Vascular permeability factor/vascular endothelial growth factor stimulates endothelial proliferation, angiogenesis, and increased vascular permeability in vivo. We investigated mechanisms of vascular permeability factor-mediated endothelial monolayer permeability changes in vitro. [14C]Albunin flux across endothelial monolayers was measured following a 90-min exposure to vascular permeability factor (660 pM). Vascular permeability factor increased albumin flux to 3.4 times that of control albumin flux. Endothelial monolayers were also incubated for 90 min with vascular permeability factor plus Go6976 (10 nM), staurosporine (1 nM), wortmannin (10 nM), AG126 (1 and 2.67 μM), and PD98059 (20 μM). Vascular permeability factor-mediated permeability was not blocked by Go6976, an antagonist of “classical” protein kinase C, staurosporine, a pan-protein kinase C antagonist, nor wortmannin, a PI3-kinase blocker, but was blocked by incubation with AG126 or PD98059, inhibitors of mitogen-activated protein kinase activation. Immunofluorescent staining of the junctional proteins VE-cadherin and occludin showed a loss of these proteins from the endothelial junction that was prevented by co-incubation with AG126 or PD98059. These data demonstrate that vascular permeability factor increases albumin permeability across endothelial monolayers in vitro and suggests that permeability increases through rearrangement of endothelial junctional proteins involving the mitogen-activated protein kinase signal transduction pathway.

Vascular permeability factor/vascular endothelial growth factor (VPF) is a family of homodimeric glycoproteins (34–46 kDa) secreted by a wide variety of cells. VPF plays key roles in several diverse physiologic processes including tumor angiogenesis, diabetic retinopathy, wound healing, and lung repair following oxidant or ischemic injury (1–4).

Human VPF has at least four transcriptionally spliced mRNA isoforms that code for polypeptides of 206, 189, 165, and 121 amino acids (5). Hypoxia potently stimulates VPF induction through stabilization of VPF mRNA as well as transcriptional activity (6). Increases in VPF mRNA levels have also been described following stimulation by reactive oxygen intermediates (7). Moreover, there is evidence that in certain cell lines VPF is also translationally regulated (8). Physiologically, VPF is a potent stimulus of angiogenesis, endothelial proliferation, and increased microvascular permeability (3, 8). VPF demonstrates high affinity binding to Flt-1 and Flk-1/KDR on endothelial cells, which mediates endothelial cell proliferation in vitro (2, 9, 10). In contrast, in vitro models of VPF-mediated endothelial permeability are lacking, as are data on the mechanisms and targets of VPF in permeability regulation.

To date, most studies on VPF have mainly examined mitogenic effects of VPF-receptor interactions. Waltenberger et al. (11), using transfected porcine aortic endothelial cells and human umbilical vein endothelium cells (HUVEC), suggested different signal transduction properties for each of the two VPF receptors. Flt-1/KDR expressing cells demonstrated changes in cell morphology, actin reorganization, chemotaxis, and mitogenic activity in response to VPF. Moreover, neither Flt-1 or Flk-1/KDR stimulation affected the activity of phosphatidylinositol 3'-kinase (PI3-kinase) or tyrosine phosphorylation of phospholipase Cγ (PLCγ). Conversely, Xia et al. (12) demonstrated that VPF can cause the activation of protein kinase C (PKC), PLCγ, and PI3-kinase. Tyrosine phosphorylation of mitogen-activated protein (MAP) kinase by Flt-1 activation has also been demonstrated in trophoblast-like choriocarcinoma cell line BeWo (13). Unfortunately, studies examining these signal transduction pathways in VPF-mediated increases in endothelial monolayer permeability are lacking.

We investigated which signal transduction pathways are involved in the VPF-mediated endothelial permeability response and examined whether these pathways affect the structural organization of the endothelial adherens and occludens junctions as the basis for increased endothelial permeability. We observed that VPF appears to increase endothelial permeability through a MAP kinase-dependent pathway, which leads to disorganization of the cell-cell junctions and results in increased endothelial monolayer permeability.

MATERIALS AND METHODS

Cell Culture—Primary cultures of HUVEC were harvested from fresh human umbilical cords using 0.25% type II collagenase, pooled, and propagated from passages 1 to 3 (14). They were grown to confluence in T-75 tissue culture flasks filled with 15 ml of EGM (Clonetics, San Diego, CA), trypsinized with 2 ml of trypsin-EDTA, and seeded at a density of 105 cells/ml onto fibronectin-coated 12-mm diameter polycarbonate filters (Snapwell, Costar, Cambridge, MA). Seeded filters were allowed to grow to confluence (5–7 days).

Measurement of Permeability—Endothelialized filters were mounted into prewarmed diffusion chambers (Costar, Cambridge, MA) filled with 6 ml/side warmed DMEM (Life Technologies, Inc.) without serum. A circulating water bath (model FJ, Haake, Saddlebrook, NJ) was used to maintain a temperature of 37°C. The filters were incubated at 37°C and 100% humidity in diffusion chambers placed in a humidified incubator (21% O2, 5% CO2, 94% N2). The filters were incubated at 37°C and 100% humidity in diffusion chambers placed in a humidified incubator (21% O2, 5% CO2, 94% N2).
to maintain the diffusion chambers at 37 °C throughout the experiments. A bubble-lift system (5% CO₂, balance room air) was used to stir each side of the filter, and the entire system was allowed to equilibrate for 30 min prior to initiation of experiments. 500 μl of 0.023 mg/ml [14C]albunin was added to the luminal ("hot") chamber, and an equimolar amount of unlabeled albumin was added to the abluminal ("cold") chamber. During the 1-h control period, 500-μl samples were removed from the abluminal chamber at 1, 15, 30, 45, and 60 min and replaced with equivalent amounts of warmed DMEM. At 1 and 60 min, 50-μl samples are taken from the luminal side and replaced with equivalent amounts of warmed DMEM. During the 1.5-h experimental period, VPF (vascular endothelial growth factor) (Collaborative Biomedical Products, Bedford, MA) was added to each diffusion chamber (luminal and abluminal) to give a final concentration of 660 μM. Samples from the abluminal side were removed at 65, 75, 90, 105, 120, and 150 min and replaced with warmed DMEM. Samples from the luminal side were removed at 65 and 150 min.

In the second series of experiments, various agents were added during the 1-h control period: 1 μM staurosporine (Sigma), 2.67 or 1 μM AG126 (Biomol, Plymouth Meeting, PA), 20 μM PD98059 (Biomol), 10 nM wortmannin (Sigma), or 10 nM G66976 (Calbiochem, San Diego, CA). DMEM was added to some chambers as a control. During the experimental portion of these series of experiments various concentrations of VPF were added to the chambers (660, 0.660, or 660 μM VPF heated to 100 °C for 30 min). All samples were placed in scintillation vials in 10 ml of scintillation fluid (Aquasol, New England Nuclear, Boston, MA) and stirred gently. Samples were read for 10 min each in a Wallac 1409 liquid scintillation counter (Wallac, Inc., Gaithersburg, MD).

Calculation of Permeability—The flux of [14C]albumin across the monolayer was calculated by using the following formula (15).

$$P_{app} = \frac{\text{slope} \times \text{cpm/min}}{60 \times A \times \text{total volume}}$$

where $P_{app}$ = apparent permeability in cm/s, slope = (cpm/ml) is y intercept at time ± 0 min and is obtained by plotting cumulative total counts (cum tot) against time and performing linear regression to obtain an x coefficient, hot = hot or luminal chamber (14C[albumin added], cum tot = (cpm - bkg/sample volume/total volume) + bak add), cpm = counts/min, bkg = background cpm (of a blank sample), bak add = Σ (cpm - bkg) at t, . (where t is the cpm obtained at the previous time), A = membrane area (1.13 cm²), and (cpm/ml)/hot = y/cm² = (hot cpm - bkg)/Acm².

Immunofluorescence Staining of Junctional Proteins—HUVEC were grown on coverslips and exposed to 660 μM VPF with and without AG126 (2.67 μM) or PD98059 (20 μM). Samples were fixed according to Kevil et al. (16) and stained for the junctional proteins VE-cadherin and occludin. Mouse anti-VE-cadherin (Hemesis, Sassanne, France) was used at a 1:500 dilution, whereas rabbit anti-occludin (Zymed, San Francisco, CA) was used at a 1:200 dilution. Cy3 goat anti-mouse and anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories Inc., Westgrove, PA) were used at a 1:250 dilution. Photomicrographs were taken at 100× with 12-s exposure times using T-Max 400 film.

Statistical Analysis—All data are reported as “relative permeability” compared with controls and statistical analysis was performed using Graphpad Instat software (San Diego, CA). Samples were compared using a one-way analysis of variance with Tukey post-testing. Significance was determined for all treatments compared with control. Data are shown with standard error.

RESULTS AND DISCUSSION

VPF is angiogenic for endothelial cells and is a potent stimulant for increased vascular permeability (7, 17–19). Initial studies show that VPF increases microvascular permeability in vivo with a potency 50,000 times greater than histamine on an equimolar basis (20). VPF has been shown to be released by monocytes and, more recently, by eosinophils and neutrophils. These observations suggest that VPF may participate in the acute cardiovascular phases of inflammation-associated permeability mediated by these cell types (21–25).

Although the role of VPF in angiogenesis is well documented, its role in endothelial permeability regulation remains more obscure (3). To date, we are aware of only one other study that has examined VPF-mediated changes in endothelial barrier in vitro (26). This study characterized permeability changes in brain-derived endothelial cells without investigating the signal transduction pathways responsible for these permeability changes. Moreover, this study only reported significant changes in [14C]sucrose permeability after 48 h of VPF exposure, long after VPF is reported to alter solute permeability in vivo (3, 17). Therefore, the purpose of this study was to 1) examine VPF-mediated permeability to molecules that are typically responsible for edema formation (e.g. albumin) in vivo in time frames that correlate with VPF biological activity in vivo and to 2) determine the signal transduction pathways involved and possible mechanisms in VPF-induced permeability.

It has been previously shown that VPF binds to and is readily internalized by HUVEC and that HUVEC contain both the Flt-1 and Flk-1 VPF receptors (27). Therefore, we used HUVEC to examine VPF effects on endothelial permeability to [14C]albumin. Fig. 1 illustrates changes in [14C]albumin permeability after exposure to VPF for 90 min. 660 μM VPF-treated monolayers demonstrate a significant 3.4-fold increase in [14C]albumin permeability compared with control monolayers, whereas 0.660 μM VPF-treated monolayers failed to demonstrate significant increases in [14C]albumin permeability. Moreover, this increase in albumin permeability was not observed in monolayers treated with heat-inactivated VPF (Fig. 1). These data suggest that native, intact VPF is responsible for the observed increases in [14C]albumin flux across HUVEC monolayers.

VPF stimulates several signal transduction pathways that could contribute to altered permeability. Previous studies have shown that VPF stimulates “classical” PKC isoforms through a phospholipase C/PLC-g-dependent pathway (12, 28, 29). Furthermore, Xia et al. (12) have shown that VPF-increased PKC-β activity is partially responsible for endothelial cell proliferation and may also stimulate migration. We therefore next determined the role of PKC-β in VPF-mediated endothelial permeability.

Fig. 2 demonstrates that VPF-mediated increases in [14C]albumin permeability is not inhibited by co-incubation with 10 nM G66976, a specific inhibitor of classical PKC-α and -β isoforms (30, 31). We also used staurosporine (1 μM), a general inhibitor of both classical and nonclassical PKC, to block VPF-mediated permeability. We found that PKC inhibition failed to block VPF-mediated increases in [14C]albumin permeability. These data strongly suggest that VPF does not appear to in-
increase permeability through activation of PKC isoforms.

VPF has also been shown to stimulate increased PI3-kinase activity (12, 29, 32). VPF binding to Flt-1 activates PI3-kinase by binding through both of the Flt-1 p85 SH2 domains upon ligand binding. Additionally, Gou et al. (29) showed that VPF-stimulated bovine aortic endothelial cells contained greater amounts of active, phosphorylated PI3-kinase, which was correlated with increased endothelial proliferation. We therefore next examined whether the specific PI3-kinase inhibitor, wortmannin, could block VPF-mediated permeability in our model. Fig. 3 illustrates the effects of 10 nM wortmannin and VPF co-incubation on [14C]albumin permeability. Preincubation with wortmannin followed by co-administration with VPF failed to block increased albumin permeability across endothelial monolayers and suggests that PI3-kinase pathways are not involved in VPF-mediated permeability.

Several studies have also demonstrated that VPF activates Ras GTPase-activating protein and subsequently activates the MAP kinase pathway primarily through Flk-1 and possibly through Flt-1 receptor interactions (11, 13, 29, 33–36). These studies reported VPF-mediated increased endothelial cell proliferation associated with the activation of the MAP kinase pathway. We next determined whether MAP kinase activity may be involved in regulating VPF-mediated permeability.

Fig. 4 illustrates the effect of AG126 and PD98059 on VPF-mediated endothelial permeability. AG126 is a tyrosine kinase inhibitor that has been demonstrated to block tyrosine phosphorylation of p42 MAP kinase in endothelial cells but does not inhibit EGF receptor, Her-2/neu receptor, or platelet-derived growth factor β receptor (37, 38). Incubation of monolayers with 2.67 μM AG126 and VPF significantly prevented increased endothelial monolayer permeability produced by VPF compared with control. Furthermore, co-incubation of VPF with 1 μM AG126 also attenuated VPF-mediated permeability. Co-incubation of VPF and PD98059 (20 μM), a specific inhibitor of MAP kinase kinase-1 (39, 40), also blocked VPF increases in permeability. This suggests that MAP kinase kinase activation and subsequent activation of ERK1/2 are critical for the VPF-mediated permeability effects in vitro. Control permeability measurements for PD98059 (1.93 × 10⁻⁵ ± 6.75 × 10⁻⁶ cm/s) or AG126 (7.25 × 10⁻⁶ ± 8.0 × 10⁻⁷ cm/s) versus media (1.45 × 10⁻⁵ ± 1.66 × 10⁻⁶ cm/s) did not demonstrate any significant differences, indicating that these compounds have no effect on albumin permeability alone. Importantly, at the concentrations used here, neither AG126 or PD98059 were toxic to the endothelium (data not shown). These data strongly suggest that activation of the MAP kinase pathway is involved in VPF-mediated permeability in vitro.

Still, the molecular targets responsible for VPF-mediated permeability have not been identified. Endothelial solute permeability is maintained through the endothelial junctional protein cadherin and occludin (16, 31, 41–44). We have previously shown that pulmonary artery endothelial cells exposed to hydrogen peroxide show a loss of junctional pan-cadherin staining with concomitant gap formation that results in increased endothelial monolayer permeability (31). The specific endothelial adherens junctional protein VE-cadherin has also been shown to maintain and perhaps regulate endothelial barrier properties (44). In addition to VE-cadherin, we have shown that the tight junctional protein occludin significantly contributes to endothelial monolayer barrier properties (16). Moreover, a previous electron microscopic study demonstrated that VPF could induce gap formation at endothelial cell-cell junctions (45). We therefore investigated the possibility that rear-
rangement of the junctional proteins occludin and VE-cadherin are the cause of increased endothelial monolayer permeability in response to VPF.

Fig. 5 shows immunofluorescent staining for the adherens junctional protein VE-cadherin and the tight junctional protein occludin. Panels A and E illustrate control staining for occludin and VE-cadherin, respectively. Panels B and F demonstrate occludin and VE-cadherin staining after 660 μM VPF incubation for 60 min. Note the gaps between adjacent endothelial cells and the lack of junctional proteins at these gaps (arrows). Panels C and G illustrate occludin and VE-cadherin staining of monolayers pretreated with AG126 (2.67 μM) and then simultaneously exposed to AG126 and 660 μM VPF. Panels D and H show occludin and VE-cadherin staining of monolayers pretreated with PD98059 (20 μM) and then simultaneously exposed to PD98059 and 660 μM VPF. Note that the junctional morphology of both occludin and VE-cadherin remain intact with either AG126 or PD98059 treatments.

Fig. 5. Effect of VPF on endothelial junctional proteins. Endothelial monolayers were stained for occludin and VE-cadherin as described previously. Panels A and E illustrate control staining for occludin and VE-cadherin, respectively. Panels B and F demonstrate occludin and VE-cadherin staining after 660 μM VPF incubation for 60 min. Note the gaps between adjacent endothelial cells and the lack of junctional proteins at these gaps (arrows). Panels C and G illustrate occludin and VE-cadherin staining of monolayers pretreated with AG126 (2.67 μM) and then simultaneously exposed to AG126 and 660 μM VPF. Panels D and H show occludin and VE-cadherin staining of monolayers pretreated with PD98059 (20 μM) and then simultaneously exposed to PD98059 and 660 μM VPF. Note that the junctional morphology of both occludin and VE-cadherin remain intact with either AG126 or PD98059 treatments.
zation of occludin and VE-cadherin or promote the appearance of inter-endothelial gap formation (Panels C and G, respectively). PD98059 (20 μM) and VPF (660 pm) co-incubation also failed to demonstrate disorganization of occludin and VE-cadherin staining (Panels D and H). These observations suggest that MAP kinase activity governs endothelial junction disorganization produced by VPF. Moreover, these data strongly suggest that VPF-mediated albumin permeability occurs through the loss of occludin and VE-cadherin at endothelial junctions, with concomitant gap formation and decreased barrier properties between adjacent endothelial cells.

In conclusion, these data demonstrate for the first time a rapidly responding in vitro model of VPF-mediated albumin permeability with a similar time course as that reported in vivo. We also demonstrate that VPF-induced permeability is apparently mediated through the MAP kinase signal transduction cascade. Our data further indicate that endothelial junction proteins (e.g. VE-cadherin and occludin) become disorganized upon VPF administration and that inhibitors of MAP kinase attenuate this disorganization. VPF appears to increase permeability in vitro through activation of MAP kinase, with a subsequent loss of VE-cadherin and occludin from the endothelial cell-cell junctions. Experiments are currently under way to better determine the roles of MAP kinase and its related pathways in the structural regulation of the endothelial junctional proteins governing solute permeability.

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