Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products

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Schaphorst, Kane L., Eddie Chiang, Keri N. Jacobs, Ari Zaiman, Viswanathan Natarajan, Frederick Wigley, and Joe G. N. Garcia. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. Am J Physiol Lung Cell Mol Physiol 285: L258–L267, 2003. First published March 7, 2003; 10.1152/ajplung.00311.2002.—In vitro and in vivo evidence indicates that circulating platelets affect both vascular integrity and hemostasis. How platelets enhance the permeability barrier of the vascular endothelium is not well understood. We measured the effect of isolated human platelets on human pulmonary artery endothelial cell (EC) barrier integrity by monitoring transmonolayer electrical resistance. EC barrier function was significantly increased by the addition of platelets (~40% maximum, 2.5 × 10^6 platelets/ml). Platelet supernatants, derived from 2.5 × 10^6 platelets/ml, reproduced the barrier enhancement and reversed the barrier dysfunction produced by the edemagenic agonist thrombin, which implicates a soluble barrier-promoting factor. The barrier-enhancing effect of platelet supernatants was heat stable but was attenuated by either charcoal delipidation (suggesting a vasoactive lipid mediator) or pertussis toxin, implying involvement of a Gα-coupled receptor signal transduction pathway. Sphingosine-1-phosphate (S1P), a sphingolipid that is released from activated platelets, is known to ligate G protein-coupled EC differentiation gene (EDG) receptors, increase EC electrical resistance, and reorganize the actin cytoskeleton (Garcia JG, Liu F, Verin AD, Biruakova A, Dechert MA, Gerthoffer WT, Bamberg JR, and English D. J Clin Invest 108: 689–701, 2001). Infection of EC with an adenoviral vector expressing an antisense oligonucleotide directed against EDG-1 but not infection with control vector attenuated the barrier-enhancing effect of both platelet supernatants and S1P. These results indicate that a major physiologically relevant vascular barrier-protective mediator produced by human platelets is S1P.

endothelium; lung; vasculature; injury; G protein; differentiation; cell differentiating gene

THE VASCULAR ENDOTHELIUM is a biologically complex tissue that forms a semipermeable barrier between the intravascular fluid compartment and the interstitium of various organs. Integrity of the endothelial cell (EC) monolayer is essential for homeostasis, and perturbations of the barrier function of the endothelium are now recognized as a cardinal feature of diverse and important pathobiological processes including acute lung injury and atherogenesis. The lung vasculature contains an enormous surface area and is particularly sensitive to the dynamic features of endothelial barrier dysregulation, where increased vascular permeability leads to exudation of fluid and solutes from the intravascular space into the pulmonary interstitium. Extensive increases in lung vascular permeability result in flooding of the alveolar air spaces (pulmonary edema), which is the hallmark pathophysiological derangement of the adult respiratory distress syndrome. Circulating blood platelets have been noted for many decades to be essential to the maintenance of the endothelium as a semipermeable barrier. In vitro and in vivo models have described profound defects in EC barrier function after perfusion with platelet-poor plasma (PPP) or after depletion of platelets with anti-platelet antibodies (29); these events are completely reversed by the infusion of platelet-rich plasma (PRP; Refs. 4, 17). In addition to the restorative effects of platelets, in vitro studies have demonstrated that these blood components decrease basal albumin flux across EC monolayers (44) and change the elution profile of several tracers in a cell column assay (18), which indicates the importance of platelets to normal vascular function. Although the mechanism underlying these protective effects remains elusive, it has been demonstrated that platelets as well as platelet supernatants provide similar barrier-enhancing results (18, 39, 44), which suggests a released barrier-enhancing factor. Preliminary characterization indicates that the active barrier-promoting factor is heat stable and resistant to inactivation by exposure to freeze-thaw cycles; therefore, it is likely not a peptide (1, 18, 32). Additionally, the barrier-enhancing effect of platelet-conditioned media was shown to involve signaling via the small GTPase Gi10. A variety of platelet-released products including adenosine, adenine nucleotides, serotonin, norepinephrine, and arachidonic acid metabolites have been excluded as possible factors involved in the platelet-induced decrease in endothelial permeability (1, 18, 37, 45). Although the specific identity of the platelet-de-
rived endothelial permeability-decreasing factor remains unknown, subsequent work has provided evidence that this factor is a phospholipid with albumin-coupled activity (32).

Sphingosine-1-phosphate (S1P) is a recently described serum-borne sphingolipid that is released by activated platelets. Through its interaction with the endothelial differentiation gene (EDG) family of receptors, S1P mediates numerous biological effects including EC migration (8, 9, 22, 28, 34–36, 49), adherens junction assembly (26), cell proliferation (3, 22, 41), wound healing (25), and inhibition of apoptosis (20, 24, 41). Platelets have a highly active form of sphingosine kinase that rapidly converts sphingosine into S1P and a relative deficiency of sphingosine lyase, which is the enzyme responsible for S1P catabolism and breakdown (52). As a result, platelets store abundant amounts of S1P and are the cellular component that is primarily responsible for the concentration of S1P found in plasma (51). We recently described (11) the ability of S1P to potentiate and rapidly enhance transendothelial electrical resistance (TER) and reorganize filamentous actin into a prominently thickened cortical actin band.

The ability of platelets to secrete S1P into the bloodstream, in the context of the potent biological effects that S1P has on ECs, warrants a closer examination of S1P as a potential mediator of platelet-induced barrier enhancement. We analyzed the effects of isolated washed human platelets on the transmonolayer electrical resistance of confluent human pulmonary artery ECs (HPAECs), which is a measure of endothelial integrity, as well as on actin cytoskeletal arrangement in these cells. Our data show that platelets and S1P produce similar concentration- and dose-dependent increases in EC barrier resistance and comparable reorganization of the actin cytoskeleton into thickened cortical bands. In addition, pharmacological inhibition of Gα-coupled protein signal transduction, EDG-1 receptor antisense oligonucleotide strategies, and incubation of the stimulus with activated charcoal diminish the effects of both exogenously added platelets and S1P on HPAECs. This correlation supports our hypothesis that S1P represents the major bioactive platelet-derived factor that affects the enhancement of the EC permeability barrier.

**MATERIALS AND METHODS**

**Reagents.** Unless otherwise noted, all chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO). Pertussis toxin (PTX) was purchased from Calbiochem (La Jolla, CA), and EDG-1 and EDG-3 antisense and scrambled oligonucleotides were synthesized by the DNA Analysis Facility at Johns Hopkins University (Baltimore, MD) as recently described (28). Lipofectamine, DMEM, and Opti-MEM were purchased from Life Technologies (Rockville, MD). Texas red phallolidin was obtained from Molecular Probes (Eugene, OR).

**Cell culture.** HPAECs were obtained from Clonetics (Walkersville, MD) and were cultured in endothelial basal medium (EBM)-2 growth media supplemented with 0.2 ml of hydrocortisone, 2 ml of human FGF-B, 0.5 ml of VEGF, 0.5 ml of long-arm insulin-like growth factor-1 (R3-IGF-1), 0.5 ml of ascorbic acid, 0.5 ml of human epidermal growth factor (EGF), 0.5 ml of GA-1000 (Clonetics), and 0.5 ml of heparin with 10% FBS. The cultures were grown in gelatin-coated tissue culture-treated flasks and maintained in a humidified atmosphere of 5% CO2-95% air until confluency was reached with contact-inhibited monolayers. Cells were then seeded onto gelatinized glass coverslips (for immunofluorescence studies) or gelatinized gold-coated microelectrodes (for electrical resistance measurements) and again grown to 100% confluent monolayers before use. All experiments were performed in DMEM serum-free media.

**Measurement of cell monolayer TER.** Electrical resistance across EC monolayers was measured by using an electrical cell-substrate sensor (ECIS) system (Applied Biophysics, Troy, NY) as previously described (13). Cells grown on gold microelectrodes (10–3 cm2) in polycarbonate wells act as insulating particles, and the resistance across the monolayers (TER) is measured in real time. TER increases as cells adhere on the microelectrode and intercellular cell contacts are formed or in response to agents that increase cell-to-cell adhesive interactions (11). In contrast, cell retraction, rounding, or loss of adhesion is reflected by decreases in TER (13). These measurements provide a highly sensitive biophysical assay that indicates the state of cell shape, focal adhesion, and endothelial barrier function (16, 47). 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 counter-electrode (1 cm2) were connected to a phase-sensitive lock-in amplifier (model 5301A, EG&G Instruments, Princeton, NJ) with a built-in differential preamplifier (model 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. Wells with baselines that exceeded 2 standard deviations from the pooled mean were rejected from analysis. All electrical resistance data are presented as values normalized by the basal resistance for each well.

**Platelet isolation.** Whole blood was collected from healthy donors into 10-ml vacutainers (Becton-Dickinson) that contained 15% K3-EDTA. Blood was transferred to round-bottom polypropylene tubes (Falcon) and centrifuged at 500 g (Allegra 6 centrifuge, Beckman) for 10 min to obtain PRP. The PRP was removed and spun at 1,200 g for an additional 10 min to separate platelets from plasma. The platelet button was resuspended and washed four times in a modified Tyrode’s buffer (2.37 mM KCl, 124 mM NaCl, 11 mM NaCHO3, 0.4 mM Na2HPO4, 0.3% BSA, 4.6 mM dextrose, and 10 mM EDTA). After the final washing, platelet counts were adjusted to 1.5–2.5 × 108/ml (Beckman Coulter Z1) in Tyrode’s buffer that contained 100 μM EDTA. Platelet supernatants were prepared by centrifuging platelet suspensions in a microcentrifuge for 45 s.

**Platelet function analysis.** Platelets suspended in Tyrode’s buffer that contained 100 μM EDTA were stimulated with 100 nM human α-thrombin either in the presence or absence of 1 mM CaCl2. Light transmission, a reflection of platelet aggregation, was measured by a Chrono-log aggregometer and recorded on a Chrono-log chart-recorder.

**Washed human platelets remain functional after the isolation and washing procedure.** After carefully isolating and washing human platelets, we tested their responsiveness, viability, and function by measuring platelet aggregation in response to the known platelet-aggregating agent thrombin.
Tyrode’s buffer is able to prevent platelet activation and aggregation in response to thrombin. Replenishing the amount of usable calcium (which is required for platelet aggregation) in the Tyrode’s buffer with 1 mM CaCl₂ allowed us to rapidly and effectively induce platelet aggregation after stimulation with thrombin. This data assures us that the isolation procedure does not affect platelet function or cause premature platelet activation.

Immunofluorescence. After treatment, HPAECs were washed with PBS and fixed with 3.7% formaldehyde for 15 min. Cells were then rinsed twice with PBS, permeabilized with 0.25% Triton X-100 for 5 min at room temperature, and rinsed twice with Tris-buffered saline that contained 0.1% Tween 20 (TBST). Coverslips were incubated in Texas red phallodin for 60 min at room temperature and rinsed four times with TBST before being mounted on glass slides. A Nikon Eclipse TE3000 microscope and Sony DKC-5000 digital camera connected to a personal computer were used to visualize and analyze filamentous F-actin.

Charcoal-stripped supernatants derived from washed human platelets. Supernatant derived from 2.5 × 10⁶ platelets/ml was incubated with washed activated charcoal overnight at 4°C to remove S1P and lipid growth factors (50). After incubation, suspensions were filtered through a 0.22-μm filter to remove charcoal and added to electrical cell impedance-sensor (ECIS) chambers immediately after filtration.

Construction of antisense EDG-1 adenovirus. The recombinant Adv-AS-EDG1 was constructed by using the method of He et al. (19). Briefly, with the use of pcDNA3.1/EDG1 (kindly provided by Dr. Timothy Hla, University of Connecticut Health Center, Farmington, CT) as a template, PCR primers that contained the indicated restriction enzymes were synthesized to amplify the EDG-1 sequence from human embryonic kidney (HEK)-293 cells. After 10 days, cell lysate was prepared and used to infect more HEK-293 cells. The presence of infectious virus was confirmed by green fluorescent protein (GFP) fluorescence.

Inhibition of EDG-1 receptor expression with overexpression of antisense EDG-1. HPAECs were seeded into ECIS chambers at 100,000 cells/well. Cells were infected with GFP control or antisense EDG-1 adenoviruses (multiplicity of infection of 25) 3 h after seeding. After 24 h of infection, the media was replaced. At 48 h postinfection, the cells were placed in serum-free media for 1.5 h and allowed to equilibrate. TER was monitored over time in response to 1 μM S1P or activated platelet supernatant obtained from 6 × 10⁶ platelets.

RESULTS

Platelets and platelet supernatants induce a rapid and sustained increase in EC transendothelial electrical resistance. In agreement with previously published data that show a decrease in albumin clearance after the addition of human platelets to HPAEC monolayers, Fig. 1A illustrates that platelets increase EC transendothelial electrical resistance (TER). Human pulmonary endothelium that was cultured on gold microelectrodes and grown to confluence before being trans-
ferred to serum-free growth media and serum starved for 2 h exhibited a basal transmonolayer resistance of 8,480 ± 1,132 Ω (mean ± SE). Addition of 10^4–10^6 freshly isolated intact human platelets to the wells resulted in a significant increase in TER (expressed as percentage increase over basal number) for all wells (Fig. 1A) with a maximal effect observed at 10^6 platelets. The time course of the effect of addition of intact freshly isolated platelets demonstrated that the increase in resistance achieved after the addition of platelets was abrupt and sustained for at least 2 h (Fig. 1B). To confirm that the barrier-enhancing activity of platelets was not merely due to impedance changes that resulted from layering of platelets onto the endothelial monolayer, supernatants were derived from suspensions that contained 2.5 × 10^6 platelets/ml and added to the wells. The platelet supernatants also markedly increased TER in an abrupt and sustained manner that was qualitatively identical to the addition of intact platelets (Fig. 1C). These data confirm prior reports that the barrier-enhancing effect of platelet supernatants results from a soluble platelet-associated factor.

**EC barrier enhancement induced by platelets is dependent on G_βδ-coupled receptor.** Pertussis toxin (PTX) is commonly used as a tool to inhibit G protein-coupled cell signaling by G_βδ-coupled proteins. To evaluate whether the barrier-enhancing factor present in platelet supernatants exerted its effect via ligation of a G protein-coupled receptor, we examined the effects of PTX on platelet- and platelet supernatant-induced increases in endothelial TER (Fig. 2). We previously reported that PTX (1 μg/ml) resulted in the ADP ribosylation of a range of proteins, induced stress-fiber formation, and increased transmonlayer albumin flux in bovine endothelium (14, 38). Consistent with our prior report, PTX pretreatment resulted in decreased TER but also abolished the barrier-enhancing effect of both freshly isolated intact platelets and platelet supernatant (Fig. 2). These data indicate that platelet-mediated effects on endothelial barrier properties are mediated by a G protein-coupled receptor.

**Removal of lipid growth factors from platelet supernatants attenuates increase in TER.** Activated platelets release a wide variety of bioactive molecules including fibrinogen, fibronectin, thrombospondin, transforming growth factor-β, platelet-derived growth factor, calcium, ATP, ADP, and platelet factor-4. Previous studies have established that the permeability barrier-enhancing activity in serum derived from platelets is resistant to heat denaturing, which suggests that the platelet-derived barrier-enhancing factor is not a peptide. We confirmed this observation by heating platelet supernatants to 100°C for 15 min and challenging confluent endothelial monolayers with the boiled material. Heat treatment resulted in only a minimal loss (<10%) of the maximal increase in TER (data not shown). We therefore hypothesized that the platelet-associated endothelial barrier-enhancing factor was a platelet-derived lipid, and that the barrier-promoting activity of platelet supernatants would be lost by delipidation. To examine this hypothesis, we used activated charcoal to remove lipids from platelet supernatants. This strategy has been previously validated by Lee et al. (27), who demonstrated that, after coincubation in serum, materials eluted from activated charcoal activated cells that had been transfected with the EDG-1 receptor, whereas the charcoal-treated serum did not, thereby confirming the ability of charcoal to remove the EDG-1 ligand from serum. We therefore adsorbed lipid components from platelet supernatants onto activated charcoal as previously described (9, 27, 50). Delipidation of platelet supernatants with this strategy resulted in marked attenuation in the maximal increase in TER after addition of the charcoal-treated platelet supernatants to the endothelial monolayers (Fig. 3). This data supports the conclusion that the platelet-derived barrier-promoting factor is a lipid product.

**SIP shares similar physiological effects and signal transduction pathways with human platelets.** Our previous work indicated that SIP is the primary endothelial chemotactic factor in serum (9) and that it exerts prominent effects on EC barrier function. As our data indicated a platelet-derived barrier-promoting lipid factor, we next explored whether SIP was the platelet-derived factor. When human pulmonary endothelium was cultured on gold microelectrodes to confluence and challenged with SIP (1 μM), TER was seen to increase in a similar time scope and to a similar magnitude when compared with the TER increases induced by either intact platelets or platelet supernatants (Fig. 3). To validate our usage of activated charcoal as a means to remove lipids (primarily SIP) in this model, we also incubated aliquots of SIP with the activated charcoal and tested the effects on TER. As illustrated in Fig. 3, the biological activity of SIP was abrogated by acti-
vated charcoal treatment, which is consistent with our
prior report (9) that demonstrates a loss of chemoattractive activity of both platelet-rich plasma and fetal bovine serum after delipidation.

Both platelets and S1P reverse thrombin-induced endothe
delial paracellular gap formation and barrier dys
fuction. Thrombin induces endothelial paracellular
gap formation in in vitro and in vivo models, is found at
sites of vascular injury and wounding, and is a known
platelet activator. To mimic a physiologically relevant
setting in which platelets and their released factors
can interact with thrombin in the presence of compro
mised endothelial monolayers, we tested the effects of
platelets after treatment of monolayers with thrombin.
As expected, thrombin produced a robust decrease in
TER that was reversed by the addition of S1P (Fig. 4A),
platelets (Fig. 4B), and platelet supernatants (data not
shown). These data provide convincing evidence that
platelets and S1P are important cellular mediators of
vascular barrier function.

Both platelets and S1P induce actin cytoskeletal re
arrangement in HPAECs. The actin cytoskeleton plays
a crucial role in many signaling pathways and in bar
rier regulation (12, 43). To demonstrate the critical
involvement of the cytoskeleton in the S1P- and plate
let-mediated increase in electrical resistance, we pre
treated cells with the microfilament-disrupting agent
cytochalasin B and found that both the platelet super
natant- and S1P-induced increases in electrical resis
tance were completely abolished (Fig. 5A). This sug
gested that the enhancement of endothelial barrier
function induced by both S1P and platelets was essen
tially dependent on the integrity or organization of
F-actin. To study the effect of platelet-derived vasoac
tive soluble factors on EC cytoskeletal organization,
platelet supernatant- or S1P-challenged endothelium
was stained with Texas red phalloidin to localize F-
actin. Both S1P and platelets induced a dramatic
change in actin staining found throughout the cell (Fig.
5B). In control endothelium, actin is found in a reticu-
lar pattern dispersed throughout the cell (Fig. 5B, left), whereas the addition of either S1P (middle) or platelets (right) caused actin to rearrange into a thickened cortical band especially enriched at the periphery. The manner in which actin was reorganized in endothelium by challenge with platelet supernatants was qualitatively highly similar to that induced by challenge with S1P.

EDG-1 receptor inhibition significantly blocks effects of platelets and S1P on HPAEC electrical resistance. The previous results are highly consistent with a role for S1P as the mediator of platelet-induced barrier protection. S1P binds a number of EDG receptors including EDG-1, -3, -5, -6, and -8 and increases TER in a PTX-sensitive manner (11). Our studies thus far have demonstrated a correlation between the events evoked by treatment of ECs with isolated washed human platelets, platelet-derived supernatants, and commercially available S1P. To confirm a central role for EDG-1-receptor ligation by S1P in platelet supernatant-mediated enhancement of endothelial barrier function, we constructed an adenoviral vector that expresses an antisense oligonucleotide directed against EDG-1. We have previously shown that this antisense oligonucleotide strategy successfully reduces the biological effects of S1P in ECs as measured by both EC migration (28) and TER (11). In addition, we have shown that the levels of EDG-5 and -8 expression are extremely low or undetectable compared with the levels of EDG-1 and -3 expression in our cells. Consistent with a role for S1P in the platelet-mediated barrier enhancement, infection with adenoviral EDG-1 antisense oligonucleotide significantly attenuated the HPAEC response to platelets and to S1P (Fig. 6A). We observed an incomplete attenuation of the S1P- and platelet-induced enhancement of barrier function by the adenoviral vector. We therefore examined the effect of the vector on EDG-1 protein level and observed that transduction of the EDG-1 antisense oligonucleotide resulted in a 40% reduction in the expression of EDG-1 protein (Fig. 6B). Therefore, the subtotal reduction in EDG-1 expression correlates with the incomplete attenuation of enhanced barrier function.

DISCUSSION

Mechanisms that govern increases in vascular permeability, which is a feature of both newly formed (angiogenic) and inflamed vessels, are under intense investigation. However, little is known about processes that determine barrier restoration or barrier protection. Platelets have been implicated in the maintenance of the vascular barrier, and proteins and lipids released after platelet activation have long been appreciated as enhancing the integrity of the microcirculation in vivo (17) and in vitro (42). In contrast, a reduction in circulating platelets (or thrombocytopenia) in humans accelerates capillary permeability, protein leakage, and edema formation in tissues (42), a result verified in animals (30, 31, 45). Thrombocytopenic patients have fragile and leaky vessels (15), although the exact mechanisms by which platelets and their products preserve capillary integrity and exert this barrier-protective action has not yet been precisely elucidated. The compromise in the endothelial barrier seen in thrombocytopenia can be reversed either with platelet...
infusions or by the systemic administration of platelet-released products. For example, Shepard et al. (44) described decreases in albumin permeability of pulmonary artery endothelium due to a platelet-derived factor. Haselton and Alexander (18) also confirmed the permeability-reducing properties of the 2-h releasate from unstimulated platelets. A number of putative agents (adenosine, ATP, β-agonists, serotonin, etc.) have since been excluded, because the barrier-enhancing agent released was found to be heat stable. These observations clearly establish that the platelet, via soluble platelet-derived factors, is essential in maintaining normal vascular homeostasis.

We and others have reported on vasoactive platelet-derived lipids that activate endothelium, including our first report that S1P activated EC phospholipase D (7, 10, 23, 33, 48). Subsequent studies have since demonstrated that platelet-derived phospholipids modulate the barrier function of the vascular endothelium via both endothelial barrier-perturbing lipid products such as phosphatidate (6) and barrier-stabilizing/enhancing lipid products such as lysophosphatidic acid (LPA) (8, 32) and S1P (11). Although these platelet-derived factors are implicated in the vascular endothelial barrier-enhancing effect of platelets, the specific platelet-derived factors remain incompletely characterized. Our data clearly demonstrate that both platelet supernatants and exogenously added S1P enhance endothelial barrier function (see Fig. 1) in a Gi-dependent manner (see Fig. 2), which is in agreement with earlier findings (11). We also found that both platelet supernatants and exogenously added S1P reversed agonist-mediated barrier dysfunction (see Fig. 4), and each produced a highly similar reorganization of cellular F-actin (see Fig. 5). The implicated role of S1P in mediating the barrier-enhancing effect of platelet supernatants was confirmed by demonstrating that diminished expression of the specific S1P receptor EDG-1 attenuated the barrier-enhancing effects of platelet supernatants and exogenously added S1P (see Fig. 6). Taken together, these observations support a significant role for S1P in mediating the vessel-stabilizing effect of circulating platelets.

Our data agree with published reports that platelet-released products decrease endothelial permeability (18) and increase TER (32). Although our data strongly support the concept that S1P is a platelet-derived factor that significantly underlies this effect, our data do not exclude the possibility that other platelet-derived products may also participate in the barrier-protective effect of platelets. We and others have demonstrated that LPA, released from thrombin-stimulated platelets, possesses endothelial barrier-stabilizing activity (1, 8, 32). Although in our experiments LPA-induced endothelial barrier enhancement was weak in vitro (8), LPA released from platelets may form an active complex with serum albumin that further enhances its barrier-enhancing activity (32), which may in part explain the previously observed trypsin sensitivity of the barrier-enhancing platelet-derived factor (18). Although we did not examine trypsin sensitivity or possible cooperation between S1P and other serum proteins in these studies, we have previously established that S1P, unlike LPA, induces significant enhancement of endothelial barrier function when added exogenously even in the absence of albumin or serum (11).

The mechanisms by which platelets and platelet phospholipids such as S1P enhance the vascular barrier are unknown. Although mechanistic approaches designed to understand EC paracellular gap and bar-
rrier regulation have revealed the extreme complexity of these processes, several valuable paradigms have been developed. One useful model describes paracellular gap formation as regulated by the balance of competing contractile and adhesive forces that together regulate cell shape changes (5, 12). In this paradigm, cell-cell and cell-matrix tethering forces exist in equilibrium with contractile mechanisms that generate centripetal tension via an actin and myosin motor. Both competing forces in this model are intimately linked to the actin-based endothelial cytoskeleton by a variety of actin-binding proteins that are critical to tensile force generation as well as the linkage of the actin cytoskeleton to adhesive membrane components. We have shown that platelets and S1P produce rapid and dramatic enhancement of polymerized F-actin and myosin staining that was spatially confined to the cortical cytoskeletal ring. To link this observation to human EC monolayer barrier enhancement, we used the actin-depolymerizing agent cytochalasin, which produced a prompt decline in barrier function (see Fig. 5). Unlike the dramatic protective response to S1P challenge after established thrombin-induced barrier dysfunction, neither platelets nor S1P increased barrier function when added after cytochalasin challenge, which indicates a critical requirement for dynamic cytoskeletal rearrangement and an intact actin cytoskeleton for S1P-mediated barrier protection. We speculate that platelets, via elaboration of S1P, may enhance barrier function by promoting a rearrangement of actin structures that either reduce or redistribute tensile forces. Because Rho-family small GTPases are key molecular switches for regulating actin assembly and dynamics in cells, they are likely to be essential effectors in the enhancement of endothelial barrier function by platelets and platelet-released products. We have previously demonstrated that the Rho-family GTPases Rac and Rho play a central role in S1P-mediated endothelial cytoskeletal rearrangement (11).

S1P is a biologically active lipid generated by hydrolysis of glycerophospholipids and sphingomyelin in the membranes of activated cells that is formed by the phosphorylation of sphingosine by sphingosine kinase. Whereas in most cell types the levels of S1P are tightly regulated by the rapid metabolism of S1P via sphingosine lyase and cellular lipid phosphohydrolase activities (52), platelets are unique in lacking in these catabolic activities, which thereby enables the accumulation of S1P in cells and allows for its release by activated platelets (51, 52). S1P is a normal constituent in plasma with increased (micromolar) levels in serum that are consistent with S1P release during whole blood coagulation (51). S1P exerts diverse biological effects on cells and is now recognized as an important courier of cellular information (7, 10, 23, 33, 48). S1P stimulates proliferation, calcium mobilization, adhesion molecule expression, and suppression of caspase-dependent apoptosis (7, 20, 40, 46) via ligation of G protein-coupled receptors of the EDG family (26). EDG-1 was cloned from RNA expressed in ECs stimulated to undergo angiogenic responses in vitro (21). Whereas S1P binds to EDG-1, -3, -5, and -6, LPA preferentially binds EDG-2 and -4 and perhaps other unidentified members of this family of receptors (2, 46). In cultured human artery endothelium, the expressed EDG-family receptors are EDG-1 and -3 (26, 49) with EDG-1 being significantly more abundant than EDG-3 in our system (8).

We have shown that platelet supernatants and S1P each invoke similar physiological and histochemical responses in pulmonary artery endothelium, and that the endothelial barrier-enhancing activity of platelets is EDG-1 dependent, which implicates an important role for S1P as a key platelet-derived endothelial barrier-enhancing factor. Thus the vascular endothelium is well positioned both molecularly and anatomically to respond to the barrier-modulating effects of platelets. We speculate that this intimate relationship between platelet-derived S1P and the vascular endothelium reflects a critical linkage between coagulation, inflammation, and angiogenesis that works in a concerted fashion with other factors to initiate neovascularization and potentiate the function of nascent vessels by optimizing endothelial barrier integrity.

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