Src-induced Tyrosine Phosphorylation of VE-cadherin Is Not Sufficient to Decrease Barrier Function of Endothelial Monolayers

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Alejandro P. Adam, Amy L. Sharenko, Kevin Pumiglia, and Peter A. Vincent

From the Center for Cardiovascular Sciences and Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York 12208

Activation of Src family kinases (SFK) and the subsequent phosphorylation of VE-cadherin have been proposed as major regulatory steps leading to increases in vascular permeability in response to inflammatory mediators and growth factors. To investigate Src signaling in the absence of parallel signaling pathways initiated by growth factors or inflammatory mediators, we activated Src and SFKs by expression of constitutively active Src or knockdown of Csk. Activation of SFK by overexpression of dominant negative Csk induced VE-cadherin phosphorylation at tyrosines 658, 682, and 731. However, dominant negative Csk expression was unable to induce changes in the monolayer permeability. In contrast, expression of constitutively active Src decreased barrier function and promoted VE-cadherin phosphorylation on tyrosines 658 and 731, although the increase in VE-cadherin phosphorylation preceded the increase in permeability by 4–6 h. Csk knockdown induced VE-cadherin phosphorylation at sites 658 and 731 but did not induce a loss in barrier function. Co-immunoprecipitation and immunofluorescence studies suggest that phosphorylation of those sites did not impair VE-cadherin ability to bind p120 and β-catenin or the ability of these proteins to localize to the plasma membrane. Taken together, our data show that Src-induced tyrosine phosphorylation of VE-cadherin is not sufficient to promote an increase in endothelial cell monolayer permeability and suggest that signaling leading to changes in vascular permeability in response to inflammatory mediators or growth factors may require VE-cadherin tyrosine phosphorylation concurrently with other signaling pathways to promote loss of barrier function.

Endothelial cells line the wall of all blood vessels, where they play a critical role in a number of physiological responses including regulation of vasoreactivity, hemostasis, and leukocyte recruitment. Vascular endothelial cells also act as a selective barrier that regulates the passage of fluid, macromolecules, and white cells from the vascular space to the interstitium. The proper regulation of fluid and protein flux is critical for maintaining normal tissue function. This is accomplished by a number of transmembrane cell-cell adhesion proteins that, when coupled with their binding partners, contribute to the adhesion of one endothelial cell to another. The adherens junction complex, comprised of cadherins and the catenins, is a major adhesion structure that connects to the actin cytoskeleton (1, 2). VE-cadherin is found specifically in the endothelial cell adherens junction and has been implicated in playing a fundamental role in controlling the transport across the endothelial barrier and in regulating angiogenesis (3, 4). The cytoplasmic domain of VE-cadherin binds to β-catenin and plakoglobin, both of which bind to α-catenin, a protein that supports the interaction of the VE-cadherin-catenin complex with the actin cytoskeleton. In addition to catenins, VE-cadherin has been found to interact with other signaling molecules and to serve as a scaffolding molecule that participates in a signaling network that controls endothelial cell-cell adhesion (5).

The inflammatory response associated with ischemia-reperfusion during stroke and myocardial infarction results in a loss of endothelial barrier function that contributes to infarct size and loss of tissue function. Src inhibition prior to the ischemic period showed reduced infarct size when compared with control animals due to inhibition of edema formation in both models of myocardial infarction and stroke (6, 7). In addition, infarct size was reduced by 50% in Src knock-out mice (6). In vitro studies have also implicated Src as a major signaling protein leading to a loss of barrier function (8–11) with Src and other members of the Src kinase family being shown to play a role in lipopolysaccharide (10) and VEGF (8, 11)-induced loss of endothelial integrity.

A number of studies have implicated tyrosine phosphorylation of VE-cadherin in the regulation of the trans-vascular flux of fluid and protein (12, 13). Indeed, previous studies have demonstrated an association of VE-cadherin phosphorylation and endothelial barrier function in response to inflammatory mediators and growth factors (10, 15, 17, 37). In addition, both the sources cited.

2 The abbreviations used are: VEGF, vascular endothelial growth factor; DN-Csk, dominant negative C-terminal Src kinase; cSrc, constitutively active Src; SFK, Src family kinases; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; ICAM, intercellular adhesion molecule; HDMEC, human dermal microvascular endothelial cell; EC50, electrical cell-substrate impedance sensor; TEER, trans-endothelial electric resistance; siRNA, small interfering RNA; CHO, Chinese hamster ovary; GFP, green fluorescent protein; PBS, phosphate-buffered saline; pfu, plaque-forming unit; DMSO, dimethyl sulfoxide; PIPES, 1,4-piperazinediethanesulfonic acid; pTyr, phosphotyrosine.
phosphorylation of VE-cadherin and monolayer permeability were dependent on activation of Src family kinases (SFKs). More recently, studies have begun to use mutations of specific tyrosine residues to investigate the role of VE-cadherin phosphorylation in regulating barrier function. Overexpression of VE-cadherin in CHO cells allows for these cells to form a restrictive barrier to protein flux, giving them an epithelial phenotype. Potter et al. (14) reported that overexpression of recombinant forms of VE-cadherin that mimic phosphorylation of either Tyr-658 or Tyr-731 did not develop a restrictive monolayer in CHO cells. These mutations also affected the ability of VE-cadherin to bind other adherens junction components, p120, and β-catenin. In addition, expression of activated Src increased phosphorylation on both Tyr-658 and Tyr-731 of VE-cadherin. In contrast, Wallez et al. (15) showed that Src overexpression in CHO cells induced VE-cadherin phosphorylation exclusively in Tyr-685. This site was confirmed to be a direct Src target using an in vitro kinase assay. Furthermore, they could also detect this phosphorylation site in human umbilical vein endothelial cells after VEGF stimulation. Recent studies have found that tyrosine phosphorylation of VE-cadherin is required for regulating leukocyte trans-endothelial cell migration (16, 17) and that this requires activation of SFKs. In addition, it has also been shown that mutation of Tyr-658 or Tyr-731 will attenuate VEGF-induced decreases in barrier function (37). Similar to studies investigating permeability, various tyrosines have also been implicated in trans-endothelial cell migration (16, 17).

Although the literature points to an important role of VE-cadherin phosphorylation in the regulation endothelial function, including barrier formation, further investigations are needed to fully understand the mechanisms of this process. The experiments presented here are a direct examination of the role of Src-mediated VE-cadherin phosphorylation in the regulation of endothelial barrier function and junctional assembly. To limit the activation of other confounding signaling pathways known to be initiated by growth factors or inflammatory mediators, SFKs were activated by expression of a dominant negative C-terminal Src kinase (DN-Csk), constitutively active Src (caSrc), or knockdown of Csk. In the studies that follow, we demonstrate that changes in endothelial permeability and the phosphorylation of specific tyrosine residues in VE-cadherin are dependent on the method used to increase active Src. More importantly, our data demonstrate that although SFK activity may be required for the loss of barrier function, SFK activation and the subsequent VE-cadherin tyrosine phosphorylation are not sufficient to increase endothelial cell permeability and junctional disassembly.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human dermal microvascular endothelial cells (HDMECs) were isolated from neonatal foreskin by incubating the obtained cells with magnetic beads coated with an antibody to CD31 (DYNAL) as described previously (18). Cells were then assessed for VE-cadherin and lectin binding to confirm their endothelial origin and then frozen and stored in liquid nitrogen. Cells were grown in EGM-2MV (Lonza Group Ltd.) containing 5% fetal bovine serum and were used for experiments between passages 6 and 11. For all experimental protocols, cells were seeded at $1 \times 10^5$ cells/cm$^2$ and incubated for 48–72 h to obtain mature cell-cell junctions.

**Adenoviral Constructs**—Dominant negative Csk adenovirus containing a lysine to arginine substitution at position 222 (DN-Csk) was a generous gift from Dr. S. Tanaka (Faculty of Medicine, University of Tokyo) (19). A pUSEamp vector containing CDNA encoding caSrc containing an Y527A mutation was purchased from Upstate Biotechnology. The coding sequence was removed from pUSEamp using NotI and EcoRV and inserted into the pShuttle-ires-hrGFP-1 vector (Stratagene). Adenovirus was then produced using the pAdEasy system as described by He et al. (20). All infections were accompanied by a control GFP and/or β-galactosidase infection with a multiplicity of infection at or above the greatest multiplicity of infection used in the experimental groups.

**VEGF Treatments**—HDMECs were seeded at confluence ($10^5$ cells/cm$^2$) and incubated for 3 days to allow the formation of a mature monolayer. Then, cells were washed and serum-starved for 18 h (in EBM-2 medium with 0.3% fetal bovine serum with no additional factors). Then, cells were treated with 50 ng/ml VEGF-165 (R&D Systems) or vehicle only (0.1% bovine serum albumin in PBS) for the time indicated for the particular experiment.

**Electrical Cell-Substrate Impedance Sensor (ECIS)**—Monolayer permeability was determined by measuring changes in electrical resistance using an electric cell-substrate impedance sensor (ECIS, Applied BioPhysics, Inc.) (21) as published previously (3, 22). Briefly, HDMECs (80,000 cells) were seeded onto ECIS 10E cultureware (0.8 cm$^2$/well) precoated with 0.1% gelatin and incubated for 3 days. The electrical impedance across the monolayer was measured at 1 V, 4000 Hz with current flowing through 10 small gold electrodes per well plus one large counterelectrode, using the culture medium as the source of electrolytes. Impedance was monitored by the lock-in amplifier, stored, and then used to calculate resistance and capacitance by the manufacturer’s software. Data are presented as a plot of resistance versus time as this best represents the barrier provided by endothelial cell-cell junctions.

**Gel Electrophoresis and Immunoblotting**—Cells growing on 6-well plates were scraped after lysis with 250 μl of Laemmli buffer containing Complete protease inhibitor mixture (Roche Applied Science), PhosSTOP phosphatase inhibitor mixture (Roche Applied Science), and 0.1 mM pivanate and boiled. A total of 20 μl of cell lysate per lane was loaded on standard SDS-PAGE gels and transferred to nitrocellulose membranes. Immunoblots were performed by blocking the membranes with 3% skim milk in PBS containing calcium and magnesium (PBS+) and incubating overnight at 4°C with any of the following antibodies: anti-VE-cadherin (intracellular domain, Santa Cruz Biotechnology), anti-VE-cadherin (extracellular domain, Strategic Diagnostics Inc.), anti-pTyr-658 VE-cadherin (Chemicon), anti-p-Tyr-685 VE-cadherin (ECM Biosciences), anti-p-Tyr-731 VE-cadherin (Chemicon), anti-p120 (S-19, Santa Cruz Biotechnology), anti-β-catenin (BD Biosciences), anti-Src (GD11, Upstate Biotechnology), anti-p-Tyr-416 Src (Cell Signaling), anti-p-Tyr-527 Src (Cell Signaling), anti-p-Tyr-731 VE-cadherin (Chemicon), anti-p120 (S-19, Santa Cruz Biotechnology), anti-β-catenin (BD Biosciences), anti-Src (GD11, Upstate Biotechnology), anti-p-Tyr-416 Src (Cell Signaling), anti-p-Tyr-527 Src (Cell Signaling),
anti-Csk (C-20, Santa Cruz Biotechnology), anti-paxillin (349, BD Biosciences), anti-p-Tyr-118 paxillin (Cell Signaling), anti-FAK (A-17, Santa Cruz Biotechnology), anti-p-Tyr-861 FAK (BIOSOURCE), or anti-pTyr (4G10, Upstate Biotechnology). Secondary anti-mouse, anti-rabbit, or anti-goat antibodies conjugated with peroxidase (Jackson ImmunoResearch Laboratories) were incubated for 1 h at room temperature. Membranes were developed using SuperSignal West Pico or Femto chemiluminescent substrate (Pierce) and Fujifilm LAS-3000 imaging system (Fujifilm).

**Immunoprecipitation**—Cells were scraped from 10-cm plates in 1 ml of immunoprecipitation buffer (0.1 M NaCl, 0.3 M Sucrose, 30 mM MgCl2, 10 mM PIPES, 0.5 mM EDTA, 0.1% Nonidet P-40) containing complete protease inhibitor mixture (Roche Applied Science), PhosSTOP phosphatase inhibitor mixture (Roche Applied Science), and 0.5 mM pervanadate. After clearing by centrifugation, an aliquot was stored for total cell lysis control, and the rest was divided evenly into two tubes for incubation with the specific antibody (5 μg/tube, 30 min at 4 °C, sources described above) and protein A- or protein G-agarose beads (50 μL/tube, 90 min at 4 °C, Santa Cruz Biotechnology) or beads alone. After washes with immunoprecipitation buffer, the tubes were centrifuged and boiled in 60 μL of 2× Laemmli buffer containing Complete protease inhibitor mixture (Roche Applied Science), PhosSTOP phosphatase inhibitor mixture (Roche Applied Science), and 0.1 mM pervanadate. The samples were then processed for immunoblotting as described above.

**Immunofluorescence Microscopy**—Immunofluorescence studies were performed by seeding 80,000 cells on Biocoat 8-well glass culture slides (BD Falcon) precoated with 0.1% gelatin. Three days after seeding, cells were infected with adenovirus to express β-galactosidase, GFP, DN-Csk, or caSrc or treated as indicated. Cells were then fixed with 4% p-formaldehyde (USB Corp.) in PBS for 30 min at 4 °C, washed twice with PBS, and processed for immunofluorescence at room temperature. Briefly, cells were permeabilized with 0.1% Triton X-100 (Sigma) in Tris-buffered saline for 15 min, treated with Image-iT FX signal enhancer (Invitrogen) for 30 min and then blocked with 5.5% bovine serum in Tris-buffered saline containing 0.1% Triton X-100. Antibodies (described above) were incubated for 2 h at room temperature. Secondary anti-goat, anti-rabbit, or anti-mouse antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 (Molecular Probes), or Cy2 (Jackson ImmunoResearch Laboratories) were incubated for 1 h at room temperature in the presence or absence of Alexa Fluor 488- or Alexa Fluor 594-conjugated phalloidin and 4′,6-diamidino-2-phenylindole (Molecular Probes). Slides were mounted using Gel Mount aqueous mounting medium (Biomed Corp.). Images were obtained using a Zeiss Axio Observer Z1 inverted microscope with the Apotome module and Zeiss AxioVision software.

**Statistical Analyses**—ECIS data were analyzed by two-way analysis of variance of repeated measurements and post hoc Bonferroni tests against GFP-infected cells. Error bars show S.D. (n = 3) in Figs. 1, 2, 4, 5, and 7. An α < 0.05 was considered statistically significant.

**RESULTS**

There is controversy in the literature with respect to which tyrosine residues of VE-cadherin are phosphorylated by VEGF treatment (14, 15). Therefore, we first wanted to define which tyrosine residues of VE-cadherin were phosphorylated by VEGF in cultures of HDMECs and to determine whether the tyrosine phosphorylation was temporally associated with VEGF-induced changes in the integrity of the endothelial monolayer. As shown in Fig. 1, the addition of VEGF to a mono-
layer of HDMECs resulted in a rapid increase in VE-cadherin phosphorylation on three different sites, namely, Tyr-658, Tyr-685, and Tyr-731. Furthermore, the increase in VE-cadherin tyrosine phosphorylation was temporally associated with the VEGF-induced decrease of resistance across the HDMEC monolayer. As expected, the VEGF-induced loss of monolayer barrier function and VE-cadherin phosphorylation was dependent on Src or other SFK activity because it was completely blocked by pretreatment with 2 μM PP1 (Fig. 1).

Together, these results suggest that SFK-dependent tyrosine phosphorylation of VE-cadherin may be required for VEGF-induced loss of barrier function in HDMECs. Nevertheless, VEGF may induce the activation of other signaling pathways that may also lead to increased permeability. To better understand the regulation of endothelial monolayer permeability in the absence of other confounding signals induced by inflammatory mediators or growth factors, we initiated Src activation by expressing DN-Csk. Adenoviral delivery of the DN-Csk plasmid produced a dose- and time-dependent increase in DN-Csk expression and an increase in the total phosphotyrosine content, consistent with activation of Src by DN-Csk. Adenoviral delivery of the DN-Csk plasmid produced a dose- and time-dependent increase in DN-Csk expression and an increase in the total phosphotyrosine content, consistent with activation of Src by DN-Csk (Fig. 2, A and B). DN-Csk-induced tyrosine phosphorylation was severalfold stronger than that induced by VEGF treatments (see supplemental Fig. 1). To confirm Src activation, we established that there was a decrease in the phosphorylation of Tyr-527 (Fig. 2B) and an increase in the phosphorylation of Tyr-416 in the endogenous Src (see Figs. 3A and 4A). In addition, phosphorylation of paxillin at Tyr-118 and of FAK at Tyr-861 was also increased, consistent with an increase in the activation of Src family kinases (supplemental Fig. 6). Surprisingly, DN-Csk expression did not induce a decrease in barrier function, as assessed by changes in trans-endothelial electrical resistance (TEER). As shown in Fig. 2C, doses...
of adenovirus shown to induce Src activation in panels A and B did not decrease the resistance across the endothelial monolayer, as would be expected with a loss of barrier function. Importantly, DN-Csk expression failed to increase endothelial monolayer permeability, although it promoted SFK activation at the sites of cell-cell contact, as shown by immunofluorescence to phosphorylated Tyr-416 Src (Fig. 3A). Consistent with the ECIS data, cell-cell junctions can still be observed at cell-cell contacts, as assessed by VE-cadherin labeling (Fig. 3B). However, we did observe a change in the morphology of the VE-cadherin-mediated junction, as characterized by an increase in cell overlap with a net-like structure of labeled VE-cadherin at these overlapping regions (Fig. 3B and supplemental Fig. 5).

We next tested whether the overexpression of caSrc resulted in a loss of barrier function. We detected caSrc expression in HDMECs starting at 6–8 h after adenoviral infection (Fig. 4A). Expression of caSrc promoted total tyrosine phosphorylation to levels that were comparable with those achieved with DN-Csk expression. As opposed to DN-Csk expression, caSrc induced a marked decrease in barrier function (Fig. 4B). Interestingly, the decrease in TEER occurred between 12 and 16 h, whereas the increase in active Src was observed as early as 8 h after infection with the caSrc construct, a time frame similar to that of DN-Csk (Fig. 4A, pY416 Src). Consistent with the decrease in TEER, expression of caSrc produced large gaps between cells (supplemental Fig. 2).

The difference in altering barrier response following DN-Csk and caSrc expression led us to test whether there was a difference in the localization of active Src following expression of these two constructs. As shown in Fig. 3A, DN-Csk expression induced SFK activation that was localized at the cell-cell borders, whereas caSrc expression induced phospho-Tyr-416 staining that was more widely distributed and not restricted to cell periphery (supplemental Fig. 2). This difference was not due to total Csk or Src localization because we observed that DN-Csk and caSrc virus induced Csk and Src expression, respectively, that localized throughout the cell (supplemental Fig. 3). The appearance of gaps between caSrc-infected cells led us to test whether caSrc induced changes in the actin cytoskeleton in these cells. Consistent with the relocalization of active Src, caSrc induced a drastic rearrangement of actin stress fibers (supplemental Fig. 4). These fibers were shorter and thicker and appeared to be bundled together. Contrary to caSrc expression, DN-Csk-expressing cells did not show any overt actin reorganization when compared with control cells (supplemental Fig. 4).

Because VE-cadherin phosphorylation may participate in changes in endothelial cell barrier function, we tested whether differences in tyrosine phosphorylation of VE-cadherin in cells expressing either DN-Csk or caSrc could explain differences in the regulation of barrier function by these two constructs. As shown in Fig. 5, both caSrc and DN-Csk expression induced VE-cadherin phosphorylation on Tyr-658 and Tyr-731, whereas only DN-Csk expression promoted Tyr-685 phosphorylation. Time course experiments showed a close relationship between SFK activation and VE-cadherin tyrosine phosphorylation (compare Fig. 4A with Fig. 5A). We detected VE-cadherin phosphorylation as early as 6 h after adenoviral infection of either DN-Csk-containing or caSrc-containing virus. Tyrosine phosphorylation of VE-cadherin was increased at both 8 and 12 h after infection with caSrc adenovirus; however, there was no change in TEER until 15 h after caSrc infection. Thus, the increase in VE-cadherin tyrosine phosphorylation was not temporally associated with decrease in TEER.

We also observed that in most cases, caSrc expression induced a stronger Tyr-658 and Tyr-731 VE-cadherin phosphorylation level than DN-Csk, which suggested that maybe DN-Csk infection was not inducing a level of phosphorylation sufficient to promote the loss of barrier function in these cells. To determine whether increased levels of tyrosine phosphorylation of VE-cadherin would induce changes in permeability, we infected endothelial monolayers with higher levels of adenovirus to increase the amount of expressed DN-Csk. In addi-
Src Activity in Endothelial Barrier Function

It has been suggested that VE-cadherin phosphorylation may reduce its ability to bind to p120 and β-catenin at the adherens junction complex. Thus, we determined whether the changes in VE-cadherin phosphorylation induced by DN-Csk expression altered VE-cadherin attachment to catenins. Co-localization of VE-cadherin with p120 in areas of cell-cell overlap suggests that there is an intact adherens junction complex in DN-Csk-expressing cells (Fig. 6A), consistent with the lack of induction of permeability by this construct. Confocal microscopy images showed that VE-cadherin staining at the cell-cell overlap regions is in the same plane as the VE-cadherin located at the cell-cell borders, suggesting that this overlap may be due to thin cell extensions (supplemental Fig. 5A). We also observed that VE-cadherin additionally co-localized at the cell borders with β-catenin and γ-catenin (supplemental Fig. 5B). Furthermore, by immunoprecipitation of VE-cadherin followed by immunoblotting for either p120 or β-catenin, we found that both p120 and β-catenin are associated with VE-cadherin in cells expressing DN-Csk despite VE-cadherin phosphorylation (Fig. 6B). Although gaps were found between EC monolayers expressing caSrc, β-catenin and p120 could also be co-immunoprecipitated with VE-cadherin in lysates from these cells (Fig. 6B), and p120, β-catenin, and γ-catenin were all co-localized at the cell-cell junction (supplemental Fig. 5).

We then tested the possibility that DN-Csk overexpression may sequester important proteins required to promote an increase in monolayer permeability, thus preventing SFK-mediated loss in barrier function. If this was the case, then Csk knockdown would not only induce SFK activation but also a permeability increase. Electroporation of siRNAs specific to Csk promoted VE-cadherin phosphorylation at tyrosines Tyr-658 and Tyr-731 but not at Tyr-685 (Fig. 7A). Nevertheless, the same siRNAs did not prevent the monolayer from forming a restrictive monolayer, as assessed by TEER (Fig. 7B), demonstrating that SFK activation alone is not sufficient to alter barrier function.

Baumeister et al. (23) reported that Csk binds to VE-cadherin in a Tyr-685-dependent fashion. Furthermore, the same group proposed that Csk binding to VE-cadherin may prevent the access of phosphatases to tyrosine 685. Consistent with this finding, overexpression of DN-Csk, but not Csk knockdown, induced VE-cadherin Tyr-685 phosphorylation (Fig. 7A). Regardless of the method employed to activate Src, the subsequent increase of VE-cadherin tyrosine phosphorylation was dependent on continuous SFK activity because the addition of PP1 Src inhibitor for 6 h reversed DN-Csk- or caSrc-induced VE-cadherin phosphorylation in cells overexpressing either DN-Csk or caSrc (Fig. 8). PP2, another pharmacological inhibitor, also prevented caSrc-induced TEER and the phosphorylation of VE-cadherin (supplemental Fig. 6).

A VE-cadherin-Csk complex has been postulated to serve as a scaffold for other signaling molecules. In fact, VE-cadherin and Csk are required for VEGF-mediated Akt phosphorylation (5). Supporting this notion, we observed that DN-Csk, but not caSrc, induced the phosphorylation of Akt (Fig. 8). Interestingly, this phosphorylation was not completely dependent on SFK activity, a finding consistent with a role for Csk as a scaffolding protein. On the other hand, caSrc, but not DN-Csk, promoted a mild increase in Erk1/2 phosphorylation that was
dependent on SFK activity. Inhibition of either Akt or Erk activation by pretreatment with the pharmacological inhibitors LY294002 or U0126 did not have any effect on TEER nor showed any modulation of TEER in the presence of DN-Csk or caSrc (supplemental Fig. 7).

DISCUSSION

Previous studies have demonstrated that Src signaling is required for receptor-induced increase in vascular permeability (6, 24, 25). We confirmed this requirement in HDMECs as the Src inhibitor PP1 was able to prevent VEGF-induced changes in permeability. In addition, the decrease in permeability was temporally associated with an increase in phosphorylation of VE-cadherin on three tyrosine residues that have been implicated in the regulation of endothelial barrier function. Nevertheless, receptor activation by VEGF, as well as other inflammatory mediators and growth factors, is known to stimulate many different signaling pathways in parallel (26), raising the question of whether Src signaling was not only required but also sufficient to regulate endothelial monolayer permeability. To answer this question, we used three systems to directly increase activity of SFKs independent of receptor activation.

Src is the prototypical member of a family of tyrosine kinases with a high degree of conservation (27). Inhibition of Csk activity by either gene knockdown or expression of a dominant negative construct drastically promotes SFK signaling (28). Interestingly, expression of Csk in endothelial cells increases VE-cadherin phosphorylation (23), and Csk interaction with VE-cadherin has been implicated in VEGF signaling and endothelial cell function (5). Surprisingly, we found that Src activation and the subsequent tyrosine phosphorylation of VE-cadherin following expression of dominant negative Csk or by decreasing the levels of Csk did not result in an increase in endothelial permeability as assessed by changes in TEER.

FIGURE 5. DN-Csk expression and downstream SFK signaling induce VE-cadherin phosphorylation but do not induce change in cell permeability. A, HDMECs were infected with adenovirus to overexpress GFP, DN-Csk, or caSrc and lysed 6–12 h later. Cell lysates were assayed by Western blot for VE-cadherin phosphorylation at tyrosines 658 (pY658), 685 (pY685), and 731 (pY731). Total VE-cadherin blots served as loading control. B, confluent HDMEC monolayers were infected with 6.8 × 10⁸ pfu/ml GFP virus, 7.2 × 10⁹ pfu/ml DN-Csk virus (labeled DN-Csk), 2.88 × 10⁹ pfu/ml DN-Csk virus (labeled 4X DN-Csk), or 3.2 × 10⁹ pfu/ml caSrc virus and lysed 24 or 48 h later. Lysates were immunoblotted for VE-cadherin phosphorylation, phospho-Tyr-118 paxillin, or Csk to document infection levels. Total VE-cadherin and -actin blots served as loading controls; NI, non-infected. C, HDMEC cells were infected with adenovirus to express GFP or increasing amounts of DN-Csk at time 0. Monolayer resistance was measured by ECIS at 4000 Hz. D, HDMEC cells were infected with adenovirus to express GFP or different amounts of caSrc at time 0. Monolayer resistance was measured as above. A two-way repeated measures analysis of variance was performed with Bonferroni post tests versus GFP-infected wells values (n = 3). No significant differences were observed at any DN-Csk infection amount at any time point. Results are representative of three independent experiments. Error bars show S.D. (n ≥ 3).
This finding suggests that Src activity may be required for mediator-induced increases in permeability but that an increase in Src-induced VE-cadherin tyrosine phosphorylation is not sufficient to decrease endothelial barrier function.

In contrast to activation of Src through a loss of Csk activity, forced expression of caSrc resulted in a decrease in barrier function. The discrepancy between caSrc and DN-Csk expression effects may lie in the mode of Src activation by these constructs. Although DN-Csk expression induces activation of endogenous SFKs, expression of caSrc does not depend on the endogenous SFKs but instead provides active Src throughout the cell. DN-Csk, however, would promote the activation of SFK at their normal subcellular localization. Thus, the induction of endothelial cell permeability in caSrc-expressing cells may be due to the activation of Src signaling at sites where activated Src would not typically be found in the cell. This is supported by immunofluorescence data showing that in DN-Csk-expressing cells, active (pTyr-416) Src is localized at the plasma membrane at the sites of cell-cell contact, whereas in caSrc-expressing cells, active Src is localized mainly at intracellular compartments, rather than just at cell-cell borders.

Alternatively, the observed changes in TEER with forced expression of caSrc may not be a direct effect of protein phosphorylation but may involve changes in gene expression that are not found with DN-Csk expression. This is supported by the data showing a 4-6 h delay between increases in protein tyrosine phosphorylation, including VE-cadherin, and the decrease in TEER induced by expression of caSrc. Although changes in gene expression may participate in the loss of TEER, this is not mediated by activation of Erk1/2 as inhibition of Erk1/2 phosphorylation by the inhibitor U0126 did not prevent the decrease in TEER following caSrc expression.

Using synthetic peptides of the VE-cadherin cytoplasmic domain, Wallez and co-workers (29) demonstrated that Tyr-685 of VE-cadherin can be phosphorylated by Src. Furthermore, these investigators demonstrated that VEGF increased phosphorylation of Tyr-685 in human umbilical vein endothelial cells. We observed that the phosphorylation of Tyr-685 in HDMECs treated with VEGF was temporally associated with a decrease in TEER. We also observed an increase in Tyr-685 phosphorylation when Src was activated by expression of DN-Csk. This is consistent with the findings of Baumeister et al. (23), who found increases in VE-cadherin phosphorylation in CHO cells coexpressing VE-cadherin and either DN-Csk or wild type Csk. Our findings that Tyr-685 is not phosphorylated upon siRNA-mediated Csk knockdown supports the concept that Csk presence is required for VE-cadherin phosphorylation at Tyr-685. These results also show that Src activation in the absence of Csk is not sufficient to phosphorylate Tyr-685, suggesting that Tyr-685 phosphoryla-

FIGURE 6. DN-Csk and caSrc overexpression does not prevent VE-cadherin binding to p120 and β-catenin or co-localization to the adherens junctions. A, HDMECs were infected with adenovirus to overexpress GFP, DN-Csk, or caSrc and fixed 24 h later. Cells were then stained with antibodies against p120 (green) and VE-cadherin (red). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) (blue). The white arrows point to the sites of cell-cell overlapping, which still showed p120 and VE-cadherin co-localization. B, HDMECs were infected with adenovirus to overexpress GFP, DN-Csk, or caSrc and lysed 16 h after infection. Immunoprecipitates (IP) of p120, β-catenin (β-cat), or VE-cadherin (VE-cad) were run on SDS-PAGE together with beads only precipitates (IP Ctrl) and total cell lysates (TCL) and immunoblotted as described in the figure. β-actin was used as loading control in the total cell lysates, whereas Src and Csk immunoblots served as infection controls. Results are representative of three independent experiments.
tion requires events other than Src activation. This is further supported by our data showing that Tyr-685 is not phosphorylated with forced expression of caSrc. However, the increase in Tyr-685 phosphorylation by expression of DN-Csk was inhibited by PP1 or PP2, showing that Src activation is indeed required for VE-cadherin phosphorylation on this tyrosine residue. This finding supports the model that SFK can phosphorylate Tyr-685 but that Csk may serve to inhibit dephosphorylation of Tyr-685 by phosphatases. This is consistent with the hypothesis that VEGF-induced phosphorylation of Tyr-685 requires Csk-VE-cadherin interaction (5).

Our findings that caSrc did not increase Tyr-685 phosphorylation are not consistent with those of Wallez et al. (15), who found increased Tyr-685 phosphorylation with expression of caSrc in CHO cells expressing VE-cadherin. However, our findings that overexpression of caSrc in endothelial cell monolayers increases both Tyr-658 and Tyr-731 phosphorylation are consistent with the findings of Potter et al. (14), who demonstrated that these are the two primary tyrosine residues in VE-cadherin that are phosphorylated when caSrc is expressed in CHO cells.

SFK-mediated TEER induction. Nevertheless, this hypothesis is not compatible with two other observations: 1) that the siRNA-mediated knockdown of Csk does not induce the phosphorylation of Tyr-685 nor promote TEER and 2) that VEGF-mediated increase in TEER is concurrent with an increase in VE-cadherin phosphorylation at Tyr-685.

**FIGURE 7.** siRNA-mediated Csk knockdown promotes Tyr-658 (pY658) and Tyr-731 (pY731) but not Tyr-685 (pY685) VE-cadherin phosphorylation and does not increase monolayer permeability. A, HDMEC cells were electroporated with 2 μg of luciferase (Luc)- or Csk-specific siRNAs, and 72 h later, Csk expression and VE-cadherin phosphorylation were assessed by Western blot. Total VE-cadherin blots served as loading control. B, after siRNA electroporation, HDMEC cells were seeded onto ECIS chambers for monolayer resistance monitoring. Notice the increase in resistance after seeding, due to cell spreading, proliferation, and cell-cell contact maturation, that reaches a plateau after ~96 h after seeding. Csk-siRNA-carrying cells show the same increase in resistance as control cells. Error bars show S.D. (n = 3).

**FIGURE 8.** DN-Csk- and caSrc-induced signaling depends on continuous SFK activity. HDMEC cells were infected with adenovirus to overexpress GFP, DN-Csk, or caSrc for 18 h and maintained in EBM-2 medium with 0.3% fetal bovine serum and no other added factors. Cells were then incubated for 6 h in the presence of 10 μM PP1 or 0.1% DMSO prior to lysis and Western blot analysis to assess VE-cadherin phosphorylation (A) and Erk and Akt activation (B). Total VE-cadherin, total Erk, and total Akt blots served as loading control. Results are representative of at least three independent experiments. pY658, phospho-Tyr-658; pY685, phospho-Tyr-685; pY731, phospho-Tyr-731; pErk1/2, phospho-Erk1/2; pAkt, phospho-Akt.
also expressing VE-cadherin. We show that Tyr-658 and Tyr-731 of VE-cadherin are also phosphorylated when Src is activated by expression of DN-Csk or knockdown of Csk. We also found increases in Tyr-658 and Tyr-731 phosphorylation following VEGF treatment of HDMEC monolayers. These two sites have also been shown to be phosphorylated in response to intercellular adhesion molecule-1 (ICAM-1) ligation (16) and to require Src activation.

A number of recent studies have begun to use the phenylalanine substitutions at the different tyrosine residues in VE-cadherin as a method to investigate the role of VE-cadherin phosphorylation in the regulation of cell function. Studies using VE-cadherin-expressing CHO cells have shown that substitution of Y685F prevented contact inhibition of growth (23). Potter et al. (14) showed that substitution of Y658F and Y731F prevented vanadate-induced decreases in the barrier function of CHO cells expressing mutant forms of VE-cadherin. These investigators also demonstrated that CHO cells expressing mutant VE-cadherin with glutamic acid substitutions of Tyr-658 or Tyr-731 did not form a restrictive barrier when compared with CHO cells expressing wild type VE-cadherin. The study of Potter et al. (14) supports the hypothesis that tyrosine phosphorylation of VE-cadherin following treatment with inflammatory mediators results in a loss of barrier function, and many of these studies have implicated Src as a major signaling molecule in the pathway leading to VE-cadherin phosphorylation and a loss of barrier function (reviewed in Refs. 30 and 31).

In contrast, our data demonstrate that VE-cadherin tyrosine phosphorylation is not sufficient to decrease the barrier function of endothelial cell monolayers. Indeed, Src activation by expression of DN-Csk or knockdown of Csk resulted in the phosphorylation of VE-cadherin on Tyr-658 and Tyr-731 but did not change TEER across the monolayers. Although caSrc resulted in a decrease in resistance, this change occurred well after the phosphorylation of Tyr-658 and Tyr-731 was observed (6–8 h for phosphorylation versus 12–15 h for decreased TEER). Thus, the tyrosine phosphorylation of VE-cadherin following caSrc expression is not temporally associated with the decrease in barrier function. The discrepancy between our findings and those of Potter et al. (14) may be the result of the different cell types as previous investigations have demonstrated that cell context is important between CHO cells and endothelial cells with respect to VE-cadherin and cell signaling (32). It is also possible that in HDMECs, the pool of active Src induced by DN-Csk expression or siRNA-mediated Csk knockdown is not capable of activating a VAV2-Rac-p21 activated kinase pathway that may result in the phosphorylation of serine 665 of VE-cadherin, binding to β-arrestin and VE-cadherin endocytosis (33). However, we did not see an increase in VE-cadherin endocytosis in our fluorescence experiments, even after caSrc overexpression, suggesting that this pathway may not be essential in this model. Also, it is possible that other GTPases regulating permeability may be differentially regulated (see Ref. 34 and references therein).

Surprisingly, we also did not find a change in the association of VE-cadherin to either β-catenin or p120. Activation of SFK signaling resulted in phosphorylation of VE-cadherin and p120, although these phosphorylations did not prevent the co-localization of adherens junction proteins at the cell-cell junction or the ability to co-immunoprecipitate VE-cadherin, p120, and β-catenin. Moreover, although caSrc expression induced a marked increase in permeability, co-localization, and co-immunoprecipitation of VE-cadherin, p120 and β-catenin were not impaired, suggesting that even in extreme circumstances, the adherens junction protein complex may be intact.

Both Akt and Erk were activated by VEGF treatments in HDMECs. DN-Csk expression induced Akt activation, whereas caSrc expression promoted Erk activation. Nevertheless, neither LY294002 nor U0126 treatments were able to modulate TEER in these conditions. This suggests that phosphatidylinositol 3-kinase-Akt and mitogen-activated protein kinase/Erk kinase (MEK)-Erk may be parallel pathways not involved in TEER or that the activation of the pathways upstream of these kinases, rather than the endpoints tested, are responsible for the changes in barrier function.

Our data show that Src-induced VE-cadherin phosphorylation at Tyr-658, Tyr-685, and Tyr-731 is not sufficient to increase barrier function. Furthermore, our results suggest that VE-cadherin phosphorylation is not always followed by junction disassembly. This does not mean that VE-cadherin phosphorylation is not required for mediator-induced decreases in endothelial barrier function. For example, VE-cadherin phosphorylation at Tyr-658 and Tyr-731 has been shown to be required for trans-endothelial migration of leukocytes as Tyr to Phe mutation of either Tyr-658 or Tyr-731 decreases trans-endothelial cell migration (35, 36). The phosphorylation of VE-cadherin on these residues is the result of ligation of ICAM-1 and the activation of SFK and Pyk2. These tyrosine residues may not be the only important residues phosphorylated in VE-cadherin as attachment of antigen-activated lymphocytes to ICAM-1 induced the phosphorylation of tyrosines 645, 731, and 733, a process mediated by Rho GTPase, Ca²⁺, and actin rearrangement but not by Src activation (17). In addition, it has also been shown that mutation of Tyr-658 or Tyr-731 attenuate VEGF-induced decreases in barrier function (37). These studies coupled with our results would suggest that activation of multiple signaling pathways by inflammatory mediators, including those resulting in VE-cadherin phosphorylation, may all be required to produce a change in permeability. In this model, VE-cadherin would serve a permissive role in which phosphorylation would be required for the change in permeability to occur but would not be sufficient to change permeability by itself. This is supported by our data that demonstrate that activation of a single pathway leading to VE-cadherin tyrosine phosphorylation may not be sufficient to promote disassembly of the adherens junction and suggest that further research is needed to elucidate the role of VE-cadherin phosphorylation in the regulation of endothelial barrier function.

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Src-induced Tyrosine Phosphorylation of VE-cadherin Is Not Sufficient to Decrease Barrier Function of Endothelial Monolayers
Alejandro P. Adam, Amy L. Sharenko, Kevin Pumiglia and Peter A. Vincent

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