Blood flow modulates endothelial cell (EC) functions through specific signaling events. Previous data show that flow stimulates SHP2 translocation to cell membranes and binding to phosphotyrosine proteins. Flow-induced ERK1/2 phosphorylation depends on SHP2 phophatase activity and SHP2 binding to phospho-PECAM1 (platelet endothelial adhesion molecule 1), suggesting that SHP2 forms a signaling module with PECAM1. We hypothesized that flow induces assembly of the multi-protein complexes with SHP2 that are required for downstream signaling. ECs were exposed to flow for 10 min, and endogenous SHP2 was immunoprecipitated. SHP2-associated proteins were analyzed by SDS-PAGE and identified by mass spectrometry. Tie2 and several known SHP2-binding proteins were identified in flow-induced SHP2 complexes. Flow significantly increased tyrosine phosphorylation of both Tie2 and PECAM1 and their association with SHP2. To evaluate their functional roles, ECs were treated with Tie2 or PECAM1 small interfering RNA (siRNA). Tie2 and PECAM1 expression decreased >80% after siRNA treatment, and flow-stimulated phosphorylation of ERK1/2, Akt, and endothelial nitric oxide synthase was significantly inhibited by Tie2 and PECAM1 siRNA. Tie2 phosphorylation by flow was significantly inhibited by PECAM1 siRNA treatment. These results establish Tie2 transactivation via PECAM1 as an early event in flow-mediated mechanotransduction and suggest an important role for a PECAM1-SHP2-Tie2 pathway in flow-mediated signal transduction.

Fluid shear stress, the frictional force from blood flow acting on the vessel wall, regulates vascular remodeling, arterial tone, and atherosclerosis (1–3). Atherosclerotic lesions occur in regions of low and disturbed flow, whereas steady laminar flow is atheroprotective (4, 5). Flow modulates the physiology, gene expression, and morphology of endothelial cells (ECs) by rapidly activating mechanosensitive pathways (4), including receptor tyrosine kinases (such as VEGFR2 (6)), members of the mitogen-activated protein kinase family (including extracellular signal-regulated kinase-1/2 (ERK1/2) (7) and big mitogen-activated protein kinase 1 (B MK1) or ERK5 (8)), and nonreceptor tyrosine kinases (such as focal adhesion kinase, proline-rich tyrosine kinase-2, and Src (9, 10)). However, the proximate membrane mechanisms by which ECs respond to flow remain poorly characterized.

An important role for cell-cell junction proteins in mechanotransduction has been proposed. Data suggest key roles for vascular endothelial cadherin, junctional adhesion molecules (11, 12), and PECAM1 (platelet endothelial adhesion molecule 1) (13). PECAM1 is a 130-kDa transmembrane glycoprotein present on the surface of monocytes, granulocytes, platelets, and ECs (14). The PECAM1 knock-out mouse is viable (15) but displays defects in angiogenesis, indicating an important role in vessel function (16). PECAM1 is a homophilic adhesion receptor that mediates leukocyte/EC interactions and has been shown to be rapidly tyrosine-phosphorylated in ECs exposed to flow (13, 17) and other stimuli (18). The PECAM1 cytoplasmic domain contains two distinct immunoreceptor tyrosine-based inhibitory motifs centered around tyrosines 663 and 686 (19), which, upon phosphorylation, recruit Src homology 2 domain-containing signaling proteins (20). Recent data show that tyrosine-phosphorylated PECAM1 binds to the phosphatase SHP2 and that the PECAM1/SHP2 complex is required for activation of ERK1/2 by flow (13).

In addition to its phosphatase activity, SHP2 serves as a scaffold and binds to several membrane proteins, including the epidermal growth factor receptor (21), the platelet-derived growth factor receptor (22), and PECAM1 (in response to flow) (13). We reasoned that characterization of SHP2-binding proteins would reveal proteins that act as membrane sensors for downstream signaling. Using a proteomic approach, we found several known SHP2-associated membrane proteins and other adaptor proteins. Among these proteins we focused on Tie2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2), which is a receptor tyrosine kinase expressed predominantly on ECs and hematopoietic cells. Angiopoietin-1 and angiopoietin-2 are ligands for Tie2. Mice deficient in angiopoietin-1 and Tie2 show defective vascular development, demonstrating that Tie2 is crucial for vascular development (23, 24). A recent paper showed that flow increased Tie2 phosphorylation in ECs (25). Here we show that flow-induced Tie2 phosphorylation requires PECAM1 and that interactions among PECAM1, Tie2, and SHP2 mediate activation of ERK1/2, Akt, and eNOS by flow.
Materials and Methods

Cell Culture and Flow Experiments—Bovine aortic endothelial cells (BAECs) were purchased from Clonetics and cultured in medium 199 supplemented with 10% fetal calf serum (FCS), 50 units/ml penicillin, and 0.05 mg/ml streptomycin at 37 °C in a 5% CO2 incubator. Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (8) and maintained in Medium 200 (Cascade Biologies) with low serum growth supplement. Cells were used at passages 1–4. Experiments were performed with confluent cells grown in 60-mm dishes (growth-arrested for 1 day by serum deprivation) to decrease basal kinase activity. Cells were exposed to laminar flow (shear stress of 24 dyn/cm²) in a cone and plate viscometer.

Antibodies—The SHP2 monoclonal antibody (B1), polyclonal antibodies (C18 and N16), the PECAM1 antibody, and ERK2 were bought from Santa Cruz Biotechnology. The phosphoserine 473-Akt, phospho-p44/42 ERK1/2, and Akt antibody were purchased from Cell Signaling Technologies. The eNOS antibody, phospho-serine 473-Akt, phospho-p44/42 ERK1/2, and the Akt antibody were purchased from Cell Signaling Technologies. The eNOS antibody was from BD Biosciences. The phosphotyrosine antibody (4G10) and the Tie2 antibody were bought from Upstate Biotechnology.

Immunoprecipitation and Western Blot Analysis—Cells were harvested in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na3VO4, and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation. The protein concentration was determined by the Bradford assay (Bio-Rad). Equal amounts of proteins were incubated with a specific antibody overnight at 4 °C with gentle rotation. Protein A Plus-agarose beads (Santa Cruz Biotechnology) were added to pull down the antibody complexes. Afterward, beads were washed with lysis buffer, and immune complexes were separated by SDS-PAGE. Total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were incubated with appropriate primary antibodies. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the Odyssey infrared imaging system (LI-COR Biotechnology). Densitometric analyses of immunoblots were performed with Odyssey software (LI-COR Biotechnology).

Identification of SHP2 Immune Complex Proteins by MALDI-TOF Mass Spectrometry—Immune complexes were separated by SDS-PAGE, and the proteins on the gel were visualized by Coomassie staining (Bio-Rad). The stained protein bands at molecular masses of ~200, ~150, and ~100 kDa were excised and subjected to tryptic hydrolysis. Peptides were fixed as matrix and analyzed by using a MALDI-TOF mass spectrometer (PF Biosystems VOYAGER System 4187). Data acquisition was computer-controlled using a program written in instrument control language that automatically provided data-dependent ion selection and varied capillary electrophoretic voltage. The partial mass spectrometry data for proteins identified as SHP2-associated proteins on the basis of correct molecular mass are presented in Supplemental Table I (available in the on-line version of this article), which provides protein names, molecular mass values, functions, accession numbers, and association with SHP2.

PECAM1 and Tie2 siRNA and Transfection—Bovine (5′-GGAGGACAAGAGAAAGUUAU-3′) and human (5′-GAUUUCUGACAGCA-GAAU-3′) PECAM1 siRNA were designed by our laboratory and made by Integrated DNA Technologies. Human Tie2 Smart Pool siRNA was purchased from Dharmaco. Subconfluent BAECs were treated with 100 nM PECAM1 siRNA by using Lipofectamine 2000 (Invitrogen) for 2 h, and then the siRNA was removed. Cells were incubated overnight and transfected with PECAM1 siRNA a second time. Cells were used for experiments 16 h after the second transfection. Subconfluent HUVECs were transfected with Tie2 siRNA as described for PECAM1, but transfection was performed once.

Statistical Analysis—All experiments were performed at least three times. Data are expressed as mean ± S.E. Where indicated, analysis of variance was performed, and p < 0.05 (indicated by an asterisk in Figs. 1, 3, 5, and 6) was considered significant in all experiments.

Results

SHP2-associated Proteins Induced by Laminar Flow—SHP2 binds directly to receptor tyrosine kinases and phospho-PECAM1 via the immunoreceptor tyrosine-based inhibitory motif domain (13). The interaction of SHP2 with these membrane proteins is essential for ERK1/2 activation by growth factors and mechanical force, such as flow (13). We hypothesized that characterizing SHP2-associated proteins regulated by flow would identify novel mechanosensors involved in flow-mediated signaling. To elucidate components of the SHP2 signaling pathway, we used a proteomic approach based on co-precipitation of proteins interacting with SHP2. HUVECs were treated with flow for 10 min, and cell lysates were immunoprecipitated (IP) with the mouse monoclonal SHP2 (B1) and rabbit polyclonal SHP2 (C18 and N16) antibodies (SHP2 Ab). Phosphotyrosine proteins were detected by immunoblots (IB) with the 4G10 antibody. Arrows show that SHP2 was immunoprecipitated by SHP2 antibodies. Bottom section, the blot was reprobed with SHP2 antibodies. B, top section, HUVECs were treated with flow for 5 min (+), and cell lysates were immunoprecipitated (IP) with mouse IgG (mIgG) and anti-SHP2 monoclonal antibody B1. Phosphotyrosine proteins were detected by immunoblotting (IB) with the 4G10 antibody. Arrows show proteins identified that change with flow. Bottom section, the blot was reprobed with SHP2 antibodies. C, quantitative analysis of flow-induced changes in phosphotyrosine proteins that co-precipitate with SHP2. The protein bands at 100–200 kDa, indicated by arrows 1–5 in panel B, were analyzed for changes in response to flow by densitometry using Odyssey software. For each band, the intensity was normalized to control, which was arbitrarily set to 1. Values are mean ± S.E., n = 3; *, p < 0.05.

Fig. 1. Flow stimulated binding of tyrosine-phosphorylated proteins to endogenous SHP2. A, top section, HUVECs were treated with flow for 10 min, and cell lysates were immunoprecipitated (IP) with the mouse monoclonal SHP2 (B1) and rabbit polyclonal SHP2 (C18 and N16) antibodies (SHP2 Ab). Phosphotyrosine proteins were detected by immunoblots (IB) with the 4G10 antibody. Arrows show that SHP2 was immunoprecipitated by SHP2 antibodies. Bottom section, the blot was reprobed with SHP2 antibodies. B, top section, HUVECs were treated with flow for 5 min (+), and cell lysates were immunoprecipitated (IP) with mouse IgG (mIgG) and anti-SHP2 monoclonal antibody B1. Phosphotyrosine proteins were detected by immunoblotting (IB) with the 4G10 antibody. Arrows show proteins identified that change with flow. Bottom section, the blot was reprobed with SHP2 antibodies. C, quantitative analysis of flow-induced changes in phosphotyrosine proteins that co-precipitate with SHP2. The protein bands at 100–200 kDa, indicated by arrows 1–5 in panel B, were analyzed for changes in response to flow by densitometry using Odyssey software. For each band, the intensity was normalized to control, which was arbitrarily set to 1. Values are mean ± S.E., n = 3; *, p < 0.05.
bands that co-precipitated with SHP2 and exhibited increased tyrosine phosphorylation in response to flow (Fig. 1A; from 220 to 97 kDa). We found that the B1 antibody, which recognizes the C terminus of SHP2, precipitated more proteins than the other two antibodies. Mouse IgG was used as a negative control to show specific B1 antibody immunoprecipitation (Fig. 1A and B). To identify these proteins, the flow experiment was repeated, and cell lysates were immunoprecipitated with the B1 SHP2 antibody, fractionated by SDS-PAGE, and stained with Coomassie Blue. Protein bands of 97–220 kDa were cut out of the gel and sent for sequence analysis by mass spectrometry. Known SHP2-associated proteins were identified in two out of the gel and sent for sequence analysis by mass spectrometry. We identified several proteins not previously reported to bind SHP2 including, mitogen-activated protein kinase kinase kinase (MAP4K; 90 kDa), a protein kinase A anchoring protein (180 kDa), and a small GTPase-activating protein (90 kDa).

**Tie2 Interacts with SHP2 in Response to Flow**—We decided to study the role of Tie2 in flow-induced SHP2 signaling because Tie2 was shown previously to be phosphorylated by flow (25), and it is known that Tie2 binds to SHP2 (26). The interaction of Tie2 and SHP2 was confirmed by immunoprecipitating with the SHP2 antibody and immunoblotting with the Tie2 antibody (Fig. 2A). Although the Tie2 interaction with SHP2 was basally minimal, it was significantly increased at 5 min and sustained for 10 min (Fig. 2A, top). To confirm that Tie2 was activated by flow, BAECs were exposed to flow for different periods of time, and Tie2 was immunoprecipitated and its phosphorylation analyzed by immunoblot with 4G10 (Fig. 2B). Tie2 phosphorylation increased within 5 min and peaked at 10 min. The phosphorylation of Tie2 temporarily corresponded with binding to SHP2 induced by flow. These results show that flow rapidly stimulates the phosphorylation of Tie2 that interacts with SHP2.

**Tie2 Is Required for Flow Stimulation of ERK1/2, Akt, and eNOS**—The Tie2 ligand, angiopoietin-1 has been shown to activate phosphorylating 3-kinase, Akt, and ERK1/2 in ECs (27, 28). Although flow activates the same signaling pathways (25), the role of Tie2 in flow-mediated signaling remains uncharacterized. To address this question, we designed Tie2 siRNA to study the specific role of Tie2 in flow-induced ERK1/2 and Akt-eNOS pathways. After transfection with Tie2 siRNA for 1 day, Tie2 expression was reduced by >90%, whereas control siRNA had no significant effect (Fig. 3A). Tie2 siRNA had no significant effect on expression of ERK1/2, Akt, eNOS (Fig. 3B–D), or PECAM1 (data not shown). Treatment with Tie2 siRNA and control siRNA did not increase cell death or cause apparent changes in cell morphology (not shown).

Flow also rapidly stimulated PECAM1 phosphorylation in response to flow (Fig. 4). Phosphorylation of PECAM1 was significantly stimulated by flow (~8-fold at 10 min) in cells treated with control siRNA. In contrast, phosphorylations of ERK1/2, Akt, and eNOS were significantly inhibited (50 ± 2.2, 40 ± 3.1, and 70 ± 1%, respectively) at 10 min in siRNA-treated cells as compared with control cells (Fig. 3, B–D). In contrast, flow-induced BMK1 activation (measured by band shift denoted by arrow in Fig. 3E) was not inhibited by Tie2 siRNA treatment. These results indicate that Tie2 is required for flow activation of ERK1/2, Akt, and eNOS.

**Flow Stimulates PECAM1 Tyrosine Phosphorylation and Interaction with SHP2**—We next performed a series of experiments to define the specific role of PECAM1 in flow-stimulated SHP2-Tie2 interactions and the signaling pathway, because it is known that SHP2-PECAM1 interaction is required for ERK1/2 activation (13). Previous studies showed that flow induced PECAM1 tyrosine phosphorylation within 2 min that was sustained for 30 min (13). In our experimental system we exposed HUVECs to flow (shear stress of 24 dyn/cm²) for 2–30 min and assayed PECAM1 tyrosine phosphorylation and PECAM1 association with SHP2 (Fig. 4). In response to flow, SHP2 associated with PECAM1 within 2 min and remained associated for 30 min (Fig. 4, top). Flow also rapidly stimulated tyrosine phosphorylation and SHP2 association with PECAM1, which was sustained for 30 min (Fig. 4, bottom). These results suggest that Tie2 is required for flow activation of ERK1/2, Akt, and eNOS.
PECAM1 tyrosine phosphorylation (Fig. 4, middle) without change in PECAM1 expression (Fig. 4, bottom).

**PECAM1 Is Required for Flow Activation of ERK1/2, Akt, and eNOS**—To confirm the importance of PECAM1 in the Tie2-SHP2 signaling pathway, we used siRNA to knock down PECAM1 expression specifically. We designed three PECAM1 siRNAs for bovine and one for human ECs. As shown in Fig. 5A, all PECAM1 siRNAs were highly effective at decreasing PECAM1 expression to <20% of normal levels, and siRNA number 3 was used for experiments (Fig. 5). The expression of ERK1/2, Akt, and eNOS was not changed by PECAM1 siRNA. The morphology of cells also remained unchanged after transfection with PECAM1 siRNA. For the experiments shown in Fig. 5, BAECs were pretreated with PECAM1 siRNA for 2 days followed by flow for 10 min. Flow-induced phosphorylation of ERK1/2, Akt, and eNOS was significantly decreased (by 60 ± 9%, 39 ± 8%, and 68 ± 2%, respectively, with p < 0.05) after PECAM1 siRNA treatment (Fig. 5B; n = 4). Similar inhibition of phosphorylated ERK1/2, Akt, and eNOS (by 54 ± 4, 52 ± 5, and 82 ± 2%, respectively, with p < 0.05) were observed in HUVECs (Fig. 5C; n = 3). These results show that PECAM1 is required for flow-induced activation of ERK1/2, Akt, and eNOS, suggesting PECAM1 activation in the Tie2-SHP2 pathway.

**Tie2 Transactivation by Flow**—To elucidate the hierarchy of the PECAM1-SHP2-Tie2 pathway, we next investigated whether Tie2 transactivation in response to flow required PECAM1. HUVECs were treated with PECAM1 siRNA and exposed to flow for 10 min. Tie2 phosphorylation was significantly decreased (52 ± 9%, p < 0.05, n = 4) in PECAM1 siRNA-treated cells as compared with control cells (Fig. 6). These results show that Tie2 activation by flow is likely downstream of PECAM1-dependent signaling events. No direct binding between PECAM1 and Tie2 was observed using several antibodies for co-precipitation experiments, suggesting that SHP2 may act as an adaptor for these two proteins.

**DISCUSSION**

The major findings of the present study define a new mechanotransducing pathway by which flow activates ERK1/2, Akt, and eNOS in endothelial cells. Specifically, by using proteomics and siRNA knockdown strategies we found that flow stimulates tyrosine phosphorylation of PECAM1, promoting SHP2 binding to PECAM1 and, subsequently, tyrosine phosphorylation of Tie2. Importantly, we demonstrate for the first time that PECAM1 is required for flow-induced Tie2 phosphorylation, establishing a novel pathway for Tie2 activation. The present results, combined with previous work from our laboratory (29) and others (12) showing activation of VEGFR2 by flow, demonstrate that the transactivation of receptor tyrosine kinases is an important mechanism for mechanotransduction.

An unanswered question is how flow activates Tie2 and VEGFR2. Three non-exclusive mechanisms appear likely. First, flow may inhibit the activity of a phosphatase that normally keeps Tie2 and VEGFR2 inactive. A possible mechanism would be localized generation of reactive oxygen species (especially H₂O₂) that inhibit tyrosine phosphatases by oxidizing the cysteine present in the catalytic site. This mechanism has been shown to mediate propagation of epidermal growth factor receptor signaling (30), and SHP2 cysteine oxidation has been specifically shown to be involved in platelet-derived growth factor receptor signaling (31). This mechanism seems likely, because proteins that immunoprecipitated with SHP2 (a tyrosine phosphatase) paradoxically exhibited increased tyrosine phosphorylation in response to flow (see Figs. 1 and 2), suggesting that SHP2 phosphatase activity was inhibited. Second, flow may promote clustering of Tie2 and VEGFR2 with amplification of signaling by autophosphorylation, as was also shown for the epidermal growth factor receptor (32). Receptor clustering could be enhanced by association with scaffold proteins such as Gab1 (or

**Fig. 4.** PECAM1 phosphorylation and interaction with SHP2 were increased by flow. HUVECs were exposed to flow for the indicated times, and cell lysates were immunoprecipitated (IP) with PECAM1 antibodies followed by immunoblotting (IB) with 4G10, PECAM1, or SHP2 antibodies.

**Fig. 5.** Knockdown of PECAM1 by PECAM1 siRNA decreased flow-induced phosphorylation of ERK1/2, Akt, and eNOS. A, BAECs were treated with control (C) or three different PECAM1 siRNAs. The expression of PECAM1 was measured by Western blots (IB, immunoblotting) with an anti-PECAM1 antibody. siRNA number 1 inhibited PECAM1 expression by 50%, whereas siRNA number 2 and siRNA number 3 inhibited expression by 80%. A similar 80% decrease was obtained for the human siRNA when used with HUVECs. B, BAECs were pretreated with control or PECAM1 siRNA for 2 days and then exposed to flow for 10 min. The immunoblots (IB) show the expression of the indicated proteins. Phosphorylation of ERK1/2, Akt, and eNOS was analyzed by phosphospecific antibodies. *, p < 0.05 versus control; n = 4. C, knockdown of PECAM1 by PECAM1 siRNA decreased flow-induced phosphorylation of ERK1/2, Akt, and eNOS in HUVECs. Samples were analyzed as described above. *, p < 0.05 versus control; n = 3. Quantitative analysis of phosphorylation was performed with Odyssey software and normalized to 1 for control siRNA at time 0 min. The numbers below each lane of phosphoblots (pERK1/2, pAkt, and peNOS) correspond to the relative band intensities in the representative experiment.
perhaps SHP2). Finally, SHP2 may play a direct role as suggested by the finding that SHP2 increases Src activity by controlling phosphorylation of Csk, a negative regulator of Src (33). The third mechanism requires activation of SHP2, which logically must occur in a different location or at a different time than that for the inactivation of SHP2 discussed in the first mechanism. In fact, Fujiwara and colleagues (13) have previously shown a requirement for SHP2 activity in flow-mediated ERK1/2 activation. Thus, we propose that SHP2 initially functions as a scaffold to activate Tie2 at the membrane, whereas SHP2 subsequently functions as a phosphatase to mediate downstream signal events such as ERK1/2 activation.

An important finding in the present study is the stimulation of PECAM1 and SHP2 tyrosine phosphorylation by flow. Although PECAM1-SHP2 interaction exists prior to the initiation of flow, there is a dramatic increase in tyrosine phosphorylation of both PECAM1 and SHP2 in response to flow. Previously, we found that detectable amounts of SHP2 and Gab1 were translocated from the cytoplasm to the EC junction in response to flow (13). Thus, we propose that flow stimulates both SHP2 translocation and activation of a kinase that can phosphorylate both SHP2 and PECAM1. Previous studies of PECAM1 have suggested that the Fer kinase is an important PECAM1 kinase (34) because it specifically phosphorylates PECAM-1 at the immunoreceptor tyrosine-based inhibitory motif. Fer also induces tyrosine phosphorylation of SHP2 and Gab1 (34).

The present study defines novel roles for PECAM1 and Tie2 in flow-mediated mechanotransduction. Although it was previously shown that PECAM1 mediated ERK1/2 activation, the role of PECAM1 in Akt and eNOS activation is novel. Pertinent to our data is a recent report that eNOS binds to PECAM1, although the interaction was reported to inhibit eNOS activity (35). More intriguing is the role of Tie2 in EC mechanotransduction. We found that Tie2 siRNA did not affect PECAM1 expression, indicating that the decreased phosphorylation of ERK1/2, Akt, and eNOS by Tie2 knockdown was not due to decreased PECAM1 expression. Based on these data, the most likely flow-activated mechanosignaling pathway appears to be PECAM1 to SHP2 to Tie2.

Physiologically, Tie2 activation by flow is likely to provide a tonic survival signal based on the well established data showing that both Tie2 and flow protect EC from apoptotic stimuli (36, 37). The fact that both Tie2 and VEGFR2 are transactivated by flow may also be important in angiogenesis and EC migration. It is therefore attractive to speculate that changes in blood flow may regulate the activity and the subcellular location of these receptors. Because PECAM1 also binds β-catenin and γ-catenin and because the catenins bind vascular endothelial cadherin (38), it appears that flow may target Tie2 to cell junctions as suggested for the VEGFR2 by Shay-Salit et al. (12). This would be an ideal location for controlling the local signaling required for cell locomotion, because actin filament formation is highly regulated at cell junctions. Future studies will be required to define the specific roles of the Tie2 and VEGFR2 pathways in flow-mediated signal transduction.