Vascular endothelial growth factor stimulates differential signaling pathways in in vivo microcirculation


Vascular endothelial growth factor stimulates differential signaling pathways in vivo microcirculation. Am J Physiol Heart Circ Physiol 287: H1590–H1598, 2004. First published May 20, 2004; 10.1152/ajpheart.00767.2003.—Vascular endothelial growth factor (VEGF) induces mild vasodilation and strong increases in microvascular permeability. Using intravital microscopy and digital integrated optical intensity image analysis, we tested, in the hamster cheek pouch microcirculation, the hypothesis that differential signaling pathways in arterioles and venules represent an in vivo regulatory mechanism in the control of vascular diameter and permeability. The experimental design involved blocking specific signaling molecules and simultaneously assessing VEGF-induced changes in arteriolar diameter and microvascular transport of FITC-Dextran 150. Inhibition of Akt [indirectly via phosphatidylinositol 3-kinase with LY-294002 or wortmannin] or PKC (with bisindolylmaleimide) reduced VEGF-induced hyperpermeability. However, phosphatidylinositol 3-kinase/Akt inhibition enhanced the early phase and attenuated the late phase of VEGF-induced vasodilation, whereas blocking PKC had no effect. Inhibition of extracellular signal-regulated kinase (ERK)-1/2 (with PD-98059 or AG-126) also reduced VEGF-induced hyperpermeability but did not block VEGF-induced vasodilation. Blockade of endothelial nitric oxide synthase (with Nω-monomethyl-l-arginine) inhibited VEGF-induced changes in both permeability and diameter. Furthermore, immunofluorescence studies with human umbilical vein endothelial cells revealed that bisindolylmaleimide, PD-98059, and l-NMMA attenuate VEGF-induced reorganization of vascular endothelial cadherin. Our data demonstrate that 1) endothelial nitric oxide synthase is a common convergence pathway for VEGF-induced changes in arteriolar diameter and microvascular permeability; 2) PKC and ERK-1/2 do not play a major role in VEGF-induced vasodilation in the hamster cheek pouch microcirculation; and 3) Akt, PKC, and ERK-1/2 are elements of the signaling cascade that regulates VEGF-stimulated microvascular hyperpermeability. Our data provide evidence for differential signaling as a regulatory step in VEGF-stimulated microvascular dynamics.

Address for reprint requests and other correspondence: W. N. Durán, Dept. of Pharmacology and Physiology, UMDNJ-New Jersey Medical School, 185 S. Orange Ave., MSB H-633, PO Box 1709, Newark, NJ 07101-1709 (E-mail: duran@umdnj.edu).

MICROVASCULAR PERMEABILITY to macromolecules is controlled mainly at the postcapillary venules and is modulated in vivo by several vasoactive substances that alter vascular tone. Both vasodilators (4, 19, 39, 56) and vasoconstrictors (33, 44, 45) are able to increase microvascular permeability. An analysis of the literature indicates that vasodilators and vasoconstrictors share the same signaling pathways. Key molecular points of differential signaling have not yet been identified for in vivo discrimination between vasodilation and hyperpermeability responses.

We (51) demonstrated earlier that the presence of different receptors on arterioles and postcapillary venules accounts at least in part for the vasoconstrictor and hyperpermeability responses to platelet-activating factor in the hamster cheek pouch. On this basis, we advanced the hypothesis that differential signaling mechanisms in arterioles and venules may represent an in vivo regulatory step in the control of vascular tone and permeability. In the present report, we tested this hypothesis using vascular endothelial growth factor (VEGF) as an agonist. We chose VEGF for three reasons: 1) VEGF is a powerful stimulus for hyperpermeability as well as a mild vasodilator; 2) the VEGF cellular signaling pathways in angiogenesis are well studied in vitro (16, 21, 24, 25, 50); and 3) VEGF and anti-VEGF strategies have been tested in animal models and in recent clinical studies (14, 40, 47, 53).

Nitric oxide (NO) has emerged as an important regulator of vascular function. Its roles in the control of vascular tone (26, 49) and in angiogenesis (24, 50) are well established; however, there is controversy regarding its participation in the modulation of microvascular permeability. Evidence in tissues and isolated venules indicates that the activity of endothelial NO synthase (eNOS) increases microvascular permeability to macromolecules in response to inflammatory agents (27, 38, 39, 44, 45, 56). Other reports indicate that NOS activity prevents increases in permeability (34, 35). The reasons for the discrepancies are unknown.

We tested the scheme shown in Fig. 1 as a backbone template of the signaling pathways that regulate microvascular permeability. We simultaneously tested the impact of our experimental interventions on VEGF-induced arteriolar relaxation. The basic scheme in Fig. 1 is similar to the pathways involved in controlling angiogenesis in vitro and is easily amenable to experimental testing in vivo. Studies in human umbilical vein endothelial cells (HUVECs) support the links among the elements of the proposed scheme (36, 52). Stimulation of Akt and fluid shear stress lead to phosphorylation of eNOS in endothelial cells (7, 10) and in cells transfected with eNOS (7, 18). VEGF, an agonist that activates Akt, causes phosphorylation of eNOS in HUVECs and increases the permeability of isolated coronary venules (34, 55). We demonstrated in vivo that phosphorylation of eNOS is associated with...
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Increased release of NO in the hamster cheek pouch (11) and with hyperpermeability (8, 45) in response to \(10^{-7}\) M platelet-activating factor, whereas translocation of eNOS to the cytosol precedes ACh-induced vasodilation (15). We report here the novel observation that the phosphatidylinositol-3-kinase (PI3-K)/Akt complex regulates signaling for VEGF-induced hyperpermeability, but is not essential for the initial stages of VEGF-induced vasodilation in the in vivo hamster cheek-pouch microcirculation.

METHODS

Hamster cheek pouch preparation. Male golden Syrian hamsters (body weight, 80–110 g) were anesthetized with pentobarbital sodium (50 mg/kg ip), and the right cheek pouch was prepared for direct observation by intravital microscopy as previously described (8, 9, 11). The experimental protocols were approved by the New Jersey Medical School’s Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health’s guidelines for the use of animals.

Assessment of microvascular permeability. FITC-Dextran 150 (FITC-Dx150; mol mass, 150 kDa; Sigma Chemical; St. Louis, MO) served as a tracer for microvascular permeability to macromolecules. It was administered intravenously as a 100 mg/kg bolus with subsequent continuous infusion (0.15 mg·kg\(^{-1}\)·min\(^{-1}\)) to maintain a steady plasma concentration. Microvascular transport was assessed by measuring integrated optical intensity (IOI) via computer-assisted digital image analysis (1, 30, 31, 46). Three or four fields were randomly selected in the cheek pouch and were recorded on an Image-1 computer system (Universal Imaging; West Chester, PA). Each field included 4–6 postcapillary venules that ranged from 15 to 30 \(\mu\)m in diameter and were relatively free of capillaries. The maximal IOI was measured 20–25 min after topical VEGF application.

Determination of vasodilation. Arteriolar luminal diameter was measured as the width of the transilluminated blood column using computer-assisted digital analysis. Four or five arterioles with diameters of 20–40 \(\mu\)m were studied per animal. Baseline diameter measurements were normalized to a value of 1 (9). For each vessel, the experimental diameter was expressed as a ratio of baseline diameter (relative luminal diameter).

Vascular endothelial growth factor. Recombinant human VEGF\(_{165}\) (R & D Systems; Minneapolis, MN) was dissolved from stock solution to a concentration of \(10^{-6}\) M with bicarbonate buffer (pH 7.35 at 37°C). VEGF was administered topically via a side port into the subcutaneous bicarbonate buffer line at a concentration necessary to achieve the desired final concentration.

Inhibitors. All inhibitors were purchased from Sigma Chemical. Wortmannin was applied at concentrations of 0.1–100 nM (18, 23). \(\alpha\)-monomethyl-L-arginine (\(\alpha\)-NMMA) was applied at \(10^{-5}\) M, which is a dose proven previously to block NOS with minimal direct influence on arteriolar diameter in the hamster cheek pouch (44, 45). The PKC inhibitor bisindolylmaleimide (BIM) was administered at \(10^{-7}\) and \(10^{-6}\) M. The MEK inhibitor PD-98059 was applied at a concentration of \(20 \times 10^{-6}\) M. The ERK-1/2 inhibitor AG-126 was applied at concentrations of 0.3, 3.0, and \(30 \times 10^{-6}\) M. All inhibitors were applied topically.

Experimental protocol. After baseline data were collected, VEGF was applied topically to the cheek pouch for 3 min. The bicarbonate suffusion was then reestablished, and the experiment was continued for an additional 40-min period. Each hamster received only one application of VEGF. In separate experiments, each inhibitor was applied topically for 10 min before VEGF administration.

Immunoﬂuorescence localization of vascular endothelial cadherin in endothelial cells. HUVECs (passages 3 and 4; Clonetics; San Diego, CA) were seeded (10⁴ cells in 250 \(\mu\)l of medium) on fibronectin-coated, round, 20-mm-diameter, no. 1 glass coverslips (Carolina Biologicals; Burlington, NC) and were grown for 4–5 days in endothelial growth medium supplemented with 10% FBS (Clonetics) to form a tightly confluent monolayer with very few dividing cells. The cells were incubated in endothelial basal medium that contained 1.5% FBS for 5 h before treatment with the indicated inhibitors and 1 nM VEGF. Cells were fixed and permeabilized in ice-cold methanol for 5 min and were then incubated in a 1% BSA blocking buffer for 30 min at room temperature. This was followed by an overnight incubation at 4°C with goat anti-vascular endothelial (VE)-cadherin antibody (Santa Cruz Biologicals; Santa Cruz, CA) and subsequent incubation for 1 h at room temperature with Cy3-conjugated anti-goat IgG (Sigma Chemical). Coverslips were mounted on glass slides using Vectashield (Vector Laboratories; Burlingame, CA). The cells were viewed using a Zeiss LSM 410 inverted laser-scan confocal microscope (Carl Zeiss; Thornwood, NY) equipped with a krypton-argon laser and a \(\times 40\) magnification water immersion lens. An excitation wavelength of 568 nm was used.

Data analysis. All data are presented as means ± SE. Statistical analysis was performed using one-way ANOVA and subsequent Student-Newman-Keuls test. Differences were considered significant for values of \(P < 0.05\). The changes in vasodilation were analyzed on the basis of VEGF-induced peak or maximal diameter change. As indicated in Figs. 2–6, changes in diameter were expressed as ratios of the experimental to the control diameters. In all experiments, when present, VEGF-induced peak diameter changes occurred at the first 5-min measurement point after topical application.

RESULTS

As a first step, we characterized the time courses and dose responses of the hamster cheek pouch microvasculature to topically applied VEGF (Fig. 2). Arterioles exhibited a dose-related maximal (peak) vasodilation to VEGF administration. The vasodilation had a rapid onset, reached a peak by 5 min, declined quickly, and returned to baseline in 20–25 min (Fig. 2A). VEGF at \(10^{-10}\) M concentration induced changes in vascular tone (1.10 ± 0.01) that were not significantly different.
from control, whereas $10^{-7}$ M VEGF caused a 1.40 $\pm$ 0.01-fold increase in vasodilation (Fig. 2B). The time course of the hyperpermeability response demonstrated by postcapillary venules is shown in Fig. 2C. VEGF at $10^{-10}$ M caused no significant changes in IOI ($3.2 \pm 1.0$ units), whereas VEGF at $10^{-8}$ and $10^{-7}$ M induced maximal responses ($35.0 \pm 2.9$ and $36.0 \pm 4.7$ units, respectively; Fig. 2D). Because our results show that $10^{-8}$ M VEGF is an efficacious dose to induce hyperpermeability in vivo in the hamster cheek pouch while causing a slight vasodilation, this concentration was applied in all other experiments.

The activity of Akt and its ability to phosphorylate eNOS are stimulated by PI3-K (10, 17, 48). Therefore, to investigate the role of Akt in the in vivo regulation of microvascular transport, we blocked PI3-K/Akt activity by topically applying two structurally different recognized inhibitors of PI3-K, namely, wortmannin and LY-294002, to the hamster cheek pouch in separate experiments. When applied alone, these agents did not stimulate changes in either vessel diameter or transport in the microcirculation. Interestingly, neither inhibitor significantly blocked the initial arteriolar vasodilation induced by VEGF; rather, a trend for enhancement of vasodilation 5 min after VEGF application was observed in the presence of 10 and 100 nM LY-294002 (Fig. 3, A and B). After this initial vasodilation, however, a significant attenuation of VEGF-induced vasodilation was observed in the presence of 10 nM LY-294002 15 min after the addition of VEGF (Fig. 3, A and C). No significant changes in VEGF-induced vasodilation were observed after wortmannin treatment. However, a similar but insignificant ($P = 0.06$) trend at the 5-min time point was observed with the application of 100 nM wortmannin (Fig. 3D). In contrast, both wortmannin and LY-294002 effectively and efficaciously blocked VEGF-induced hyperpermeability (Fig. 3, E and F).

Because PLC and PKC are involved in eNOS-related hyperpermeability in vivo (32, 33, 44, 45), we speculated that PKC might be a signaling step in VEGF-mediated hyperpermeability. To test this concept, before topical application of VEGF to the hamster cheek pouch, we administered the PKC inhibitor BIM at 0.1 and 1.0 $\mu$M in separate experiments. Neither of these concentrations blocked VEGF-induced vasodilation (Fig. 4A). Importantly, BIM at 0.1 and 1.0 $\mu$M blocked microvascular transport of FITC-Dx150 in a reverse dose-related manner (Fig. 4B).

We subsequently tested whether VEGF-induced hyperpermeability required eNOS activity. Topical application of 10 $\mu$M l-NAME alone caused vasoconstriction. More importantly, topical application of 10 $\mu$M l-NAME inhibited both the hyperpermeability and the vasodilation induced by $10^{-8}$ M VEGF (Fig. 5).

Having shown that signaling molecules considered to be upstream of eNOS influence VEGF-induced hyperpermeability, we explored the potential regulation of the arteriolar and postcapillary venular responses by signaling molecules presumed to operate downstream of eNOS. It is acknowledged that NO stimulates the production of cGMP by activating soluble guanylate cyclase. In turn, cGMP activates PKG and the phosphorylation of MAP kinases. Thus it is plausible that the signaling pathways for hyperpermeability include activation of MAP kinases. To elucidate this possibility, we used PD-98059 (an inhibitor of MEK-1/2 or MAP kinase kinase) and AG-126 (a tyrosine kinase inhibitor shown to block phosphorylation of ERK-1/2). We applied PD-98059 topically at a concentration of 20 $\mu$M, and AG-126 was applied at concentrations of 0.3, 3.0, and 30 $\mu$M. Topical application of these agents alone did not induce changes in either postcapillary permeability or arteriolar diameter with the exception of 30 $\mu$M AG-126: when applied alone, it increased arteriolar diameter $\sim$20% (Fig. 6B). Neither PD-98059 nor AG-126 blocked the arteriolar vasodilation induced by VEGF (Fig. 6, A and B). In fact, VEGF-induced vasodilation was enhanced in the pres-
ence of AG-126 (Fig. 6B). In contrast, on the postcapillary microvascular segment, topical application of PD-98059 as well as topical application of AG-126 inhibited VEGF-induced hyperpermeability (Fig. 6C and D).

To further assess that the mechanism of VEGF-induced increases in permeability operate at the level of endothelial cells, we applied VEGF to HUVEC monolayers and evaluated changes in VE cadherin organization using immunofluorescence microscopy. Although VE cadherin had a relatively smooth, continuous appearance at intercellular junctions in control cells, VEGF (1 nM for 10 min) reorganized intercellular VE cadherin to yield a jagged appearance with several fingerlike projections; this event has been associated with decreased adhesiveness between endothelial cells and disruption of the endothelial barrier (12). Pretreatment of cells with either 10 μM BIM, 20 μM PD-98059, or 10 μM L-NMMA attenuated the VEGF-induced changes in VE cadherin organization (Fig. 7).

**DISCUSSION**

Our results demonstrate an in vivo dissociation of the signaling elements that control vascular tone in arterioles and microvascular permeability in venules. We report that blocking PI-3K/Akt inhibits VEGF-induced hyperpermeability in postcapillary venules and simultaneously enhances an early phase (within 5 min) and attenuates a later phase (after ~10 min) of VEGF-induced vasodilation in arterioles. We also report the novel findings that PKC inhibition selectively blocks VEGF-induced hyperpermeability but not VEGF-induced vasodilation. These observations strongly suggest the existence of a differential signaling mechanism upstream of eNOS in venules and arterioles. In addition, we show that inhibition of MAP kinases also selectively blocks VEGF-induced hyperpermeability but not VEGF-induced vasodilation. Furthermore, we report that selective inhibition of either PKC or MAP kinase blocks VEGF-induced reorganization of VE cadherin at endo-
thelial intercellular junctions, which is an event associated with increases in endothelial permeability (12).

We report here that VEGF signals hyperpermeability through PI3-K/Akt in the in vivo microcirculation. These data are in agreement with in vitro experiments in HUVECs in our laboratory (36, 52). Our results, however, differ from in vitro data reporting that PI3-K/Akt is not involved in the regulation of permeability of HUVEC monolayers (29). The main reason for the discrepancy may reside in the different pharmacological approaches employed to investigate the role of PI3-K/Akt in vitro and in vivo. In particular, we used LY-294002 at concentrations in the nanomolar range, whereas previous investigators applied concentrations in the micromolar range because of their interest in vasomotor activity (18). We speculate that our exploration of lower doses may have uncovered different sites of action of LY-294002 on the PI3-K/Akt molecules.

Interestingly, pharmacological blockade of PI3-K/Akt with LY-294002 in the hamster cheek pouch augmented VEGF-stimulated vasodilation at an early time point (5 min) and attenuated VEGF-stimulated vasodilation at later time points. This finding was surprising, because it is generally accepted that endothelium-derived vasodilation is mediated by a pathway that involves PI3-K, Akt, and eNOS (37). However, there is recent evidence that Akt activation is not a prerequisite for the rapid NO production that occurs in the first 10 min after stimulation with VEGF, but rather that Akt is involved in maintaining elevated NO production 10–30 min after VEGF stimulation (20). Although our data support this idea of early, Akt-independent NO signaling in arterioles, it is not presently clear why inhibition of PI3-K/Akt caused a transient augmentation of VEGF-induced vasodilation. It is important to note that our findings with wortmannin did not match those with LY-294002. This is likely because wortmannin, in addition to blocking PI3-K/Akt, is also capable of inhibiting inositol 1,4,5-trisphosphate formation, MAP kinase activation, and myosin light-chain kinase activity (3, 13, 42). Although the lack of specificity of wortmannin makes it a less useful tool for studying PI3-K/Akt, we nevertheless chose to use this compound because 1) it is reported in the literature as commonly used to inhibit PI3-K, 2) it is structurally distinct from LY-294002, and 3) its ability to attenuate VEGF-induced microvascular hyperpermeability in vivo was not previously described.

The action of PKC on eNOS is controversial. Studies in vitro (in bovine artery endothelial cells [BAECs]) indicate that PKC inhibits eNOS activity (43), whereas studies in vivo are consistent with the concept that activation of PKC stimulates eNOS and increases microvascular permeability (27, 33, 44, 45). This controversy might depend on experimental designs and protocols. The experiments on BAECs were performed on cells derived from large vessels that do not regulate microvascular permeability; in addition, the authors stimulated PKC for a prolonged period (43). On the other hand, the in vivo studies were performed directly on vessels involved in microvascular permeability and the researchers used short-duration stimulation of PKC (33, 44, 45). In this study, we provide additional compelling evidence that inhibition of PKC reduces the hyperpermeability response to VEGF without affecting its mild...
capacity for vasodilation. Our in vivo data agree with results reported for coronary venules and in HUVECs (5, 54, 55).

We also present data that show involvement of the ERK-1/2 MAP kinase pathway in VEGF-induced hyperpermeability in vivo, whereas inhibition of ERK-1/2 failed to attenuate VEGF-induced vasodilation. Our data support previous studies that show that inhibition of ERK-1/2 diminishes VEGF-stimulated increases in HUVEC monolayer permeability (5, 29, 36). On the in vivo arteriolar side, our data show that inhibition of ERK-1/2 with PD-98059 does not affect VEGF-induced vasodilation. Interestingly, AG-126, a tyrphostin family-derived inhibitor that blocks VEGF-induced activation of ERK-1/2 (6), caused vasodilation when applied alone and augmented the increase in vasodilation after VEGF stimulation. This difference between PD-98059 and AG-126 may reflect a broader spectrum of AG-126 targeting of signaling molecules and/or a difference in the level of additional inhibition of each agent in the signaling cascade.

Our data provide compelling evidence for the existence of differential signaling steps for vascular tone and permeability upstream (PI3-K/Akt, PKC) and downstream (MEK and MAP kinase) of eNOS. Unfortunately, there is insufficient information regarding the precise mechanisms of action of the inhibitors applied to establish or even speculate about the potential molecular differences in arterioles and venules. In addition, approaches based on molecular biology tools such as oligonucleotide transfer are not yet easily applicable in the in vivo microcirculation. Using oligonucleotide transfer in HUVECs, we demonstrated a role for ERK-1/2 in VEGF-induced hyperpermeability. Moreover, we showed that inhibiting the phosphorylation of ERK-1/2 on threonine 183 and tyrosine 185 attenuated both VEGF- and NO donor-stimulated increases in endothelial permeability (5).

We cannot exclude the possibility that some experimental interventions that affect local blood flow may alter tissue metabolism, which may in turn influence arteriolar tone and venular barrier function. However, it is unlikely that these secondary effects occurred in our experiments because 1) the hamster cheek pouch was continuously superfused with bicarbonate buffer, which would wash out or dilute diffusible vasoactive metabolites; and 2) our experimental interventions only caused transient changes in arteriolar diameter. We also cannot eliminate the possibility that the pharmacological inhibitors used may have caused secondary effects on cells other than vascular endothelium, particularly vascular smooth muscle. It is important to note, however, that VEGF is a selective agonist for vascular endothelial cells, and the pathways we tested in vivo are activated in cultured endothelial cells upon stimulation with VEGF.

Considering that pharmacological inhibitors have served to yield successful scientific insights in biology, our data point out that 1) eNOS activity is common to both hyperpermeability and vasodilation, and 2) vasodilation is not required for VEGF-induced hyperpermeability. The latter statement is supported indirectly by our earlier observation that platelet-activating factor, which is a powerful vasoconstrictor in arterioles, causes a large increase in microvascular permeability in hamster cheek pouches (8). We have also shown previously (19) that the ability of adenosine to increase microvascular permeability in hamster cheek pouches (8). We have also shown previously (19) that the ability of adenosine to increase microvascular permeability in hamster cheek pouches (8). We have also shown previously (19) that the ability of adenosine to increase microvascular permeability in hamster cheek pouches (8). We have also shown previously (19) that the ability of adenosine to increase microvascular permeability in hamster cheek pouches (8). We have also shown previously (19) that the ability of adenosine to increase microvascular permeability in hamster cheek pouches (8).
Fig. 7. VEGF-induced reorganization of vascular endothelial (VE) cadherin. Human umbilical vein endothelial cells grown on coverslips were treated as indicated, and VE cadherin was immunofluorescently labeled. Shown are cells treated with no inhibitor or with inhibitors alone (left) and cells treated with $10^{-8}$ M VEGF for 10 min (right). A and B: cells were treated with no inhibitor. C and D: BIM was applied at 1 µM concentration. E and F: PD-98059 was applied at 20 µM concentration. G and H: L-NMMA was applied at 10 µM concentration. Fingerlike projections of VE cadherin were observed upon stimulation with VEGF (arrows). Bar, 20 µM.
shared by several biological processes, the specific codes for cell signaling and their possible functional significance in vivo remain to be elucidated.

ACKNOWLEDGMENTS

Present address of H. Aramoto: Department of Surgery, Sakakibara Memorial Hospital, Tokyo, Japan.

Present address of J. W. Breslin: Department of Surgery, Texas A&M University Health Science Center, Temple, TX 76504.

REFERENCES


