Published in final edited form as:

Am J Physiol Heart Circ Physiol. 2006 September ; 291(3): H1058–H1064.

Functional significance of differential eNOS translocation

Fabiola A. Sánchez¹, Nirav B. Savalia¹, Ricardo G. Durán¹, Brajesh K. Lal¹, Mauricio P. Boric², and Walter N. Durán¹

¹Program in Vascular Biology, Departments of Pharmacology and Physiology and Surgery, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, New Jersey

²Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

Abstract

Nitric oxide (NO) regulates flow and permeability. ACh and platelet-activating factor (PAF) lead to endothelial NO synthase (eNOS) phosphorylation and NO release. While ACh causes only vasodilation, PAF induces vasoconstriction and hyperpermeability. The key differential signaling mechanisms for discriminating between vasodilation and hyperpermeability are unknown. We tested the hypothesis that differential translocation may serve as a regulatory mechanism of eNOS to determine specific vascular responses. We used ECV-304 cells permanently transfected with eNOS-green fluorescent protein (ECV-eNOS-GFP) and demonstrated that the agonists activate eNOS and reproduce their characteristic endothelial permeability effects in these cells. We evaluated eNOS localization by lipid raft analysis and immunofluorescence microscopy. After PAF and ACh, eNOS moves away from caveolae. eNOS distributes both in the plasma membrane and Golgi in control cells. ACh (10^{-5} M, 10^{-4} M) translocated eNOS preferentially to the trans-Golgi network (TGN) and PAF (10^{-7} M) preferentially to the cytosol. We suggest that PAF-induced eNOS translocation preferentially to cytosol reflects a differential signaling mechanism related to changes in permeability, whereas ACh-induced eNOS translocation to the TGN is related to vasodilation.

Keywords

endothelial nitric oxide; endothelial cells; microvascular permeability; endothelial nitric oxide synthase translocation; acetylcholine; platelet-activating factor

ENDOTHELIAL NITRIC OXIDE (NO) synthase (eNOS) is the prime source of NO in the cardiovascular system. The main emphasis of NO vascular research has focused on its role in vasodilation, and less is known about the function of eNOS in the regulation of microvascular permeability. eNOS regulates the postcapillary venular hyperpermeability response to different stimuli (18,23,33); however, the exact signaling mechanisms of eNOS regulation of permeability have not been elucidated completely.

Phosphorylation of eNOS is a determinant of its activity. Shear stress, hormones and autacoids activate eNOS by phosphorylation/dephosphorylation (2,5,10,20,21,24,32). In addition to

Address for reprint requests and other correspondence: F. A. Sánchez, Dept. of Pharmacology and Physiology, UMDNJ-New Jersey Medical School, 185 South Orange Ave., MSB H-638, PO Box 1709, Newark, NJ 07101-1709 (e-mail: sanchefa@umdnj.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported in part by National Heart, Lung, and Blood Institute Grants 5-R-01-HL-70634, Fondo Nacional de Ciencias y Tecnología (Chile) No. 1040816, and a grant from the American College of Surgeons.

phosphorylation, the location of eNOS is important for its activation (12,25,26). In the plasma membrane, eNOS is mainly targeted to the caveolae (12,26), where it is inhibited by binding to caveolin-1 (cav-1) through a consensus site (8,11). Calcium-calmodulin and intracellular calcium can dissociate eNOS from cav-1, allowing activation of the enzyme (11,19). On application of different stimuli, eNOS shuttles between caveolae and subcellular compartments such as cytosol, Golgi apparatus, and/or perinuclear structures (6,13,19,22,30,31). Therefore, in addition to phosphorylation, eNOS movement and subcellular localization may play an important role in the regulation of its activity (6,13,22,30,31).

We hypothesized that if translocation of eNOS regulates its vascular actions, then eNOS should 1) move away from caveolae and 2) preferentially translocate to distinct subcellular compartments in response to agonists that stimulate different vascular functions. To test the hypothesis, we chose ACh and platelet-activating factor (PAF) as the agonists. ACh is a calcium-mobilizing, endothelium-dependent vasodilator that activates eNOS and has no in vivo influence on microvascular permeability (9). Conversely, PAF is a potent inflammatory phospholipid that causes vasoconstriction, phosphorylates eNOS, and increases microvascular permeability in vivo (23).

MATERIALS AND METHODS

Cell culture

Dermal microvascular endothelial cells (DMVEC), human umbilical vein endothelial cells (HUVEC) (Cambrex Bio Science), and ECV-304 cells transfected with eNOS-green fluorescent protein (ECVeNOS-GFP; initial batch kindly provided by Dr. William Sessa, Yale University) (28) were grown in EGM-2MV; EGM (Cambrex Bio Science); and DMEM supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 100 IU/ml penicillin and 400 mg/ml G418 (Invitrogen).

Monolayer permeability experiments

We determined whether PAF and ACh stimulated permeability changes across the cellular monolayer by using an established method (15). Fluorescein-isothiocyanate-dextran (70 kDa) (FITC-Dx-70) was determined with a LS-3 Perkin-Elmer Fluorescence Spectrophotometer.

Immunoblot analysis

Cells were grown to confluence in 100-mm plates. We extracted protein according to Breslin et al. (1) to detect eNOS, PAF-receptor (PAF-r), and eNOS phosphorylation. To detect muscarinic receptors, we biotinylated cells with Sulfo-NHS-Biotin (Pierce) according to manufacturer instructions. We precipitated biotinylated proteins with Neutravidin (Pierce) and ran them in SDS-PAGE gels. Proteins were detected by enhanced chemiluminescence (ECL; Pierce) with antibodies against eNOS, phospho-eNOS(Ser1177) [p-eNOS(Ser1177)], p-eNOS(Thr495) (BD Biosciences), PAF-r (Cayman, kindly provided by Dr. Tamas Jilling, Northwestern Medical School), and M2 (Affinity BioReagents) and M3 muscarinic receptors (Biogenesis). Anti- β -actin (Santa Cruz Biotechnologies) was used as control for loading. To inhibit muscarinic and PAF receptors, 1 μ M atropine or 1 nM ABT-491 (Sigma) was added to the cells 30 min before treatment with ACh or PAF and maintained thereafter. Afterward, the cells were processed for detection of eNOS phosphorylation.

Detergent-free purification of caveolae-enriched fractions

Caveolae-enriched fractions were prepared following published protocols (17,27). Each fraction was analyzed by Western blot by using anti-eNOS and anti-cav-1 antibodies (BD Biosciences).

Immunofluorescence microscopy

Cells were grown on glass coverslips to form a confluent monolayer. Cells were serum-starved for at least 5 h or overnight and then treated with 10^{-4} – 10^{-5} M ACh or 10^{-7} M PAF. Cells were fixed in paraformaldehyde for 15 min, permeabilized in 0.5% Triton X-100 (5 min), and labeled with anti-eNOS, anti-zonula occludens-1 (anti-ZO-1), anti-cav-1 (BD Biosciences), or anti-TGN46 (Serotec). The secondary antibodies were Alexa-Fluor-conjugated antibodies (Santa Cruz Biotechnology). Alternatively, ECVeNOS-GFP cells were fixed and permeabilized in methanol at -20°C and observed directly for GFP fluorescence. Cells were viewed with a Zeiss Axiovert 100 Microscope and a Bio-Rad Confocal Microscope model Radiance 2100 MP.

Hamster cheek pouch preparation-in vivo translocation

The experimental protocols were approved by the Pontificia Universidad Católica de Chile's and the New Jersey Medical School's Institutional Animal Care and Use Committees and conducted in accordance with National Institutes of Health's Guidelines for the Use of Animals. Ten male golden Syrian hamsters (*Mesocricetus aureatus*) weighing 100 – 110 g were anesthetized with pentobarbital sodium (60 mg/kg ip, supplemented with 15 mg/kg iv as necessary). The right cheek pouch was prepared for PAF application and processed for Western blotting (5,7). After a 30-min equilibration period, the chamber volume was replaced with fresh bicarbonate buffer (control) or buffer containing 10^{-7} M PAF. Buffer and PAF were applied for 3 min, based on studies demonstrating vasopermeability responses in vivo (3,23). At the end of the exposure to buffer or PAF, the exposed cheek pouch area (80–100 mg of wet tissue) was quickly excised and homogenized for 15 s with an Ultraturrax in 500 μl cold detergent-free antiproteolysis buffer [aprotinin (1 $\mu\text{g}/\text{ml}$), benzamidine (1 mM), leupeptin (10 $\mu\text{g}/\text{ml}$), phenylmethylsulfonyl fluoride (1 mM), soybean trypsin inhibitor (200 $\mu\text{g}/\text{ml}$), EGTA (5 mM), Tris (100 mM, pH 7.4)]. The homogenate was centrifuged initially at 1,000 g to remove nuclei. Subsequently, separation of the membrane and cytosol fractions was achieved by centrifugation at 100,000 g. Each pellet was resuspended in 100 μl of 100 mM Tris, pH 7.4, containing 1% SDS. Samples were separated by 7.5% SDS-PAGE and analyzed by Western blot as described above.

Statistical analysis

Data are presented as means $\pm$ SD or SE. Groups were analyzed for differences by one-way ANOVA followed by Tukey-Kramer's test. Significance was accepted at $P < 0.05$.

RESULTS

eNOS-GFP is expressed in stably transfected cell line ECV304

We used ECV304 stably transfected with eNOS-GFP expression plasmid as a model to assess the trafficking of eNOS in endothelial cells (28). We corroborated that eNOS-GFP is expressed in transfected ECV304 cells but not in nontransfected ECV304 cells (Fig. 1A). The protein is recognized by anti-eNOS and anti-GFP antibodies. Figure 1B shows that eNOS-GFP distributes preferentially in the plasma membrane and in perinuclear Golgi-like structures, in agreement with its typical distribution in endothelial cells (Fig. 1C) (12,25,26).

ECVeNOS-GFP cells are adequate models for endothelial cell physiology

To validate our model and strategy, we investigated eNOS activation by ACh or PAF by measuring phosphorylation at Ser1179 and dephosphorylation at Thr495. PAF and ACh increased phosphorylation at Ser1179 and decreased it at Thr495 in ECVeNOS-GFP cells (Fig. 2A). We also demonstrated the presence of functional muscarinic M3-ACh and PAF receptors by examining the effect of pharmacological inhibition of these receptors on eNOS

phosphorylation at Ser1179 (Fig. 2B). Both inhibitors greatly reduced eNOS phosphorylation. We also demonstrated muscarinic M3 receptors and PAF receptors by Western blot (Fig. 2C). The cells also express M2-ACh receptors (not shown).

To assess ECVeNOS-GFP functionality, we investigated their permeability to FITC-Dx-70. PAF at 10^{-7} M induced a statistically significant increase in permeability to FITC-Dx-70 from $1.1 \pm 0.1 \times 10^{-6}$ to $2.0 \pm 0.1 \times 10^{-6}$ cm/s, while ACh (baseline = $1.5 \pm 0.2 \times 10^{-6}$ cm/s) had no significant impact (Fig. 3). These results indicate that ECVeNOS-GFP cells are an adequate model for endothelial cells with regard to PAF and ACh receptors and to permeability to macromolecules.

ACh and PAF displace eNOS from caveolae in ECVeNOS-GFP cells. Because eNOS is compartmentalized and regulated by interactions with cav-1 in caveolae, we examined the effect of PAF or ACh on eNOS localization in caveolae. We used sodium carbonate extraction of cells, followed by a discontinuous sucrose gradient. In control, we found eNOS mainly in fractions containing 11–20% sucrose (Fig. 4, A and B, *top*) that represent the caveolae fractions as indicated by the location of caveolin in the same fractions (Fig. 4, A and B, *bottom*). Exposure to 10^{-7} M PAF or 10^{-4} M ACh induced eNOS and cav-1 relocation to heavier fractions. Quantifying the blots as the percentage of eNOS protein in each fraction of the density gradient confirms the displacement of eNOS to heavier fractions. We stripped the blots and reprobed them against CD71, a marker for non-lipid raft fractions. We found CD71 protein only in the heavier fractions and confirmed that neither ACh nor PAF changed its distribution (data not shown). These results indicate that PAF and ACh induced movement of eNOS out of caveolae.

ACh and PAF promote translocation of eNOS to different subcellular locations

To determine the subcellular destination of eNOS after it leaves the caveolae, we investigated the influence of ACh and PAF on eNOS subcellular distribution by using regular and confocal fluorescence microscopy. ECVeNOSGFP cells were stimulated with 10^{-4} M ACh or 10^{-7} M PAF for 1 and 5 min, respectively. In control (Fig. 5A), eNOS distributed preferentially in the plasma membrane and Golgi. ACh caused diminished staining in the plasma membrane and an increase in the perinuclear region of the cells. Stimulation with PAF also resulted in reduced staining in the plasma membrane, but the label appeared more diffuse in the cytosol. To assess specificity of treatment, we investigated whether ZO-1 (a plasma membrane marker) was influenced by ACh or PAF. Figure 5B shows colocalization of eNOS and ZO-1 in discrete regions of the plasma membrane. ACh and PAF promoted the internalization of eNOS from the plasma membrane. ACh induced redistribution of eNOS to a perinuclear Golgi-like compartment, while PAF promoted eNOS redistribution to the cytosol. Neither ACh nor PAF influenced the distribution of ZO-1. To determine whether the perinuclear stains in the cells treated with ACh correspond to Golgi, we studied the colocalization of eNOS and TGN46, a marker for the trans-Golgi network (Fig. 6A). In control, TGN46 is observed around the nucleus. Treatment with ACh or PAF did not alter TGN46 localization. The merged control image demonstrates partial colocalization of eNOS and TGN46. After treatment with ACh, eNOS is concentrated in the Golgi region, and the colocalization is almost complete. Treatment with PAF resulted in eNOS and TGN46 colocalization comparable with control, confirming the PAF induces preferential eNOS translocation to the cytosol.

We obtained similar results in DMVEC and HUVEC treated with ACh and PAF for periods of 30 s to 3 min. Figure 6B shows in DMVEC that, in control, eNOS is preferentially located in the plasma membrane, while caveolin is homogeneously distributed. After ACh, eNOS leaves the plasma membrane and relocates to the Golgi-like perinuclear region, where it colocalizes with caveolin. PAF induces preferential translocation of eNOS to the cytosol. Distribution of caveolin is not significantly influenced either by PAF or ACh.

We performed image analysis in ECVeNOS-GFP cells by using Adobe Photoshop tools. To minimize possible experimental variability, we evaluated the fluorescence intensity of each compartment as a percentage of the total fluorescence intensity in the analyzed regions (Table 1).

PAF promotes eNOS translocation to the cytosol in vivo

ACh promotes translocation of eNOS to the Golgi in vivo in the hamster cheek pouch (7). To confirm in vivo our findings with PAF in cultured cells, we performed subcellular fractionation experiments in the hamster cheek pouch. PAF application resulted in a significant increase in cytosolic eNOS compared with control (Fig. 7). These data reinforce in vivo the results obtained in vitro with ECVeNOS-GFP, HUVEC, and DMVEC.

DISCUSSION

Our data demonstrate that 1) ECVeNOS-GFP cells are an adequate model to investigate functionally relevant characteristics of eNOS; 2) these cells express functional M2- and M3-ACh and PAF receptors; 3) PAF and ACh cause phosphorylation of eNOS at Ser1179 and dephosphorylation at Thr495; 4) ACh and PAF dissociate eNOS from caveolae to non-lipid raft domains; and 5) ACh and PAF promote the preferential movement of cell membrane-bound eNOS to distinct intracellular locations.

Our results support the use of ECVeNOS-GFP cells to study eNOS biology (16,28). We provide evidence that 10^{-7} M PAF, a concentration that increases transmonolayer transport of FITC-Dx-70, acting on its receptors, leads to eNOS phosphorylation at Ser1179 and dephosphorylation at Thr495 in ECVeNOS-GFP cells. To the best of our knowledge, this is the first such report, and it confirms our findings that 10^{-7} M PAF increases eNOS phosphorylation, enhances release of NO, and increases permeability in the hamster cheek pouch (4,5). We also demonstrate that ACh induces phosphorylation of eNOS at the Ser1179 in ECVeNOS-GFP and dephosphorylation at Thr495. These data confirm previous reports demonstrating that Ser1177 (the corresponding residue in the human protein) is important in the activation of eNOS, which mediates ACh-induced vasodilation in vivo (24). While PAF and ACh induce eNOS phosphorylation at Ser1179 and dephosphorylation at Thr495, they lead to different physiological outcomes in vivo. Our study suggests a functional significance for the molecular movement of eNOS.

We demonstrate that eNOS and cav-1 move away from lipid rafts to a non-lipid raft domain in response to PAF and ACh. Our confocal microscopy images in endothelial cells indicate these molecules may move independently from each other. Because colocalization of eNOS and cav-1 favors inhibition of eNOS, these images suggest that cytosolic eNOS most likely represents an activated state of the enzyme.

The ACh-induced eNOS translocation from cell membrane to the Golgi suggests that this preferential movement is a signal element important for vasodilation. Because translocation occurred after ACh applications as short as 30 s, we interpret these data as an indication that translocation signals for the onset of vasodilation. This interpretation is in agreement with our earlier in vivo data in the hamster cheek pouch (7).

The PAF-induced translocation could be a signal for vasoconstriction or hyperpermeability or both. We were unable to measure constriction in ECVeNOS-GFP cells or DMVEC. It is unlikely that PAF induces constriction in these cells. In addition, NOS blockade in the hamster cheek pouch microcirculation did not inhibit PAF-induced arteriolar constriction (23), suggesting that eNOS is not involved directly in PAF-induced vasoconstriction. The PAF-induced increase in permeability to FITC-Dx-70 in ECVeNOS-GFP supports our in vivo

studies detailing the important role of eNOS in PAF-induced hyperpermeability in the hamster cheek pouch (3,4,14,23,29). It is important to consider that our in vivo subcellular distribution results in the hamster cheek pouch correlate very well with the cell culture (ECV, HUVEC, and DMVEC) transport studies in demonstrating preferential translocation of eNOS to cytosol. This remarkable correlation between in vivo and in vitro data supports the concept that preferential movement of eNOS to the cytosol may serve to promote immediate availability of substrates for the production of NO and enhance interaction with actin and other structural cell proteins that participate in the onset of hyperpermeability. Our results constitute a strong experimental basis for a biochemical and functional correlation among 10^{-7} M PAF stimulation, eNOS mobilization away from lipid raft domains, eNOS phosphorylation, eNOS preferential translocation to cytosol, and endothelial hyperpermeability to macromolecules. Thus we suggest that PAF-induced translocation of eNOS preferentially to the cytosol is a step in the signaling mechanism encoding for endothelium-mediated microvascular hyperpermeability.

Why is eNOS location important for specific signaling? It could be argued that location is irrelevant because endothelial cells produce large amounts of NO. In fact, NO is easily measured in perivascular compartments. On the basis of this simple observation, one may speculate that relatively high concentrations of NO are necessary to provide the correct signal to the specific intracellular acceptor protein. We propose that high local NO concentrations can be best achieved by correct location of the enzyme.

In summary, PAF and ACh induce eNOS activation and dissociation from lipid raft domains. However, ACh induces preferential movement of eNOS to the TGN while PAF moves eNOS to the cytosol. The selective movement of eNOS to different cellular compartments may be an essential step in the signaling mechanism encoding for the distinct physiological outcomes of the two agonists. Our results, which combine physiological and molecular biological approaches, contribute to advance the understanding of the regulatory mechanisms by integrating in a systematic fashion the molecular basis of the regulation of microvascular transport.

REFERENCES

1. Breslin JW, Pappas PJ, Cerveira JJ, Hobson RW II, Durán WN. VEGF increases endothelial permeability by separate signaling pathways involving ERK-1/2 and nitric oxide. *Am J Physiol Heart Circ Physiol* 2003;284:H92–H100. [PubMed: 12388327]
2. Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res* 1996;79:984–991. [PubMed: 8888690]
3. Dillon PK, Durán WN. Effect of platelet-activating factor on micro-vascular permselectivity: dose-response relations and pathways of action in the hamster cheek pouch microcirculation. *Circ Res* 1988;62:732–740. [PubMed: 2450695]
4. Durán WN, Dillon PK. Acute microcirculatory effects of platelet-activating factor. *J Lipid Mediat* 1990;2(Suppl):S215–S227. [PubMed: 2133284]
5. Durán WN, Seyama A, Yoshimura K, González DR, Jara PI, Figueroa XF, Boric MP. Stimulation of NO production and of eNOS phosphorylation in the microcirculation in vivo. *Microvasc Res* 2000;60:104–111. [PubMed: 10964584]
6. Feng Y, Venema VJ, Venema RC, Tsai N, Caldwell RB. VEGF induces nuclear translocation of Flk-1/KDR, endothelial nitric oxide synthase, and caveolin-1 in vascular endothelial cells. *Biochem Biophys Res Commun* 1999;256:192–197. [PubMed: 10066445]
7. Figueroa XF, González DR, Martínez AD, Durán WN, Boric MP. ACh-induced endothelial NO synthase translocation, NO release and vasodilatation in the hamster microcirculation in vivo. *J Physiol* 2002;544:883–896. [PubMed: 12411531]
8. Fulton D, Fontana J, Sowa G, Gratton JP, Lin M, Li KX, Michell B, Kemp BE, Rodman D, Sessa WC. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in

- Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem* 2002;277:4277–4284. [PubMed: 11729179]
9. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;288:373–376. [PubMed: 6253831]
 10. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, Corson MA. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem* 1999;274:30101–30108. [PubMed: 10514497]
 11. Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S, Lisanti MP, Sessa WC. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the NOScaveolin binding domain in vivo. *J Biol Chem* 1997;272:25437–25440. [PubMed: 9325253]
 12. Garcia-Cardena G, Oh P, Liu J, Schnitzer JE, Sessa WC. Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci USA* 1996;93:6448–6453. [PubMed: 8692835]
 13. Goetz RM, Thatte HS, Prabhakar P, Cho MR, Michel T, Golan DE. Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1999;96:2788–2793. [PubMed: 10077589]
 14. Kobayashi I, Kim D, Hobson RW II, Durán WN. Platelet-activating factor modulates microvascular transport by stimulation of protein kinase C. *Am J Physiol Heart Circ Physiol* 1994;266:H1214–H1220.
 15. Lal BK, Varma S, Pappas PJ, Hobson RW II, Durán WN. VEGF increases permeability of the endothelial cell monolayer by activation of PKB/akt, endothelial nitric-oxide synthase, and MAP kinase pathways. *Microvasc Res* 2001;62:252–262. [PubMed: 11678628]
 16. Liu J, Hughes TE, Sessa WC. The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. *J Cell Biol* 1997;137:1525–1535. [PubMed: 9199168]
 17. Lungu AO, Jin ZG, Yamawaki H, Tanimoto T, Wong C, Berk BC. Cyclosporin A inhibits flow-mediated activation of endothelial nitric-oxide synthase by altering cholesterol content in caveolae. *J Biol Chem* 2004;279:48794–48800. [PubMed: 15383526]
 18. Mayhan WG. Role of nitric oxide in leukotriene C₄-induced increases in microvascular transport. *Am J Physiol Heart Circ Physiol* 1993;265:H409–H414.
 19. Michel JB, Feron O, Sacks D, Michel T. Reciprocal regulation of endothelial nitric-oxide synthase by Ca²⁺-calmodulin and caveolin. *J Biol Chem* 1997;272:15583–15586. [PubMed: 9188442]
 20. Michell BJ, Chen Z, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, Kemp BE. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem* 2001;276:17625–17628. [PubMed: 11292821]
 21. Montagnani M, Chen H, Barr VA, Quon MJ. Insulin-stimulated activation of eNOS is independent of Ca²⁺ but requires phosphorylation by Akt at Ser(1179). *J Biol Chem* 2001;276:30392–30398. [PubMed: 11402048]
 22. Prabhakar P, Thatte HS, Goetz RM, Cho MR, Golan DE, Michel T. Receptor-regulated translocation of endothelial nitric-oxide synthase. *J Biol Chem* 1998;273:27383–27388. [PubMed: 9765266]
 23. Ramírez MM, Quardt SM, Kim D, Oshiro H, Minnicozzi M, Durán WN. Platelet activating factor modulates microvascular permeability through nitric oxide synthesis. *Microvasc Res* 1995;50:223–234. [PubMed: 8538502]
 24. Scotland RS, Morales-Ruiz M, Chen Y, Yu J, Rudic RD, Fulton D, Gratton JP, Sessa WC. Functional reconstitution of endothelial nitric oxide synthase reveals the importance of serine 1179 in endothelium-dependent vasomotion. *Circ Res* 2002;90:904–910. [PubMed: 11988492]
 25. Sessa WC, Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM, Desai KM. The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J Biol Chem* 1995;270:17641–17644. [PubMed: 7543089]

26. Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG, Michel T. Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* 1996;271:6518–6522. [PubMed: 8626455]
27. Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M, Lisanti MP. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J Biol Chem* 1996;271:9690–9697. [PubMed: 8621645]
28. Sowa G, Liu J, Papapetropoulos A, Rex-Haffner M, Hughes TE, Sessa WC. Trafficking of endothelial nitric-oxide synthase in living cells. Quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *J Biol Chem* 1999;274:22524–22531. [PubMed: 10428829]
29. Tomeo AC, Durán WN. Resistance and exchange microvessels are modulated by different PAF receptors. *Am J Physiol Heart Circ Physiol* 1991;261:H1648–H1652.
30. Venema VJ, Marrero MB, Venema RC. Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. *Biochem Biophys Res Commun* 1996;226:703–710. [PubMed: 8831678]
31. Wang Q, Patton WF, Hechtman HB, Shepro D. A novel anti-inflammatory peptide inhibits endothelial cell cytoskeletal rearrangement, nitric oxide synthase translocation, and paracellular permeability increases. *J Cell Physiol* 1997;172:171–182. [PubMed: 9258338]
32. Wang Y, Nagase S, Koyama A. Stimulatory effect of IGF-I and VEGF on eNOS message, protein expression, eNOS phosphorylation and nitric oxide production in rat glomeruli, and the involvement of PI3-K signaling pathway. *Nitric Oxide* 2004;10:25–35. [PubMed: 15050532]
33. Yuan Y, Granger HJ, Zawieja DC, DeFily DV, Chilian WM. Histamine increases venular permeability via a phospholipase C-NO synthase-guanylate cyclase cascade. *Am J Physiol Heart Circ Physiol* 1993;264:H1734–H1739.

Fig. 1.

Expression of endothelial nitric oxide synthase (eNOS) in ECV-304 cells permanently transfected with eNOS-green fluorescent protein (ECVeNOS-GFP) and in dermal microvascular endothelial cells (DMVEC). *A*: Western blot of protein extracted from ECVeNOS-GFP cells. The eNOS-GFP protein is expressed in the transfected cells but not in the control. *B* shows high expression of eNOS-GFP protein in cell membrane and Golgi in ECVeNOS-GFP. *C* shows similar eNOS distribution in DMVEC.

Fig. 2.

ECVeNOS-GFP cells express functional receptor-mediated eNOS activation. Cell lysates from control, ACh-, and platelet-activating factor (PAF)-treated cells were immunoblotted against phospho-eNOS (p-eNOS) and β -actin. *A*: ACh and PAF activate eNOS by Ser1177 phosphorylation and Thr495 dephosphorylation. *B*: inhibition of muscarinic ACh and PAF receptors (PAF-r) blocks Ser1177 phosphorylation. *C*: ECVeNOS-GFP cells express muscarinic M3 ACh-receptors (M3-r) and PAF-r. Atr, atropine; ABT, PAF-receptor inhibitor.

Fig. 3.

PAF increases permeability in ECVeNOS-GFP cells. Monolayers of ECVeNOS-GFP cells were treated with PAF (A) or ACh (B). Permeability to FITC-Dextran-70 was measured. Data are expressed as permeability coefficients (means $\pm$ SE) for control, PAF-, and ACh-stimulated monolayers. * $P < 0.05$; PAF, $n = 6$; ACh, $n = 5$.

Fig. 4.

ACh and PAF move eNOS away from the caveolae. Isolation of lipid raft domains was done in ACh- or PAF-treated cells. Fractions were probed against caveolin-1 and eNOS. *A*: ACh treatment. *B*: PAF treatment. Blots represent 3 independent experiments.

Fig. 5.

A: influence of ACh and PAF on the distribution of eNOS-GFP was assessed by fluorescence of GFP. *B:* colocalization between eNOS and zonula occludens-1 (ZO-1). Confocal images were obtained in ECVeNOS-GFP cells treated with ACh and PAF. Arrows point to disappearance of eNOS from plasma membrane. Images are representative of 3 independent experiments.

Fig. 6.

A: colocalization of eNOS and TGN46 (a marker for the trans-Golgi network) in ECVeNOS-GFP cells treated with ACh and PAF. B: immunofluorescence microscopy in DMVEC treated with ACh and PAF, respectively. Red, eNOS; green, caveolin. Images are representative of 3 independent experiments.

Fig. 7. PAF stimulates eNOS translocation in vivo. PAF (10^{-7} M) was applied to the hamster cheek pouch and subsequently processed for Western blot. eNOS band intensities were evaluated by integrated optical density (IOD). $*P < 0.05$, $n = 5$.

Table 1

Analysis of confocal images

	M-C	M-PAF	M-ACh	Cyt-C	Cyt-PAF	Cyt-ACh	G-C	G-PAF	G-ACh
Mean	49.3 [*]	34.2	35.2 [†]	12.8 [*]	22.6	10.1 [*]	37.8	42.9	54.5 [*]
SD	2.8	4.4	1.0	2.4	2.1	1.0	2.7	4.7	1.6
n	5	5	4	5	5	4	5	5	4

Location of endothelial nitric oxide synthase (eNOS) in ECV-304 cells permanently transfected with eNOS-green fluorescent protein (ECV-eNOS-GFP), expressed as a percentage of total GFP fluorescence; n = no. of regions analyzed. Each region = 4–6 cells. M, membrane; Cyt, cytosol; G, Golgi; C, control; PAF, platelet-activating factor.

^{*} $P < 0.05$ compared with corresponding PAF data;

[†] $P < 0.05$ compared with control (ANOVA + Tukey-Kramer test).