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Induction of Endothelial Monolayer Permeability by Phosphatidate

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Abstract Released into the vasculature from disrupted cells or transported to the surface of adjacent effectors, phosphatidate and related lipids may potentiate endothelial cell activation. However, the effect of these lipids on endothelial monolayer barrier integrity has not been reported. The present study documents the induction of endothelial monolayer permeability by phosphatidate. Both long (di-C18:1) and medium (di-C10; di-C8) chain length phosphatidates increased permeability of bovine pulmonary artery endothelial cell monolayers assessed using a well characterized assay system in vitro. Barrier disruption effected by dioctanoyl (di-C8) phosphatidate was markedly potentiated by the addition of propranolol, an inhibitor of endothelial cell "*ecto*"-phosphatidate phosphohydrolase (PAP), a lipid phosphate phosphohydrolase (LPP) that efficiently hydrolyzes extracellular substrate. Disruption of barrier function by phosphatidate did not result from its non-specific detergent characteristics, since a non-hydrolyzable but biologically inactive phosphonate analog of dioctanoyl phosphatidate, which retains the detergent characteristics of phosphatidate, did not induce permeability changes. Furthermore, neither diacylglycerol nor lyso-PA effected significant increases in monolayer permeability, indicating the observed response was due to phosphatidate rather than one of its metabolites. Phosphatidate-induced permeability was attenuated by preincubation of endothelial cells with the tyrosine kinase inhibitor, herbimycin A (10 μg/ml), and enhanced by the tyrosine phosphatase inhibitor, vanadate (100 μM), implicating a role for activation of intracellular tyrosine kinases in the response. In addition, phosphatidate increased the levels of intracellular free Ca^{2+} in endothelial cells and ligated specific binding sites on endothelial cell plasma membranes, consistent with the presence of a phosphatidate receptor. Since phosphatidate generated within the plasma membrane of adherent effectors potentially interacts with endothelial membranes, we evaluated the influence of phosphatidate-enriched neutrophil plasma membranes on endothelial monolayer integrity. The effects of ectopic phosphatidate on endothelial monolayer permeability were mimicked by phosphatidate confined to neutrophil plasma membranes. We conclude that phosphatidate may be a physiologic modulator of endothelial monolayer permeability that exerts its effects by activating a receptor-linked, tyrosine kinase-dependent process which results in mobilization of intracellular stored Ca^{2+} and consequent metabolic activation. J. Cell. Biochem. 75:105–117, 1999.

Key words: phosphatidic acid; endothelial cells; vascular permeability; lipid mediators

The role of neutrophils in vascular edema and capillary permeability has been apparent for some time [Brigham et al., 1982; Harlan, 1985; Kaslovsky et al., 1990; Gie et al., 1990; Hocking et al., 1990; Lewis and Granger, 1986] but the exact mechanisms involved remain undefined. Several reactants derived from stimulated neutrophils have been identified as barrier disruptive agents, including oxidants [Lewis and Granger, 1986; Kaslovskiet al., 1991; Varani et al., 1991; Loyd et al., 1983; Newman et

Abbreviations used: LPP, lipid phosphate phosphohydrolase; LPA, lyso-phosphatidic acid; PAP, phosphatidate phosphohydrolase.

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al., 1983; Welbourn et al., 1991; Goldman et al., 1992], proteases [Welbourn et al., 1991; Goldman et al., 1992], lysosomal contents [Mavat and Wasi, 1985], defensins [Nygaard et al., 1993], and other cationic proteins [Rosengren et al., 1989; Peterson et al., 1987; Peterson, 1989; Rosengren et al., 1989]. Permeability is increased reversibly by the directed, or chemotactic, migration of neutrophils through either epithelial [Nash et al., 1987, 1989] or endothelial [Huber and Weiss, 1989; Smith et al., 1991] monolayers. Irreversible increases in permeability result from interaction of the endothelium with stimulated adhering neutrophils [Lum et al., 1994; Lampugnani et al., 1993; Kurose et al., 1994; Harlan et al., 1985; Edwards et al., 1992], but adherence per se does not appear to effect barrier disruption [Rosengren et al., 1989; Rosengren et al., 1991; Siflinger-Birnoboim et al., 1993].

There has been considerable confusion and disagreement regarding the specific mediators involved in permeability disruption effected by stimulated neutrophils. For example, Kaslovsky et al. [1991] found that neutrophils from patients with chronic granulomatous disease failed to increase endothelial monolayer permeability upon stimulation, indicating an involvement of superoxide-derived free radicals and oxidants. However, Harlan et al. [1985] previously observed that cells from these patients disrupted permeability at rates similar to that of cells from healthy individuals upon exposure to the chemoattractant, FMLP. In the latter in vitro model as well as in certain biological systems, free radical inhibitors were not effective in inhibiting barrier disruption [Rosengren et al., 1988], but in other models, these agents are strongly protective [Loyd et al., 1983; Newman et al., 1983; Goldman et al., 1992]. The relative contribution of proteases, cationic proteins, and other intracellular reactants in neutrophil-mediated barrier disruption is also unclear. Moreover, it is likely that the contribution of oxidants and other mediators to neutrophil-dependent increases in permeability is highly dependent upon the system used and the manner of cellular stimulation. Barrier disruption appears to be a multi-faceted response, and the effect cannot be attributed to the generation and release of any single mediator by stimulated neutrophils.

Several investigators have emphasized the importance of adherence in barrier disruption [Lum et al., 1994; Lampugnani et al., 1993; Kurose et al., 1994; Harlan et al., 1985; Edwards et al., 1992; Rosengren et al., 1988, 1991; Siflinger-Birnoboim et al., 1993; Goldman et al., 1992; Williams et al., 1984; Fingar et al., 1992], but in the absence of stimulation, adherence alone does not result in increased permeability. However, when adherence is inhibited, the ability of stimulated neutrophils to increase permeability is markedly attenuated, if not completely lost. Adherence may increase the permeability disruptive potential of stimulated neutrophils in two ways. First, adherence obviously provides an efficient mechanism to localize inflammatory mediators generated and released by stimulated neutrophils close to the surface of attached targets, where the toxic effects of the released reactants are enhanced. Second, adherence brings membrane components of the two cell types together, where they can interact to trigger cell function. Thus, during attachment, endothelial cell membranes become intimately exposed to components of the membrane of stimulated neutrophils. Presented in this manner, membrane-bound agonists can focus their effects efficiently while limiting the area of stimulation, thereby increasing the efficacy and efficiency of the inflammatory response [Zimmerman et al., 1993; Siddiqui et al., 1994].

A potential agonist generated in cellular membranes upon metabolic stimulation is phosphatidate, which is generated by cellular processes activated during the inflammatory response [English et al., 1996; English, 1996]. The present study explores the permeability enhancing properties of phosphatidate and related lipids, and investigates the mechanism of this effect. Our finding that phosphatidate enhances endothelial monolayer permeability increases our understanding of the mechanisms of neutrophil-mediated permeability and provides new insights into mechanisms by which adherent cells potentially disrupt the integrity of endothelial monolayers, an early event in the inflammatory response.

MATERIALS AND METHODS

Reagents

Chemicals and reagents including Hanks' balanced salt solution, ficoll-Hypaque, HEPES, d,l propranolol, sodium fluoride, N-ethylmaleimide, herbimycin-A, and *Streptomyces chromofuscus* phospholipase D were obtained from Sigma

Chemical Company (St. Louis, MO). Lipids were from Avanti Polar Lipids (Alabaster, AL). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR). A nonhydolyzable phosphonate analogue of diC8 phosphatidate, phosphonate 1, was prepared as described in [English et al., 1997] by Dr. Theodore Widlanski, Indiana University (Bloomington, IN) for use in this study. ^{32}P-dioctanoyl phosphatidate was prepared by the phosphorylation of dioctanoylglycerol with ATP-γ-^{32}P (3,000 Ci/mmol; New England Nuclear, Boston, MA) as previously described [English et al., 1991]. Silica gel thin layer chromatography plates (type K6F, 20 × 20 cm) were from Whatman, Inc. (Clifton, NJ). Bovine pulmonary artery endothelial cells (BPAEC) were from the America Type Culture Collection (Rockville, MD). Transwell chambers used for assays of endothelial monolayer permeability were from Costar, Inc. (Cambridge, MA).

Bovine Pulmonary Artery Endothelial Cell Culture and Permeability Assays

Endothelial cells were cultured within a humidified atmosphere of 5% CO_2 in air as described previously [Garcia et al., 1995; Verinet al., 1995]. After 16–20 passages in gelatin coated T-75 flasks, the cells were trypsinized and seeded onto 0.45 µm pore size polycarbonate filters at the base of inserts of 6.5 mm diameter Transwell chambers at an average density of approximately 10^4 cells/well. The inserts were returned to wells containing 0.5 ml of endothelial cell growth media and cultured until confluence was obtained (3–5 days). The permeability characteristics of the resulting confluent monolayers were assessed by following the diffusion of albumin-conjugated Evan's blue dye from the upper to lower compartments of the Transwell chambers, as described previously [Garcia et al., 1995]. After addition of dye, stirred chambers were allowed to equilibrate for 10–30 min at 37°C. After equilibration, stimuli were added and dye levels in lower compartments intermittently assayed by spectrophotometric analysis. Results reflect those obtained within individual experiments which were repeated at least twice for confirmation.

Phosphatidate Dephosphorylation

Endothelial cell phosphatidate phosphohydrolase (PAP), which has recently been renamed *lipid phosphate phosphohydrolase* (LPP) [see Brindley and Waggoner, 1998], was assayed by following the release of ^{32}Pi from ^{32}P-labeled dioctanoyl phosphatidate in the absence of detergent as previously described [English et al., 1997]. Hydrolysis of medium chain phosphatidates such as dioctanoyl (C8) phosphatidate effected by intact cells in the absence of detergent is mediated by a plasma membrane associated *"ecto"*-enzyme that has been well-characterized on neutrophilic leukocytes [English et al., 1997; Perry et al., 1993]. This activity is neither Mg^{2+}-dependent nor inhibited by N-ethylmaleimide, differentiating it from the cytosolic PA-Pase, PAP-1. Plasma membrane PAPases, formerly referred to as a collection of as PAP-2 enzymes, are Mg^{2+}-independent and resistant to inhibition by millimolar levels of N-ethylmaleimide [Brindley and Waggoner, 1998]. The ability of these enzymes to hydrolyze lipids other than phosphatidate, including LPA, sphingosine phosphate and ceramide phosphate, as well as their unknown function and physiological substrates has promoted application of the new name, LPP [Brindley and Waggoner, 1998]. While several LPP subtypes exist, it is clear that at least some of these enzymes are able to hydrolyze substrates presented extracellularly in the absence of detergent, consistent with the view that they are functional *ecto*-enzymes which may regulate cellular responses to either their substrates or their products.

LPP was quantified using either intact or sonically disrupted cells in suspension or cultured endothelial monolayers. Suspensions of endothelial cells were prepared by washing monolayers in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution followed by addition of 10 mM EDTA. Cation depleted monolayers were disrupted with a rubber policeman and drawn through a 1 ml pipette tip several times to produce single cell suspensions, which were resuspended in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution at a concentration of 10^7 cells/ml in the absence of EDTA. Where indicated, resuspended cells were disrupted by three short (5 sec) bursts of sonic energy delivered from the microtip of a fisher Dismembrator Model 300. LPP assays of suspended or disrupted cells were carried out in a total volume of 100 µl and initiated by addition of 10 µl of radiolabelled substrate used at a final concentration of 100 µM unless indicated otherwise. Assays were terminated with 0.5 ml of chloroform/methanol/HCl (30/60/1). For assays of phosphatidate hydrolysis by intact cells within

endothelial monolayers, cells were grown on 22 × 22 mm glass cover slips until confluent, and assays were performed after flooding the slips with 90 µl of LPP assay buffer (10 mM HEPES in 0.15 M NaCl, pH 7.0). Reactions were initiated with 10 µl of radiolabelled substrate and terminated with chloroform/methanol/HCl. After phase separation effected by addition of 0.5 ml water and 0.5 ml chloroform to the chloroform/methanol/HCl extracts, water soluble material was isolated and assayed for radioactivity by Cherenkov counting. The identity of the radioactive species in the aqueous extracts as $^{32}P_i$ was confirmed by molybdenum extraction and anion exchange HPLC, as described previously [English et al., 1997].

Metabolic Activation

Ca^{2+} mobilization in endothelial cells was assayed by fluorescence after loading the cells with fura-2 [Garcia et al., 1993]. For assays of Ca^{2+} mobilization within intact endothelial monolayers, cells were cultured on 5 × 22 mm plastic strips cut from 22 × 22 mm plastic microscopic cover slips. Ca^{2+} mobilization of cells in suspension was performed using 5 × 10^5 cells/ml of HEPES buffered Hanks' BSS containing approximately 120 µM Ca^{2+} at 37°C. The concentration of free Ca^{2+} was maintained at 120 ± 20 µM by using 2 mM EGTA and 1.25 mM $CaCl_2$ in Hanks' BSS that was otherwise free of Ca^{2+} and other cations. The concentration of free Ca^{2+} in the resultant buffers was routinely monitored by aequorin chemiluminescence [Siddiqui and English, 1997]. Cell suspensions were assayed for fluorescence at 340 nm excitation and 510 nm emission using a thermostatted Perkin Elmer LS50B spectrofluorometer, as described previously [Siddiqui and English, 1997]. After baseline fluorescence values were established, agonists were added as indicated and fluorescence was monitored continuously for approximately 5 min to determine relative changes in intensity. In some experiments, Ca^{2+} mobilization within cultured endothelial monolayers was determined by analysis of 340/380 fluorescence ratios [Natarajan et al., 1998]. After incubation of monolayers grown on glass cover slips with 5 µM fura-2 AM for 15 min at 37°C, cells were rinsed and submerged in Ca^{2+} assay buffer. Baseline fluorescence ratios were determined and cells were stimulated as indicated below.

For assays of tyrosine phosphorylation, endothelial monolayers were extracted with buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10% glycerol, 1% Nonidet P-40, 2 mM PMFS, 1 µg/ml leupeptin and 0.15 units/ml aprotinin, as previously described. In some experiments, extracts were immunoprecipitated with anti-focal adhesion kinase antibody, using conditions described by Natarajan et al. [1998]. Extracts (100 µl) or immunoprecipitates recovered from extracts of approximately 5 × 10^6 cells were partitioned into wells of precast 8% SDS polyacrylamide gels, resolved electrophoretically, and transferred onto PVDF (Millipore Corp., Bedford, MA). Membranes were blocked with TTBS (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 2% bovine serum albumin (BSA), incubated with anti-phosphotyrosine (clone 4G10, 1:1000 in 1% BSA in TTBS) for 3 h at room temperature, rinsed, and incubated with alkaline phosphatase or horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000 dilution in 1% fetal calf serum). After rinsing, alkaline phosphatase localization was determined by developing blots with NBT/5-bromo-4-chloro-3-indolyl phosphate color reagent and horseradish peroxidase was localized by enhanced chemiluminescence.

Phosphatidate-Binding Assays

Binding of phosphatidate to endothelial cells was assessed using "carrier free" ^{32}P-labeled dioctanoyl phosphatidate (3,000 Ci/mmol) in the presence and absence of varying concentrations of unlabelled dioctanoyl phosphatidate. High specific activity ^{32}P-phosphatidate was prepared by the diglyceride kinase mediated phosphorylation of dioctanoyl glycerol in the presence of high specific activity ^{32}P-γ-ATP (3,000 Ci/mM, New England Nuclear) without any additional unlabeled phosphatidate [English et al., 1997]. Binding was assessed in 50 × 13 mm glass tubes containing 100 µl of binding buffer (10 mM HEPES, pH 7.4 in 150 mM NaCl) above 5 × 10^5 endothelial cells, deposited at the base of each tube by centrifugation of monolayer suspensions prepared as described above for assays of LPP activity. Binding tubes were gently positioned in a 37°C water bath and equilibrated for 2 min prior to addition of ^{32}P-labeled phosphatidate in the absence and presence of unlabeled carrier to assess total

and non-specific binding, respectively. LPP inhibitors were added to binding assays to prevent degradation of the radioactive ligand as indicated. After addition of phosphatidate, binding was allowed to proceed for various times and supernatants were carefully and completely removed by aspiration. ^{32}P remaining with cell pellets was determined by Cherenkov counting. Specific binding was assessed by subtraction of non-specific binding (assessed in the presence of excess unlabelled phosphatidate) from total binding as described in the results.

Phosphatidate-Enriched Plasma Membrane Preparations

Plasma membranes were prepared from neutrophilic leukocytes that had been treated to alter the content of phosphatidate on the outer leaflet of the plasma membrane using a well characterized sucrose density gradient centrifugation method [Graves et al., 1992]. Prior to disruption and fractionation, neutrophils (1.0 ml, 3×10^7 cells/ml of Hanks' BSS containing 1.0 mM Ca^{2+} and 1.0 mM $MgCl_2$) were preincubated for 90 min at 37°C with 50 U of *Streptomyces chromofuscus* phospholipase D in the presence and absence of 10 mM NaF, added to inhibit neutrophil plasma membrane LPP [English et al., 1997]. For reference values, neutrophils were incubated with NaF alone or with no agent. After incubation, cell suspensions were cooled on melting Ice and the cells disrupted by sonication, as previously described. After a brief centrifugation to remove intact cells and debris (250*g*, 2 min), disrupted cells (1.0 ml) were layered onto a cushion of 40% sucrose in 3×15 mm plastic ultracentrifuge tubes and centrifuged for 20 min at 45,000*g* in a Beckman desk top TL 100 Ultracentrifuge. Following centrifugation, the plasma membrane rich area at the top of the sucrose layer was rinsed several times with water and the membranes in the top third of the sucrose cushion recovered for subsequent analysis. Phosphatidate levels in recovered membrane preparations was estimated after organic extraction and thin layer chromatography as previously described [Siddiqui and English, 1997].

RESULTS

Effect of Phosphatidate on Monolayer Permeability

Phosphatidate was found to induced profound alterations of endothelial monolayer permeability, as assessed using a well characterized assay for monolayer integrity (Fig. 1a). These effects were optimally induced by didecanoyl (diC10) phosphatidate. Long chain phosphatidate (diC18:1) was also effective in inducing monolayer permeability, but not as effective as didecanoyl phosphatidate (not shown). Dioctanoyl (diC8) phosphatidate also induced changes in barrier function, again somewhat less effectively than diC10 phosphatidate (Fig. 1a). The response to diC8 PA was markedly potentiated by propranolol, an effective LPP inhibitor. Propranolol had little effect on permeability induced by longer chain phosphatidates (not shown), which are hydrolyzed much less efficiently than diC8 phosphatidate by *ecto*-LPP [English et al., 1997]. Thus, the ability of certain phosphatidates to increase endothelial cell monolayer permeability may be regulated by substrate hydrolysis effected by *ecto*-LPP.

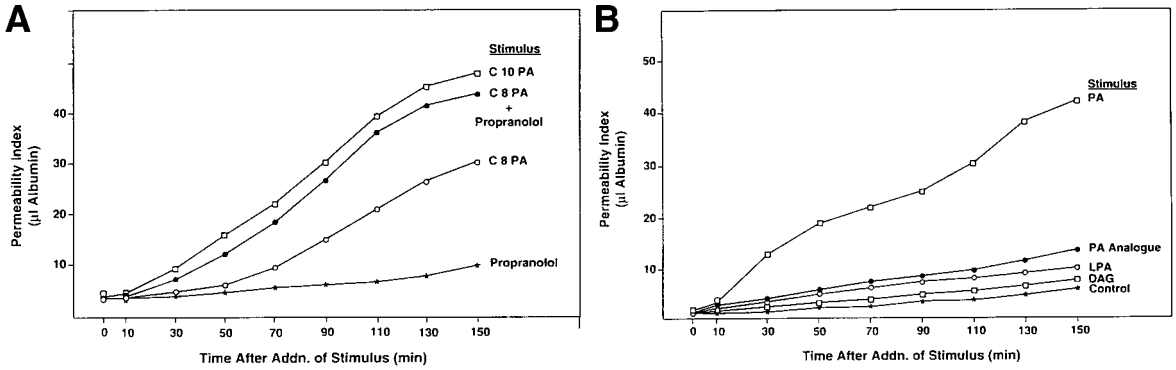

Fig. 1. Induction of endothelial monolayer permeability by phosphatidate. Transport of Evans Blue dye across endothelial monolayers was continuously assessed at 37°C. **A:** monolayers were exposed to 20 µM of the indicated phosphatidate in the presence or absence of 0.5 mM propranolol. **B**: Cells were exposed to 40 µM diC8-phosphatidate, phosphonate 1 (analogue), 1-octanyl-2-lysophosphatic acid (LPA) or 1,2 dioctanyl-rac-glycerol (DAG).

Experiments were undertaken to assess the presence of endothelial cell *ecto*-LPP directly. Exogenous diC8 phosphatidate was rapidly hydrolyzed by intact endothelial cells in suspension (Table I), suggesting the presence of *ecto*-LPP. Sonication of suspended cells increase LPP activity only slightly, demonstrating that most of the total cellular activity was available for hydrolysis of extracellular substrate. Viable cells grown on confluent monolayers also effectively hydrolyzed diC8-phosphatidate (Table I). This activity was not inhibited by 5 mM N-ethylmaleimide. Thus, endothelial cells in cultured monolayers expressed N-ethylmaleimide-insensitive *ecto*-LPP, consistent with the hypothesis that the enzyme's active site is exposed on the luminal surface of blood vessels, where it potentially influences the ability of phosphatidate or related lipids to effect alterations in vascular permeability. Diacylglycerol, the hydrolysis product generated by the action of LPP on exogenous phosphatidate, did not induce significant alterations in membrane monolayer permeability (Fig. 1B). LPA and a non-hydrolyzable analogue of phosphatidate, phosphonate 1, were also ineffective (Fig. 1B).

Induction of Ca^{2+} Mobilization and Tyrosine Kinase Activation in Endothelial Cells

Experiments were undertaken to define cellular processes activated by extracellular phosphatidate that result in increased monolayer permeability. We first assessed the influence of phosphatidate on Ca^{2+} mobilization in cultured endothelial monolayers. Exogenous phosphatidate rapidly induced a pronounced increase in intracellular free Ca^{2+} in monolayer endothelial cells (Fig. 2). Permeability induction by phosphatidate was attenuated by preincubation of endothelial cells with herbimycin A and enhanced by the tyrosine phosphatase inhibitor vanadate, implicating a role for activation of intracellular tyrosine kinases in the response (Table II). To further explore the role of tyrosine kinases activation in the response of endothelial cells to phosphatidate, tyrosine phosphorylation in endothelial cells exposed to phosphatidate was assessed by Western analysis of tyrosine phosphorylated proteins. Phosphatidate activated tyrosine kinases in cultured endothelial cells as assessed by anti-phosphotyrosine immunoblotting. Several proteins including a dominant band at approximately 125 kDa in extracts of cells exposed to diC10 phosphatidate displayed enhanced reactivity with anti-phosphotyrosine antibody (Fig. 3A). Long chain (diC18:1) phosphatidate also effectively induced tyrosine phosphorylation of high molecular weight substrates in endothelial cells. One of these substrates was p125 focal adhesion kinase, as demonstrated by the dose dependent phosphorylation of this substrate in immunoprecipitates of endothelial cells exposed to diC18:1 phosphatidate (Fig. 3B).

TABLE I. Phosphatidate Hydrolysis by Intact and Disrupted Endothelial Cells*

Cell preparation	Addition	PA hydrolysis
Intact EC	None	2.53 ± 0.28
Disrupted EC	None	3.13 ± 0.13
Monolayer cells	None	3.14 ± 0.31
Monolayer cells	5 mM NEM	3.31 ± 0.35

*Endothelial cells grown in wells in 24-well culture dishes (approximately 3×10^4 cells/well) were tested for phosphohydrolase activity after treatment with EDTA and resuspension (intact EC) or in intact monolayers (monolayer cells). Cells were incubated with 224 μM [^{32}P]-diC8 phosphatidate (PA) for 15 min at 37°C. PA hydrolysis indicates nmol Pi released/15 min. Results are means and standard deviations of three determinations of one experiment that was repeated twice. NEM, N-ethylmaleimide.

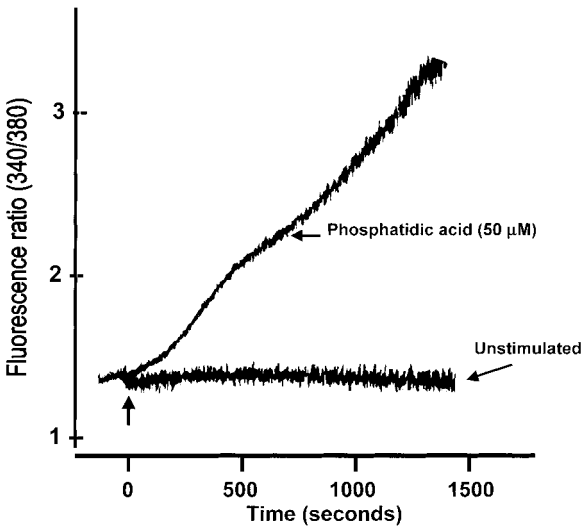

Fig. 2. Ca^{2+} mobilization in endothelial cells exposed to phosphatidate. Cells grown on a cover slip and pre-incubated in a buffer containing 2.0 mM EGTA and 0.5 mM Ca^{2+} were stimulated with diC10 phosphatidate as indicated by the arrow. The ratio of fluorescence excited at 340 and 380 nm is shown. Under the conditions used, the concentration of free Ca^{2+} in the extracellular media was approximately 100 μM. The results shown were repeated twice for verification.

TABLE II. Influence of Herbimycin and Vanadate on Phosphatidate-Induced Permeability*

Stimulus	Vanadate (100 µM)	Herbimycin A (10 µg/ml)	Permeability response (nl albumin/min)
None	−	−	49.8 ± 4.4
20 µM diC10 PA	−	−	125.1 ± 9.3
20 µM diC10 PA	+	−	261.6 ± 17.2
40 µM diC10 PA	−	−	203.3 ± 11.5
40 µM diC10 PA	+	−	347.9 ± 29.4
40 µM diC10 PA	−	+	118.4 ± 33.0

*Endothelial monolayer permeability was assessed by following the transport of Evans blue dye conjugated to albumin across intact monolayers. Results are expressed as the average rate of albumin transport (±S.D., n = 3) determined over a 60 min period.

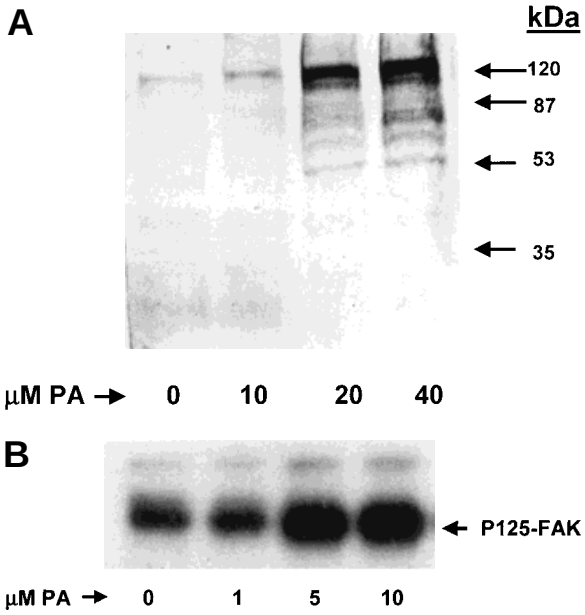

Fig. 3. Induction of tyrosine phosphorylation in endothelial cells exposed to phosphatidate. **A:** Cells were exposed to 0, 10, 20, and 40 µM diC10 phosphatidate for 5 min at 37°C. **B:** Cells were exposed to 0, 1, 5 1n 10 µM diC18:1 phosphatidate for 10 min at 37°C. After stimulation, monolayers were extracted, separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies. The arrow in B indicates the migration position of p125 focal adhesion kinase (FAK).

Specific Binding of Phosphatidate to Endothelial Cell Membranes

Experiments were undertaken to determine if we could demonstrate specific binding of phosphatidate to endothelial cell membranes, a prerequisite for implicating receptor binding in functional activation. Initial experiments revealed several problems in analysis of phosphatidate binding to endothelium. First, binding could not be demonstrated at low temperatures (<4°C). Thus, rapid metabolism of the ligand by cellular enzymes presented a potential problem. Second, neither simple centrifugation nor filtration effectively separated free from bound ligand, due to micelle formation and the absorbent properties of the substrate. An assay was therefore developed which allowed measurement of [^{32}P]-phosphatidate incorporation onto endothelial cells deposited at the base of wells within a 96-well culture plate. Using this assay, little activity became cell associated over an extended time interval in the absence of LPP inhibitors. However, in the presence of LPP inhibitors, *saturable* binding of phosphatidate by endothelial cells was readily apparent (Fig. 4), as demonstrated by decreased incorporation of radiolabeled ligand in the presence of increasing concentrations of competing, non-labeled

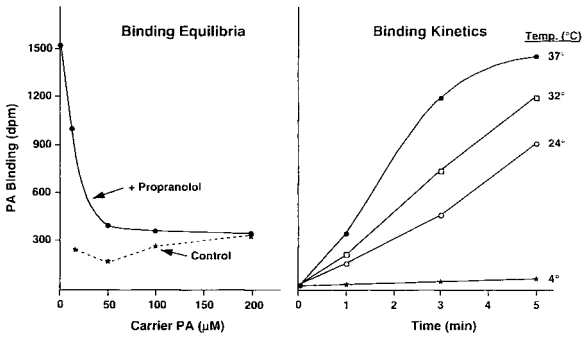

Fig. 4. Specific binding of phosphatidate by endothelial cells. Cells were deposited at the base of wells within a 96-well culture dish without or with indicated concentrations of additional carrier in the presence or absence (control) of 5.0 mM propranolol, added to inhibit LPPases (left panel). After incubation with gentle agitation, the media above the cells was aspirated and free and bound radioactivity determined by liquid scintillation counting. Binding kinetics (right panel) were assessed by incubating cells with "carrier free" [^{32}P]-diC8 phosphatidate in the presence of 5.0 mM propranolol for the indicated time intervals at the temperatures shown. After incubation, supernatants were removed, the cells recovered and assayed for radioactivity by liquid scintillation counting. Results are means of duplicate determinations which varied from one another by <10%. The experiments shown were repeated twice for verification.

substrate. Binding occurred rapidly at 37°C, and was considerably slowed by temperature reduction (Fig. 4). In the experiment shown, total and non-specific binding were assessed by determination of the kinetics of [^{32}P]-phosphatidate association with cells in the absence and presence of excess, non-radioactive substrate, respectively. Specific binding (total-nonspecific binding) was evident within minutes after addition of ligand at 37°C. Little if any specific binding was observed at 4°C.

Responses of Endothelial Cells to Phosphatidate-Enriched Plasma Membranes

In biological systems, endothelial cells may be exposed to phosphatidate confined to the plasma membrane of neutrophilic leukocytes responding to infectious or inflammatory stimuli. While phosphatidate is presumably generated on the inner leaflet of neutrophil membranes by the activation of phospholipase D or diglyceride kinase, it may readily translocate to the outer leaflet when plasma membrane phospholipid asymmetry collapses as a result of cellular activation, cytotoxicity, or exposure to inflammatory mediators [Zwaal and Schroit, 1997]. We therefore investigated the ability of phosphatidate-enriched neutrophil plasma membranes to alter permeability of cultured endothelial monolayers. We used a well-characterized discontinuous density gradient method to isolate plasma membranes from disrupted neutrophils. Membranes were isolated from untreated cells and from cells preincubated with exogenous phospholipase D to increase outer leaflet phosphatidate levels. No phosphatidate band was observed in chromatographs of extracts of membranes (~10 mg protein) from untreated cells, indicating a phosphatidate concentration of <100 ng/mg membrane protein. Membranes from cells pretreated with 50 U of *Streptomyces chromofuscus* phospholipase D for 90 min at 37°C displayed only a moderate elevation in phosphatidate levels, evident as a faintly detectable band judged to reflect phosphatidate levels of between 100–150 ng/mg membrane protein. Addition of 10 mM NaF to inhibit membrane LPPases increased phosphatidate levels in resultant membrane preparations of over 200 ng/mg membrane protein. Fluoride alone also potentiated phosphatidate levels to between 150–200 ng/mg protein, perhaps because of its ability to directly activate neutrophil phospholipase D [English et al., 1991] as well as its ability to inhibit LPP activity (English et al., 1997].

Plasma membranes isolated from neutrophils treated with phospholipase D in the presence of NaF were remarkably effective in enhancing the permeability characteristics of cultured endothelial monolayers (Fig. 5). The permeability enhancing characteristics of membranes of cells treated with phospholipase D alone were not markedly greater than those of membranes of resting cells. Membranes from cells treated with fluoride alone possessed intermediate permeability enhancing characteristics.

Preliminary experiments were undertaken to explore biochemical responses induced by phosphatidate enriched plasma membranes in order to determine if these responses resulted from activation of the signaling pathway induced by ectopic phosphatidate. We explored Ca^{2+} mobilization in stimulated endothelial cells, since this response presumably plays a key role in transducing signals transmitted by phosphatidate. Like phosphatidate, phosphatidate-enriched plasma membranes induced a rapid rise in free Ca^{2+} levels in cells within cultured endothelial monolayers (Fig. 6). This response was inhibited by addition of the tyrosine kinase inhibitor herbimycin A (5 µg/ml), and in this respect was similar to the characteristics of the permeability response induced by exogenous phosphatidate. Thus, phosphatidate confined to plasma membranes may exert its

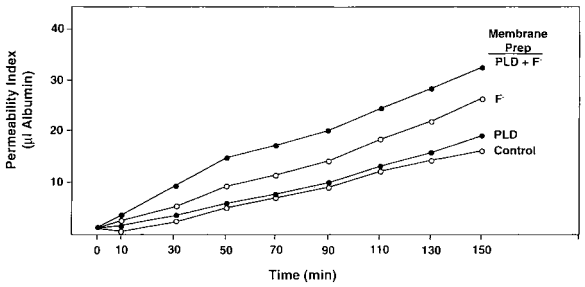

Fig. 5. Induction of endothelial monolayer permeability by neutrophil plasma membranes. Neutrophils were exposed to phospholipase D to cause phosphatidate generation on the outer leaflet of their plasma membranes in the presence and absence of the phosphatidate phosphohydrolase inhibitor, NaF (10 mM). After treatment, cells were cooled, washed and disrupted by sonication. Plasma membranes were prepared from the sonicates by sucrose density gradient centrifugation and washed extensively. Graphs show permeability changes observed when these membranes were added to endothelial monolayers. In the experiment shown, 300 µg of membrane protein were added to each 800 µl permeability chamber.

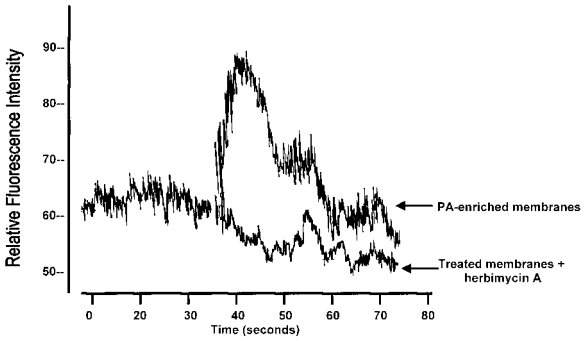

Fig. 6. Induction of Ca^{2+} mobilization in endothelial cells exposed to phosphatidate-enriched plasma membranes. Endothelial cells were loaded with fura-2 and Ca^{2+} mobilization induced by neutrophil plasma membranes prepared as described in the legend to figure 5 was assessed by following fluorescence changes excited at 340 nm. Membrane preparations were used at a final concentration of approximately 350 µg membrane protein/ml. The experiment shown was repeated once for verification.

influence on membrane monolayer permeability by activating the same signalling pathway that is activated by exogenous phosphatidate, a pathway which appears to be initiated by receptor ligation and driven by the activation of a tyrosine kinase-dependent process which results in Ca^{2+} mobilization and consequent cellular activation.

DISCUSSION

Phosphatidate and LPA are biologically active phospholipids that play diverse roles in cellular signaling. Released from activated platelets and other cells, LPA induces cellular proliferation, differentiation, and migration by activating a G-protein receptor linked signalling system which results in activation of key intracellular mediators, Ca^{2+} mobilization and cellular function [Jalink et al., 1990; 1994]. While the influence of both phosphatidate and LPA has been carefully examined in a number of physiologically relevant systems, little published information documents the effects of these lipids on endothelial cell structure and function. Cross et al. [1996] demonstrated the tyrosine kinase-dependent activation of endothelial cell phospholipase D by LPA, an event that resulted in intracellular phosphatidate accumulation and actin stress fiber formation. Addition of phosphatidate to these cells also resulted in stress fiber formation by a process that was sensitive to inhibition by both tyrosine kinase inhibitors and micro-injected C3 exotoxin, a potent inhibitor of the small molecular weight G protein, Rho. Rho activation is becoming increasing recognized as a key event in signalling pathways which regulate endothelial cytoskeletal changes and alterations in barrier function in response to a diverse array of physiological agonists [Cross et al., 1996; Hippenstiel et al., 1997; Wojciak-Stothard et al., 1998; Aepfelbacher et al., 1997].

The present report demonstrates the induction of endothelial monolayer permeability by phosphatidate. This response was not induced by LPA or the product of *ecto*-PAPase mediated phosphatidate hydrolysis, diacylglycerol, consistent with the hypothesis that phosphatidate and not one of its metabolites was the activating ligand. Moreover, the response appeared to be susceptible to regulation by endothelial cell *ecto*-LPP, since the LPP inhibitor propranolol potentiated permeability produced by medium chain (diC8) phosphatidate. Short and medium chain phosphatidates are preferentially hydrolyzed by *ecto*-LPP's in other systems, as a consequence of the increased solubility of these substrates in comparison to longer chain phosphatidates [English et al., 1997]. Cellular responses to medium chain phosphatidates may therefore be limited by substrate hydrolysis effected by *ecto*-LPP. The present report provides direct experimental evidence that confirms the existence of LPP that can hydrolyze extracellular substrates on cultured endothelial cells. Thus, intact cells in suspension and in cultured monolayers were able to hydrolyze extracellular phosphatidate. This response was not Mg^{2+} dependent nor was it inhibited by N-ethylmaleimide, properties associated with plasma membrane *ecto*-LPP of other cells [Brindley and Waggoner, 1998]. Inhibition profiles and kinetic considerations further attest to the similarity of endothelial cell *ecto*-LPP to previously described enzymes. Thus, endothelial cells possess a potent *ecto*-PAP that may regulate responses of the endothelium to phosphatidate and related lipids. Since endothelial cells in cultured monolayers expressed phosphatidate hydrolyzing activity, ecto-LPP may regulate the ability of phosphatidate and related lipids to enhance permeability of intact blood vessels.

The signalling pathway initiated when endothelial cells are exposed to phosphatidate is not entirely defined, but appears to involve activa-

tion of a tyrosine kinase-dependent process that results in Ca^{2+} mobilization and cellular activation. The tyrosine phosphorylation of several endothelial cell proteins including p125 focal adhesion kinase (FAK) was enhanced upon exposure of the cells to phosphatidate. In addition to FAK, phosphatidate enhanced the tyrosine phosphorylation of several smaller proteins, which possibly correspond to lower molecular weight intracellular mediators that, upon tyrosine phosphorylation, govern the responses of neutrophils and other cells to phosphatidate and related lipids. Work is currently underway to identify these proteins and determine their relationship to the tyrosine and lipid kinases we have shown to direct neutrophil migratory responses to phosphatidate [Siddiqui and English, 1997].

Phosphatidate may initiate this permeability-disrupting signalling cascade in endothelial cells by ligating a specific plasma membrane receptor. If so, it may share another unique characteristic with neutrophilic leukocytes, namely the presence of a phosphatidate receptor. Many other cell types, including fibroblasts, neurons and certain tumor cells appear to be specifically responsive to LPA as compared to phosphatidate [Jalink et al., 1990, 1994]. In contrast, neutrophils appear to be quite unresponsive to LPA administered under a variety of experimental conditions, a fact which led early workers to discount the existence of an LPA receptor on neutrophilic leukocytes [Jalink et al., 1990]. Our research has confirmed this early conclusion [Siddiqui and English, 1997], and extended them by demonstrating specific binding of phosphatidate by neutrophil plasma membranes [Siddiqui and English, 1996]. In the present study, endothelial cells displayed specific binding of phosphatidate under activating conditions. Thus both endothelial cells and neutrophils may possess a plasma membrane receptor for phosphatidate. The failure of the non-hydrolyzable phosphatidate analog phosphonate 1 to induce permeability does not negate this hypothesis, since ligand hydrolysis may be necessary for receptor recycling and maximal biological activity. In this respect, it is of interest that non-hydrolyzable phosphonate analogs of lysophosphatidate possess markedly less biological activity than their parent compounds [Jalink et al., 1995]. Further work needs to be done to confirm or negate this hypothesis. It is noteworthy that unlike neutrophils, endothelial cells do appear to possess LPA receptors. While our results shown little or no response to LPA as assessed by permeability increases and Ca^{2+} mobilization, the cells vigorously respond to LPA with Rho-dependent phospholipase D activation and stress fiber formation [Cross et al., 1996]. In addition, LPA apparently activates processes in endothelial cells which result in strengthening rather than disruption of the permeability barrier [Alexander et al., 1998]. Thus, PA and LPA appear to activate different signaling pathways in endothelial cells which result in markedly divergent effects on endothelial monolayer permeability.

The physiological manner of presentation of phosphatidate to the endothelium in biological systems is unknown. Unlike LPA, phosphatidate is not known to be released as a soluble mediator by activated cells, preferring instead to remain confined to cellular membranes. However, like other anionic phospholipids, phosphatidate may translocate between plasma membrane leaflets as a result of either the activation or inhibition of specific transporting enzymes. In most viable mammalian cells, phosphatidylserine and other phospholipids are not normally found on the outer membrane leaflet because they are rapidly moved to the inner leaflet by the continuous activity of specific transporting enzymes that function to maintain plasma membrane phospholipid asymmetry [Zwaal and Schroit, 1997; Seigneuret and Devaux, 1984; Williamson et al., 1992; Fadoket al., 1992]. Processes activated during cellular stimulation as well as processes inactivated by cell death or diminished vitality can rapidly compromise the efficiency of these transporting systems, resulting in the collapse of phospholipid asymmetry and the exposure of anionic phospholipids to the cells' exterior. Indeed, one of the earliest events associated with apoptosis is appearance of reactive phosphatidylserine in the outer leaflet of cellular plasma membranes [Fadoket al., 1992; Martin et al., 1995]. It is therefore likely that internally generated phosphatidate also appears on the outer leaflet of stimulated, damaged, or dying cells, including neutrophilic leukocytes. While our experiments do not demonstrate this directly, they do show that phosphatidate confined to cellular membranes is biologically active; membrane bound phosphatidate appeared to efficiently disrupt

endothelial monolayer integrity. This effect was associated with the ability of these plasma membrane preparations to mobilize intracellular Ca^{2+}. Like responses to ectopic phosphatidate, Ca^{2+} mobilization effected by membrane-bound phosphatidate was inhibited by the tyrosine kinase inhibitor herbimycin A, indicating similar signaling pathways may be involved. Recently, endothelial barrier properties were linked to tyrosine kinase dependent cellular activation [Gilbert-McMlain et al., 1998]. Thus, membrane-associated phosphatidate may activate endothelial cell phosphatidate receptors, initiating a tyrosine kinase-dependent process that culminates in Ca^{2+} mobilization and cellular activation. The phosphatidate that causes this effect may be generated within stimulated neutrophils and other cells by phospholipase D and transported to the outer leaflet of the plasma membrane as a result of cellular stimulation, cytotoxicity or apoptosis. This method of cellular communication potentially operates during the inflammatory process to increase the permeability characteristics of endothelial monolayers and regulate the egress of cells, fluids and mediators from the vasculature to sites of infection, tissue damage, and inflammatory reactivity.

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REFERENCES

Aepfelbacher M, Essler M, Huber E, Sugai M, Weber PC. 1997. Bacterial toxins block endothelial wound repair. Evidence that Rho GTPases control cytoskeletal arrangements in migrating endothelial cells. Arter Thromb Vasc Biol 17:1623–1629.

Alexander JS, Patton WF, Christman BW, Cuiper LL, Haselton FR. 1998. Platelet-derived lysophosphatidic acid decreases endothelial permeability in vitro. Am J Phys 274:H115–122.

Brigham KL, Teloy D, Newman JM, Snapper JR, Ogletree ML, English D. 1982. Granulocytes in acute lung vascular injury in unanesthetized sheep. Chest 81:556–559.

Brindley DN, Waggoner DW. 1998. Mammalian lipid phosphohydrolases. J Biol Chem 273:1–4.

Cross MJ, Roberts S, Ridley AJ, Hodgkin MN, Stewart A, Claesson-White L, Wakelam MJO. 1996. Stimulation of actin stress fiber formation mediated by activation of phospholipase D. Curr Biol 6:588–597.

Edwards MJ, Miller FN, Sims DE, Abney DL, Schuschke DA, Corey TS. 1992. Interleukin 2 acutely induces platelet and neutrophil endothelial adherence and macromolecular leakage. Cancer Res 52:3425–3431.

English D, Martin M, Harvey KA, Akard LP, Allen R, Widlanski TS, Garcia JGN, Siddiqui RA. 1997. Character-ization and purification of neutrophil ecto-phosphatidate phosphohydrolase. Biochem J 324:941–950.

English D, Taylor G, Garcia JG. 1991. Diacylglycerol generation in fluoride-treated neutrophils: involvement of phospholipase D. Blood 77: 2746–2756.

English D, Cui Y, Siddiqui RA. 1996. Messenger functions of phosphatidic acid. Adv Chem Phys Lipids 80:117–132.

English D. 1996. Phosphatidic acid as an intracellular and extracellular messenger. Cell Signal 8:349–354.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal be macrophages. J Immunol 148:2207.

Fingar VH, Wieman TJ, Wiehle SA, Cerrito PB. 1992. The role of microvascular damage in photodynamic therapy: the effect of treatment on vessel constriction, permeability and leukocyte adhesion. Cancer Res 52:4914–1921.

Garcia JGN, Paterson C, Bahler C, Aschner J, Hart CM, English D. 1993. Thrombin receptor activating peptides induce Ca^{2+} mobilization, barrier dysfunction, prostaglandin synthesis, and platelet derived growth factor mRNA expression in cultured endothelium. J Cell Phys 156:541–549.

Garcia JGN, Davis H, Patterson CE. 1995. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. J Cell Phys 163:510–520.

Gie RP, Doerschuk CM, English D, Coxson HO, Hogg JC. 1990. Neutrophil-associated lung injury after the infusion of activated plasma. J Appl Phys 70:2471–2478.

Gilbert-McClain LI, Verin AD, Shi S, Irwin RP, Garcia JGN. 1998. Regulation of endothelial cell Light chain phopsphorylation by vanadate. J Cell Biochem 70:141–155.

Goldman G, Welbourn R, Rothlein R, Wiles M, Kobzik L, Valeri CR, Shepro D, Hechtman HB. 1992. Adherent neutrophils mediate permeability after atelectasis. Ann Surg 216:372–378.

Goldman G, Welbourn R, Kobzik L, Valeri CR, Shepro D, Hechtman HB. 1992. Reactive oxygen species and elastase mediate lung permeability after acid aspiration. J Appl Phys 73:571–575.

Graves VT, Gabig TL, McCarthy L, Strour EF, Leemhuis T, English D. 1992. Simultaneous mobilization of Mac-1 (CD11b/CD18) and formyl peptide chemoattractant receptors in human neutrophils. Blood 80:776–787.

Harlan JM, Schwartz BR, Reidy MA, Schwartz SM, Ochs HD, Harker LA. 1985. Activated neutrophils disrupt endothelial monolayer integrity by an oxygen radical-independent mechanism. Lab Invest 52:141–150.

Harlan JM. 1985. Leukocyte-endothelial interactions. Blood 65:513–525.

Hippenstiel S, Tannert-Otto S, Vollrath N, Krull M, Just I, Aktories K, von Eichel-Streiber C, Suttorp N. 1997. Glucosylation of small GTP-binding Rho proteins disrupts endothelial barrier function. Am J Phys 272:L38–L43.

Hocking DC, Phillips PG, Ferro TJ, Johnson A. 1990. Mechanisms of pulmonary edema induced by tumor necrosis factor-alpha. Circ Res 67:68–77.

Huber AR, Weiss SJ. 1989. Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel wall. J Clin Invest 83:1122–1136.

Jalink K, Van Corven EJ, Moolenaar WH. 1990. Lysophosphatidic acid, but not phosphatidic acid, is a potent $Ca2^{+}$-mobilizing stimulus for fibroblasts. J Biol Chem 265:12232–12239.

Jalink K, Hordijk PL, Moolenaar WH. 1994. Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. Biochim Biophys Acta 1198:185–196.

Jalink K, Hengeveld T, Mulder S, Postma FR, Simon MF, Chap H, van der Marel GA, van Boom JH, van BlitterswijkWE J, Moolenaar WH. 1995. Lysophosphatidic acid-induced Ca2+ mobilization in human A431 cells: Structure-activity analysis. Biochem J 307:609–616.

Kaslovsky RA, Gibbs L, Malik AB. 1991. Neutrophils from chronic granulomatous disease fail to increase endothelial permeability. Am J Phys 261:H1226–1230.

Kaslovsky RA, Horgan MJ, Lum H, McCandless BK, Gilboa N, Wright SD, Malik AB. 1990. Pulmonary edema induced by phagocytosing neutrophils. Protective effects of monoclonal antibody against phagocyte CD18 integrin. Circ Res 67:795–802.

Kurose I, Granger DN, Evans DJ, Evans DG, Graham DY, Miyasaka M, Anderson DC, Wolf RE, Cepinskas G, Kvietys PR. 1994. Heliocobacter pylori-induced microvascular protein leakage in rats: role of neutrophils, mast cells, and platelets. Gastroenterology 107:70–79.

Lampugnani MG, Caveda L, Breviario F, Del Maschio A, Dejana E. 1993. Endothelial cell-to-cell junctions. Structural characteristics and functional role in the regulation of vascular permeability and leukocyte extravasation. Baillieres Clin Haemtol 6:539–558.

Lewis RE, Granger HJ. 1986. Neutrophil-dependent mediation of microvascular permeability. Federation Proc 45: 109–113.

Loyd JE, Newman JH, English D, Ogeltree ML, Brigham KL. 1983. Lung vascular effects of phorbol myristate acetate in awake sheep. J Appl Phys 54:267–276.

Lum H, Gibbs L, Lai L, Malik AB. 1994. CD18 integrin-dependent endothelial injury: effects of opsonized zymosan and phorbol ester activation. J Leuko Biol 55:58–63.

Martin SJ, Reutelingsperger CPM, AcGahon AJ, Rader JA, VanSchie RCAA, Laface DM, Greene DR. 1995. Early redistribution of phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 182:1545.

Movat HZ, Wasi S. 1985. Severe microvascular injury induced by lysosomal releasates of human polymorphonuclear leukocytes. Increase in vasopermeability hemorrhage, and microthrombosis due to degradation of subendothelial and perivascular matrices. Am J Pathol 121:404–417.

Nash S, Staffors J, Madara JL. 1987. Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. J Clin Invest 80:1104–1113.

Nash S, Staffors J, Madara JL. 1988. The selective and superoxide-independent disruption of intestinal epithelial tight junctions during leukocyte transmigration. Lab Invest 59:531–537.

Natarajan V, Scribner WM, Al-Hassani M, Vepa S. 1998. Reactive oxygen species signaling through regulation of protein tyrosine phosphorylation in endothelial cells. Environ Health Pers 106 supp 5:1205–1212.

Newman JH, Loyd JE, English D, Ogeltree ML, Fulkerson W, Brigham KL. 1983. Effects of 100% oxygen on lung vascular function in awake sheep. J Appl Phys 54:1379–1386.

Nygaard SD, Ganz T, Peterson MW. 1993. Defensins reduce the barrier integrity of a cultured epithelial monolayer without cytotoxicity. Am J Resp Cell Mol Biol 8:193–200.

Perry DK, Stevens VL, Widlanski T, Lambeth JD. 1993. A novel ecto-phosphatidate phosphohydrolase activity mediates activation of neutrophil superoxide generation by exogenous phosphatidate. J Biol Chem 265:25302–25310.

Peterson MW. 1989. Neutrophil cathepsin G increases transendothelial albumin flux. J Lab Clin Med 113:297–208.

Peterson MW, Stone P, Shasby DM. 1987. Cationic neutrophil proteins increase transendothelial albumin movement. J Appl Phys 62:1521–1530.

Rosengren S, Ley K, Arfors KE. 1989. Dextran sulfate prevents LTB-4 induced permeability increases, but not neutrophil emigration, in the hamster cheek pouch. Microvasc Res 38:243–254.

Rosengren S, Bjork J, Smedegard G. 1988. Oxygen radicals are not a prerequisite for neutrophil-mediated increased vascular permeability. Lab Invest 58:559–564.

Rosengren S, Olofsson AM, von Andrian UH, Lundrgen-Akerlund E, Arfors KE. 1991. Leukotriene B4-induced neutrophil-mediated endothelial leakage in vitro and in vivo. J Appl Phys 71:1322–1330.

Seigneuret M, Devaux PF. 1984 Asymmetric distribution of spin labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc Natl Acad Sci USA 81: 3751.

Siddiqui R, English D, Garcia JGN. 1994. Rationale for juxtacrine regulation of neutrophil interactions with the stimulated endothelium. J Lab Clin Med 125:18–27.

Siddiqui RA, English D. 1996. Phosphatidic acid binding to human neutrophils: effects on tyrosine kinase-regulated intracellular Ca++ mobilizaton. Cell Signal 8:349–354.

Siddiqui RA, English D. 1997. Phosphatidic acid elicits calcium mobilization and actin polymerization through a tyrosine kinase-dependent process in human neutrophils: a mechanism for induction of chemotaxis. Biochem Biophys Acta 1349:81–95.

Siflinger-Birnoboim A, Bode DC, Malik AB. 1993. Adenosine 3′,5′-cyclic monophosphate attentuates neutrophil-mediated increase in endothelial permeability. Am J Phys 264:H370–375.

Smith WB, Gamble JR, Clark-Lewis I, Vadas MA. 1991. Interleukin-8 induces neutrophil transendothelial migration. Immunology 72:65–72.

Varani J, Ginsburg I, Gibbs DF, Mukhopadhyay PS, Sulavik C, Johnson KJ, Weinberg JM, Ryan US, Ward PA. 1991. Hydrogen peroxide-induced cell and tissue injury: Protective effects of Mn^{2+}. Inflammation 15:291–301.

Verin AD, Patterson CE, Day MA, Garcia JGN. 1995. Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. Am J Physiol 269:L99–108.

Welbourn CR, Goldman G, Paterson IS, Valeri CR, Shepro D, Hechtman HB. 1991. Neutrophil elastase and oxygen radicals: synergism in lung injury after hindlimb ischemia. Am J Phys 260:H1852–1856.

Williams TJ, Jose PJ, Forrest MJ, Wedmore CV, Clough GF. 1984. Interactions between neutrophils and microvascular endothelial cells leading to cell emigration and plasma protein leakage. Kroc Found Ser 16:195–208.

Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF. 1992. Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocyte. Biochemistry 31:6355.

Wojciak-Stothard B, Entwistle A, Garg R, Ridley AJ. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc-42 in human endothelial cells. J Cell Phys 176: 150–165.

Zwaal RFA, Schroit AJ. 1997. Pathological implications of membrane phospholipid asymmetry in blood cells. Blood 89:1121–1132.

Zimmerman GA, Lorant DE, Mcintyre TM, Prescott SM. 1993. Juxtacrine intracellullar signalling: another way to do it. Am J Resp Cell Mol Biol 9:923–927.