Diperoxovanadate alters endothelial cell focal contacts and barrier function: role of tyrosine phosphorylation

JOE G. N. GARCIA,1 KANE L. SCHAPHORST,1 ALEXANDER D. VERIN,1 SURYANARAYANA VEPA,1 CAROLYN E. PATTERSON,2 AND VISWANATHAN NATARAJAN1

1Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21224; and 2Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202

Received 10 January 2000; accepted in final form 21 July 2000

Garcia, Joe G. N., Kane L. Schaphorst, Alexander D. Verin, Suryanarayana Vepa, Carolyn E. Patterson, and Viswanathan Natarajan. Diperoxovanadate alters endothelial cell focal contacts and increases permeability: role of tyrosine phosphorylation. J Appl Physiol 89: 2333–2343, 2000.—Diperoxovanadate (DPV), a potent tyrosine kinase activator and protein tyrosine phosphatase inhibitor, was utilized to explore bovine pulmonary artery endothelial cell barrier regulation. DPV produced dose-dependent decreases in transendothelial electrical resistance (TER) and increases in permeability to albumin, which were preceded by brief increases in TER (peak TER effect at 10–15 min). The significant and sustained DPV-mediated TER reductions were primarily the result of decreased intercellular resistance, rather than decreased resistance between the cell and the extracellular matrix, and were reduced by pretreatment with the tyrosine kinase inhibitor genistein but not by inhibition of p42/p44 mitogen-activating protein kinases. Immunofluorescent analysis after DPV challenge revealed dramatic F-actin polymerization and stress-fiber assembly and increased colocalization of tyrosine phosphoproteins with F-actin in a circumferential pattern at the cell periphery, changes that were abolished by genistein. The phosphorylation of focal adhesion and adherens junction proteins on tyrosine residues was confirmed in immunoprecipitates of focal adhesion kinase and cadherin-associated proteins in which dramatic dose-dependent tyrosine phosphorylation was observed after DPV stimulation. We speculate that DPV enhances endothelial cell monolayer integrity via focal adhesion plaque phosphorylation and produces subsequent monolayer destabilization of adherens junctions initiated by adherens junction protein tyrosine phosphorylation catalyzed by p60src or Src-related tyrosine kinases.

adherens junctions; cadherin; catenin; electrical resistance

BECAUSE OF ITS SPATIAL ORIENTATION between blood and tissue, the vascular endothelium maintains a semi-selective permeability barrier to circulating proteins. Vascular barrier function is altered, however, by diverse circulating vasoactive proteins, cytokines, and inflammatory mediators, including reactive oxygen species released from stimulated leukocytes, which contribute to the physiological derangement observed in various vascular syndromes. Many of these diverse physiologically relevant, barrier-disrupting agents share the ability to enhance the activity of specific serine-threonine and tyrosine kinases and to increase cytosolic Ca2+, thereby evoking a signaling cascade that produces paracellular gap formation and enhanced organ edema formation. For example, activation of the Ca2+-calmodulin-dependent, serine-threonine kinase, myosin light chain kinase (MLCK), increases endothelial cell contraction (22) and endothelial cell permeability (50). The role of tyrosine kinases in regulating endothelial cell barrier properties has not been studied extensively; however, tyrosine protein phosphorylation has been noted to participate in regulation of Ca2+ capacitive pathways in endothelium (7, 8). Whereas tyrosine kinase inhibitors attenuated bradykinin-induced Ca2+ transients (8), treatment with a protein tyrosine phosphatase inhibitor directly increased cytosolic Ca2+ in cultured human endothelium (8). Tyrosine kinases may also target specific cytoskeletal effectors as substrates, thereby regulating nonmuscle contraction. In prior work, our laboratory described the ability of the tyrosine kinase inhibitor genistein to attenuate thrombin-induced tyrosine kinase activity, Ca2+ transients, and endothelial cell barrier dysfunction, suggesting a role for Src family kinases and tyrosine phosphorylation in endothelial cell barrier regulation (41). These findings were further supported by additional studies employing vanadate, an inhibitor of tyrosine phosphatases (16), which directly increased endothelial permeability without an increase in cytosolic Ca2+.

The precise tyrosine kinase targets that regulate endothelial cell permeability are not known; however, an increase in endothelial cell contraction or a decrease in endothelial cell tethering, either to adjacent cells or to the extracellular matrix, appears to be an essential step in specific models of agonist-induced vascular permeability and tissue edema. Our laboratory’s earlier work (41), as well as ongoing studies (11) utilizing the

http://www.jap.org 8750-7587/00 $5.00 Copyright © 2000 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cell-permeable tyrosine kinase activator and protein tyrosine phosphatase inhibitor diperoxovanadate (DPV), strongly suggested that tyrosine phosphorylation participates in the regulation of endothelial cell contractile forces via direct effects on MLCK activity and myosin light chain (MLC) phosphorylation. Proteins that promote endothelial cell tethering to each other or to the extracellular matrix include adherens proteins, such as the homotypic cadherins and α-, β-, and γ-catenins, which, in some systems, are regulated by protein tyrosine phosphorylation (4, 45). Similarly, the integrity of matrix-integrin-cytoskeleton linkages is dependent on focal contacts through which signaling occurs by the activation of p125 focal adhesion kinase (FAK), a tyrosine kinase that mediates phosphorylation of focal adhesion plaque proteins such as paxillin (3, 36, 48).

In this study, we examined the participation of intracellular signaling cascades initiated by protein tyrosine phosphorylation in the disruption of the endothelial cell barrier, utilizing DPV as an edemagenic agent. Our results indicate that DPV produces significant dose-dependent biphasic alterations in endothelial electrical resistance with the initial enhancement of barrier function being followed by increases in endothelial cell permeability. DPV evokes large increases in phosphotyrosine accumulation in the endothelial cell focal contact protein, p125 FAK, as well as the adherens junction proteins, β- and γ-catenins. The temporal sequence of biochemical events indicates that the early DPV-induced enhancement of barrier function is unlikely to be related to the phosphorylation of adherens junction components. However, the subsequent DPV-induced disruption of endothelial barrier function was characterized by strong enhancement of tyrosine phosphorylation in adherens junction proteins. Together, these studies suggest a role for protein tyrosine phosphorylation in causing barrier-protective and barrier-disruptive effects. In addition to contractile properties, vascular endothelial cell barrier regulation appears to depend on the specific sites of tethering protein phosphorylation and the strength and duration of activation.

MATERIALS AND METHODS

Materials. Bovine pulmonary artery endothelial cells (CCL-209, passage 16) were obtained from American Type Culture Collection (Rockville, MD). MEM, DMEM, nonessential amino acids, FBS, and Hanks’ balanced salt solution without phenol red were purchased from Gibco (Grand Island, NY). Colostrum-free bovine serum was from Irvine Scientific (Santa Ana, CA). Endothelial cell growth supplement was obtained from Collaborative Research (Bedford, MA). Pencillin/streptomycin, hydrogen peroxide, sodium orthovanadate, sodium metavanadate, and fatty acid-free bovine serum albumin were procured from Sigma Chemical (St. Louis, MO). Affinity-purified monoclonal antiphosphotyrosine antibody (4G10) was purchased from Upstate Biotech (Lake Placid, NY). Polycrylamide ready-to-use gels were obtained from Bio-Rad (Hercules, CA). Polycarbonate micropore membranes were obtained from Nucleopore (Pleasanton, CA).

**Bovine pulmonary artery endothelial cell cultures.** Bovine endothelium was cultured in complete DMEM supplemented with 20% (vol/vol) colostrum-free bovine serum, 15 μg/ml endothelial cell growth supplement, 1% antibiotic and antimycotic solution (10,000 U/ml penicillin, 10 μg/ml streptomycin, and 25 μg/ml amphotericin B), and 0.1 mM nonessential amino acids, as previously described (12). The endothelial cell cultures (passages 19–24) were maintained at 37°C in a humidified atmosphere of 5% CO₂:95% air and grew to contact-inhibited monolayers with typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh culture medium, and passaged into polycarbonate filters for permeability studies, into 100-mm² dishes for immunoprecipitation studies, into 60-mm² dishes for tyrosine kinase activity determination and MLC phosphorylation studies, or into 11-mm wells for electrical resistance determination.

**Cytotoxicity assays.** Assessment of cytotoxicity to DPV or other agents was determined by measuring release of[^H]deoxyglucose as described previously (29). The percentage of[^H]deoxyglucose released was calculated by using the formula

\[
\frac{[^{3}H]DG_{\text{exp}} - [{^3H}]DG_{\text{spont}}}{[^{3}H]DG_{\text{total}} - [{^3H}]DG_{\text{spont}}} \times 100
\]

as an index of cytotoxicity, where DG is deoxyglucose, exp is experimental, and spont is spontaneous.

**Albumin clearance measurement of endothelial cell permeability.** Macromolecular permeability of cultured endothelial cell monolayers was performed as previously described (13), with some modifications (31). Briefly, to measure the albumin flux across the monolayer, a system consisting of two compartments, the upper compartment (luminal) and the lower compartment (abluminal), separated by polycarbonate micropore membrane filter on which the endothelial cells are grown, was used. The lower compartment was stirred continuously and kept at a constant temperature of 37°C by a thermally regulated water bath. Medium M199 containing 25 mM HEPES (pH 7.4) and 4% bovine serum albumin was used in both compartments. Bovine serum albumin (4% final concentration) complexed to Evans blue dye was added to the luminal compartment, and samples were taken from the abluminal compartment at 5-min intervals for 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 Evans blue dye in abluminal chamber samples at 620 nm in a Vmax multiplate 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.** Endothelial cells were grown to confluence in wells containing small, evaporated gold microelectrodes (10^-3 cm²) in series with a large gold counterelectrode through the tissue culture media, as previously described (9, 37, 45). Measurements of transendothelial electrical resistance (TER) were performed utilizing an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY). Briefly, current was applied across the electrodes by a 4,000-Hz AC voltage source with an amplitude of 1 V in series with a 1 MΩ resistance to approximate a constant current source (~1 μA). The small gold electrode and the larger counterelectrode (1 cm²) were connected to a phase-sensitive lock-in amplifier (5301A; EG&G Instruments, Princeton, NJ) with a built-in differential preamplifier (5316A; EG&G Instruments). The
in-phase and out-of-phase voltages between the electrodes were monitored in real time with the lock-in amplifier and converted to scalar measurements of transendothelial impedance, of which resistance was the primary focus. TER was monitored for 30 min to establish a baseline resistance ($R_0$), which, for bovine lung endothelium, was typically between 8 and 12 × 10$^3$ Ω (wells with $R_0 < 7 × 10^3$ Ω or $R_0 > 15 × 10^3$ Ω were rejected). DPV was then added, and real-time transendothelial resistance measurements were collected. As cells adhere and spread out on the microelectrode, the TER (maximal at confluence) increased, whereas cell retraction, rounding, or loss of adhesion is reflected by a decrease in resistance (14). These measures provide a highly sensitive biophysical assay that indicates the state of cell shape and focal adhesion (45). For some experiments, total TER was resolved into components reflecting resistance to current flow beneath the cell layer ($\alpha$) and resistance to current flow between adjacent cells ($\beta$), utilizing the method of Giaever and Keese (15), which models the endothelial monolayer mathematically. Thus changes in $\alpha$ reflect alterations in the net state of cell-matrix adhesion, whereas changes in $\beta$ reflect alterations in the integrity of cell-cell adhesion. TER values from each microelectrode were pooled at discrete time points and plotted vs. time as the mean ± SE.

**Mitogen-activating protein kinase activation.** Determination of mitogen-activating protein (MAP) kinase activation was assessed by the immunoblotting of endothelial cell lysates with specific phospho-extracellular signal-regulated kinase (ERK) antibodies (New England Biolab) that indicate the enhanced catalytic activity of the enzyme. Briefly, after DPV challenge, cell lysates were prepared by extracting cells into a 1% Triton X-100 buffer for 20 min at 4°C. The lysates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes for Western immunoblotting, as our laboratory has previously described (37). After incubation with phospho-ERK or pan-ERK primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies, membranes were developed by using an enhanced chemiluminescence protocol, according to the manufacturer’s instructions (Amersham).

**Immunofluorescence.** The fluorescent imaging of endothelial cell gap formation and F-actin organization was performed on endothelial cell monolayers grown to confluence on glass coverslips. After treatment, cells were fixed by exchanging the media with 5% paraformaldehyde, 50 mM phosphate, 75 mM NaCl and 50 mM Tris, pH 7.4, and then permeabilized by treatment with 0.2% Triton for 4 min in rinse buffer. Cells were then rinsed three times and incubated at room temperature for 1 h with 1% BSA in rinse buffer and then with 1 U/ml rhodamine phalloidin (Molecular Probes, Eugene, OR) to identify F-actin. Time-dependent changes in intracellular distribution of the actin cytoskeleton before and after 5 μM DPV challenge were analyzed on a Zeiss Axiosplan fluorescent microscope with MC100 camera, as our laboratory has previously described (11). To study colocalization of actin and phosphotyrosine proteins, the fixed and permeabilized cells were exposed overnight at 4°C to 1:50 dilution of 4G10 antibody (UBI, Lake Placid, NY) in 150 mM NaCl-50 mM Tris (pH 7.4) buffer containing 4% BSA. After being rinsed to remove unbound primary antibody, cells were incubated for 1 h at room temperature with labeled secondary antibody (3 μg/ml) (FITC-conjugated donkey anti-rabbit IgG; Jackson, West Grove, PA) and rhodamine phalloidin. Cells were examined by using a ×60 oil objective with the Bio-Rad MRC 1024 confocal microscope and excitation with Ar-Kr laser at 568-nm excitation and 598-nm emission for rhodamine and 488-nm excitation and 522-nm emission for FITC at a 3-mm aperture. Data were collected for 7–17 planar sections at 0.5-μm intervals by Bio-Rad LaserSharp acquisition software, processed by MetaMorph Imaging software (Universal, West Chester, PA), and printed on a thermal dye diffusion printer (Kodak, Rochester, NY). Endothelial cell monolayers that were not exposed to primary antibody showed no staining with the secondary antibody.

**Immunoprecipitation and Western blotting.** Endothelial cell monolayers were washed once with serum-free DMEM medium and stimulated in serum-free MEM for specified time periods. The cells were washed once in ice-cold PBS and were washed again in ice-cold PBS containing 1 mM sodium orthovanadate. Cells (5 × 10$^6$) were scraped into 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, containing 0.5% deoxycholic acid, 0.5% SDS, 1% Triton X-100, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate). The samples were cleared by centrifugation at 14,000 rpm for 10 min at 4°C, and the supernatants were used for immunoprecipitation with either anti-FAK (Santa Cruz), or anti-pan-cadherin (2–4 μg/ml; Sigma Chemical) at 4°C for 4–18 h. Protein A/G agarose (20 μl) was then added and incubated for an additional 2–4 h at 4°C. The antigen-antibody complex was pelleted, washed three times with ice-cold lysis buffer, and dissociated by boiling in 1× SDS sample buffer for 5 min. The samples were then analyzed on 8 or 10% SDS-PAGE gels. After SDS-PAGE, proteins were transferred to Immobilon-P membranes by electrophoreticing, blocked with blocking buffer (Gibco), and incubated for 18–24 h at 4°C with either anti-FAK (1:1,000 dilution) or anti-pan-cadherin (1 μg/ml). Membranes were washed four times with PBS containing 0.1% Tween 20, followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:13,000 dilution) for 1 h at room temperature, and blots were developed by using enhanced chemiluminescence. Densitometric scanning of the blots was carried out by using a Bio-Rad model GS-700 densitometer and quantified by using the Molecular Analyst software program.

**Statistics.** Linear regression analysis was performed for determination of albumin clearance rates in individual wells with Epistat 2.0 public domain software. These slopes were then averaged from at least $n = 6$. Paired t-tests were used to compare pretreatment and posttreatment slopes within the same control membrane of each endothelial cell chamber. ANOVA with Student-Newman-Keuls test was used to compare means of clearance rates of two or more different treatment groups. Significance level was taken to be $P < 0.05$, unless otherwise stated. Data are expressed as means ± SE.

**RESULTS**

**Effect of DPV on endothelial cell barrier function.** Our laboratory previously demonstrated that DPV, the major peroxovanadium compound generated when equimolar amounts of H$_2$O$_2$ and sodium ortho- or metavanadate are mixed at neutral pH (2, 18, 40), dramatically increases endothelial cell protein tyrosine phosphorylation via modulation of tyrosine kinase and protein tyrosine phosphatase activities (11). The marked increase in protein tyrosine phosphorylation results in endothelial cell activation, as reflected by activation of phospholipase A$_2$, phospholipase C, and phospholipase D (PLD) (30). We have previously noted...
that 100 μM H₂O₂ and 100 μM vanadate individually produce modest alterations in endothelial cell permeability, beginning after 1 h, whereas lower concentrations (10 μM) of either agent do not alter endothelial cell barrier properties. Our initial experiments defined the effects of DPV on endothelial cell barrier function utilizing two complementary indexes of endothelial cell permeability.

Figure 1A depicts the dose-dependent DPV-induced increased albumin clearance across confluent endothelial cell monolayers (1 μM to 10 μM). Interestingly, this increase in albumin clearance was not observed to be significant within the first 60 min of DPV addition. Figure 1B demonstrates that a combination of 100 μM H₂O₂ and 10 μM vanadate produced dramatic increases in albumin clearance, whereas either agent alone was less effective. Another sensitive index of endothelial cell barrier integrity is the extent of TER generated across endothelial cell monolayers grown on gold microelectrodes (14, 45). Consistent with the integrated effect of DPV on endothelial cell albumin clearance depicted in Fig. 1A, DPV produced concentration-dependent alterations in TER compared with controls (Fig. 2). DPV-induced reductions in TER dropped below basal values after 30 min and were consistently preceded by a brief, but highly reproducible, increase in TER. This barrier enhancement peaked ~10–15 min postchallenge, lasted ~20–30 min (Fig. 2), and was followed by prolonged and sustained declines in normalized TER. Both the peak of the amplitude of the early DPV-induced increase in TER and the subsequent rate of TER decline were positively correlated with the DPV dose. We speculate that this increase in TER from baseline explains the lack of overall permeability change seen in the albumin clearance assays within the first hour after DPV.

The response to 5 μM DPV was mathematically resolved into parameters reflecting the TER to Rᵦ and α (Fig. 3). Before DPV was added (Fig. 3A), 60% of the resting total TER was contributed by Rᵦ, 20% by α, and the balance by the resistance of the electrode (not plotted). When the endothelium was challenged by DPV, the major alteration in barrier function could be...
DPV alters endothelial junctions and barrier function

A fundamental property of focal contacts is that they appear to modulate endothelial barrier function, both in the early enhancement and subsequent disruptive phases, through effects on intercellular adhesion. In the mathematical model used by Giaver and Keese (15), the total resistance mediated by DPV challenge. Thus DPV appears to modulate endothelial barrier function, both in the early enhancement and subsequent disruptive phases, through effects on intercellular adhesion. The DPV-mediated changes in endothelial resistance suggested the possibility that DPV alters the levels of tyrosine phosphorylation of specific target proteins present at peripheral sites of cell-matrix adhesion. The DPV-mediated changes in endothelial cell function were not due to cytotoxicity, assessed either by [3H]deoxyglucose release in the absence or presence of DPV (Table 1) or by light microscopic evaluation (data not shown).

**Effect of DPV on endothelial cell actin and phosphotyrosine immunofluorescence.** Having established that DPV is a potent barrier-disrupting agent that appears to potently affect the state of intercellular adhesion in endothelium, we next examined the effect of DPV on endothelial cell cytoskeletal architecture. It is generally recognized that an actin-containing dense peripheral band is normally present in a circumferential distribution in resting confluent endothelial cells (52, 53). Confocal immunohistochemical studies revealed that DPV treatment resulted in a time-dependent dissolution of the dense peripheral band and a rapid assembly of actin-based stress fibers, a strong indication of the presence of a contractile phenotype (Fig. 4A). Figure 4B also demonstrates the presence of phosphotyrosine proteins in a circumferential pattern under vehicle-stimulated conditions. In contrast, DPV dramatically increases the level of phosphotyrosine staining with significant aggregation of phosphotyrosine proteins colocalized with the actin cytoskeleton. These changes in the actin stress fibers and the association with phosphotyrosine proteins paralleled the presence of paracellular gap formation. DPV also produced a dramatic increase in phosphotyrosine protein detection, which remained in a circumferential pattern of distribution in association with paracellular gap formation (Fig. 4). Furthermore, these experiments indicated significant colocalization of actin and the circumferentially distributed phosphotyrosine proteins 10 min after DPV challenge, which persisted at 30 min, at which time F-actin itself appeared to accumulate phosphotyrosine immunoreactivity.

**Effect of DPV on phosphorylation of focal adhesion and adherens junction proteins.** The dramatic increase in circumferential tyrosine phosphorylation in concert with DPV-mediated reductions in intercellular resistance suggested the possibility that DPV alters the levels of tyrosine phosphorylation of specific target proteins present at peripheral sites of cell-matrix and actin distribution in association with paracellular gap formation.

---

**Table 1. Effect of DPV on endothelial cell viability**

<table>
<thead>
<tr>
<th>DPV, μM</th>
<th>[3H]deoxyglucose Released, %control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.2 ± 1.2</td>
</tr>
<tr>
<td>1</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>2.5</td>
<td>4.0 ± 5.8</td>
</tr>
<tr>
<td>5.0</td>
<td>-2.8 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>31.4 ± 5.2</td>
</tr>
</tbody>
</table>

Values are means ± SE of average of triplicate determination. DPV, diperoxovanadate. Bovine pulmonary artery endothelial cells labeled with [2-3H]deoxyglucose (1 mCi/dish, specific activity 10 Ci/mmol) for 24 h were rinsed with DMEM and challenged with DMEM or DMEM containing different concentrations of DPV for 2 h. [2-3H]deoxyglucose released was quantified as described in MATERIALS AND METHODS. Results are exposed as [3H]deoxyglucose released as %control.
cell-cell adhesion. The focal adhesion protein p125 FAK was next immunoprecipitated from unchallenged endothelial cells, which demonstrated a detectable level of basal tyrosine phosphorylation of p125 FAK (Fig. 5A). DPV exposure did not significantly change the total amount of immunoprecipitatable p125 FAK; however, there was a dramatic increase in tyrosine phosphorylation of p125 FAK detected as early as 5 min after DPV. The effect of DPV on adherens junction proteins was also examined by using antisera raised against the conserved C terminus of classical cadherins. Cadherin-associated proteins were immunoprecipitated under nondenaturing conditions in unchallenged endothelial cells with little detectable evidence of basal phosphotyrosine immunoreactivity (Fig. 5B). DPV challenge, however, resulted in dramatic time-dependent phosphorylation of cadherins and β-catenins (Fig. 5B). This augmentation of tyrosine phosphorylation was delayed compared with p125 FAK phosphorylation but corresponded well with the immunohistochemical pattern of tyrosine phosphotyrosine staining noted in Fig. 4.

Effect of MAP kinase inhibition on DPV-induced barrier dysfunction. As the disassembly of adherens junctions proteins has been linked to the growth factor-induced activation of MAP kinases, we speculated that...
DPV-mediated activation of MAP kinases may be an upstream event that leads to the potent effects on intercellular adhesion that we observed. The activity of p42 and p44 MAP kinases, also collectively known as extracellular regulated kinases (ERKs), are recognized as being readily responsive to stresses evoked by reactive oxygen species and osmotic stress (25, 46). As shown in Fig. 6A, DPV challenge resulted in a dose-dependent increase in ERK phosphorylation on tyrosine, a well-recognized index of ERK catalytic activity. ERK activation was maximal after 30 min of DPV stimulation (0.5–5 μM) and then subsequently declined toward baseline, with higher doses of DPV resulting in a more sustained ERK activation (Fig. 6A).

To assess the role of ERK activation in DPV-induced endothelial cell permeability, endothelial cells were pretreated with PD-98054, an inhibitor of the upstream ERK activator MAP kinase kinase (MEK), and then challenged with DPV (Fig. 6B). Although PD-98054 pretreatment produced a substantial attenuation of DPV-induced ERK phosphorylation, pretreatment of endothelial cell monolayers with PD-98054 did not significantly affect DPV-induced alterations in TER (Fig. 6C). These data suggest that, whereas DPV is a potent activator of endothelial MAP kinases, neither the early barrier-protective response nor the subsequent barrier-disruptive effects elicited by DPV evolve in an ERK-dependent manner.

**Effect of tyrosine kinase inhibition on DPV-mediated alterations in electrical resistance.** The time-dependent enrichment of phosphotyrosine-containing proteins at the cell periphery indicated that DPV-mediated barrier dysfunction may be mediated by tyrosine kinases that modulate endothelial cell-cell adhesive properties. To examine the role of DPV-mediated tyrosine phosphorylation in altering the activity of endothelial cell-cell and cell-matrix adhesive structures, endothelial cells were challenged with DPV with or without the tyrosine kinase inhibitor genistein (100 μM), and the effect on TER was assessed (Fig. 7). Similar to other experiments (Fig. 2), DPV alone produced an early increase in resistance (barrier enhancement) that was maximal by 15 min postchallenge, followed by a decline in TER that became different from control at ~40 min (Fig. 7A). Whereas pretreatment of the endothelial cell monolayer with genistein alone had no discernable effect on basal endothelial barrier function, pretreatment with genistein significantly attenuated DPV-induced decreases in TER (Fig. 7A).

As we noted in Fig. 3, the major DPV-mediated alteration in electrical resistance is due to major disturbances in the endothelial barrier provided by intercellular adhesion (Rb). Genistein had no effect on basal endothelial cell electrical resistance or its derived components, Rb and α (Fig. 7B). However, pretreatment with genistein resulted in ablation of the early DPV-mediated barrier enhancement previously shown to be due to increased Rb and attenuated the subsequent decline in Rb (Fig. 7C), suggesting that a genistein-sensitive tyrosine kinase is involved in both limbs of the DPV-mediated biphasic response in endothelial cell barrier regulation. Finally, we noticed that inhibition of tyrosine kinase activities with genistein abolished both DPV-mediated actin rearrangement and phosphotyrosine colocalization (Fig. 8). These results are consistent with the role of Src family tyrosine kinase-mediated tyrosine phosphorylation and actin basal cytoskeletal rearrangement.

![Fig. 6. Effect of DPV on extracellular regulated kinase (ERK) activation in bovine endothelium. A: homogenates of confluent endothelial cells challenged with varying doses of DPV (0–5 μM) for 0 to 120 min were separated by SDS-PAGE and immunoblotted with antibodies (ab) for phospho (p-) and pan-ERK. Maximum phosphorylation of ERK occurred 30 min after DPV challenge. B: pretreatment of endothelial cells with the mitogen-activating protein kinase kinase (MEK) inhibitor PD-98054 (50 μM × 60 min) significantly attenuated the level of DPV-induced ERK phosphorylation and activation in endothelial cell homogenates after DPV exposure (30 min). C: confluent endothelial monolayers grown on gold microelectrodes were pretreated with vehicle (V; DMSO) or PD-98054 (PD; 50 μM × 60 min) and then challenged with 5 μM DPV while continuous measurements of TER were made, as described in MATERIALS AND METHODS. For each experiment, resistance values at discrete time points were pooled at 6-min intervals and plotted as the mean ± SE (n = 4). These results indicate that DPV activates endothelial cell ERK in a MEK-dependent manner. However, ERK activation does not appear to modulate DPV-induced barrier dysfunction.](http://jap.physiology.org/Content/10.1152/jappl.00898.2016)
Inflammatory responses are characterized by increases in vascular permeability and enhanced leukocyte infiltration, responses that reflect compromise of the endothelial cell barrier. Current concepts of endothelial cell barrier regulation postulate that, under basal or unstimulated conditions, a fine balance exists between competing contractile forces (determined by the extent of MLC phosphorylation) and endothelial cell tethering forces (determined by the activity of focal contact and adherens junction complexes). Reduction in endothelial cell contractile forces via endothelial cell MLCK inhibition significantly attenuates thrombin-mediated increases in endothelial cell permeability (12), edema formation in isolated ischemia-reperfused rat lungs (21), and transendothelial leukocyte migration in regulating lung inflammation (10). In contrast, the inability of MLCK inhibitors to completely attenuate increases in endothelial cell permeability produced by other edemagenic agents (9, 32) indicates the presence of alternate permeability-producing pathways that do not rely entirely on increases in MLC phosphorylation (9, 32). In prior studies, we utilized the cell-permeable oxidant and potent tyrosine kinase activator/phosphatase inhibitor DPV to study signaling cascades, which regulate the endothelial cell barrier through tyrosine phosphorylation. The mixture of equimolar amounts of H$_2$O$_2$ and vanadate to generate DPV produces a rise in endothelial cell cytosolic Ca$^{2+}$ and activates endothelial cell PLD in a dose-, time-,

**Fig. 7.** Effect of tyrosine kinase inhibition on DPV-mediated alterations in electrical resistance. Endothelial cells grown on gold microelectrodes were pretreated with either vehicle (0.1% DMSO) or genistein (Gen; 100 μM) for 60 min and then challenged with DPV (2 μM) and followed with real-time measurements of TER, as described in MATERIALS AND METHODS. DPV elicits a rapid but brief increase in TER with a peak effect at 10–15 min postchallenge that is attenuated by genistein. A: the subsequent DPV-induced decline in TER is also attenuated by genistein pretreatment. Time points from each experiment were pooled at discrete 6-min intervals and plotted as the mean transendothelial resistance ± SE (n = 4). Total TER was resolved into $R_b$ (B) and $\alpha$ (C), as described in MATERIALS AND METHODS. B: genistein blocked the DPV-induced increase in $R_b$ and attenuated the subsequent decline. C: the contribution to total resistance made by $\alpha$ was not significantly altered by either DPV or genistein.

**Fig. 8.** Inhibition of DPV-induced protein tyrosine phosphorylation and stress fiber formation by genistein. Bovine pulmonary artery endothelial cells were stimulated with 2 μM DPV for 30 min (A–D) after 1-h preincubation with either vehicle (A and C) or genistein (B and D). Immunofluorescent staining for F-actin (A and B) was detected by staining cells with Texas red-conjugated phalloidin. Similar staining was performed with anti-tyrosine phosphorylation antibodies (C and D), as described in MATERIALS AND METHODS. These studies demonstrate clear inhibition of DPV-mediated actin rearrangement and phosphorylation accumulation by genistein.
and tyrosine kinase-dependent fashion (30). Chelators of intracellular Ca\(^{2+}\) and antioxidants attenuated DPV-mediated stimulation of PLD and protein tyrosine phosphorylation in endothelial cells, indicating that the redox state of the cell is important to DPV-mediated endothelial cell activation (28, 30). In the present study, we have demonstrated that DPV produces substantial disruption of the endothelial cell barrier and increases tyrosine phosphorylation of endothelial cell tethering proteins, which may also provide relevant molecular signaling mechanisms that underlie DPV-induced physiological alterations. Although albumin clearance assays confirmed that DPV increases endothelial permeability (Fig. 1) in a time course that agrees with TER monitoring (Fig. 2), the much greater time sensitivity of the electric cell-substrate impedance sensor system (14) enabled the reproducible detection of the early enhancement of endothelial barrier function invoked by DPV that could not be demonstrated by the albumin clearance assays. The ablation by genistein of both the DPV-evoked barrier enhancement and the subsequent deterioration of endothelial barrier function (Fig. 7B) indicates that both phases of the response are dependent on tyrosine kinase-dependent cell signaling pathways. These findings are highly consistent with a major role of tyrosine phosphorylation in the regulation of endothelial cell barrier properties.

MAP kinases, including ERK1 and ERK2, are common targets for activation by tyrosine kinase-mediated cell signaling and are activated by DPV, as we (Fig. 6A) and others (23) have shown. Recently, we have noted diverse, biologically relevant mediators, such as lysophosphatidic acid (LPA), with significant increases in TER. As both LPA and DPV share an ability to rapidly activate p42 and p44 ERK (6), the biphasic effect of DPV on electrical resistance was of particular interest. Our findings suggest, however, that neither the increase in the integrity of the monolayer nor the subsequent decline in electrical resistance are MEK and/or ERK dependent. The disassembly of adherens junctions in growth factor-challenged cells occurs in a Rho-dependent manner (33), leading us to speculate that the DPV-induced TER alterations seen in our studies (Fig. 3) could be mediated through endothelial cell ERK1 and ERK2. Although DPV-induced activation of ERK1 and ERK2 (Fig. 6A) correlates temporally with the termination of the initial phase of DPV-induced endothelial barrier enhancement (Fig. 2), studies with PD-98059, which effected a significant reduction in DPV-mediated ERK activation, did not alter either phase of DPV-induced TER alterations. This is of interest because our laboratory has recently shown that MEK inhibition in this manner significantly attenuates protein kinase C-dependent endothelial cell permeability (49). Thus, although ERK1 and ERK2 are strongly activated by DPV challenge, these studies indicate that ERKs are unlikely to be major upstream effectors that modulate the activity of cell-cell or cell-matrix adhesion and endothelial barrier function in this model.

Endothelial cell structures that mediate cell-matrix (focal contacts) and cell-cell (adherens junctions) adhesion are both composed of cytoplasmic complexes of proteins that contain substrates for protein tyrosine kinases and can, therefore, be directly phosphorylated on tyrosine residues after DPV challenge. Immunocytochemically, the major site of phosphotyrosine enrichment after 10 min of DPV exposure, the point at which endothelial barrier enhancement is maximal, occurs at the ends of actin stress fibers, which corresponds cytoarchitecturally with focal adhesion plaques (Fig. 4). At 30 min after DPV exposure, the timepoint at which barrier disruption is developing, phosphotyrosine reactivity is notably prominent at the cell periphery at sites of intercellular attachment corresponding to adherens junctions (Fig. 4). Phosphotyrosine immunoblotting confirmed that DPV resulted in rapid (5 min) enhancement in the tyrosine phosphorylation of immunoprecipitated p125 FAK (Fig. 5A), whereas increases in phosphotyrosine content of cadherin-associated proteins do not develop until after 15 min (Fig. 5B).

This extremely rapid induction of tyrosine phosphorylation of p125 FAK (Fig. 5) suggests that this may be a relevant mechanism for barrier enhancement. FAK is a signaling molecule with a rich inventory of protein-protein interaction domains and, when activated by tyrosine phosphorylation, could potentially serve as an upstream effector that coordinates the enhancement of the intercellular barrier through small G protein-dependent mechanisms. LPA, which increases endothelial resistance (6), also enhances the tyrosine phosphorylation of p125 FAK (49–52), leading us to speculate that p125 FAK may participate in a barrier-protective capacity in this function, an observation that we are presently investigating. Although increases in intercellular resistance (\(R_{\text{c}}\)) after DPV accounts for nearly all of the enhancement of endothelial barrier function (Fig. 3), tyrosine phosphorylation in adherens junctions correlates poorly with endothelial barrier enhancement, indicating that tyrosine phosphorylation substrates in adherens junctions are unlikely to underlie DPV-mediated barrier enhancement (Fig. 5). Our observations appear to temporally correlate with the evolution of DPV-mediated barrier disruption to the tyrosine phosphorylation of the cadherin-associated proteins \(\beta\)- and \(\gamma\)-catenin. Whereas the tyrosine phosphorylation-specific pathways invoked by DPV that alter endothelial barrier properties are not known, pervanadate was noted to induce the association of multiple phosphotyrosine-containing proteins with the Src homology SH2 and SH3 domains of phospholipase C\(_{\gamma}\) and activation of Src-family kinases (20, 34, 38) and to rapidly increase tyrosine phosphorylation of potentially important adaptor proteins such as growth-associated protein (35). Tyrosine phosphorylation of adherens junction proteins in Rous sarcoma virus-transfected cells (1, 17, 26, 43) and in cells treated with tyrosine phosphatase inhibitors (42) has previously been associated with the loss of intercellular adhesiveness and represents a plausible mechanism for the development of DPV-induced endothelial
barrier dysfunction. Resolving TER vectors illustrates that both phases of the endothelial barrier response to DPV occur predominantly through changes in $R_w$, and that changes in $\alpha$ during the time course of DPV challenge contribute little to total resistance (Fig. 3); again, this is consistent with a decrease in the physiological integrity of the intercellular barrier via the tyrosine phosphorylation of adherens junction proteins. It is interesting to note that the disassembly of adherens junctions has recently been shown to be induced by $p60^{src}$-mediated tyrosine phosphorylation (5, 47, 51). These results are unlikely to be related to DPV-mediated increases in $Ca^{2+}$, as our laboratory has recently reported that a similar endothelial barrier disruption occurs after vanadate challenge of endothelial cell monolayers and is not associated with any significant alteration of intracellular $Ca^{2+}$ homeostasis (16).

In summary, DPV produces biphasic alterations in endothelial cell integrity and barrier properties. The initial phase is characterized by increased MAP kinase activity, increases in p125 FAK phosphorylation, and barrier protection. The more delayed response involves increases in MLCK activity and endothelial cell MLC phosphorylation (11), coupled with intense tyrosine phosphorylation of p125 FAK and cadherin-associated proteins. We speculate that these biochemical events are important contributors to subsequent endothelial cell paracellular gap formation and reduction in barrier properties observed after DPV challenge. Future studies that clarify the involvement of Src kinase and related family members and their targets may increase our understanding of endothelial cell barrier integrity and edema formation through focal contact or adherens junction disassembly.

The authors gratefully acknowledge Lakshmi Natarajan and Steve Durbin for superb technical assistance and Ellen G. Reather for expert manuscript preparation.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-50533, HL-58064, HL-03666, HL-57260, and HL-47671, by the National American Heart Association, and by awards from the American Lung Association.

REFERENCES


24. Kumagai N, Morii N, Fujisawa K, Yoshimasa T, Nakao K, and Narumiya S. Lysophosphatidic acid induces tyrosine phosphorylation and activation of MAP-kinase and focal adhesion