Cell Culture and Exposure to Flow
Bovine aortic ECs (BAECs) were purchased from Clonetics and were cultured in medium 199 supplemented with 10% fetal bovine serum (Invitrogen).26 Confluent cells cultured in 60-mm dishes were serum-starved for 24 hours and exposed to laminar flow (shear stress/12 dyne/cm²) in a cone and plate viscometer26 or stimulated by VEGF (25 ng/mL). For the inhibitor studies, cells were pretreated with various inhibitors for 30 minutes before exposure to flow or VEGF.

Antisense Oligonucleotide Treatment
To determine the contribution of VEGFR2 in flow-stimulated signaling, we treated ECs with antisense S-oligonucleotide against bovine VEGFR2 mRNA. The same sequence of antisense S-oligonucleotide as reported by Bernatchez et al.28 against bovine VEGFR2 (AS-VEGFR2: 5’-GCTGCTCTGATTGTTGGG-3’) and as negative control, scrambled VEGFR2 (SCR-VEGFR2: 5’-TGGCGCATGTCGTTGTT-3’) were purchased from custom DNA synthesis service of Integrated DNA Technologies.
Technologies, Inc. BAECs were transfected with AS-VEGFR2 and SCR-VEGFR2 as described.

**Immunoprecipitation and Western Blot Analysis**

Cells were harvested in lysis buffer (0.5% Triton X-100, 0.5% Nonidet P-40, 10 mmol/L Tris, pH 7.5, 2.5 mmol/L KCl, 150 mmol/L NaCl, 30 mmol/L β-glycerophosphate, 50 mmol/L NaF, 1 mmol/L Na3VO4, and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation. The protein concentration of the lysate was determined using the Bradford method (BioRad). Equal amounts of protein were incubated with specific antibody overnight at 4°C with gentle rotation. Then, protein A/G PLUS-agarose (Santa Cruz) was added and incubated for an additional 2 hours. Afterward, beads were washed extensively with lysis buffer and immune complex were eluted in SDS-PAGE sample buffer. Total immune complex samples or protein samples from total cell lysate were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with appropriate primary antibodies. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the ECL detection system (Amersham). Where indicated, the membranes were stripped and reprobed with another antibody. Densitometric analyses of immunoblots were performed by NIH image. Results were normalized by arbitrarily setting the densitometry of control cells to 1.0.

**Identification of Immune Complex Protein by MALDI-TOF Mass Spectrometry**

Immune complexes were separated on a SDS-PAGE gel, and the proteins on the gel were visualized by silver staining. The stained protein band at molecular weight of 230 kDa was excised and subjected to tryptic hydrolysis. Tryptic peptides were spotted with α-cyano-4-hydroxycinnamic acid as matrix and analyzed using a MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometer (PE Biosystems VOYAGER System 4187). The protein is identified by search of MS Fit with the resulting peptide masses in NCB database.

**Measurement of Nitric Oxide Concentration**

NO released by BAECs was measured by nitrogen oxide (NOx) metabolites that accumulated in the medium, using a chemiluminescence detector. Samples (1 mL) were collected from the dishes after flow exposure and kept dark on ice until NO assay. For each experiment, a standard curve was constructed using 50 to 250 nmol/L NaNO3 for calculation of NOx content per sample. The background signal in the Hanks’ buffer was subtracted from each measured value, and the results were normalized by arbitrarily setting the value of control cells to 1.0.

**Evaluation of Flow-Mediated Vasodilation**

The study was approved by the University Committee on Animal Resources at the University of Rochester. In brief, the left cheek pouch in anesthetized hamster was prepared for in vivo microcirculation observations with transillumination using a modified Nikon microscope connected with a video image system. Arteriole dilatation at the entrance to the network was induced by increasing blood flow with the micropipette application of 10 µg/mL antibody LM609 (α,β integrin agonist) on downstream sites at the end of arteriolar...
Inhibiting VEGFR2 kinase attenuates flow-induced eNOS activation. BAECs were pretreated with vehicle (DMSO) or SU1498 or VTI at different concentrations indicated for 30 minutes before exposure to flow or VEGF (25 ng/mL) for 5 minutes (A through D) or pretreated with vehicle (DMSO) or SU1498 (10 μmol/L) or VTI (10 μmol/L) 30 minutes before exposure to flow for the time periods indicated (E through H). Cell lysates were analyzed by immunoblot as described above. The same blots were reprobed with antibody detecting total VEGFR2, Akt, eNOS, and ERK1/2, respectively. *P<0.05 vs flow alone; **P<0.05 vs VEGF alone (n=4 for A through D, and n=3 for E through H). I, Effect of SU1498 and VTI on association of PI3K with VEGFR2. ECs were pre-treated with SU1498 (30 μmol/L) and VTI (30 μmol/L) for 30 minutes and then exposed to flow for 5 minutes, then cell lysate were immunoprecipitated with antibodies of VEGFR2 or anti-p85. Association of VEGFR2 with PI3K were detected by immunoblot with p85 and VEGFR2.
network and videotaped with a video caliper system for further analysis. For inhibition experiments, dilation response to LM609 was determined at 30 minutes after onset of inhibitors, which were applied continuously at the observation sites.

Statistical Analysis
Group differences were analyzed using the standard Student’s t test and one-way AVOVA and Bonferroni correction. All values are expressed as mean±SE. A value of P<0.05 was considered statistically significant.

Results
VEGFR2 Is Associated With Flow-Dependent PI3K-Akt-eNOS Signaling
We first examined the role of tyrosine kinases in flow-mediated phosphorylation and activation of Akt and eNOS in cultured BAECs. Exposure of BAECs to flow (an arterial level of laminar shear stress=12 dynes/cm²) elicited phosphorylation and activation of Akt, eNOS, and extracellular signal-regulated kinase (ERK1/2) in a time-dependent manner (Figure 1). Inhibiting tyrosine kinases by genistein (100 μmol/L) or herbimycin A (1 μmol/L), significantly attenuated flow-induced phosphorylation of Akt and eNOS at all time points (Figure 1A). The finding that herbimycin but not genistein inhibited ERK1/2 activation by flow is consistent with our previous report,24 suggesting that different tyrosine kinases are involved in Akt-eNOS activation and ERK1/2 activation.

A PI3K-Akt dependent mechanism for NO synthesis by flow,17,21,22 was confirmed by treatment with the PI3K inhibitors LY294004 and wortmannin, which completely blocked flow-induced phosphorylation of Akt and eNOS but not ERK1/2 (Figures 1C and 1D). Taken together, the data indicate that both genistein- and herbimycin-sensitive tyrosine kinases are involved in regulation of the PI3K-Akt-eNOS signaling pathway by flow.

It is well known that the activation of PI3K is triggered by the binding of its p85 regulatory subunit to phosphorylated tyrosine residues of activated tyrosine kinases. Therefore, to understand the mechanism of activation of the PI3K-Akt-eNOS pathway by flow, it is important to know the nature of tyrosine-phosphorylated proteins interacting with p85. Immunoprecipitations of total cell lysates from control or flow-exposed ECs with anti-p85 antibody, followed by immunoblotting with an anti-phosphotyrosine (pY-4G10) antibody, showed that several tyrosine-phosphorylated proteins were associated with endogenous p85, especially lysates from flow-stimulated cells (Figure 1E). Among them, a band of 230 kDa was most prominent. To further identify the 230-kDa protein, we separated the PI3K bound proteins on a SDS-PAGE gel and excised the visualized 230-kDa band after silver staining. The proteins from the excised gel were digested by trypsin, and the resulting peptides were analyzed by the MALDI-TOF mass spectrometer, which yielded several mass peaks (data not shown). By performing a search of MS Fit with the resulting peptide masses in the NCBI database, we unambiguously identified the 230-kDa band as the 230-kDa band.

VEGFR2 Mediates eNOS Phosphorylation and Activation by Flow
To determine the role of flow-induced VEGFR2 activation in triggering the PI3K-Akt-eNOS signaling pathway in ECs, we inhibited VEGFR2 kinase. Inhibiting VEGFR2 activation with two selective, but structurally different antagonists, SU1498 and VTI, greatly attenuated phosphorylation of VEGFR2, Akt, and eNOS in response to flow and VEGF in a dose-dependent manner (Figures 3A through 3D). SU1498 and VTI inhibited VEGFR2 tyrosine phosphorylation and Akt and eNOS activation to similar extent (Figure 3). Of interest, there was no significant effect of SU1498 and VTI on ERK1/2 activation by flow (Figures 3B and 3D). At times up to 30 minutes (Figures 3E through 3H), VEGFR2 inhibition by SU1498 and VTI significantly decreased activation of Akt and eNOS. The phosphorylation of VEGFR2, Akt, and eNOS by flow was also observed in human umbilical vein ECs and bovine lung microvascular ECs and was blocked by SU1498 and VTI (data not shown), suggesting a general phenomenon for ECs in response to flow. Based on these results, it appears very likely that activation of VEGFR2 is required for flow-induced phosphorylation of Akt and eNOS but not ERK1/2 in ECs. To demonstrate the link between
VEGFR2-PI3K and activation of Akt and eNOS, ECs were pretreated with SU1498 and VTI followed by flow for 5 minutes. The immunoprecipitation from total cell lysates was performed with antibodies of VEGFR2 and p85, and Western blotting was performed with antibodies for p85 and VEGFR2. Pretreatment with SU1498 and VTI almost completely inhibited flow-mediated interaction of VEGFR2 with PI3K (Figure 3I).

To further confirm the involvement of VEGFR2 in flow-stimulated Akt and eNOS activation, ECs were pretreated with antisense VEGFR2 oligonucleotides and stimulated with flow or VEGF. Because of low transfection efficiency of ECs, VEGFR2 protein expression was decreased by about 50%, similar to the data reported by Bernatchez et al.30 (Figures 4A and 4B). The expression of Akt and eNOS did not change (Figure 4A). After antisense oligonucleotide treatment, both flow- and VEGF-stimulated phosphorylation of VEGFR2, Akt, and eNOS were inhibited to a similar extent (Figures 4A and 4C through 4E). Whereas VEGF-induced phosphorylation of ERK1/2 was also inhibited, flow-stimulated ERK1/2 phosphorylation was not significantly affected by antisense oligonucleotide treatment (Figures 4A and 4F). There was no change in cells treated with scrambled oligonucleotides (Figure 4). Taken together, these data demonstrate that activation of VEGFR2 is required for phosphorylation and activation of Akt and eNOS by flow.

Src Kinases Are Involved in Flow-Induced Activation of VEGFR2 and Downstream Signaling

Having shown that activation of VEGFR2 mediates flow-induced PI3K-Akt-eNOS activation, we investigated the upstream mechanisms. To determine whether flow-mediated VEGFR2 activation involves release of endogenous VEGF, we treated ECs with a specific VEGF neutralizing antibody 577B11. Treatment with this VEGF neutralizing antibody had no effect on flow-stimulated phosphorylation of VEGFR2, Akt, and eNOS although it almost completely inhibited VEGF-induced phosphorylation (Figure 5A), indicating that activation of VEGFR2 by flow is VEGF ligand-independent. Because we have previously shown that flow rapidly stimulated Src kinases26 and Src kinases transactivate growth factor receptors,34,35 we examined whether Src kinases are involved in flow-induced VEGFR2 activation. Inhibiting Src kinases with PP2 significantly reduced flow-stimulated phosphorylation of VEGFR2, Akt, and eNOS although it almost completely inhibited VEGF-induced phosphorylation (Figure 5A)
activation of VEGFR2 and its downstream signaling is Src kinase dependent.

**VEGFR2 Is Involved in Flow-Induced NO Production and Vasodilation**

We next determined the role of VEGFR2 in flow-induced NO production in cultured ECs. As previously reported, flow rapidly increased NO production in cultured ECs (Figure 6). Pretreatment of ECs with VEGFR2 kinase inhibitors (SU1498 or VTI), markedly reduced flow-stimulated NO production (Figure 6). To determine the involvement of VEGFR2 in flow-mediated dilation of intact blood vessels, we examined the effect of SU1498 and VTI on flow-induced NO-dependent arteriolar dilation in the anesthetized hamster cheek pouch microcirculation. By applying α,β3-integrin antibody LM 609 to downstream sites at the end of the arteriolar network, arteriolar flow was increased. The increase in flow precedes upstream arteriolar dilation, which is NO dependent. Pretreatment with VEGFR2 kinase inhibitors (VTI or SU1498) for 30 minutes, significantly reduced upstream flow-induced arteriolar dilation in a dose-dependent manner (Figures 7A and 7B). The concentrations of SU1498 and VTI necessary to inhibit vasodilation were lower than required to inhibit eNOS activation in cultured ECs, suggesting differences in uptake, metabolism, and VEGFR2 signaling in ECs in intact vessels compared with ECs in culture.

**Discussion**

The major finding of the present study is that flow-mediated NO production and vasodilation occurs via ligand-independent activation of the VEGFR2 in ECs. We characterized a pathway requiring Src kinase family–dependent activation of VEGFR2, followed by activation of PI3K, Akt, and eNOS. We found that VEGFR2 is associated with PI3K and rapidly tyrosine phosphorylated in response to flow. Inhibition of VEGFR2 kinase with specific inhibitors or downregulation of VEGFR2 by antisense oligonucleotides significantly reduced flow-stimulated phosphorylation and activation of Akt and eNOS. Furthermore, inhibition of Src kinases reduced flow-induced VEGFR2 activation and downstream signaling. Finally, VEGFR2 kinase inhibition attenuated flow-induced NO production from ECs and arteriolar...
dilation in vivo. This is the first report to show that VEGFR2 links mechanical forces to eNOS activation and vasodilation. Several groups have previously reported activation of VEGFR2 by flow; however, the physiological significance has not been established. Chen et al reported that flow induced a rapid and transient tyrosine phosphorylation of VEGFR2 and its concomitant association with the adapter protein Shc. Recently, Shay-salit et al showed that flow induced a rapid induction and nuclear translocation of VEGFR2 and promoted its binding to adherens junction molecules, VE-cadherin, and β-catenin. In the present study, we show an important role for VEGFR2 in regulation of vascular function by fluid shear stress because VEGFR2 kinase inhibitors block flow-induced eNOS activation, NO production in cultured ECs, and arterial dilation in hamster cheek pouch microcirculation in vivo. Unfortunately, because of limited tissue in the preparation, we cannot definitively prove a role for VEGFR2 in vivo because we were unable to show changes in VEGFR2 phosphorylation and eNOS activation. Regardless, it is well documented that VEGF stimulates VEGFR2 and activates PI,K-Akt-eNOS. Thus, our findings suggest that mechanical force can utilize the same signaling pathway triggered by hormonal factors to regulate vascular function by activating these receptors in a ligand-independent manner.

We also found that Src kinases are involved in VEGFR2 activation by flow. Wang et al reported that integrins are involved in the activation of VEGFR2 by flow but not for activation of VEGFR2 by VEGF. Consistent with our results, Src kinases are required for activation of epidermal growth factor (EGF) receptor (EGFR) by integrins. Because activation of integrin receptors is associated with activation of Src kinases, it is likely that Src kinases transduce signals from potential mechanosensors such as integrins to mechanotransducers including VEGFR2, which via downstream signaling cascades trigger physiological responses.

Both fluid shear stress and VEGF stimulate multiple signaling pathways, including PI,K-Akt-eNOS and protein kinase C (PKC)-Raf-ERK1/2. We observed that VEGFR2 kinase inhibitors (SU1498 and VTI) and the Src kinase inhibitor (PP2 at relatively low concentration) blocked flow-induced activation of Akt and eNOS but not ERK1/2. This finding suggests a role for different mechanotransducers and/or signaling pathways in activation of Akt-eNOS and ERK1/2 by flow. In terms of tyrosine kinases, our results suggest that the tyrosine kinase(s) involved in PI,K-Akt-eNOS activation by shear stress is both herbimycin-sensitive and genistein-sensitive, whereas the tyrosine kinase(s) for shear stress-induced ERK1/2 activation is herbimycin-sensitive but not genistein-sensitive (Figures 1A and 1B). Indeed, we have shown that VEGFR2 tyrosine phosphorylation induced by flow is inhibited by both herbimycin and genistein (Figure 2H). Although VEGFR2 appears key for activation of Akt and eNOS, there are other pathways activated by flow that are likely responsible for ERK1/2 activation. Consistent with this concept, it has been reported that integrin-induced FAK activation mediates ERK1/2 activation by flow. In addition, we found that phosphorylation of VEGFR2, Akt, and eNOS induced by flow were sustained longer than that induced by VEGF, suggesting that the mechanisms involved in flow-induced and VEGF-induced phosphorylation and dephosphorylation of VEGFR2 are different. A likely explanation is that VEGF binding increases VEGFR2 phosphorylation at several tyrosine residues that mediate VEGF-induced signaling such as phospholipase C-γ (PLC-γ) to ERK1/2 and PI,K to Akt, and also recruits...
tyrosine phosphatases such as SH2 protein-tyrosine phosphatases (SHIP-1 and SHP-2). In contrast, we speculate that flow increases VEGFR2 phosphorylation of only a few tyrosine residues necessary for the activation of the PI3-K-Akt-eNOS pathway. Recently, Takahashi et al. reported that phosphorylation of one tyrosine (Tyr-1175) in VEGFR2 is essential for VEGF-stimulated activation of PLC-γ and ERK1/2. As yet unidentified tyrosine residue(s) of VEGFR2 responsible for PI3-K-Akt activation by VEGF and flow merit further investigation, which may provide insight into the nature of fluid shear stress-induced activation versus ligand-dependent activation of receptor tyrosine kinases.

In summary, the data show that ligand-independent activation of VEGFR2 by fluid shear stress increases NO production and possibly vasodilation. As multiple pathways are involved in mechanotransduction, the results presented in this study identify VEGFR2 as a mechanotransducer for eNOS activation by flow. VEGFR2-mediated eNOS activation by flow may be involved in many aspects of vascular function. For example, NO is a powerful inhibitor of NF-κB activation and contributes to the antiinflammatory effects of steady laminar flow. Furthermore, NO is necessary for vascular remodeling, suggesting that flow-mediated VEGF2 activation via Src kinases may be involved in vascular remodeling associated with angiogenesis, hypertension, and atherosclerosis.

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References


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