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Vascular Permeability Induced by VEGF Family Members in Vivo: Role of Endogenous PAF and NO Synthesis

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Abstract We previously reported that vascular endothelial growth factor (VEGF) increases vascular permeability through the synthesis of endothelial platelet-activating factor (PAF), while others reported the contribution of nitric oxide (NO). Herein, we addressed the contribution of VEGF receptors and the role played by PAF and NO in VEGF-induced plasma protein extravasation. Using a modified Miles assay, intradermal injection in mice ears of VEGF-A_{165}, VEGF-A_{121}, and VEGF-C (1 μM) which activate VEGFR-2 (Flk-1) receptor increased vascular permeability, whereas a treatment with VEGFR-1 (Flt-1) analogs; PlGF and VEGF-B (1 μM) had no such effect. Pretreatment of mice with PAF receptor antagonist (LAU8080) or endothelial nitric oxide synthase (eNOS) inhibitor (L-NAME) abrogated protein extravasation mediated by VEGF-A_{165}. As opposed to PAF (0.01-1 μM), treatment with acetylcholine (ACh; up to 100 μM; inducer of NO synthesis) or sodium nitroprusside (SNP; up to 1 μM; NO donor) did not induce protein leakage. Simultaneous pretreatment of mice with eNOS and protein kinase A (PKA) inhibitors restored VEGF-A_{165} vascular hyperpermeability suggesting that endogenous NO synthesis leads to PKA inhibition, which support maintenance of vascular integrity. Our data demonstrate that VEGF analogs increase vascular permeability through VEGFR-2 activation, and that both endogenous PAF and NO synthesis contribute to VEGF-A_{165}-mediated vascular permeability. However, PAF but not NO directly increases vascular permeability per se, thereby, suggesting that PAF is a direct inflammatory mediator, whereas NO serves as a cofactor in VEGF-A_{165} proinflammatory activities. J. Cell. Biochem. 100: 727–737, 2007.

Key words: VEGF analogs; VEGF receptors; PAF; NO; vascular permeability

Vascular endothelial growth factor (VEGF) is a key modulator of angiogenesis promoting endothelial cell (EC) migration, proliferation, and survival. In addition, VEGF possesses inflammatory properties by its capacity to mediate microvascular permeability increase and adhesion of leukocytes [Senger et al., 1983; Melder et al., 1996; Detmar et al., 1998]. There are at least five different VEGF homodimeric isoforms of 206, 189, 165, 145, and 121 amino acids, termed as VEGF-$A_{206,\ 189,\ 165,\ 145,\ and\ 121}$ which are produced from a single gene by alternative splicing [Jussila and Alitalo, 2002]. VEGF family also includes several members such as placental growth factors (PlGF-1 and -2), VEGF-A, -B, -C, -D, and a viral homolog, VEGF-E. The actions of VEGF family members are mediated by the activation of tyrosine kinase receptors including VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed majoritary on EC, and VEGFR-3 (Flt-4), which is mainly limited to the lymphatic endothelium [Jussila and Alitalo, 2002]. VEGF-A binds to VEGFR-1 and VEGFR-2; PlGF-1, PlGF-2 and VEGF-B bind to VEGFR-1; VEGF-C and D bind to VEGFR-2 and R-3; whereas VEGF-E interacts only with VEGFR-2 [Jussila and Alitalo, 2002]. Recent studies also reported that neuropilin-1 (NRP-1), a transmembrane receptor, acts as a coreceptor, complexing with VEGFR-1 and VEGFR-2. NRP-1 specifically enhances the binding of VEGF-A_{165} to VEGFR-2 and potentiates various VEGF-A_{165} biological activities [Soker et al., 1997; Bernatchez et al., 2002; Rollin et al., 2004]. Such selectivity is attributable to the presence of

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VEGF-A exon 7 in VEGF-A_{165}, a domain that is lacking in VEGF-A_{121}, VEGF-C, VEGF-D, and PlGF-1 [Soker et al., 1997].

We previously reported that VEGF-A_{165} increases vascular permeability through the synthesis of endothelial platelet-activating factor (PAF). Under in vivo condition, VEGF-A_{165}-mediated protein extravasation was prevented by a pretreatment with a PAF receptor antagonist. Our observation was supported by the capacity of VEGF-A_{165} to induce a rapid and transient PAF synthesis in cultured endothelial cells [Sirois and Edelman, 1997]. However, there is little information defining how PAF might contribute to the modulation of endothelial integrity under VEGF stimulation. The endothelial cell–cell interaction is maintained by junctional proteins and focal adhesion complexes that are anchored to the cytoskeleton. Vascular endothelial (VE)-cadherin is connecting adjacent EC through a calcium-dependent homophilic binding of its extracellular domain whereas its intracellular domain interacts with the actin cytoskeleton via a family of catenins, including α-, β-, and γ-catenins, α-actinin, and vinculin [Bazzoni and Dejana, 2004]. Interestingly, stimulation of EC either with VEGF-A_{165} or PAF induces a rapid and transient phosphorylation of VE-cadherin and the dissociation of adherens junction leading to gap formation [Esser et al., 1998; Kevil et al., 1998; Hudry-Clergeon et al., 2005; Potter et al., 2005], thereby, suggesting that endogenous PAF formation is involved in VEGF-mediated VE-cadherin phosphorylation.

On the other hand, it has also been reported that nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) production are contributing to VEGF-A_{165}-induced vascular hyperpermeability [Murohara et al., 1998; Mayhan, 1999]. However, as opposed to PAF, the capacity of intracellular NO synthesis to promote vascular permeability is more ambiguous. For instance, by using NO donors, different studies reported that NO promote vascular leakage [Gimeno et al., 1998; Fujii et al., 1999], whereas others did not detect such effect [Feletou et al., 1996; Murohara et al., 1998; Klabunde and Anderson, 2000]. Similar conflicting results are also observed with cGMP analogs, which increase basal permeability in isolated vessels, but attenuate vascular hyperpermeability mediated by various inflammatory agents. Such discrepancies remain elusive, but might result from differences in species, organs, involvement of leukocytes and hemodynamic factors.

In the present study, we wanted to define how VEGF analogs are modulating vascular permeability increase and investigate the contribution of PAF and NO.

MATERIALS AND METHODS

Materials

Vascular endothelial growth factor-A_{165} (VEGF-A_{165}) was purchased from PeproTech, Inc., (Rocky Hill, NJ), VEGF-A_{121}, VEGF-B, VEGF-C, VEGF-D, placental growth factor-1 (PlGF-1), and basic fibroblast growth factor (bFGF) were purchased from R&D (Minneapolis, MN). Sodium nitroprusside (SNP), acethylcholine (ACh), phenylephrine, Evans blue dye (EB), formamide, PAF, and the following inhibitors: H-89 (ODQ) and N_{ω}-Nitro-L-arginine methyl ester hydrochlorine (L-NAME) were purchased from Sigma-Aldrich (Oakville, ON, Canada). SU1498 and CV3988 were purchased from Calbiochem (La Jolla, CA) and BIOMOL (Plymouth Meeting, PA), respectively. LAU8080 (formerly known as BN50730) was generously provided by Dr. Nicolas Bazan (Louisiana State University Health Sciences Center, New Orleans, LA).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment from fresh umbilical cords, seeded on gelatin-coated (0.25%) plates and cultured in endothelial growth medium-2 (EGM-2; Cambrex, Walkersville, MD) containing 10% fetal bovine serum (FBS) (Medicorp, Montreal, QC, Canada) and antibiotics. HUVEC were used at passage 1.

Vascular Permeability Studies

EB dye binds specifically to albumin and is used to study vascular permeability to macromolecules [Rogers et al., 1989]. The procedures were performed by one trained operator in accordance to the guidelines set by the Montreal Heart Institute animal care committee and the Canadian Council for Animal Protection. CD1 mice (18–22 g body weight) (Charles River Breeding Laboratories; Saint-Constant, Quebec, Canada) were anesthetized with an intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine HCl (10 mg/kg). The mice

received a bolus injection of EB dye (20 mg/kg) in the caudal vein. This was followed 30 min later by an intradermal injection in mice ears of VEGF-A_{165} (4, 40, or 400 ng/10 μl; final concentration 0.01, 0.1, or 1 μM, respectively), VEGF-A_{121}, -B, -C, -D, PlGF-1 (1 μM), bFGF (1 μM), PAF (0.01, 0.1, and 1 μM), SNP (1 μM), ACh (up to 100 μM), or PAF plus SNP (0.01 and 1 μM, respectively). In another set of experiments, mice were treated with PAF receptor antagonist (LAU8080; 20 mg/kg [Ma et al., 2004]), VEGFR-2 inhibitor (SU1498; 20 mg/kg; [Weis et al., 2004]), nitric oxide inhibitor (L-NAME; 20 mg/kg; [Murohara et al., 1998]), guanylate cyclase inhibitor (ODQ; 20 mg/kg; [Cerwinka et al., 2002]), and protein kinase A inhibitor (H-89; 4 and 40 μg/kg [Sanada et al., 2001]) co-administered with EB dye. Mice were sacrificed 60 min after the injection of agonists in the ears. The ears were dissected and EB dye extracted in formamide (4 ml/g wet weight for 24 h). The concentration of EB dye was determined by spectrophotometry (620 nm wavelength) against a standard curve and expressed as micrograms of EB dye.

Microscopy and Image Analyses

Pictures of the arteriovenous system of mice ears were taken at different magnifications (8.4×, 12×, 24×, 38.4×, and 48×) with a color videodigital camera (Sony DKC 5000) adapted to a stereomicroscope (Olympus SZX12). The surface of each vessel was calculated by computerized digital planimetry with a custom software (NIH image 1.6). Vascular tone was assessed at 0, 30, and 60 min following a bolus injection of L-NAME (20 mg/kg). The vasoconstrictor effect of L-NAME on the arteriovenous system of mice ears was reproduced by an intravenous infusion of phenylephrine (Phe; 0.1 mg/ml) at a constant rate (0.8 ml/h). Phenylephrine infusion was initiated, and when similar constriction was reached as compared to L-NAME, VEGF-A_{165} was injected in mice ears.

Western Blot Analysis VE-Cadherin Phosphorylation

HUVEC were starved for 6 h with EBM-2 containing 1% FBS and antibiotics. HUVEC were stimulated with VEGF-A_{165} (1 nM) or SNP (1 μM) for various periods of time. In another set of experiments, HUVEC were pretreated with either SU1498 (10 μM), LAU8080 (10 μM), L-NAME (100 μM), or ODQ (10 μM) for 15 min prior to stimulation with VEGF-A_{165} (1 nM) for 45 min. Western blots analyses were performed as described previously [Rollin et al., 2004]. Briefly, cells were rinsed with cold PBS containing depolymerized Na_3VO_4 (2 mM) and lysed in ice cold lysis buffer supplemented with phosphatase and protease inhibitors. Cell lysates (500 μg) were immunoprecipitated with a mouse monoclonal VE-cadherin IgG (2 μg; Santa Cruz Biotechnology, Santa Cruz, CA) and separated by SDS–PAGE. Proteins were transferred onto a PVDF membrane, and probed with a mouse monoclonal anti-phosphotyrosine IgG (clone 4G10, 1:1,000 dilution; Upstate Biotechnology, Inc., Lake Placid, NY). Membranes were stripped using Re-Blot Plus Strong stripping solution (Chemicon International, Temecula, CA) and reprobed with a mouse monoclonal VE-cadherin IgG (1:1,000 dilution; Santa Cruz Biotechnology). Bands were visualized using LumiGlo™ (Cell Signaling Technology, Inc., Beverly, MA).

Statistical Analysis

Data are presented as the mean + SE. Statistical comparisons were made by analysis of variance followed by a Bonferroni's *t*-test for multiple comparisons. Differences were considered significant when $P < 0.05$.

RESULTS

Effect of VEGF Analogs and Corresponding Receptors on Microvascular Permeability

Using VEGF analogs, we assessed the contribution of VEGF receptors on the modulation of vascular permeabilty in a modified EB dye Miles assay. Intradermal injection of VEGF-A_{165} (0.01, 0.1, and 1 μM) in mice ears increased within 60 min EB dye extravasation by 47, 160, and 320% as compared to control PBS-treated mice. VEGF-A_{121} (1 μM), which like VEGF-A_{165} binds to VEGFR-1 and VEGFR-2 but not to NRP-1 coreceptor increased microvascular permeability by 109% (Fig. 1). Treatment with VEGF-C and VEGF-D (1 μM; VEGFR-2 and R-3 analogs) increased EB dye exsudation by 107% and 79%, respectively, whereas a treatment with VEGF-B and PlGF-1 (1 μM; VEGFR-1 analogs) had no significant effect. Treatment with bFGF (1 μM) was used as negative control, and did not promote vascular permeability increase (Fig. 1).

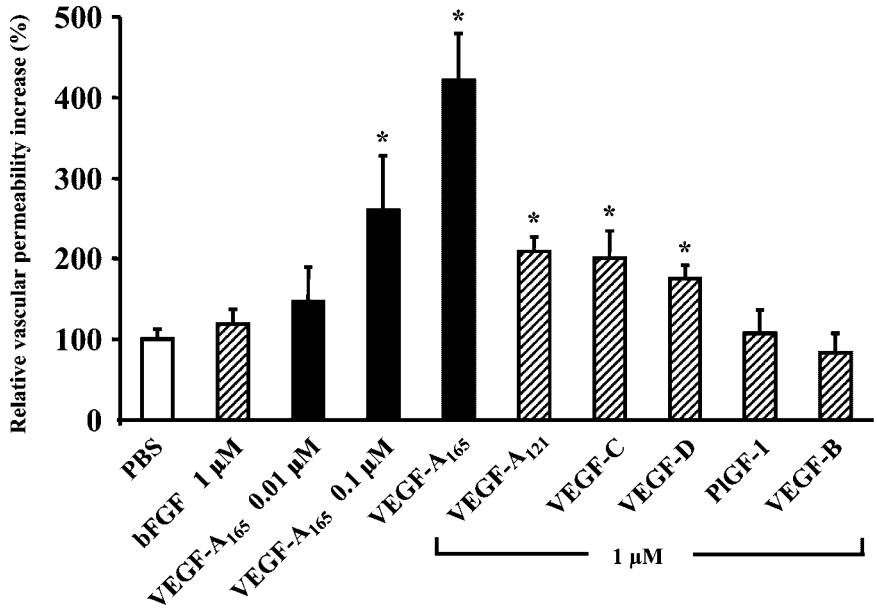

Fig. 1. Vascular permeability mediated by VEGF analogs. Evans blue dye (EB; 20 mg/kg) was injected in the caudal vein of mice and 30 min later PBS (control vehicle), VEGF-A_{165} (4, 40, or 400 ng/10 μl; final concentration 0.01, 0.1, or 1 μM, respectively), VEGF-A_{121}, -B, -C, -D, PlGF-1 (1 μM), or bFGF (negative control; 1 μM) were injected in the ears. Animals were sacrificed 60 min after agonists injection. Values are mean ± SEM of at least five independent experiments. $^*P < 0.05$ compared to PBS.

Implication of PAF and NO in VEGF-Induced Microvascular Permeability

We previously showed that PAF is involved in VEGF-A_{165}-mediated vascular permeability increase whereas others showed the contribution of NO [Murohara et al., 1998; Mayhan, 1999]. In addition, VEGF-A_{165} can promote a rapid and transient synthesis of PAF and NO in EC [Sirois and Edelman, 1997; Murohara et al., 1998; Montrucchio et al., 2000]. Thus, we wanted to delineate how PAF and NO are contributing to VEGF-A_{165}-mediated vascular permeability increase. Intradermal injection of PAF (0.01, 0.1, 1 μM) led to a concentration-dependent extravasation of EB dye by 363, 1,005, and 1,263% as compared to PBS-treated mice (Fig. 2). Treatment with PAF (0.01 μM) resulted in a similar vascular permeability increase as observed with VEGF-A_{165} (1 μM), and was used for subsequent studies implying stimulation with PAF. Pretreatment with PAF receptor antagonist (LAU8080 or CV3988; 20 mg/kg, i.v.) 30 min prior to subcutaneous injection of PAF (0.01 μM), abrogated PAF-mediated vascular permeability increase. We also wanted to assess the contribution of NO production on PAF-mediated hyperpermeability. Pretreatment with eNOS inhibitor (L-NAME; 20 mg/kg; i.v.) 30 min prior to subcutaneous injection of PAF (0.01 μM) abrogated as well PAF-mediated vascular permeability increase. In another set of experiments, we combined PAF (0.01 μM) with an exogenous NO donor (SNP; 1 μM), and observed that SNP did not synergize PAF inflammatory activity (Fig. 2).

Treatment with a nitric oxide donor (SNP up to 1 μM) [Murohara et al., 1998] or with an inducer of NO production (ACh up to 100 μM) [Feletou et al., 1996] were unable to promote vascular permeability increase (Fig. 3). We then assessed the contribution of PAF and NO on microvascular permeability increase mediated by VEGF analogs (VEGF-A_{165} and VEGF-C; 1 μM). Pretreatment with either LAU8080 (20 mg/kg) or L-NAME (20 mg/kg) prevented vascular permeability increase mediated by VEGF-A_{165} and VEGF-C (Fig. 3).

Assessment of L-NAME Vasoconstrictor Effect on VEGF-Induced Vascular Permeability

Inhibition of eNOS activity with L-NAME obliterates NO vasodilatory effect over basal tone and results in an arteriovenous constriction [Rees et al., 1990]. One might suggest that L-NAME inhibitory effect on vascular permeability increase was the result of L-NAME

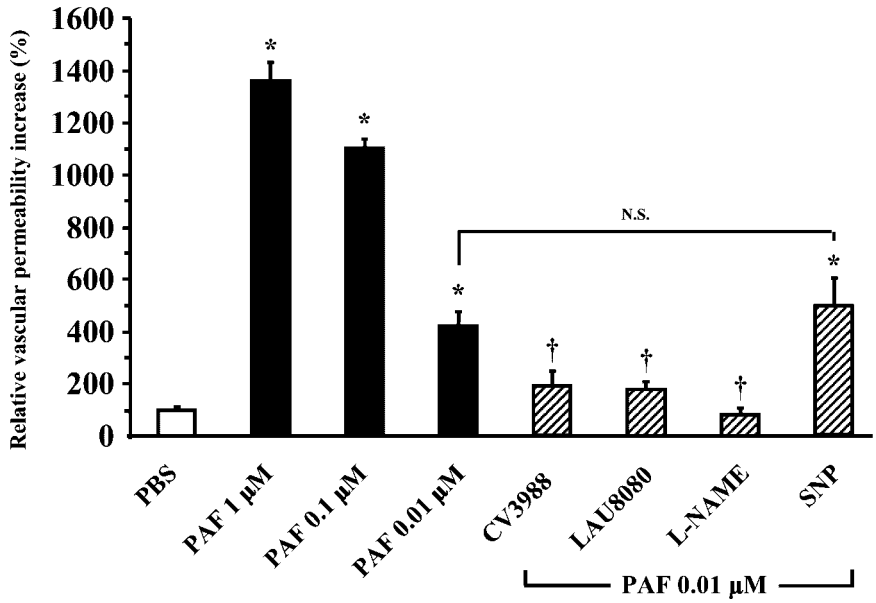

Fig. 2. Dose-dependent effect of PAF on vascular permeability. EB dye injection in the caudal vein was followed 30 min later by an intradermal injection of PBS or PAF (1, 0.1, and 0.01 μM, respectively) or PAF (0.01 μM) plus SNP (1 μM). In another group, mice were pretreated with LAU8080, CV3988, or L-NAME (20 mg/kg) by concomitant intravenous injection with EB prior to PAF injection (0.01 μM). The animals were sacrificed 60 min upon PBS or PAF injection. Values are mean ± SEM of at least three independent experiments. $^{*}P < 0.05$ compared to PBS, $^{\dagger}P < 0.05$ compared to PAF.

vasocontractile activity. Thus, we assessed how a bolus injection of L-NAME affected the arteriovenous tone in mice ears. Thirty minutes following a bolus injection of L-NAME (20 mg/kg, i.v.), arterioles and venules surfaces were reduced, representing 77.0 ± 5.5 and 81.0 ± 0.5% of the initial surface area, respectively. Sixty minutes post-infusion, corresponding

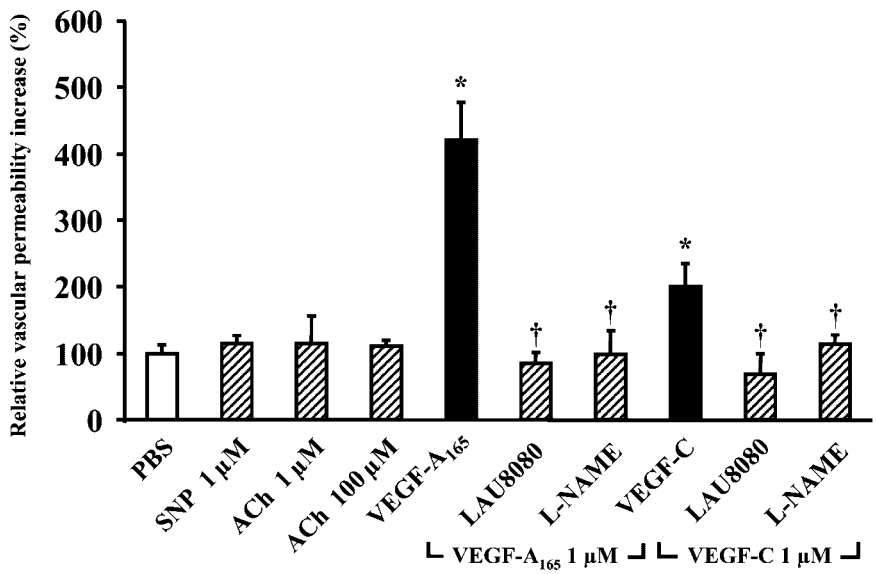

Fig. 3. Implication of NO and PAF on VEGF-induced protein extravasation. EB dye injection in the caudal vein was followed 30 min later by an intradermal injection of PBS, SNP (1 μM), ACh (1 and 100 μM), VEGF-A_{165}, and VEGF-C (1 μM). In another group, mice were pretreated with either L-NAME (20 mg/kg) or LAU8080 (20 mg/kg) combined with EB dye (20 mg/kg) and followed a subcutaneous injection of VEGF-A_{165} or VEGF-C (1 μM). In another group, mice were pretreated either with L-NAME or LAU8080 (20 mg/kg, respectively) by concomitant intravenous injection with EB dye 30 min prior to VEGF-A_{165} or VEGF-C injection (1 μM) in mice ears. The animals were sacrificed 60 min later. Values are mean ± SEM of at least four independent experiments. $^{*}P < 0.05$ compared to PBS, $^{\dagger}P < 0.05$ compared to VEGF-A_{165} or VEGF-C.

TABLE I. Effect of Vasoconstriction on VEGF-A_{165}-Induced Protein Extravasation

Vascular tone modulation					Vascular permeability (EB dye extravasation; μg)	
					Treatment	
Pretreatment	Time (min)	−60	−30	0	PBS	VEGF-A_{165}
None	Arteries (%)	N/A	N/A	N/A	1.34 ± 0.16	5.64 ± 0.78*
	Venules (%)	N/A	N/A	N/A		
L-NAME	Arteries (%)	100	77.0 ± 5.5	74.2 ± 4.2	0.76 ± 0.26	1.03 ± 0.16††
	Venules (%)	100	81.0 ± 0.5	86.0 ± 5.4		
Phe	Arteries (%)	100	74.2 ± 15.1	68.4 ± 13.2	1.92 ± 0.19	5.90 ± 0.16*
	Venules (%)	100	76.1 ± 6.2	82.1 ± 10.2		

Vascular tone of arteries and venules in mice ears was assessed by taking pictures with a videocamera adapted to a stereomicroscope at 0, 30 and 60 minutes following a bolus intravenous injection of L-NAME (20 mg/kg), or sustained infusion of Phe (0.1 mg/ml; at a constant rate of 0.8 ml/h). PBS or VEGF-A_{165} (1 μM) were injected subcutaneously in mice ears and the animals were sacrificed 60 min later. Values are mean ± SEM of three independent experiments.
*$P < 0.05$ compared to its control PBS.
††$P < 0.01$ compared to VEGF-A_{165}.

surfaces were at 74.2 ± 4.2 and 86.0 ± 5.4%, respectively as compared to basal surface (Table I). Based on these values, we performed a similar vascoconstriction of the arteriovenous system in mice ears by performing a sustained intravenous infusion of Phe (0.1 mg/ml at 0.8 ml/h) (Table I). Treatment with Phe did not alter the basal vascular permeability as compared to control mice (1.9 μg ± 0.19 vs. 1.34 μg ± 0.16, respectively), and did not affected VEGF-A_{165}-mediated vascular permeability (5.90 μg ± 0.16 vs. 5.64 μg ± 0.78, respectively) as opposed to L-NAME pretreament (Table I).

To identify the contribution of NO downstream effectors, a group of mice were pretreated with a NO-sensitive guanylate cyclase inhibitor (ODQ; 20 mg/kg), which completely abolished VEGF-A_{165}-induced microvascular permeability. Since protein kinase A (PKA) has a barrier-tightening function on basal microvascular permeability, we assessed its role in VEGF-A_{165}-induced protein leakage. Pretreatment of mice with a PKA inhibitor (H89; 40 μg/kg) did not further increase VEGF-A_{165}-induced permeability. However, coadministration of H89 (40 μg/kg) with L-NAME restored almost completely VEGF-A_{165}-induced microvascular permeability (Fig. 4). Since a treatment with H-89 restored VEGF-A_{165}-induced microvascular permeability in the presence of L-NAME, we wanted to assess if H-89 in presence of PAF receptor antagonist (LAU8080) would restore VEGF-A_{165}-mediated vascular permeability increase. The combination of H-89 with LAU8080 did not affect the blockade of VEGF-A_{165} hyperpermeability mediated by LAU8080 (data not shown).

Role of PAF and NO in VEGF-A_{165}-Mediated VE-Cadherin Phosphorylation

Microvascular hyperpermeability is associated with a redistribution of endothelial cell *adherens* junction proteins [Bazzoni and Dejana, 2004]. Since, both VEGF-A_{165} and PAF can induce phosphorylation of VE-cadherin and, that NO is implicated in VEGF-A_{165}-mediated VE-cadherin redistribution, we assessed the contribution of PAF and NO in VE-cadherin phosphorylation induced by VEGF-A_{165}. Treatment of HUVEC with VEGF-A_{165} (10^{-9} M) induced a transient phosphorylation of VE-cadherin starting at 30 min, reaching maximal phosphorylation at 45 min, and returning to basal level within 120 min (Fig. 5A). Treatment of HUVEC with a VEGFR-2 inhibitor (SU1498; 10^{-6} M) 15 min prior to VEGF-A_{165} stimulation for 45 min abrogated VE-cadherin phosphorylation (Fig. 5B). Treatment with LAU8080 (10^{-5} M) abolished VE-cadherin phosphorylation induced by VEGF-A_{165} whereas a treatment with L-NAME (10^{-4} M) or with ODQ (10^{-5} M) provided a partial inhibition (68 and 62%, respectively) of VE-cadherin phosphorylation induced by VEGF-A_{165} (Fig. 5B).

DISCUSSION

In the current study, we observed that the increase of vascular permeability mediated by VEGF analogs was maximal under VEGF-A_{165}

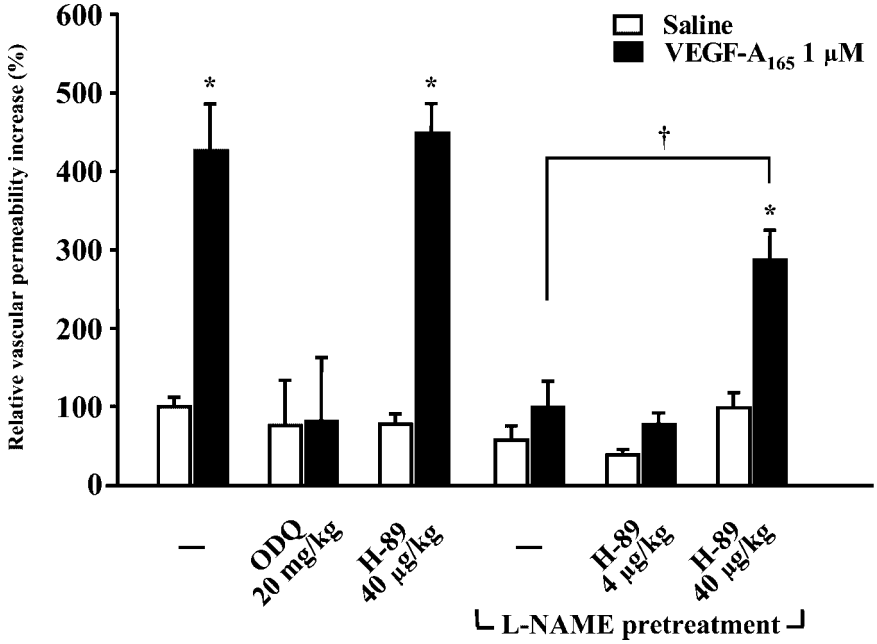

Fig. 4. Implication of NO signaling pathway on VEGF-A_{165}-induced protein extravasation. EB dye injection in the caudal vein was followed 30 min later by an intradermal injection of PBS, or VEGF-A_{165} (1 μM) in mice ears. In a second group, mice were pretreated with L-NAME (20 mg/kg), ODQ (20 mg/kg), or H-89 (40 μg/kg). In a third group, mice were pretreated with a combined injection of L-NAME (20 mg/kg) and H-89 (4 or 40 μg/kg) prior to intradermal injection of VEGF-A_{165} (1 μM), and the animals were sacrificed 60 min later. Values are mean ± SEM of at four independent experiments. $^{*}P < 0.05$ compared to PBS, $^{\dagger}P < 0.05$ compared to VEGF-A_{165} pretreated with L-NAME.

stimulation through the activation of VEGFR-2/NRP-1 complex. Using selective PAF receptor antagonists and inhibitors of NO/cGMP pathway, we observed that both PAF and NO are essential to VEGF-mediated vascular hyperpermeability, despite that only PAF showed a direct inflammatory activity.

Contribution of VEGF Receptors in Vascular Hyperpermeability

To assess the contribution of VEGF receptors and NRP-1 coreceptor in VEGF-induced vascular permeability, we used a selective receptor-agonist approach. Intradermal injection of VEGFR-2 agonists (VEGF-A_{165}, -A_{121}, -C, and -D) promoted exsudation of vascular macromolecules, whereas a treatment with selective VEGFR-1 agonists (PlGF-1 and VEGF-B) had no such effect. Since VEGF-C and -D have the capacity to bind as well to VEGFR-3, one might suggest that VEGF-C and -D inflammatory activities can be mediated through VEGFR-3 activation. However, it has been reported that VEGF-C mutant (ΔNΔC156S-VEGF), a selective VEGFR-3 agonist, lost its property to promote vascular permeability [Joukov et al., 1998]. Thus, our data and previous reports demonstrate that VEGFR-2 activation is essential to vascular permeability increase [Kondo et al., 1995; Joukov et al., 1998; Cao et al., 2004].

Intradermal injection of VEGF-A_{165} was about three times more potent than VEGF-A_{121}, -C, and -D to promote EB dye leakage, demonstrating that NRP-1 enhances VEGF-A_{165}-induced microvascular hyperpermeability. Our data are in agreement with previous reports demonstrating that NRP-1 potentiates VEGF-A_{165} effects upon VEGFR-2 activation, such as EC migration, proliferation, PGI_2, and PAF synthesis [Soker et al., 1997; Bernatchez et al., 2002; Neagoe et al., 2005].

Contribution of PAF and NO/cGMP Pathway in VEGF Vascular Hyperpermeability

We previously showed that a pretreatment of rats with PAF receptor antagonist BN52021 (10 mg/kg) abolished EB dye leakage mediated by VEGF-A_{165} intravenous injection. On the other hand, Murohara et al. [1998] reported that another PAF receptor antagonist; CV6209 (2 mg/kg) failed to prevent vascular permeability increase upon intradermal injection of

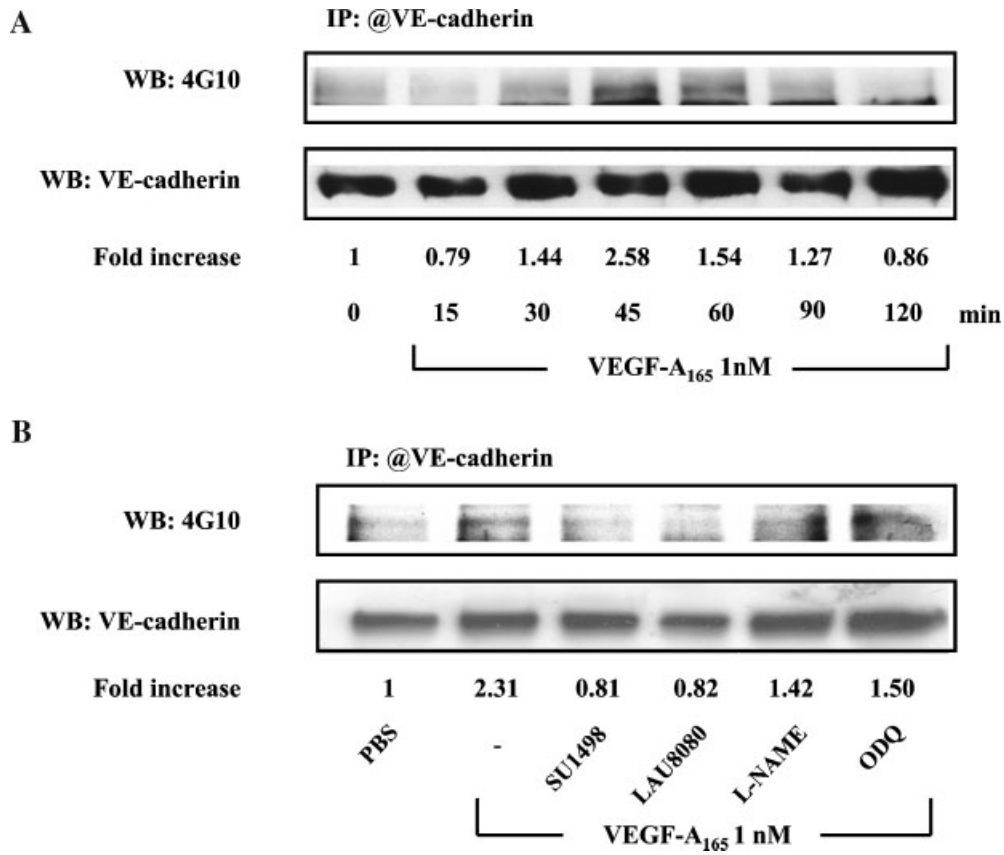

Fig. 5. Phosphorylation of VE-cadherin by VEGF-A_{165}. **A**: HUVEC were stimulated with PBS or VEGF-A_{165} (1 nM; 0–120 min). Cell lysates were immunoprecipitated with anti-VE-cadherin IgG. PhosphoVE-cadherin was detected by immunoblotting using an anti-phosphotyrosine IgG (**upper bands**). Membranes were stripped and the detection VE-cadherin protein expression was performed with an anti-VE-cadherin IgG. **B**: HUVEC were pretreated with SU1498 (10 μM), LAU8080 (10 μM), L-NAME (100 μM), or ODQ (10 μM) for 15 min prior to stimulation with VEGF (10^{-9} M). WB, Western blotting, IP immunoprecipitation.

VEGF-A_{165} in guiney pig skin. In the present study, two additional PAF receptor antagonists, CV-3988 and LAU8080 (20 mg/kg) (Figs. 2 and 3) were used prior to VEGF-A_{165} intradermal injection in mice ears, and both antagonists abrogated the hyperpermeability. The difference between our previous and current findings as compared to Murohara et al., observations might be explained by the type, the dosage, and the potency of PAF receptor antagonist selected. Another explanation may reside in the species and/or tissues investigated in those studies.

Considering the discrepancies regarding the capacity of NO to modulate vascular permeability, we assessed the contribution of NO donor (SNP) and inducer (ACh) in our vascular permeability model and observed that intradermal injection of SNP or ACh did not alter basal vascular permeability. However, pretreatment with L-NAME prevented EB dye exsudation mediated by VEGF-A_{165} and PAF. Since L-NAME abrogated protein extravasation mediated by VEGF-A_{165} and PAF, and that NO per se had no such effect, our data suggest that NO is a cofactor in the signaling pathway of vascular hyperpermeability.

Since the basal formation of NO maintains an arteriovenous dilation, we delineated if the blockade of VEGF-A_{165}-mediated vascular permeability by a pretreatment with L-NAME was due to vasocontractile or anti-inflammatory properties. To do so, we reproduced L-NAME-mediated vasoconstriction with Phe, which is deprived of inflammatory properties. Our data showed that a similar arteriovenous constriction mediated by phenylephrine as compared to L-NAME did not reduce VEGF-A_{165}-mediated microvascular hyperpermeability. These data are strengthening our hypothesis that NO serves as an intracellular cofactor of VEGF-

A_{165} inflammatory effect, independently from the modulation of the vasomotor tone.

The proinflammatory contribution of NO resides in its capacity to activate endothelial soluble guanylate cyclase (sGC) to produce cGMP which participate in vascular hyperpermeability induced by inflammatory mediators [Michel and Curry, 1999]. Thus, we pretreated mice with a sGC inhibitor (ODQ). Selective inhibition of sGC prevented protein leakage mediated by VEGF-A_{165} supporting that an intact eNOS/NO/sGC/cGMP pathway is essential to support VEGF-A_{165} hyperpermeability activity.

It is proposed that cGMP production induced by inflammatory mediators increases vascular permeability by a mechanism reducing cAMP level through activation of phosphodiesterase subtypes. The protective effect of cAMP is mediated by PKA activation which modulates cytoskeletal rearrangement associated with microvascular leakage [Liu et al., 2001; Birukova et al., 2004]. Thus, we hypothesized that the activation of NO/cGMP pathway by VEGF-A_{165} leads to the inhibition of cAMP/PKA activity, thereby setting a proinflammatory environment. We observed that the blockade of NO/cGMP production and PKA activity under quiescent conditions did not modulate basal vascular permeability. However, the blockade of NO or cGMP production, with L-NAME or ODQ abrogated VEGF-A_{165}-mediated vascular permeability, whereas the blockade of PKA activity with H-89 did not alter VEGF-A_{165} inflammatory activity. One might suggest that blockade of PKA activation should have increased VEGF-A_{165}-mediated vascular permeability, however based on our hypothesis, PKA inhibition is already warranted by endogenous cGMP production. To confirm our hypothesis, mice were pretreated with a concomitant injection of L-NAME and H-89, which restored the capacity of VEGF-A_{165} to increase vascular permeability. This series of experiments supports our hypothesis that the activation of NO/cGMP pathway is inhibiting cAMP/PKA pathway, thereby permitting the development of an inflammatory response.

Since a treatment with H-89 in presence of L-NAME restored VEGF-A_{165}-hyperpermeability activity, we assessed if H-89 would restore VEGF-A_{165} inflammatory activity in presence of LAU8080. H-89 did not rescue VEGF-A_{165}-hyperpermeability activity, suggesting that PAF activity is not mediated through the inhibition of cAMP/PKA pathway.

Contribution of PAF and NO in VEGF-Induced VE-Cadherin Phosphorylation

The induction of VE-cadherin phosphorylation by VEGF-A_{165} and PAF is leading to an intercellular redistribution of VE-cadherin, a disruption of cell–cell barrier function and vascular permeability increase [Esser et al., 1998; Dejana et al., 1999; Hudry-Clergeon et al., 2005]. However, there are no studies reporting whether PAF or NO/cGMP contribute to VE-cadherin phosphorylation upon VEGF-A_{165} stimulation. Pretreatment of HUVEC with LAU8080 abrogated VEGF-A_{165}-mediated VE-cadherin phosphorylation, whereas a pretreatment with L-NAME or ODQ had an intermediate effect. This latter observation is in agreement with Aramoto et al. [2004] who reported that the blockade of NO synthesis attenuated VEGF-A_{165}-mediated VE-cadherin redistribution. In addition, we observed that a treatment of HUVEC with SNP was unable to promote VE-cadherin phosphorylation (data not shown), as compared to PAF [Hudry-Clergeon et al., 2005]. Our data, might explain why SNP itself as opposed to PAF is unable to provide a direct vascular permeability increase. Thus, suggesting that NO serves as cofactor of VEGF-A_{165}-mediated permeability increase.

In conclusion, VEGF analogs increase vascular permeability through VEGFR-2 activation, maximal protein leakage is induced under VEGF-A_{165} stimulation, and involves NRP-1/VEGFR-2 complex formation. The blockade of PAF and NO/cGMP pathway abolished VEGF-A_{165}-mediated permeability, however, PAF but not NO directly increases vascular permeability per se, thereby, suggesting that PAF is a direct inflammatory mediator, whereas NO serves as a cofactor in VEGF-A_{165} proinflammatory activities.

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