Involvement of c-Src in diperoxovanadate-induced endothelial cell barrier dysfunction

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The endothelium functions as a semiselective barrier between the plasma and the interstitium to circulating bioactive agents, inflammatory cells, and macromolecules (6, 8, 9, 38). Maintenance of this vascular barrier represents a critical physiological process for vessel homeostasis and organ function. During acute lung injury, however, impaired barrier function leads to the exudation of fluids and proteins into the interstitium (29), alveolar flooding, and subsequent derangements in lung compliance and gas exchange, a characteristic feature of acute respiratory distress syndrome (2). Development of acute lung injury in many settings has been tightly coupled to the activation of polymorphonuclear leukocytes in the lung microvasculature, with the subsequent release of proteases, inflammatory mediators, and reactive oxygen and nitrogen intermediates (14, 15, 54). As a result of its extensive surface area, the pulmonary endothelium is a prime target for the inflammatory mediators and reactive oxygen species (ROS), resulting in cellular damage and barrier dysfunction (3, 27).

ROS generated during ischemia-reperfusion lung injury or the exogenous addition of either hydrogen peroxide ($H_2O_2$) or xanthine/xanthine oxidase to endothelial cell (EC) monolayers resulted in morphological, biochemical, and physiological perturbations such as barrier dysfunction (24, 26, 41, 44). Although the mechanism(s) of ROS-induced EC barrier dysfunction is not well understood, earlier studies (5, 7, 27, 31, 49, 54) suggested that in addition to potential ROS-induced cytotoxicity, modulation of protein kinases or phosphatases and generation of intracellular second messengers may be responsible for ROS-mediated changes in vascular permeability. Exposure of bovine pulmonary artery ECs (BPAECs) to ROS increased permeability to albumin (41) that was dependent on protein kinase C (PKC) activation (22) and increased $Ca^{2+}$ availability (39, 44). Similarly, inhibition of PKC with H-7 prevented $H_2O_2$-induced pulmonary edema in isolated perfused guinea pig lungs (20). In Madin-Darby canine kidney cells, treatment with orthovanadate or pervanadate, potent inhibitors of protein tyrosine phosphatases (PTPases), increased the levels of phosphotyrosine proteins that colocalized with adherens junction proteins, with disruption of cell junction-matrix contacts and increased tight junction permeability (28, 46). A recent study (42) in BPAECs suggested a role for protein tyrosine phosphorylation in thrombin-induced EC contraction and permeability via a non-receptor tyrosine kinase (TyK) that was sensitive to genistein. Although these studies with vanadate, pervanadate, and thrombin suggested a potential role for contractile and cytoskeletal proteins in gap formation and paracellular transport, very little is known regarding the mechanism(s) of ROS-induced endothelial barrier dysfunction and signaling pathways that regulate the contractile and tethering forces.
The aim of the present study was to determine the role of protein tyrosine phosphorylation in endothelial barrier dysfunction and to identify specific TyKs involved in the regulation of EC permeability. We employed diperoxovanadate (DPV), a potent inhibitor of PTPases and activator of TyKs (32, 37), as a model agent to investigate the mechanism(s) of endothelial barrier dysfunction. Our results in BPAECs demonstrate that DPV-mediated protein tyrosine phosphorylation is involved in permeability changes. Our data also show for the first time that the activation and tyrosine phosphorylation of the Src family of non-receptor TyKs regulate vascular permeability. Furthermore, because DPV increased the association of Src with actin binding protein, cortactin, and myosin light chain (MLC) kinase (MLCK), these proteins may represent cytoskeletal targets involved in DPV-mediated EC barrier dysfunction.

METHODS

Materials. Minimum essential medium (MEM), H2O2, sodium orthovanadate, nonessential amino acids, trypsin-EDTA, penicillin-streptomycin, and fetal bovine serum were obtained from Sigma (St. Louis, MO). BPAECs (passage 16) were purchased from American Type Culture Collection (Manassas, VA). Genistein, Brij 35 detergent (polyoxyethylene-glycol dodecyl ether), and PP-2 were obtained from Calbiochem (San Diego, CA). Endothelial cell growth supplement, affinity-purified monoclonal anti-phosphotyrosine antibody (4G10), Src cDNAs (pUSE src wild type, pUSE src activated, pUSE src kinase mutant, and empty vector), and monoclonal antibody to Src were obtained from Upstate Biotechnology (Lake Placid, NY). An enhanced chemiluminescence kit was purchased from NEN (Boston, MA). Crystallized DPV (potassium sodium orthovanadate, nonessential amino acids, trypsin-EDTA, penicillin-streptomycin, and Brij 35 detergent). DC coupled with 0.05% trypsin, resuspended in fresh medium, and cultured on either polycarbonate filters for permeability determinations (42), or 100-mm dishes for Src immunoprecipitation, were precleared with protein A/G plus agarose (20 μl) at 4°C. Protein A/G plus agarose (20 μl) was then added, incubated for an additional 2 h at 4°C, and centrifuged at 14,000 rpm in a microfuge (4°C for 5 min). Protein concentrations of the supernatants were determined with a Pierce protein assay kit. The supernatants, adjusted to 1 mg protein/ml for immunoprecipitation, were precleared with protein A/G plus agarase at 4°C for 60 min and incubated overnight with rabbit polyclonal anti-Src antibody (Santa Cruz) at 4°C. Protein A/G plus agarase (20 μl) was then added, incubated for an additional 2 h at 4°C, and centrifuged at 14,000 rpm in a microfuge for 5 min. The precipitates were washed three times with ice-cold PBS and dissociated by boiling in 1× SDS sample buffer for 5 min. The samples were then analyzed on 10% SDS-PAGE gels (52), and densitometric scanning of the blots was carried out with a Bio-Rad model GS-700 densitometer and quantified with Molecular Analyst software.

Preparation of cell lysates, immunoprecipitation, and immunoblot analysis. BPAECs grown in 100-mm culture dishes were stimulated with 5 μM DPV for various times and rinsed with ice-cold PBS to stop stimulation. ECs were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 150 mM NaCl, 10 μg/ml of apro tin, 10 μg/ml of leupeptin, and 1 μg/ml of pepstatin), scraped off the dishes, sonicated on ice with a probe sonicator (3 times for 15 s each), and centrifuged at 14,000 rpm in a microfuge (4°C for 5 min), and protein concentrations of the supernatants were determined with a Pierce protein assay kit. The supernatants, adjusted to 1 mg protein/ml for immunoprecipitation, were precleared with protein A/G plus agarase at 4°C for 60 min and incubated overnight with rabbit polyclonal anti-Src antibody (Santa Cruz) at 4°C. Protein A/G plus agarase (20 μl) was then added, incubated for an additional 2 h at 4°C, and centrifuged at 14,000 rpm in a microfuge for 5 min. The precipitates were washed three times with ice-cold PBS and dissociated by boiling in 1× SDS sample buffer for 5 min. The samples were then analyzed on 10% SDS-PAGE gels (52), and densitometric scanning of the blots was carried out with a Bio-Rad model GS-700 densitometer and quantified with Molecular Analyst software.

Measurement of EC permeability. Macromolecule permeability of albumin across cultured EC monolayers was performed as previously described (36). Briefly, the system consisted of two compartments, upper (luminal) and lower (abluminal), which were separated by a polycarbonate micro pore membrane filter (Nuclepore, Pleasanton, CA) on which the ECs were seeded to confluence. For measurement of albumin flux, the lower compartment was stirred continuously and kept at a constant temperature of 37°C by use of a thermally regulated water bath. Medium 199 with 25 mM HEPES was used in both compartments. Bovine serum albumin (4% final concentration) complexed to Evans blue (EB) dye was added to the luminal compartment, and samples were taken from the abluminal compartment at 10-min intervals for the first 60 min to establish the basal albumin clearance rate (baseline) and then for an additional 60- to 120-min period after each specific intervention. Transendothelial cell albumin transport was determined by measuring the absorbance of EB dye in abluminal chamber samples at 620 nm in a spectrophotometer (Vmax Multilplate Reader, Molecular Devices, Menlo Park, CA). Albumin clearance rates were calculated by linear regression analysis for control and experimental groups.

Measurement of transendothelial cell electrical resistance. Transendothelial cell electrical resistance was measured as described earlier (42), with minor modifications. Briefly, in this electrical cell-substrate impedance-sensing system (Applied Biophysics, Troy, NY), ECs were cultured on a small gold electrode (10 × cm2), and the culture medium was used as the electrolyte. The total electrical resistance, measured dynamically across the monolayer, was determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion, and the resistance between cells. Thus a change in electrical resistance represents a change in cell-cell adhesion and/or cell-matrix adhesion. A 1-V 4,000-Hz AC signal was supplied through a 1-MΩ resistor to approximate a constant-current source. Voltage and phase data were stored and processed with a pentium 100-MHz computer that controlled the output of the amplifier and relay switches to different electrodes. Experiments were conducted only on wells that achieved >5,000 Ω of steady-state resistance. Resistance is expressed as the inphase voltage (proportional to the resistance) that was normalized to the initial voltage and as a fraction of the normalized resistance value, similar to that previously described (42).

Preparation of cell lysates, immunoprecipitation, and immunoblot analysis. BPAECs grown in 100-mm culture dishes were stimulated with 5 μM DPV for various times and rinsed with ice-cold PBS to stop stimulation. ECs were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 150 mM NaCl, 10 μg/ml of apro tin, 10 μg/ml of leupeptin, and 1 μg/ml of pepstatin), scraped off the dishes, sonicated on ice with a probe sonicator (3 times for 15 s each), and centrifuged at 14,000 rpm in a microfuge (4°C for 5 min), and protein concentrations of the supernatants were determined with a Pierce protein assay kit. The supernatants, adjusted to 1 mg protein/ml for immunoprecipitation, were precleared with protein A/G plus agarase at 4°C for 60 min and incubated overnight with rabbit polyclonal anti-Src antibody (Santa Cruz) at 4°C. Protein A/G plus agarase (20 μl) was then added, incubated for an additional 2 h at 4°C, and centrifuged at 14,000 rpm in a microfuge for 5 min. The precipitates were washed three times with ice-cold PBS and dissociated by boiling in 1× SDS sample buffer for 5 min. The samples were then analyzed on 10% SDS-PAGE gels (52), and densitometric scanning of the blots was carried out with a Bio-Rad model GS-700 densitometer and quantified with Molecular Analyst software.

p60Src kinase activity. To evaluate Src kinase activity, p60Src immunoprecipitates were washed three times in ice-cold PBS as described in Preparation of cell lysates, immunoprecipitation, and immunoblot analysis and once in kinase assay buffer (50 mM HEPES, pH 7.5, containing 0.1 mM EDTA and 0.015% Brij 35 detergent). The kinase activity in the p60Src-immunoprecipitated complex was determined in a final reaction volume of 40 μl of 50 mM HEPES, pH 7.5, containing 0.1 mM EDTA, 0.015% Brij 35 detergent, 15 mM MgCl2, 1 mM Na3VO4, 150 μM ATP, and 33 μCi of [γ-32P]ATP
with and without raytide peptide, which acts as a substrate for Src tyrosine kinase (42). The reaction mixture was incubated at 30°C for 30 min, and the reaction was terminated by the addition of 6× Laemmli sample buffer or 10% phosphoric acid. The samples were boiled for 5 min and subjected to SDS-PAGE or spotted on P81 filter paper, washed five times with 10% phosphoric acid, and counted in a scintillation counter.

EC transfection. Src DNA plasmids (wild type, constitutively active, or dominant negative) were transfected into ECs at 50–80% confluence with the FuGENE 6 transfection reagent. The constitutively active Src cDNA carries a Tyr-to-Ala substitution at amino acid 529; the dominant negative cDNA has two point mutations, Lys-to-Arg substitution at residue 296 and Tyr-to-Phe substitution at residue 528, whereas wild-type Src cDNA encodes the wild-type p60Src. FuGENE 6 reagent (3–6 µl) was added directly into 100 µl of serum-free MEM and incubated for 5 min at room temperature, and the diluted FuGENE 6 transfection reagent was added dropwise to a tube containing Src cDNA (3 µg/ml). The contents of the tube were incubated for 15 min at room temperature, transferred to 35-mm dishes containing BPAECs (50–80% confluent), and incubated for 5 h. At the end of the transfection, the Src cDNA-FuGENE 6 complex was removed by aspiration, 2 ml of MEM containing 10% serum were added, and the cells were incubated in 95% air-5% CO2 chamber for 48 h. Protein expression was determined with Western blotting 48 h posttransfection.

Statistics. Linear regression analysis was performed for determination of clearance rates in individual wells with Epistat 2.0 public domain software, and these slopes were then averaged from at least six determinations. Paired t-test was used to compare pretreatment and posttreatment slopes within the same control membrane or BPAEC chamber. ANOVA with Student-Newman-Keuls test was used to compare means of clearance rates of two or more different treatment groups. The level of significance was taken to be \( P < 0.05 \) unless otherwise stated. Data are expressed as means ± SE.

RESULTS

DPV induced EC barrier dysfunction. \( \text{H}_2\text{O}_2 \) increased albumin flux across EC monolayers via a PKC-dependent pathway (45) and enhanced protein tyrosine phosphorylation through modulation of TyKs and PTPases (32). However, the role of protein tyrosine phosphorylation in ROS-mediated EC barrier dysfunction is poorly understood. To assess the potential involvement of protein tyrosine phosphorylation in EC barrier regulation, BPAECs were treated with either \( \text{H}_2\text{O}_2 \) (100 µM), vanadate (10 µM), or \( \text{H}_2\text{O}_2 \) (100 µM) plus vanadate (10 µM), and changes in albumin clearance were measured. \( \text{H}_2\text{O}_2 \) (100 µM) altered albumin clearance from 70 ± 15 (control) to 240 ± 34 nl/min after a 2-h challenge, representing a threefold increase in albumin flux. Interestingly, pretreatment of cells with vanadate (10 µM) had no effect on albumin clearance; however, it potentiated the albumin clearance induced by \( \text{H}_2\text{O}_2 \) from 240 to 580 nl/min (data not shown). The effect of vanadate on \( \text{H}_2\text{O}_2 \)-induced permeability change is consistent with the notion that vanadate not only acts as an inhibitor of phosphatases but reacts with \( \text{H}_2\text{O}_2 \) to generate peroxovanadium compounds like DPV (37, 40). Peroxovanadium compounds exhibit dual properties as potent activators of TyKs and inhibitors of PTPases (32, 37). We therefore investigated the effect of DPV on EC barrier function. DPV increased EB-albumin clearance (Fig. 1A) and decreased electrical resistance across EC monolayers (normalized resistance after 3 h of treatment: vehicle, 0.94 ± 0.08; 1 µM DPV, 1.36 ± 0.12; 5 µM DPV, 0.62 ± 0.09; 10 µM DPV, 0.40 ± 0.05) in a dose-dependent fashion (Fig. 1B). DPV-induced decreases in electrical resistance dropped below basal values 60 min after addition of DPV, which was consistently preceded by increases in electrical resistance. This barrier enhancement lasted for 15–30 min post-DPV challenge and was followed by substantial decreases in electrical resistance 2 h after addition of DPV. To exclude cytotoxicity as the mechanism of DPV-mediated barrier dysfunction, we determined 2-[\( ^{3}\text{H} \)]deoxyglucose release (33) after challenge with \( \text{H}_2\text{O}_2 \), vanadate, \( \text{H}_2\text{O}_2 \) plus vanadate, and DPV. The cytotoxic index, expressed as percent of control value, ranged from 2.8 to 9.9% with exposure to various ROS, indicating minimal cytotoxicity (data not shown).

TyK inhibition attenuated DPV-induced barrier dysfunction. Because DPV modulated TyK and PTPase activities in ECs (33), we examined the effect of TyK
inhibitors on DPV-induced protein tyrosine phosphorylation and EC barrier function. Genistein (100 μM), an inhibitor of both receptor and non-receptor TyKs, partially attenuated both basal and DPV-induced albumin clearance and electrical resistance (Fig. 2). Interestingly, herbimycin, which is known to block Src kinases (21), also partially blocked the DPV-mediated electrical resistance seen after 2 h (Fig. 2B). We next examined the effect of PP-2, a more specific inhibitor of the Src family of non-receptor TyKs (13), on DPV-induced barrier dysfunction. PP-2 (1 μM) attenuated DPV-induced albumin clearance (by ~50%) but had no significant effect on basal albumin clearance (Fig. 3). Similarly, PP-2 attenuated DPV-induced EC electrical resistance in a dose- and time-dependent manner (Table 1). In independent experiments, no change in EC morphology or cytotoxicity was observed after PP-2 (1–50 μM) treatment (data not shown). These data strongly suggest the involvement of the Src family of non-receptor TyKs in DPV-induced EC barrier dysfunction.

DPV increased Src activation. DPV (5 μM) enhanced protein tyrosine phosphorylation in a time-dependent manner as evidenced by phosphotyrosine immunofluorescence and Western blot analysis of total EC lysates (12, 34). To further study the effect of DPV on activation of specific TyKs, BPAECs were challenged with vehicle or DPV (5 μM) for varying time periods; Src immunoprecipitates from control or DPV-treated cell lysates were subjected to SDS-PAGE, and Src activation was determined by tyrosine phosphorylation, autophosphorylation with [γ-32P]ATP, or phosphorylation of raytide peptide substrate. Increased Src autophosphorylation was observed as early as 2 min after DPV treatment and returned to near basal level by 30 min, whereas increased Src protein tyrosine phosphorylation was sustained beyond 30 min (Fig. 4). Densitometric analysis of the pooled data from three experiments showed 2.6- and 10-fold increases in Src autophosphorylation and raytide peptide phosphorylation of

| Table 1. Dose-dependent response of PP-2 on DPV-induced decrease in electric resistance |
|-------------------------------|----------------------|
| Treatment | Normalized Resistance |
| Vehicle | 0.90 ± 0.10 |
| DPV (5 μM) | 0.84 ± 0.03* |
| PP-2 (1 μM) | 0.82 ± 0.07 |
| PP-2 (1 μM) + DPV | 0.49 ± 0.07 |
| PP-2 (10 μM) | 0.67 ± 0.06* |
| PP-2 (10 μM) + DPV | 0.72 ± 0.05† |
| PP-2 (50 μM) | 0.60 ± 0.06* |
| PP-2 (50 μM) + DPV | 0.75 ± 0.05† |

Values are means ± SE of 3 independent experiments 3 h after diperoxovanadate (DPV) challenge. Bovine pulmonary artery endothelial cells grown on gold microelectrodes were pretreated with medium 199 (vehicle) or PP-2 for 1 h before addition of 5 μM DPV. Changes in transendothelial electrical resistance were measured over a 3-h period as described in METHODS. *Significantly different from vehicle, P < 0.05. †Significantly different from DPV treatment, P < 0.01.
was observed as early as 2 min of DPV treatment (Table 2). SDS-PAGE of Src immunoprecipitates followed by Western blotting with Src antibody indicated almost equal loading on the gels (Fig. 4). These results show that DPV rapidly increases Src activity and tyrosine phosphorylation.

We next determined whether the barrier protective effect elicited by PP-2 was linked to modulation of Src kinase activation. PP-2 (1–50 μM) attenuated DPV-induced increases in protein tyrosine phosphorylation of total EC proteins (Fig. 5A), tyrosine phosphorylation of Src and Src autophosphorylation (Fig. 5B). Densitometric analysis of the data from three independent experiments showed that PP-2 (1 μM) treatment substantially and significantly attenuated the DPV-induced Src activation (Fig. 5C). These results are consistent with a mechanistic link between DPV-induced Src activation and EC barrier dysfunction.

To further determine the specificity of PP-2 on DPV-mediated EC barrier dysfunction, we investigated the effect of PP-2 on DPV- and phorbol 12-myristate 13-acetate (PMA)-induced decreases in electrical resistance. Treatment of BPAECs with DPV (5 μM) or PMA (100 nM) for varying time periods (60–180 min) decreased electrical resistance (Fig. 6). Pretreatment of cells with PP-2 (1 μM) had no effect on the PMA-mediated decrease in electrical resistance; however, the DPV-induced EC barrier dysfunction was partially attenuated (normalized resistance after 3 h of treatment: vehicle, 1.14 ± 0.09; 5 μM DPV, 0.72 ± 0.12; 100 nM PMA, 0.77 ± 0.04; 1 μM PP-2, 0.94 ± 0.06; 1 μM PP-2 plus 5 μM DPV, 1.06 ± 0.14; 1 μM PP-2 plus 100 nM PMA, 0.64 ± 0.07). Similarly, PP-2 (50 μM) showed no effect on PMA-mediated barrier dysfunction (data not shown). Under similar experimental conditions, the PMA-induced electrical resistance was abolished by pretreating cells with a known PKC inhibitor, bisin-dolylmaleimide (data not shown). These results suggest that the protective effect of PP-2 on EC barrier dysfunction is agonist specific.

**Src overexpression altered DPV-induced EC barrier dysfunction.** We next examined DPV-induced changes in electrical resistance in ECs transiently overexpressing either a wild-type Src construct, a constitutively active Src mutant, or a dominant negative Src mutant and compared the results to empty vector-transfected cells. The Src expression level as determined by Western blotting compared with vector alone or endogenous p60Src indicated a significant increase in expression (Fig. 7A). Interestingly, as indicated by the manufacturer (Upstate Biotechnology), in the transfected cells, the expressed Src protein exhibited a retarded mobility on SDS-PAGE compared with native p60Src (Fig. 7A). The kinase activity present in Src immunoprecipitates from cells transfected with the dominant negative Src mutant was much lower than the activity present in the wild-type Src or the constitutively active mutant of Src-transfected ECs (Fig. 7A). Also, DPV-induced tyrosine phosphorylation of Src was lower in ECs expressing dominant negative Src mutant, whereas wild-type Src and constitutively active Src increased DPV-mediated tyrosine phosphorylation (data not shown).

Next, we examined the effects of transient expression of wild-type Src, constitutively active Src, and dominant negative Src on DPV-induced changes in EC electrical resistance. As shown in Fig. 7B, expression of dominant negative Src attenuated DPV-induced electrical resistance compared with vector alone-transfected cells, whereas monolayers overexpressing wild-type Src or constitutively active Src exhibited a higher decrease in electrical resistance compared with basal or DPV treatment (normalized resistance after 3 h: vector, 1.17 ± 0.14; 5 μM DPV, 0.63 ± 0.13; wild-type Src, 0.14; 50 μM PP-2, 0.94; 100 μM PP-2 plus 100 μM PP-2, 0.94).

**Table 2. Effect of DPV on Src activation**

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<tr>
<th>DPV Treatment, min</th>
<th>Src Tyrosine Phosphorylation, %control</th>
<th>Src Kinase Activity, %control</th>
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Confluent bovine pulmonary artery endothelial cells (5 million cells/challenge) were subjected to treatment with vehicle or 5 μM DPV. Cells were washed in ice-cold PBS containing 1 mM vanadate and cell lysates prepared under native conditions with modified RIPA buffer as described in METHODS. Cell lysates (1 mg of protein) were subjected to immunoprecipitation with anti-Src polyclonal antibody and protein A/G agarose. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibody or subjected to autophosphorylation or raytide peptide phosphorylation as described in METHODS. Densitometric analysis of the data for DPV-induced tyrosine phosphorylation, Src autophosphorylation, and raytide peptide phosphorylation was carried out on Western blots with a Bio-Rad densitometer, and data were quantified with a Molecular Analyst image analyzer. Data are representative of 1 of 3 experiments and were normalized to intensity of Src bands from the Western blots.
These results provide strong evidence that Src activation is an important regulatory event in DPV-induced EC barrier dysfunction.

DPV enhances association of contractile and adherens junction proteins in Src immunoprecipitates. The Src family of non-receptor kinases are localized in focal plaques and mediate phosphorylation of focal adhesion kinases (FAKs), p130Cas, paxillin, and MLCK in response to an external stimulus (12, 19). To further define the potential regulation of DPV-induced EC barrier dysfunction by Src, we investigated the possible association of contractile and adherens junction proteins in Src immunoprecipitates before and after DPV challenge. Western blot analysis of Src immunoprecipitates obtained from control and DPV-treated ECs were subjected to Western blotting for Src tyrosine phosphorylation, Src autophosphorylation, and raytide peptide phosphorylation as described above. Densitometric analyses of the blots were performed with a Bio-Rad densitometer. Data are averages of 3 independent experiments.

Fig. 5. PP-2 attenuates DPV-induced Src activation in ECs. Confluent BPAECs in 100-mm dishes were pretreated with MEM or MEM containing varying concentrations of PP-2 for 1 h followed by treatment with (+) and without (−) DPV for 30 min. Cell lysates were prepared under native conditions with modified radioimmunoprecipitation assay buffer for electrophoresis and immunoprecipitation. A: cell lysates (5 μg of protein) were subjected to electrophoresis on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-PTyr antibody. Nos. at left, molecular mass. B: Src immunoprecipitates were subjected to Western blotting with anti-PTyr antibody (top), autophosphorylation with [γ-32P]ATP (middle), and Western blotting with anti Src antibody (bottom) as described in METHODS. C: Src immunoprecipitates from control or DPV-treated ECs were subjected to Western blotting for Src tyrosine phosphorylation, Src autophosphorylation, and raytide peptide phosphorylation as described above. Densitometric analyses of the blots were performed with a Bio-Rad densitometer. Data are averages of 3 independent experiments.

Src, 0.95 ± 0.08; wild-type Src plus 5 μM DPV, 0.51 ± 0.07; constitutively active Src, 0.99 ± 0.12; constitutively active Src plus 5 μM DPV, 0.38 ± 0.02; dominant negative Src, 0.93 ± 0.06; dominant negative Src plus 5 μM DPV, 0.80 ± 0.08). These results provide strong evidence that Src activation is an important regulatory event in DPV-induced EC barrier dysfunction.

DPV enhances association of contractile and adherens junction proteins in Src immunoprecipitates. The Src family of non-receptor kinases are localized in focal plaques and mediate phosphorylation of focal adhesion kinases (FAKs), p130Cas, paxillin, and MLCK in response to an external stimulus (12, 19). To further define the potential regulation of DPV-induced EC barrier dysfunction by Src, we investigated the possible association of contractile and adherens junction proteins in Src immunoprecipitates before and after DPV challenge. Western blot analysis of Src immunoprecipitates obtained from control and DPV-treated cells under non-denaturing conditions revealed a marked increase in tyrosine-phosphorylated proteins (60–214 kDa) associated with Src (Fig. 8). Two proteins subsequently identified by Western blotting in the Src immunoprecipitates were the novel high molecular weight 214-kDa MLCK (53) and the p80/85 actin-binding protein cortactin (Fig. 8). Immunoreactive MLCK was not associated with Src immunoprecipitates prepared from control cells prepared under native conditions. However, after DPV challenge, there was a time-dependent increase in MLCK associated with Src. In contrast to
results in impaired barrier function, with exudation of fluids and proteins into the interstitium and alveoli (29). ROS released from activated polymorphonuclear leukocytes induced changes in intracellular levels of Ca\(^{2+}\) (39), thiols (43), and high-energy nucleotides (55), resulting in cell injury and dysfunction. Although the role of ROS in EC cell injury has been well studied with cells in culture and in ischemia-reperfusion systems (24, 54), the mechanism(s) of ROS-induced EC barrier dysfunction has yet to be completely defined. Earlier studies (10, 27) with macro- and microvascular ECs suggested that rapid activation of phosphatidylinositol 4,5-bisphosphate-specific phospholipase (PL) C and generation of diacylglycerol and inositol 1,4,5-trisphosphate second messengers may be involved in thrombin- and H\(_2\)O\(_2\)-induced increases in permeability. Exposure of dermal microvascular ECs to PMA (30, 41) or H\(_2\)O\(_2\) (45) increased albumin flux across the monolayer in association with activation and translocation of PKC-\(\beta\) to the membrane (45). However, the protein target(s) involved in the regulation of ROS-induced EC barrier dysfunction is not known. Interestingly, \(\alpha\)-thrombin- or PMA-mediated activation of PKC enhanced phosphorylation of the actin-, myosin-, and calmodulin-binding protein caldesmon and the intermediate filament vimentin and also enhanced albumin permeability across EC monolayers, suggesting a role for cytoskeletal proteins in EC barrier dysfunction (47). A similar role for PKC in the thrombin-induced increase in transendothelial permeability to albumin, which was attenuated by PKC inhibitors (10, 27), was also observed.

Pretreatment of guinea pig lungs with H-7, a 

**Fig. 7.** Src expression alters DPV-induced EC barrier dysfunction. A: confluent BPAECs in 100-mm dishes were transfected with either empty vector or wild-type (wt) Src, constitutively active (+) Src, or dominant negative (–) Src cDNAs with FuGENE 6 as described in METHODS. After transfection, Src was immunoprecipitated from the cell lysates with a polyclonal anti-Src antibody under native conditions. Src immunoprecipitates were subjected to electrophoresis in 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-Src or anti-PTyr antibodies. Src activity in the immunoprecipitates was measured with \([\gamma-32P]\)ATP and raytide peptide as a substrate as described in METHODS. Nos. at left, molecular mass. B: BPAECs grown on gold microelectrodes were transfected with empty vector or wild-type Src, constitutively active Src, or dominant negative Src mutants. After transfection (48 h), cells were treated with medium 199 or medium 199 containing DPV for 4 h, and changes in electrical resistance were measured as described in METHODS. Tracings are representative of 3 independent experiments.

**Fig. 8.** DPV enhances the stable association of myosin light chain kinase (MLCK) and cortactin in Src immunoprecipitates. Confluent BPAECs in 100-mm dishes were treated with MEM or MEM containing DPV (5 \(\mu\)M) for indicated time periods. Cells were washed in ice-cold PBS, and cell lysates were prepared in lysis buffer as described in METHODS. Cell lysates (1 mg of protein) were subjected to immunoprecipitation with anti-Src antibody, and immunoprecipitates were subjected to electrophoresis on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-PTyr antibody (A), anti-MLCK antibody (B), and anti-cortactin antibody (C). Blots are representative of 3 independent experiments. Nos. at left, molecular mass.

**DISCUSSION**

The structural and functional integrity of the vascular endothelium is critical to normal lung function and vessel wall homeostasis. Injury to the endothelium MLCK, there was a significant association of p80 and p85 cortactin isoforms with Src even under basal conditions, and stimulation with DPV (5 \(\mu\)M) further enhanced the level of association (Fig. 8). These findings suggest that DPV treatment increases the association of MLCK and cortactin with Src and that MLCK and cortactin may represent important downstream targets for Src in regulating EC barrier function.
PKC inhibitor, prevented pulmonary edema in response to perfusion with H₂O₂, suggesting involvement of PKC in permeability changes (20). Similarly, changes in intracellular Ca²⁺, mediated by inositol 1,4,5-trisphosphate or by other mechanism(s), may also contribute to the EC barrier dysfunction observed with agonists or ROS (39).

The results presented in this study demonstrate for the first time a role for Src kinases in DPV-induced EC barrier dysfunction. An increase in permeability to albumin or a decrease in electrical resistance induced by DPV, a potent inhibitor of PTPases and activator of TyKs (32, 37), was attenuated by genistein, suggesting a role for protein tyrosine phosphorylation. DPV-induced reduction in electrical resistance was always preceded by a barrier enhancement that lasted for 15–30 min. (Fig. 1B). At this time, it is unclear what the significance of this initial increase in resistance to barrier function is, but it may represent an early barrier protective mechanism. DPV rapidly activated Src kinase as evidenced by increased autophosphorylation and raytide peptide phosphorylation, and changes in EC barrier dysfunction were seen 2 h later with Src activation. Pretreatment of ECs with PP-2, a recently described specific inhibitor of the Src family of non-receptor kinases (13), attenuated DPV-induced Src activation and increased permeability to albumin, strongly suggesting a role for Src in barrier dysfunction. The protective effect of PP-2 was specific toward DPV-mediated Src kinase activation and barrier dysfunction because PP-2 had no effect on PMA-mediated decrease in electrical resistance. The present study employing a pharmacological Src kinase inhibitor was complemented by additional investigations with overexpression of wild-type Src, constitutive active Src, and dominant negative Src plasmids, which also significantly altered DPV-induced EC electrical resistance. Our results also show that transient expression of constitutively active Src is not sufficient to alter basal electrical resistance. It is possible that an additional signaling pathway(s), such as changes in intracellular Ca²⁺, may be necessary to modulate DPV-induced barrier function. An earlier study (32) has demonstrated that DPV increased intracellular Ca²⁺ in ECs that was attenuated by chelators of Ca²⁺. Further experiments to demonstrate the role for Ca²⁺ in DPV-mediated EC barrier dysfunction by Src are needed.

TyKs and, in particular, the Src family of non-receptor TyKs play an important role in transducing signals from cell exterior to cell interior. In response to growth factors, oxidative and shear stress, ultraviolet light, and a variety of agonists including thrombin and angiotsin II, Src is activated, as evidenced by enhanced specific activity and increased protein tyrosine phosphorylation (35, 51). In our studies, autophosphorylation and tyrosine phosphorylation of Src were detected as early as 2 min after DPV, which was similar but not identical to that observed with thrombin stimulation of lung fibroblasts (4) and angiotsin II activation of p60⁶⁵⁷ in vascular smooth muscle cells (18). Although regulation of Src kinase family members involves phosphorylation of tyrosine-416 and dephosphorylation of tyrosine-527 (48), it is unclear which of these two tyrosine residues is involved in DPV-induced Src activation. However, because DPV is known to inhibit PTPase activity and/or to activate TyKs (34), it is reasonable to assume that the DPV-induced activation of Src involves either of the pathways. It is also known that additional phosphorylation sites, including phosphorylation of serine-12 of Src, may have functional consequences on kinase activity (48), and analyses of the phosphorylation sites on Src in control and DPV-challenged ECs should provide further insight into the mechanism of its activation under oxidative stress.

Our finding that DPV stimulates Src and that this event represents an important mechanism for EC barrier dysfunction is consistent with a previous report (42) of the possible involvement of Src in thrombin-induced EC permeability changes and electrical resistance. Because paracellular transport of macromolecules across the monolayer is regulated by contractile and tethering forces (11), activation of Src kinase by DPV may involve tyrosine phosphorylation of other downstream target proteins such as adherens junction proteins, FAKs, and actomyosin contractile proteins regulating barrier function. One potential key cytoskeletal target is the EC MLCK, the activity of which is a major determinant of tension development. EC MLCK activity is regulated by Ser/Thr phosphorylation (53), and Garcia et al. (12) and Shi et al. (42) have recently demonstrated a novel regulation of the enzyme by tyrosine phosphorylation. A recent study (12) indicated that DPV evokes significant endothelial contraction, tyrosine phosphorylation of MLCK, and MLCK activation in BPAECs (12). The DPV-induced MLCK phosphorylation and EC contraction were attenuated by either C3 exotoxin from Clostridium botulinum or MLCK inhibitors, consistent with dual mechanisms that regulate the level of MLC phosphorylation in ECs involving Rho GTPase-mediated inhibition of MLC phosphatase and regulation of MLCK activity via tyrosine phosphorylation (12). The results reported here on the association of MLCK in Src immunoprecipitates after DPV treatment implicate Src as the effector kinase in catalyzing phosphorylation of tyrosine residues in MLCK. A recent study (53) on the cloning of the 214-kDa EC MLCK indicated that this unique isoform is present predominantly in nonmuscle tissue such as ECs compared with the 130- to 160-kDa isoform in smooth muscle cells (53). This unique MLCK from ECs contains consensus sequences for Src kinases, PKC, and the calmodulin-protein kinase II region in the novel NH₂ terminus that are not present in the smooth muscle isoform (23). Enhanced tyrosine phosphorylation of MLCK may result in increased protein-protein interaction involving Src homology (SH) 2 and SH3 domains that have also been identified in the EC MLCK isoform (Garcia, unpublished data). Also, the presence of the SH3 motif could support its role as a scaffolding and adaptor protein. We also identified an association of the p80/85 actin-binding protein cortactin in Src immunoprecipitates. Cortactin is tyrosine phosphorylated by Src family of non-receptor TyKs in...
response to external stimuli (25). Although the physiological role of tyrosine phosphorylation of cortactin is not well understood, a recent study (17) indicated that cortactin cross-links filamentous actin in vitro that is downregulated by Src-dependent tyrosine phosphorylation. Src-mediated tyrosine phosphorylation of cortactin resulted in an enhanced motility of EC304 ECs, suggesting a possible role in angiogenesis (16). It is possible that DPV-mediated phosphorylation of cortactin by Src in ECs may be involved in cytoskeletal reorganization and barrier dysfunction. Further studies on the sites of tyrosine phosphorylation in cortactin by DPV and sites of interaction between cortactin and other cytoskeletal proteins would give a better understanding of the role of cortactin in EC barrier function.

In addition to Src, H₂O₂ and DPV stimulate the tyrosine phosphorylation of p125Fak, paxillin, caveolin, and mitogen-activated protein kinases in vascular ECs and smooth muscle cells (1, 52). Because rearrangement of cytoskeletal proteins and focal adhesion proteins plays an important role in determining EC shape, migration, proliferation, and barrier function, phosphorylation-dephosphorylation of focal adhesion proteins may have a role in barrier function. A recent study (18) in vascular smooth muscle cells suggested that thrombin and angiotensin II cause actin stress fibers and focal adhesion protein assembly through Src activation and increased phosphorylation of Cas, paxillin, and tensin (18). The mechanism(s) by which the FAK-associated signaling complex alters the actin cytoskeleton and barrier dysfunction is unclear but may involve small G proteins, other cytoskeletal proteins, and focal adhesion proteins. A recent study by Vepa et al. (52) showed that H₂O₂ and DPV stimulate tyrosine phosphorylation of FAK and paxillin in BPAECs and that DPV is a potent stimulator of p42/p44 mitogen-activated protein kinases, consistent with this notion. Interestingly, we have noted that although the DPV-induced EC barrier dysfunction was inhibited by both PP-2 and genistein, neither pharmacological agent reduced DPV-induced tyrosine phosphorylation of FAK or paxillin (Vepa S and Natarajan V, unpublished data), suggesting either that these effectors may not directly participate in EC barrier dysfunction or that spatial location of these proteins may not be linked to their phosphorylation status.

The present studies do not exclude other signaling pathways such as mitogen-activated protein kinases, phospholipases, and receptor TyRs in DPV-mediated EC barrier dysfunction. Preliminary studies suggest that DPV-induced phospholipase D (PLD) activation with subsequent generation of phosphatidic acid increases the permeability to albumin across EC monolayers (Natarajan and Shi, unpublished observations). The mechanism of induction of endothelial monolayer permeability by phosphatidic acid is not known. Phosphatidic acid is recognized as a second messenger and can phosphorylate intracellular proteins through activation of phosphatidic acid-dependent protein kinases. Another pathway that may be modulated by DPV and other peroxovanadium compounds is altering the cellular thiol redox status. Because peroxovanadium compounds oxidize cysteine residues of protein tyrosine phosphatases, it is conceivable that DPV also alters cysteine residues in Src and other signaling proteins regulating barrier function. Further studies on phosphatidic acid-dependent activation of protein kinases and redox regulation of Src and PLD activation should provide a better understanding of other mechanisms involved in endothelial barrier dysfunction.

In summary, the data presented here demonstrate that DPV-induced changes in EC permeability are reg-

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Fig. 9. Model for reactive oxygen species-induced regulation of EC barrier function by Src, MLCK, and cortactin. Tyr-P, tyrosine phosphorylation; P-Tyr, phosphorylated tyrosine; ML-C-P, myosin light chain phosphorylation; PPase, protein phosphatase.
ululated by protein tyrosine phosphorylation involving activation of Src kinase as an early and important upstream signaling mechanism that regulates EC barrier dysfunction. Our data also indicate that association and tyrosine phosphorylation of cortactin and MLCK by Src may represent downstream signaling pathways regulating DPV-induced EC permeability. A recent study (50) suggested the feasibility of suppression of Src activity for gene therapy in rheumatoid arthritis, and our studies also suggest that modulation of Src activity by specific Src inhibitors or dominant negative Src could represent a viable therapeutic treatment for pulmonary edema and endothelial dysfunction. A proposed model of DPV- or ROS-induced barrier dysfunction involving Src, cortactin, and MLCK is illustrated in Fig. 9.

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