Deciphering Vascular Endothelial Cell Growth Factor/Vascular Permeability Factor Signaling to Vascular Permeability

INHIBITION BY ATRIAL NATRIURETIC PEPTIDE

Ali Pedram, Mahnaz Razandi, and Ellis R. Levin

From the Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, California 90822 and Departments of Medicine and Pharmacology, University of California, Irvine, California 92717

Vascular endothelial cell growth factor (VEGF) was originally described as a potent vascular permeability factor (VPF) that importantly contributes to vascular pathobiology. The signaling pathways that underlie VEGF/VPF-induced permeability are not well defined. Furthermore, endogenous vascular peptides that regulate this important VPF function are currently unknown. We report here that VPF significantly enhances permeability in aortic endothelial cells via a linked signaling pathway, sequentially involving Src, ERK, JNK, and phosphatidylinositol 3-kinase/AKT. This leads to the serine/threonine phosphorylation and redistribution of actin and the tight junction (TJ) proteins, zona occludens-1 and occludin, and the loss of the endothelial cell barrier architecture. Atrial natriuretic peptide (ANP) inhibited VPF signaling, TJ protein phosphorylation and localization, and VPF-induced permeability. This involved both guanylate cyclase and natriuretic peptide clearance receptors. In vivo, transgenic mice that overexpress ANP showed significantly less VPF-induced kinase activation and vascular permeability compared with non-transgenic littermates. Thus, ANP acts as an anti-permeability factor by inhibiting the signaling functions of VPF that we define here and by preserving the endothelial cell TJ functional morphology.

The vascular endothelial cell growth factor (VEGF) glycoprotein is an important angiogenesis factor that was originally isolated as a vascular permeability factor (VPF) (1, 2). VEGF/VPF (henceforth designated VPF) potently stimulates fluid transgression through endothelial cell (EC) tight junctions (TJ) (3, 4). This permeability factor also modulates the formation and function of vesiculovascular organelles in venules (5) and the development of EC fenestrations (6). These mechanisms underlie the enhanced vascular permeability seen in response to VPF, which is implicated in the ascites associated with ovarian and other carcinomas (1, 7), the pathogenesis of diabetic retinopathy (8), and the ovarian hyperstimulation syndrome (9).

The angiogenesis-promoting actions of VPF result after binding and signaling through the transmembrane receptors Flk-1 (10), Flt-1 (11), and neuropilin (12). The Flk-1 tyrosine kinase receptor (VEGF-R2) has been proposed to participate in VPF permeability (13), but signaling through other related receptors also appears to be important (14). Flk-1 receptors activate membrane-associated kinases, such as Src and phosphatidylinositol 3-kinase (PI3K) (11, 15), and Src is critical to the role of the participation of VPF in the development of local edema following brain insult (16, 17). PI3K contributes to the ability of VPF to promote EC migration (18), but its precise role in permeability is unclear. Signaling to the generation of nitric oxide (NO) (13, 18) and subsequent activation of the extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (19), prostacyclin generation (13), or VPF-induced protein kinase C activity (20) have all been proposed to contribute to increased vascular permeability. How activation of various signals is integrated into a functional pathway responsible for permeability is undetermined.

Regarding NO, this gas disrupts both cytoskeletal protein complexing in epithelial cells and the management of the actin cytoskeleton (21–24). This results in the dilation of cell tight junctions due to ATP depletion (23). We previously implicated NO as participating in VPF-induced permeability in the pathogenesis of the human ovarian hyperstimulation syndrome (9). In parallel, poorly defined VPF signaling pathways can result in the phosphorylation of TJ proteins that exist in complex, such as zona occludens-1 (ZO-1) or occludin (24). Phosphorylation of TJ proteins results in a lower transcellular resistance of EC (25), serving as an index of barrier function. Upon phosphorylation, TJ proteins assume abnormal relationships with other members of this complex, thereby creating “leaky” endothelial cell-cell contacts (26, 27). Thus, VPF-induced signaling to the cytoskeleton and to associated TJ proteins is potentially important and could present therapeutic targets to prevent vascular pathobiology (7–9).

Relevant to vascular biology, the natriuretic peptides (NP), a family of small proteins that mediate salt and water balance, as well as vascular tone (reviewed in Ref. 28), atrial and brain natriuretic peptides (ANP and BNP) are produced predominantly in the heart, whereas C-type natriuretic peptide (CNP) is synthesized by the endothelial cell. These peptides inhibit vascular cell growth mainly after binding the guanylate cyclase A (reactive to ANP or BNP) or B (CNP specific) recep-
tors (29, 30). This stimulates the production of cGMP and subsequent activation of PKG, inducing target genes or the modulation of K⁺ channels (28). A second class of NP receptors is the clearance receptor (NPRC) (31), and this protein may contribute to the inhibition of cardiomyocyte, vascular endothelial cell, or astrocyte proliferation (28, 32, 33).

Because VPF-induced permeability underlies the pathobiology of several disorders (7–9, 14), we determined a relevant signaling pathway and the resulting morphological consequences in EC. Furthermore, we identify the NP as inhibitors of these processes, in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Antibodies and substrate for kinase activation/activity and for phospho-ZO-1 or occludin were from Santa Cruz Biotechnology, Santa Cruz, CA. PD98059 was a generous gift from Dr. Alan Saltiel (Parke-Davis). VPF, wortmannin, LY294002, FP2, and t-NAG, and t-AcN methyl-t-arginine acetate were from Calbiochem. LipofectAMINE was from Invitrogen. 3H]Mannitol was from PerkinElmer Life Sciences. SB203580 was from Dr. P. R. Young (Smith Kline & French Laboratories) (34).

Cell Preparation—Primary cultures of bovine aortic EC were prepared and used as described previously (35, 36). In transfection studies, EC were transfected in passages 4–5 on coverslips, based upon previous observations that greatly increases the transfection efficiency of these cells. Kinase Activity Assays—For ERK and c-Jun N-terminal kinase (JNK) activity assays, the cells were synchronized for 24 h in serum- and growth factor-free medium. The cells were then exposed to VPF, 10–20 ng/ml, for 10 (ERK) or 15 min (JNK), with or without additional substances or peptides, as described previously (36, 37). Immunoprecipitated kinases were then added to the protein myelin basic protein (for ERK), or glutathione S-transferase-c-Jun(1–79) (for JNK) for in vitro kinase assays. In addition, the VPF-induced phosphorylation of Src (5 min) and AKT (15 min) kinases were determined as indices of activation. Cultured cell lysates were pelleted and dissolved in SDS samples buffer, separated, and then transferred to nitrocellulose. Phosphorylated kinase proteins were detected using phospho-specific monoclonal antibodies (Santa Cruz Biotechnology) and the ECL Western blot kit. Equal samples from the cells were also immunoprecipitated, and immunoblots of the precipitated kinase protein from each experimental condition were determined to show equal gel loading. All experiments were repeated two to three times.

Transient Transfections—BAEC (passage 4–5) were grown to 40–50% confluence and then transiently transfected with 1.5 (each well of 6-well plates) or 10 μg of fusion plasmid DNA (100-mm dishes). Plasmids included wild type JNK-1 (pcDNA3FLAG-JNK-1) or dominant negative JNK-1 (pcDNA3 FLAG-JNK-1 APF) (kindly provided by Dr. Dr D. Downward), dominant negative Myc-tagged FMT2-AH-AKT (kindly provided by Dr. Julian Downward) (39), dominant negative PI-3K p85 subunit (pcDNA3-p85, lacking residues 478–513) (kindly provided by Dr. Barry Posner) (40), dominant negative Src constructs pRcC3V-Src289M (kindly provided by Drs. Louis Luttrell and Robert Lefkowitz) (41), and SrcK286E/R726F (kindly provided by Michael Simonson) (42). Transfection was carried out using LipofectAMINE. Cells were incubated with liposome-DNA complexes at 37 °C for 5 h, followed by overnight recovery in DMEM containing 10% fetal bovine serum, 24 h synchronization in serum-free DMEM, and then treatment with VPF ≥ NP.

Endothelial Cell Permeability Assay—Assays were performed as described previously (9). Primary cultures of BAEC were seeded onto 0.45-μm CM filters (Millipore, Bedford, MA) within plastic inserts; the inserts were placed into 24-well plates, and the cells were grown to confluence over several days. In some experiments, the EC were first transfected with dominant negative constructs of JNK, PI3K, or Akt and then transferred to chambers. Chambers were created by placing membranes over the inner insert and the outer wells, with the endothelial cell monolayer on the insert filter. Both the apical and basolateral surface of the insert containing the cells on filter was added VPF ± ANP (24 h). The electrical resistance of the filter-grown endothelial cell monolayers was measured using an EVOM resistance meter with and without labeled mannitol (no serum or added peptides) was used for control permeability assessments.

EC Monolayer Resistance Assay—Primary BAEC were grown until post-confluent on Transwell filters (Millicell) that were previously coated with rat tail collagen. The EC were then treated with VEGF ± ANP for 24 h. The electrical resistance of the filter-grown endothelial cell monolayers was measured using an EVOM resistance meter with and without labeled mannitol (no serum or added peptides) was used for control permeability assessments.

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Cytoskeletal Actin—Non-transfected or transfected EC were grown to confluence on poly-L-lysine-coated glass coverslips and then exposed to 10ng/ml VPF ± ANP or 100 nM C-ANP (4–23) for 30 min. The cells were permeabilized, washed, then stained with fluorescent-labeled phalloidin (Molecular Probes). Actin distribution was examined and photographed under a Nikon epifluorescent microscope. Western blots were carried out three times.

ZO-1 and Occludin Studies—The phosphorylation of ZO-1 or occludin was determined by Western blot. Subconfluent, transfected, or non-transfected cultured BAEC were serum-deprived overnight and then incubated under various conditions for 10 min with inhibitors followed by 30 min of treatment with stimulants. The cells were lysed and antibodies to ZO-1 or occludin (1:50 dilution) were conjugated to Sepharose beads and then added to the cell lysate for 2 h at 4 °C. After washing, samples were electrophoresed on a 7% SDS gel, transferred to nitrocellulose, and immunoblotted. Detection utilized the ECL kit (Amersham Biosciences). For immunofluorescent staining of ZO-1 and occludin proteins, BAEC were cultured on coverslips in DMEM containing 10% fetal bovine serum. Upon confluence, coverslips were treated, fixed, and stained. BAEC coverslips were either pretreated with ANP or other inhibitors and then exposed to VEGF for 30 min. Cells were fixed in 2% freshly prepared paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 3 min, and then incubated with 0.5% bovine serum albumin in phosphate-buffered saline solution for 30 min at 23 °C. After washing, primary antibodies of mouse anti-occludin (1:250) and rabbit anti ZO-1 (1:250; Zymed Laboratories Inc., San Francisco, CA) were incubated for 1 h at 37 °C. Coverslips were then washed and fluorescently labeled secondary antibodies (1:200, anti-mouse fluorescein isothiocyanate, anti-rabbit rhodamine) were incubated on a 7% SDS gel, transferred to nitrocellulose, and immunoblotted. Detection utilized the ECL kit (Amersham Biosciences). For immunofluorescent staining of ZO-1 and occludin proteins, BAEC were cultured on coverslips in DMEM containing 10% fetal bovine serum. Upon confluence, coverslips were treated, fixed, and stained. BAEC coverslips were either pretreated with ANP or other inhibitors and then exposed to VEGF for 30 min. Cells were fixed in 2% freshly prepared paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 3 min, and then incubated with 0.05% bovine serum albumin in phosphate-buffered saline solution for 30 min at 23 °C. After washing, primary antibodies of mouse anti-occludin (1:250) and rabbit anti ZO-1 (1:250; Zymed Laboratories Inc., San Francisco, CA) were added for 1 h at 37 °C. Coverslips were then washed and fluorescently labeled secondary antibodies (1:200, anti-mouse fluorescein isothiocyanate, anti-rabbit rhodamine) were incubated, then cover slips were examined under a Nikon epifluorescent microscope. Western blots were carried out three times.

In Vivo Studies—All experiments were approved by the Animal Studies Subcommittee and the Research and Development Committees at the Long Beach Veterans Affairs Medical Center. To determine the interactions of ANP and VPF in vivo, we utilized a transgenic mouse that overexpresses the ANP gene (44, 45). Founder mice were bred on a C57B1/6J background to minimize the possibility of randomly segregating alleles. Non-transgenic littermates were used for comparisons, and PCR-based genotyping of tail DNA confirmed the identity of the mice. Plasma ANP levels in these mice are elevated compared with non-transgenic littersmates.

To determine permeability, a Miles assay (46) was adapted to mice. ANP transgenic or littermate control mice were anesthetized and intravenously injected with 100 μl of 0.5% Evan’s blue dye. This was allowed 30 min later by injection of either 10 μl of VEGF (1μg/ml), 10 μl of 1μm histamine, or saline, into the ears of the mice. The mice were then imaged and the injection site was carefully dissected to encompass the same amount of area from each mouse. The dye was then eluted from the dissected samples with formamide at 56 °C, and absorbance was quantitated by spectrophotometer (A490). Injection site areas were also obtained from separate mice for kinase activity determination and were snap-frozen in liquid nitrogen. The samples were pulverized, followed by lysing in buffer containing protease inhibitors. Lysates were sonicated and microcentrifuged, and the resulting supernatants were immunoprecipitated for kinase studies.

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RESULTS

ANP Inhibits VPF-induced Src, through Both NPRC and GC Receptors—The non-membrane tyrosine kinase Src is critical to the participation of VPF in the vascular permeability/edema following cerebrovascular occlusion (17, 47). However, the signaling pathways downstream of Src that mediate permeability have not been defined. We first showed that VPF stimulates the activating phosphorylation of tyrosine 416 of Src and that ANP prevents this activation (Fig. 1A), as a potentially important function of the NP to inhibit VPF action. ANP can act at both GC-A receptors and the NPRC (28), but the VPF effect here was also significantly inhibited by C-ANP-(4–23), which only acts at the NPRC. These data therefore support both receptors as mediating the ability of ANP to inhibit Src activation, probably reflecting a common pathway downstream from each receptor.
In response to cytokines, activated Src signals to the stimulation of numerous downstream kinases (48–50). To identify downstream effectors, we first validated the inhibitory function of the PP2 antagonist for the Src family of kinases (Fig. 1A, lane 3), and we then showed that VPF-induced ERK activation was dependent upon Src family kinase phosphorylation by using this inhibitor (Fig. 1B). Src family kinase activity was also important for VPF to stimulate JNK and PI3K/AKT activity in the EC because PP2 significantly prevented this. We showed previously (37) that VPF induces ERK-dependent JNK activation and subsequent angiogenesis in vitro, linking these signaling molecules. To establish the significance of Src here, we carried out permeability studies. As seen in Fig. 1C, PP2 significantly blocked the ability of VPF to stimulate labeled mannitol flux through the EC barrier by about 65%.

To corroborate the specific importance of Src, EC were transiently transfected to express two different and well validated kinase-deficient Src constructs (pRC-c-SrcK298M (41) and K296R/Y528F (42)) that serve as dominant negative Src mutant proteins. The ability of VPF to stimulate ERK, JNK, and AKT activation was each very significantly impaired in this setting (Fig. 1D). We also carried out permeability studies and found that the mutant Src molecules significantly reduced VPF-induced EC permeability (Fig. 1E). These results identify and link downstream molecules that mediate VPF-induced permeability, emanating from Src activation.

**Both JNK and PI3K Contribute to VPF-induced Permeability**—We then determined the roles of JNK and PI3K/AKT, because these signaling molecules mediate other cellular effects of VPF (18, 51, 52). We recently showed that VPF significantly stimulates JNK activity in EC, and that is was inhibited maximally 69% by ANP or the NPRC-specific ligand, C-ANP(4–23). By using an antibody that detects the activating phosphorylation at serine 473 of AKT, we found here that VPF significantly stimulates AKT activity in EC, and that is was inhibited nearly 5-fold (Fig. 2A). Activation of AKT was significantly blocked by the soluble PI3K inhibitors, wortmannin and LY294002, indicating that activation of PI3K occurred upstream of AKT augmentation. ANP blocked AKT activation by 70% (lanes 2 and 5), and this was substantially reversed by the GC receptor antagonist, HS-142-1 (53) (lane 7). C-ANP(4–23) was less potent than ANP. These data support both the GC-A and NPRC as mediating the ability of ANP to inhibit AKT activation by VPF and indicate another level of restraint of permeability factor signaling.
We then asked whether these kinases contributed to VPF-induced EC permeability. In cultured cell monolayers, VPF caused a near 4-fold augmentation in permeability (Fig. 2B, left side). This was 70% blocked by ANP and, again, slightly less potently by C-ANP (4-23). To implicate the involvement of JNK, we transiently expressed a dominant negative Jnk-1 (JNK1-APF) that we previously showed (37) was capable of preventing JNK activation, and which we validate here (Fig. 3). In this setting, VPF-induced permeability was 51% prevented (Fig. 2B, right side), and it must be considered that our transfection efficiency is only 52%. Therefore, the ability of ANP to block JNK activation (36) contributes to the inhibition of VPF-induced permeability by this natriuretic peptide. To support this idea, we transiently expressed a mildly constitutively active JNK-1 (FLAG-JNK1) (37, 38). As a result, ANP (or C-ANP (4-23)) was significantly less capable of blocking VPF-induced permeability (Fig. 2B, right side). Thus, these data indicate the importance of this MAP kinase family member for ANP anti-permeability action. To confirm these results, the resistance to permeability was measured on the post-confluent EC monolayers (Fig. 2C). The basal resistance to permeability attributed to EC tight seal formation was 140 ohms/cm², and this was comparable with the resistance reported from microvascular EC (54). VEGF lowered resistance by 68%, and ANP significantly reversed the decreased permeability to VEGF.

A role for PI3K and AKT was examined. Wortmannin and LY294002 maximally inhibited VPF-induced permeability by 80 and 74%, respectively, indicating the importance of PI3K (Fig. 2D). To support the role of AKT, we transiently expressed a dominant negative AKT protein (37). Here VPF was 52% less

**Fig. 3. Cross-talk between PI3K/AKT and JNK.**

**A,** VPF-induced JNK requires PI3K/AKT. EC were transfected with pcDNA3 (control), dominant negative AKT, or JNK1(APF), or Δp85 PI3K (dominant negative), and incubated with VPF ± wortmannin or LY294002. JNK activity was determined at 10 min, and total Jnk-1 protein is also shown. *, p < 0.05 for pcDNA3 versus VPF; +, p < 0.05 for VPF versus VPF + condition, in three combined experiments. B, AKT activation by VPF is mediated through JNK and PI3K. EC were incubated with VPF ± wortmannin, LY294002, or transfected with Δp85 PI3K (dominant negative) or dominant negative AKT or JNK1(APF), and AKT activity was determined at 15 min. Total AKT protein is also shown. *, p < 0.05 for pcDNA3 versus VPF; +, p < 0.05 for VPF versus VPF + condition, in three combined experiments. C, nitric oxide production precedes ERK activation by VEGF. EC were incubated with VEGF ± l-NAME, 1 μM, for 8 min, and ERK activity was determined. The study was repeated three times for the bar graph. *, p < 0.05 for control versus VEGF; †, p < 0.05 for VEGF versus VEGF plus l-NAME or PD98059.
potent in stimulating permeability, despite a 58% efficiency of dominant negative AKT transfection. Thus, PI3K/AKT are important effectors of VPF-induced permeability.

Cross-talk between JNK and PI3K—Our results implicate both JNK and PI3K/AKT as playing important roles in VPF-induced permeability. It is possible that the kinases act in distinct but complementary pathways to alter EC barrier function. Alternatively, JNK and PI3K/AKT may lie along the same signaling pathway, in response to VPF. To assess this, we first determined the role of PI3K and AKT in JNK activation. As seen in Fig. 3A, incubation of the cells with wortmannin, LY294002, or expression of the dominant negative p85 subunit (lane 7) each significantly inhibited VPF-induced JNK. Similarly, expression of the dominant negative AKT also prevented JNK activation by VPF (lane 4).

We then examined the phosphorylation of AKT. The ability of VPF to phosphorylate AKT was PI3K-driven, as shown using the dominant negative p85 subunit of PI3K, wortmannin, and LY294002 (Fig. 3B). Expression of the JNK1 APF construct also substantially prevented AKT activation (lane 2 versus 3). Validation of JNK1 (APF) to block endogenous JNK activation is also shown (Fig. 3A, lane 3). Thus, we provide novel evidence that these signaling molecules are linked in response to VPF, and this interaction may involve a bi-directional augmentory loop.

Several NP Block, Whereas NO and p38 Mediate, VPF-induced Permeability—We also determined the specificity of ANP to block VPF-induced permeability, in comparison to other NP family members. BNP functions similarly to ANP in that it binds both GC-A receptors and NPRC (28), and we found it to be equipotent to ANP for inhibiting this VPF action (Table I). Interestingly, CNP also significantly prevented VPF-induced permeability; this peptide binds a second guanylate cyclase receptor, GC-B, as well as NPRC, and thus is likely to act by mechanisms similar to that of ANP or BNP. ANP and BNP circulate after being secreted mainly from the heart, whereas CNP is produced in EC. Thus, all the members of this family of vasoactive peptides can inhibit VPF action, and these data complement our previous findings that several NP family members inhibit VPF synthesis (35).

From published cell models, NO is produced after VPF binds to Flik-1 (55, 56). Flik-1 ligation causes Sre activation leading to NO production (57) and independently stimulates PI3K-dependent NO production (58). This leads to angiogenesis (58), and in part, NO-related ERK activation stimulates EC proliferation (59). NO production has also been implicated in VPF-induced permeability acting through unknown downstream targets (13). We found that the NO synthase inhibitor, l-NAME, partially but significantly (58%) blocked the ability of VPF to stimulate permeability. Additionally, the specific MEK inhibitor, PD98059, also partially blocked VPF-induced permeability (Table I), and NO inhibition partially prevented VEGF-induced ERK activation (Fig. 3C), linking these events. Finally, it has been reported that the p38 MAP kinase contributes to the ability of VPF to stimulate EC migration (60, 61). We found that upon using a specific and potent soluble inhibitor of p38, SB203580, VPF-induced permeability was partially blocked (Table I). Inhibiting either ERK or p38 MAP kinases resulted in a significant reduction of VPF-augmented permeability. Src-dependent ERK activation shown here provides upstream signaling for our previous report (37) that ERK activates JNK in response to VPF. Supporting this linkage, we found that VPF-induced JNK activity is also Src-dependent (Fig. 1B). These results further define the sequential signaling pathways involved in this specific function of the vascular growth/permeability factor.

VPF Stimulates ZO-1 and Occludin Phosphorylation via PI3K/Akt and JNK, Blocked by ANP—An important mechanism of EC barrier compromise is the disruption of the EC TJ. The ZO-1 protein maintains the structural integrity of TJ in both endothelial and epithelial cells. In response to VPF, we found that ZO-1 was strongly phosphorylated, using an antibody specific for serine/threonine residues (Fig. 4A). This was significantly prevented by the expression of JNK1(APF), dominant negative AKT, and by the PI3K inhibitors. Both AKT and JNK phosphorylate substrate Ser/Thr residues, and so the results are consistent with the known requirements of these kinases. The inhibition seen was not complete, and we cannot rule out the possibility of other Ser/Thr kinases playing a role. These results indicate that the specific kinases underlying VPF-induced permeability also phosphorylate ZO-1.

Because the phosphorylation of ZO-1 by VPF correlates with increased vascular permeability, then ANP might inhibit this function. We tested this idea, and we found that ANP (or C-ANP) inhibited ZO-1 phosphorylation by 55% (Fig. 4B). This partially resulted from GC-A activation, as the ANP effect was reversed by the specific guanylate cyclase inhibitor, HS-142-1 (lane 5), whereas the C-ANP inhibition was unaffected by this compound (demonstrating the involvement of the NPRC, as well). Our results point out a novel cross-talk activated by ANP binding the GC-A receptor, between cGMP/PKG and the inhibition of PI3K/AKT activation by the vascular permeability factor. ANP signaling through cGMP/PKG also inhibits JNK activation (see Ref. 36 and this work), and previous studies (62, 63) show that the relationship between cGMP and JNK is cell context-specific.

We next examined the possible phosphorylation of the TJ protein, occludin. As seen in Fig. 4C, VPF stimulated a 2.5-fold increased phosphorylation of this protein on Ser/Thr residues. This was significantly prevented by expressing dominant negative proteins for JNK (lane 3), Akt (lane 4), or by incubating the cells with wortmannin and LY294002 (lanes 5 and 6). Consistent with the signaling to permeability, and the phosphorylation of ZO-1 by VPF, ANP also caused a 74% inhibition of occludin phosphorylation (Fig. 4D). These effects were substantially prevented by the GC-A inhibitor (lanes 3 versus 5), but the NPRC receptor also contributed (lane 4).

Localization of ZO-1 and Occludin at the EC Tight Junction—Both occludin and ZO-1 proteins are crucial to the organization of the EC tight junction, and the two proteins physi-
cally associate with each other and with actin. By using immunohistochemistry, we identified the localization of ZO-1 at the EC cell contacts of confluent cells (Fig. 5A). The tightly organized and discrete localization of ZO-1 in the control cells (panel a) was in contrast to the disrupted localization and aggregation of this protein in response to VPF treatment (panel b). Expression of dominant negative JNK in the EC (panel c), dominant negative AKT (panel d), or soluble inhibitors of PI3K (panel e) each significantly prevented the effects of VPF. Thus, Ser/Thr phosphorylation of ZO-1 by the implicated kinases importantly contributes to the disruption of the TJ cytoarchitecture after VPF exposure, producing gaps at the EC interface boundary.

We also examined the effects of the natriuretic peptides (Fig. 5B). ANP or C-ANP substantially prevented the disruption of ZO-1 localization caused by VPF (panels c and d). Consistent with the roles of the GC-A and NPRC receptors, HS-142-1 partially but significantly reversed the effects of ANP (panel e) but not C-ANP (4–23) (panel f). This identifies a cell biological effect of the NP to explain mechanistically how these peptides prevent increased vascular permeability due to VPF.

The localization of occludin in the EC barrier function was also determined by immunostaining (Fig. 5C). Occludin positioning in the EC contact points was severely disrupted by VPF (Fig. 5C, panel b), but this was prevented by expression of dominant negative Jnk-1 (panel c), dominant negative AKT (panel d), wortmannin (panel e), and LY294001 (panel f). In composite Fig. 5D, VPF disruption of occludin localization (panel b) is reversed by ANP (panel c) or C-ANP (panel d). The ANP effect was prevented by HS-142-1 (Fig. 5D, panel e), but the C-ANP action was not affected by the GC inhibitor (panel f). Furthermore, we showed that expression of dominant negative Src constructs (Fig. 5E, panels c and d) prevented VEGF-induced disruption of occludin localization (panel b). These results support a similar mechanism of VPF action directed toward both key TJ proteins.

**VPF Signals to Actin Stress Fiber Formation That Is Prevented by ANP**—The stability of actin association with TJ proteins contributes to the distinct cytoarchitecture that is necessary for EC barrier function (64). We therefore determined the effects of VPF signaling and the NP on actin organization. In control EC, there was a peripheral pattern of actin organiza-
Fig. 5. ZO-1 and occludin localization within the tight junction of endothelial cells is altered by VPF, which is inhibited by the natriuretic peptides. A, the composite shows ZO-1 organization at the junctions of confluent EC, after staining with a specific antibody for this protein. Panel a is control EC; panel b is EC incubated with VPF 20 ng/ml; panel c is VPF added to cells expressing Jnk-1 APF (dominant negative);
tion, characteristic of cells in an unperturbed state (Fig. 6A, panel a). VPF stimulated a re-arrangement of actin into stress fibers (Fig. 6A, panel b). When the cells were transfected with dominant negative JNK-1 and then incubated with VPF, there was a significant reversion of actin alignment toward the control situation (absence of VPF) (Fig. 6A, panel c). Similarly, expression of dominant negative AKT (Fig. 6A, panel d), or co-incubation of the cells with wortmannin (panel e) or LY294002 (panel f), prevented the majority of VPF-induced actin rearrangement. Consistent with the signaling data, ANP or C-ANP-(4–23) significantly prevented VPF-induced stress fiber formation (Fig. 6B, panels a–d). Blocking GC receptor (panel d) is VPF added to cells expressing the AKT (dominant negative); panel e is VPF + wortmannin; and panel f is VPF + LY294002. The latter two inhibitors of PI3K had no effects by themselves (data not shown). Arrows show the disruption of the localization of these proteins at the apposition of the EC. B, the composite panel a is control; panel b is VPF; panel c is VPF + ANP 100 nM; panel d is VPF + C-ANP-(4–23) 100 nM; panel e is VPF + ANP + HS-142-1 (GC inhibitor); and panel f is VPF + C-ANP-(4–23) + HS-142-1. The NP or HS-142-1 by themselves had no effect. C, occludin localization is shown in control EC (panel a); panel b is EC incubated with VPF, 20 ng/ml; panel c is VPF added to cells expressing Jnk-1 (dominant negative); panel d is VPF added to cells expressing the AKT (dominant negative) protein; panel e is VPF + wortmannin; and panel f is VPF + LY294002. The latter two inhibitors of PI3K had no effect by themselves (data not shown). D, the composite is occludin staining in control EC (panel a); panel b is VPF-treated cells (panel b); VPF + ANP, 100 nM (panel c); VPF + C-ANP-(4–23), 100 nM (panel d); VPF + ANP + HS-142-1 (GC inhibitor) (panel e); and panel f is VPF + C-ANP-(4–23) + HS-142-1. E, control occludin localization (panel a) is altered by VPF (panel b); the effect is reversed by pRC-cSrc-K298M (panel c) or SrcK296R/Y528F expression (panel d). Panels e and f are the two dominant negative constructs alone. The composites are representative of three separate experiments.
activation with HS-142-1 reversed the effects of ANP (Fig. 6B, panels d versus f) but had little effect on C-ANP-(4–23) action (panels d versus f). These results provide additional structural information relevant to the mechanisms by which VPF stimulates and the NP prevent EC permeability. Re-alignment of actin to stress fibers also importantly contributes to the ability of EC to migrate (65).

In Vivo Studies—To support our in vitro findings, we utilized a mouse that overexpresses an ANP transgene (44, 45). We confirmed that the plasma levels of ANP in these mice were significantly greater than their non-transgenic littermates plasma ANP 124 ± 0.8 versus 11.2 ± 0.2 pg/ml, n = 4 per group, p < 0.05. A Miles permeability assay (46) was then carried out to detect the interactions of ANP and VPF. Evans blue dye had been delivered by intravenous injection 30 min prior to VPF injection. Careful VPF injection into the ear skin of normal mice caused a rapid (10 s) and dense extravasation of dye (Fig. 7A, left). In contrast, the extravasation of dye upon VPF injection was significantly reduced in the ANP-overexpressing mice. Ear skin saline injections into either mouse was without effect. The quantification of these results is shown in Fig. 7B and indicates 65% less permeability to VPF in the ANP transgenic mice. The decreased response to VPF in the ANP-overexpressing mice was significantly reversed by the co-injection of the GC antagonist, HS-142-1, with VPF (Table II). Similarly, in both control and ANP-tranagenic mice, inhibitors of PI3K, ERK, or NO partially prevented VPF-induced permeability. We also compared the effect of histamine injection, and we found that histamine-induced vascular permeability occurred comparably in normal or ANP transgenic mice (Fig. 7A, right).

Our in vitro data indicated the importance of VPF-induced kinase activation for permeability and that ANP inhibition of

![Fig. 6. Actin rearrangement in the EC cytoskeleton is induced by VPF and is inhibited by the natriuretic peptides. A, the composite shows actin organization in the EC, after staining with phalloidin. Panel a is control EC; panel b is EC incubated with VPF, 20 ng/ml; panel c is VPF incubated with cells expressing Jnk-1 APF (dominant negative); panel d is VPF added to cells expressing the AKT (dominant negative); panel e is VPF + wortmannin; and panel f is VPF + LY294002. The latter two inhibitors of PI3K had no effects by themselves (data not shown). B, the composite panel a is control; panel b is VPF; panel c is VPF + ANP, 100 nM; panel d is VPF + C-ANP-(4–23), 100 nM; panel e is VPF + ANP + HS-142-1 (GC inhibitor); and panel f is VPF + C-ANP-(4–23) + HS-142-1. The NP or HS-142-1 by themselves had no effect. The composites are representative of three separate experiments.](http://www.jbc.org/content/early/2006/11/17/jbc.M605104200/Figure6.jpg)
signaling provided a mechanistic understanding of the effects of this peptide. We therefore assessed the modulation of signaling at the VPF injection sites, in the transgenic and non-transgenic controls. VPF induced a clear increase in ERK, JNK, and AKT activity in normal mice (Fig. 7C). In contrast, VPF-induced kinase activation was markedly attenuated in the ANP-overexpressing mice. These data are quantified in Table III and provide in vivo support to the deduced mechanisms of VPF and ANP action.

**DISCUSSION**

VPF-induced vascular permeability is a critical contributor to the pathophysiology of diabetic retinopathy, ovarian, and other cancer-related ascites and the cerebral edema and injury following vascular insufficiency (1, 7, 8, 16, 17). Permeability of EC also contributes to angiogenesis. We report that the ability of VPF to modify 1) the actin cytoskeletal architecture, 2) TJ protein phosphorylation and localization, and 3) the permeability barrier function of vascular endothelial cells results from signaling. The tyrosine kinase activity of the Flk-1 receptor mediates the ability of VPF to signal to ERK and EC proliferation (59). Stimulation of the p38 MAP kinase or PI3K/AKT/S6 kinases by VPF underlies EC migration (54, 59, 60) and also proliferation (66). Interestingly, AKT can inhibit p38 kinase activation in some settings, thus promoting EC survival, a function of VPF (67). We showed previously that JNK (and molecules downstream from this kinase) affects VPF-induced cyclin D1 synthesis, Cdk4 activity, and G_{1}/S cell cyclin progression in EC (37). This occurs through a signaling cross-talk,
where ERK stimulation leads to the upstream activation of SEK-1 and JNK. Thus, multiple signaling proteins contribute to the various functions of this important vascular factor.

Here we define the signaling events that are responsible for VPF-induced permeability and establish that the natriuretic peptides inhibit these functions (Fig. 7). VPF-induced Src is essential to the vascular permeability and cerebral edema that follows ischemic stroke (17, 47), but what occurs downstream of Src is not clear. We find that Src activation is necessary for VPF stimulation of ERK and JNK MAP kinases and the activation of PI3K/AKT (Fig. 8). By inhibiting each of these downstream proteins, we identify and link important signaling effectors of VPF and Src. These effectors result in the egress of molecules through the EC barrier. We also find that p38 MAP kinase and NO contribute to this effect of VPF. As shown previously, Flk-1 activation of Src results in enhanced NO production (57), and activation of endothelial nitric-oxide synthase contributes to VPF-induced angiogenesis and vascular permeability (68). Here we show that inhibition of NO generation partially prevents VEGF-induced ERK. It must be appreciated, however, that AKT can directly activate NO (69) upon activation by PI3K in response to VEGF (58).

We show that Src also activates JNK in the setting of VPF, and this occurs in part from ERK activation and ERK-JNK cross-talk (37). We report here a novel auto-activation loop for VPF signaling, where JNK and PI3K/AKT bi-directionally and positively regulate each other’s activity. There is precedent that platelet-derived growth factor or epidermal growth factor activates a uni-directional cross-talk from PI3K to JNK (70). Analogous to our model, the small GTP-binding protein Rac has been found to be both upstream (71) and downstream of PI3K (72), leading to the proposal of a positive feedback loop between these two signaling proteins (71). These molecules are relevant to VPF action in that VPF-induced PI3K/AKT contributes to cell migration (18). The importance of JNK and PI3K/AKT cross-activation by VPF is demonstrated here in that signaling from these Ser/Thr kinases leads to alteration of 1) the morphological actin structure and TJ protein localization, and 2) enhanced permeability of the EC (see below). In diabetic proliferative retinopathy or the hypervascularity of prematurity, the newly formed blood vessels are “leaky,” and this has been attributed in part to the actions of VPF, stimulated by hypoxic conditions in the retina (8, 73). Increased vascular permeability can lead to retinal hemorrhage and edema. Therefore, inhibition of the VPF-induced signaling that we define here (prevented in this case by natriuretic peptides) may be therapeutically useful.

How does VPF-induced signaling result in leaky EC? This permeability factor promotes fenestrations in EC and stimulates vesiculovascular organelle formation/function in venules (5, 6). VPF also promotes the paracellular transgression of fluid through EC tight junctions (3, 4). The important barrier function of EC is highly dependent upon the architecture of the TJ. TJ proteins such as the ZO family (ZO-1, -2, and -3) physically complex with occludin (74), claudin (75), junctional adhesion molecule (76), and cingulin (77), and several of these proteins associate with actin at cell junctions to form a seal (78). Rearrangement of the actin cytoskeleton disrupts the TJ protein(s) complex formation, lowering transcellular resistance and barrier function (23, 79). This also leads to the development of endothelial cell fenestrations that are known to be induced by VPF (6).

We found that after VPF treatment, a redistribution of ZO-1 and occludin proteins at the EC contact sites occurred, and this

### Table II

**Permeability response to VPF in normal or ANP-transgenic mice**

| Dye density (A500 nm) | Control mice | ANP transgenic mice |
|-----------------------|--------------|---------------------|
| Saline                | 0.16 ± 0.03  | 0.19 ± 0.02         |
| VPF 5 ng              | 1.40 ± 0.2   | 0.62 ± 0.07         |
| VPF + H1-142-1        | 1.42 ± 0.2   | 1.10 ± 0.08         |
| VPF + LY 294002       | 0.70 ± 0.2   | 0.42 ± 0.06         |
| VPF + wortmannin      | 0.77 ± 0.09  | 0.31 ± 0.04         |
| VPF + L-NMMA          | 0.82 ± 0.09  | 0.51 ± 0.06         |
| VPF + PD98059         | 0.80 ± 0.06  | 0.37 ± 0.002        |

* a p < 0.05 for saline versus VPF in control mice.
  * b p < 0.05 for VPF versus saline and control mice versus ANP-transgenic mice.
  * c p < 0.05 for VPF versus VPF plus condition as analyzed by ANOVA plus Scheffe’s test. Inhibitor compounds by themselves were no different than saline injection.

### Table III

**Kinase activation in response to VPF in normal or ANP-transgenic mice**

| Kinase activity (pixels ×1000) |
|------------------------------|
| ERK | JNK | AKT |
|-----|-----|-----|
| C   | T   | C   | T   | C   | T   |
| Control (saline)             | 21.6 | 18.8 | 19.7 | 17.7 | 15.4 | 15.0 |
| VPF 5 ng                     | 35.7 | 19.6 | 24.8 | 19.8 | 26.6 | 17.9 |
| VPF 50 ng                    | 53.7 | 32.1 | 41.3 | 25.6 | 43.3 | 23.5 |

Densitometry data are pooled specimens from four mice subjected to each treatment. C is control mice (non-transgenic), and T is transgenic mice. Data are densitometry units.
was blocked by ANP. It has been reported recently that VPF can 1) alter ZO-1 and occludin concentrations at the EC tight junction, and 2) lower transendothelial cell electrical resistance by undetermined mechanisms (80). Here we implicate the phosphorylation of these TJ proteins by JNK and PI3K/AKT, as the basis for the morphological alteration of the TJ. Preventing this phosphorylation by expressing dominant negative signaling molecules or by exposing the cells to ANP significantly led to the inhibition of VPF-enhanced permeability. Previous work (81) implicated ERK in the increased EC permeability induced by VPF, which correlated with the loss of VE-cadherin and occludin localization at EC junctions. Also, oxidant stress induced by VPF, which correlated with the loss of VE-cadherin to the inhibition of VPF-enhanced permeability. Previous work the basis for the morphological alteration of the TJ. Preventing phosphorylation of these TJ proteins by JNK and PI3K/AKT, as by undetermined mechanisms (80). Here we implicate the specificity of soluble inhibitor of PKGI substantially prevents the actions of ANP shown here. Additionally, the signaling mechanisms for the NPRC are poorly understood but perhaps involve the inhibition of cAMP generation (reviewed in Ref. 28). The in vivo function of the NPRC is mainly to clear NP from serum (31), but it may contribute to other actions of ANP, as shown here. In summary, we have defined a signal pathway that mediates the important function of VPF to induce vascular permeability. The signaling leads to the Ser/Thr phosphorylation and architectural disruption of protein components of the EC tight junction. Natriuretic peptides inhibit all these effects, thereby qualifying as potential anti-permeability factors. These results indicate unanticipated and potential therapeutic functions for the natriuretic peptides and are in concert with the roles of these peptides to dampen the response to impact of insult to the cardiovascular system.

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