

The NO cascade, eNOS location, and microvascular permeability

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The nitric oxide (NO) cascade and endothelial NO synthase (eNOS) are best known for their role in endothelium-mediated relaxation of vascular smooth muscle. Activation of eNOS by certain inflammatory stimuli and enhanced NO release have also been shown to promote increased microvascular permeability. However, it is not entirely clear why activation of eNOS by certain vasodilatory agents, like acetylcholine, does not affect microvascular permeability, whereas activation of eNOS by other inflammatory agents that increase permeability, like platelet-activating factor, does not cause vasodilation. In this review, we discuss the evidence demonstrating the role of eNOS in the elevation of microvascular permeability. We also examine the relative importance of eNOS phosphorylation and localization in its function to promote elevated microvascular permeability as well as emerging topics with regard to eNOS and microvascular permeability regulation.

Keywords Endothelial nitric oxide synthase • Microvascular permeability • Inflammation • Protein traffic

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1. Introduction

Inflammation is an important component of host defence; however, excessive or uncontrolled inflammation can disrupt normal tissue homeostasis. Traumatic injury can produce a massive, systemic inflammatory response that complicates resuscitation and patient outcomes.¹ In addition, smaller, chronic tissue insults that occur during disease formation, such as long-term hyperglycaemia, ischaemic injuries, elevated oxidative stress, etc., can alter the expression of inflammatory mediators and receptors, as well as intracellular signal transduction pathways and structural components in the microvascular endothelium, facilitating exaggerated inflammatory responses or a chronic inflammatory state.^{2,3}

Inflammatory processes are characterized by an increase in microvascular permeability (hyperpermeability) to macromolecules. The extravasation of macromolecules is a normal ongoing process that occurs predominantly at post-capillary venules, and plays an important role in formation of lymph and immune function. However, excessive leakage of macromolecules into tissues causes oedema, which can disrupt homeostasis and cause tissue dysfunction. Several inflammatory mediators can rapidly increase microvascular permeability to macromolecules across the venular endothelium. The mechanisms by which these inflammatory stimuli elicit hyperpermeability of the endothelium have been an area of intense investigation. In this Spotlight, we will review evidence obtained about cellular regulation of enhanced microvascular permeability obtained in isolated, cultured

endothelial cells (EC) as well as in the complex cellular environment of the *in vivo* microcirculation. Application of the knowledge acquired in the *in vitro* setting to the *in vivo* interactions is fundamental to understand the integrated regulation of microvascular transport and its functional alterations in vascular disease.

Nitric oxide (NO) is an established important signalling regulator of cardiovascular function,⁴ but recognition of the significance of its role in the control of microvascular permeability has developed at a slower rate. The first experimental evidence for the involvement of NO in transport of macromolecules in intact microvessels was published in the early 1990s and caused immediate controversy.^{5–8} Studies performed in the intestinal microvasculature reported that NO serves to maintain a tight microvascular barrier; inasmuch as inhibition of nitric oxide synthases (NOS) with L-arginine analogs increased leakage of macromolecules in the intestine.^{5,9} However, evidence in other tissues, in isolated venules, single capillaries and in EC monolayers indicated that the activity of endothelial nitric oxide synthase (eNOS) increases microvascular permeability to macromolecules in response to inflammatory agents.^{6–8,10–14} These initial opposite results may have been due to species differences or to different targeted mechanisms operating under the specific experimental conditions. In both cases, the experimental evidence was derived from the ability of L-arginine analogs to block NOS or from the reliance on NO donors to mimic endogenous NO actions. Definitive evidence to settle this initial controversy was obtained in eNOS knockout mice. Studies performed in these molecularly engineered animals

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contributed unequivocally to establish that eNOS-derived NO causes hyperpermeability in response to pro-inflammatory agents such as platelet-activating factor (PAF) and vascular endothelial growth factor (VEGF).^{15,16}

PAF and VEGF are agents that have proven useful in studying microvascular endothelial permeability *in vivo* and *in vitro*. Administration of PAF rapidly and significantly increases permeability in the mouse cremaster and mesenteric microvasculature as well as in the hamster cheek pouch.^{12,16,17} Administration of PAF to organs of eNOS knockout mice fails to mount a robust hyperpermeability response. Interestingly, the data in eNOS knockout mice show that baseline permeability is not affected by the presence or absence of eNOS. Thus, the eNOS-associated signalling cascade becomes important mainly in the response to inflammatory challenges inasmuch as administration of VEGF also fails to initiate a hyperpermeability response in these genetically altered animals.¹⁵

The specificity of eNOS-derived NO in the hyperpermeability process is supported by the observation that administration of VEGF or PAF to inducible NOS (iNOS) knockout mice elicits hyperpermeability responses that are not significantly different from control normal mice.^{15,16} In addition, VEGF-induced NO production is nearly abolished in mice lacking eNOS,¹⁵ and PAF-induced NO release is largely inhibited in single-perfused microvessels treated with a fusion protein containing the eNOS-inhibitory binding domain of caveolin-1.¹⁸ Moreover, in single-perfused microvessels, the PAF-induced NO production is calcium-sensitive, a feature attributed to eNOS activity but not iNOS.¹⁸ The fundamental importance of eNOS-derived NO for the onset of hyperpermeability has also

been confirmed in EC in culture. Specific knockdown of eNOS expression using eNOS-targeting siRNA abolishes the ability of coronary venular EC to increase permeability to macromolecules in response to PAF.¹⁹ Although these studies demonstrate that eNOS-derived NO is of greater relevance in the immediate regulation of microvascular permeability, iNOS-derived NO may play a more significant role in events taking longer periods of time such as the changes in permeability associated with systemic inflammation or sustained angiogenesis.^{15,20}

2. Mechanisms of signal transduction in hyperpermeability

In vivo evidence indicates that the signalling pathways for several inflammatory agents involve the activation of protein kinase C (PKC) and eNOS.^{10,14,21–23} These reactions are presumably followed by synthesis of cGMP. Experiments in isolated microvessels and EC support the concept that stimulation of NOS and production of cGMP leads to changes in permeability to small and large solutes.^{24,25}

A simplified scheme of our understanding of the regulatory signalling in the control of microvascular permeability is shown in *Figure 1*. The diagram in *Figure 1* is based both on experiments performed *in vivo* and *in vitro*. Briefly, the interactions of pro-inflammatory agonists with their specific receptors, lead to activation of phospholipases (PL) A₂, C, and D, as well as Ca²⁺-channels. PLA₂ (not shown) exerts a dual action. Through the lipoxygenase pathway, PLA₂ activation leads to formation of leucotrienes, particularly C₄, which activate

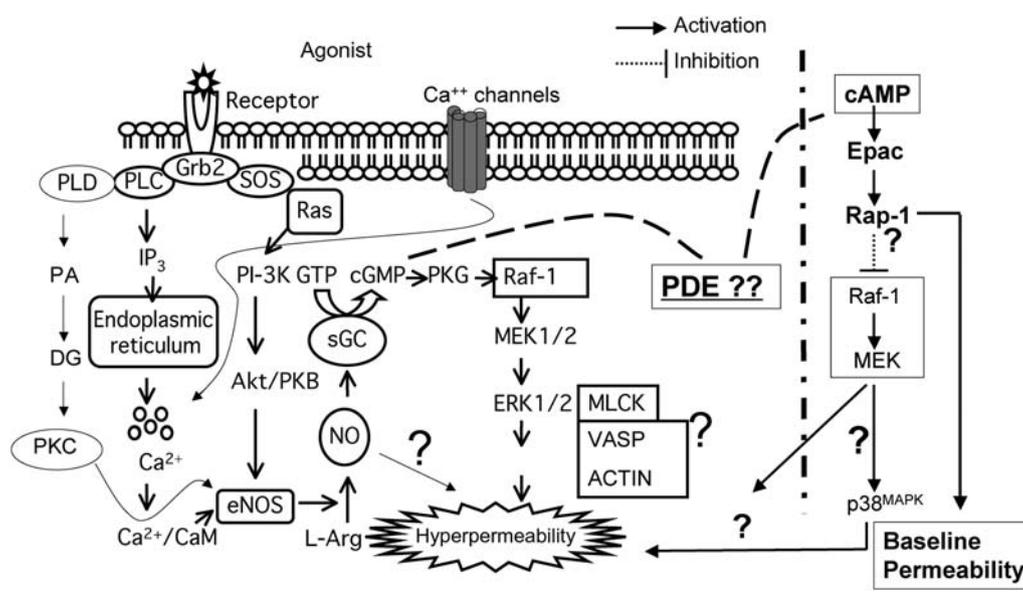


Figure 1 Simplified scheme of pro-inflammatory signal transduction pathways leading to eNOS activation and endothelial hyperpermeability. Binding of pro-inflammatory agonists to their receptors activates a myriad of intracellular second messengers, leading to release of calcium from intracellular stores, PKC activation, Akt/PKB activation, and ERK-1/2 activation. The overall result is a functional state favouring changes in phosphorylation state of eNOS and elevated production of NO and increased permeability of the microvascular barrier. Several elements of the overall signalling paradigm remain elusive, such as the downstream effectors linking eNOS-derived NO to changes in the cytoskeleton and intercellular junctions that produce the hyperpermeable state, the precise role of the balance between cGMP and cAMP [via phosphodiesterases (PDE)] in permeability regulation. The mechanisms involved in the restoration of basal permeability and/or promotion of enhanced barrier integrity are represented by the Epac/Rap1 pathway.

NOS and elevate permeability. Through the cyclooxygenases pathway, PLA₂ activates thromboxane synthase and is mainly responsible for the vasoconstrictor effect of PAF in the hamster cheek pouch.²⁶ PLC is an important phospholipase that serves as a catalyst for the synthesis of inositol triphosphate and diacylglycerol (DAG). The role of PLC γ in microvascular permeability has been evaluated with specific inhibitors. Indeed, inhibition of PLC reduces the impact of pro-inflammatory agonists on microvascular transport in isolated venules and *in vivo*^{13,27} suggesting a role for this phospholipase in the control of basal permeability. Importantly, the activity of PLC leads to activation of PKC, an important element in the signal transduction associated with the regulation of microvascular permeability.²³

2.1 Protein kinase C

The standard methods to study the impact of PKC *in vivo* consist of applying exogenous phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) or phorbol ester dibutyrate (PDBu), or exogenous DAG or DAG analogs. These agents stimulate conventional and novel PKCs to translocate from the cytoplasm to the cell membrane and become activated.^{28,29} Another standard method is to inhibit the catalytic or the regulatory domains of PKC. PKC α , PKC β , PKC δ , and PKC μ have been documented to influence endothelial permeability.^{29–31}

The role of PKC as a biochemical pathway for PAF has been evaluated *in vivo* using inhibitors of the catalytic (sphingosine, iso H-7) and regulatory (calphostin C) domains of the enzyme. These experiments demonstrate that inhibition of PKC leads to a reduction in agonist-induced hyperpermeability.^{23,32} In addition, stimulation of PKC with phorbol esters (PDBu at 10⁻⁷, 10⁻⁶, and 10⁻⁵ M and PMA at 10⁻⁶ M and 5 × 10⁻⁷ M) increases microvascular transport of macromolecules in a dose-dependent fashion. Further evidence for PKC as a signalling pathway in the *in vivo* regulation of microvascular transport is derived from the ability of calphostin C to inhibit PDBu-induced macromolecular extravasation.²³ Pharmacologic inhibition of PKC reduces the impact of agonists on the hyperpermeability induced by bradykinin and VEGF *in vivo* and *in vitro*.^{11,13,21–23,32} Importantly, inhibition of NOS blocks PDBu-stimulated hyperpermeability to FITC-dextran in the hamster cheek pouch,¹¹ which demonstrates PKC-NO interactions in the promotion of hyperpermeability.

There are controversial reports on the role of PKC in the regulation of eNOS. PKC increases phosphorylation of eNOS on threonine-495 in EC, resulting in decreased eNOS activity.³³ It has also been reported that inhibition of PKC promotes increased release of nitrogen oxides in bovine aortic EC.³⁴ The functional outcomes of these changes in phosphorylation obtained in cultured or isolated cells are unknown. In contrast, it is clear that activation of PKC *in vivo* promotes hyperpermeability.^{11,23} PKC may activate, via phosphorylation, cytoskeletal proteins such as myosin light chain kinase, caldesmon and vimentin^{35–37} as well as influence the reorganization of focal adhesions and intercellular junctions.^{21,30} On the basis of elegant studies using fluorescent resonance energy transfer, it has been suggested that an imbalance in the PKC β II/PKC δ expression and activity contributes to the endothelial hyperpermeability observed in diabetes in coronary venules.³¹ The preponderance of the evidence supports the concept that PKC increases macromolecular transport, and that the mechanism involves activation of eNOS.

3. Significance of eNOS in the regulation of microvascular hyperpermeability

Several laboratories have contributed to paint the picture of how activation of eNOS proceeds. The process is complex and involves dissociation from caveolin-1, association with heat shock protein (HSP)-90, Ca²⁺-calmodulin binding, phosphorylation on serine-1177, and dephosphorylation of threonine-495.^{33,38–41} These steps are implicit in the initial reactions summarized in Figure 1. In particular, binding of eNOS to caveolin-1 through a consensus site in caveolae regulates the basal state of eNOS production of NO.^{42,43} Several agonists activate eNOS through multiple mechanisms such as phosphorylation or dephosphorylation of specific residues, interaction with different proteins, S-nitrosylation and specific subcellular localization.^{19,39,42,44–46} In this section the mechanisms of eNOS activation, NO production, and proposed mechanisms based on current experimental evidence will be discussed.

3.1 Phosphorylation of eNOS and NO production

The genes encoding for human and bovine endothelium eNOS have been characterized and their cDNA have been cloned.^{47–49} Although the gene sequence is highly conserved among species, the post-translational regulatory modalities may vary in different species.^{40,41} The regulation of eNOS by phosphorylation is supported by its deduced amino acid sequence. However, the extent to which phosphorylation or dephosphorylation of specific sites lead to activation or inactivation of eNOS activity has been the subject of debate, particularly in bovine arterial EC.³⁴ When EC are stimulated by agonists such as VEGF, insulin-like growth factor, or elevated shear stress (which all can stimulate increased permeability of venules^{6,13,50}), Akt phosphorylates eNOS on serine-1177, which increases eNOS activity.^{38,39} Bradykinin stimulates calmodulin-dependent kinase II to phosphorylate serine-1177 of eNOS as well.³³ There are at least six phosphorylation sites identified to date that may play a role in the activity of eNOS, i.e. four sites are on serine (S116, S617, S635, S1179)^{51–53} one on threonine (T497)²⁶ and at least one on tyrosine (Tyr83).⁵² The functional significance of each of these sites is undergoing systematic evaluation. It is of interest that the phosphorylation/dephosphorylation of these sites can be stimulated by a large number of agents through closely related pathways.⁵³

The impact of each of the specific sites of eNOS phosphorylation on our knowledge of microvascular transport is difficult to evaluate. The majority of available molecular biology data comes from experiments designed to investigate the function of eNOS and of NO in control of blood flow and blood pressure, and performed in EC derived mainly from large vessels. Under these experimental conditions, investigators of the microcirculation are forced to extrapolate data from large vessel endothelia (not normally involved in blood-tissue transport) to post-capillary venular endothelium, where agonist-stimulated transport of macromolecules mainly occurs. In addition, the uncertainty of the extrapolation may be compounded by possible phenotypical changes undergone by EC grown in specifically designed and controlled culture media. In fact, the tissue culture situation may be at variance with the dynamic environment of EC in the microcirculation. Interestingly, acetylcholine (ACh), an agent

that causes vasodilation but does not alter microvascular permeability,¹² induces exactly the same changes in phosphorylation of eNOS as PAF and VEGF.⁵⁴ These observations suggest that phosphorylation of eNOS *per se* is not a major determinant of the functional consequences or outcomes of eNOS-derived NO. This prompted our investigation as to whether ACh and PAF may cause differential translocation of eNOS.

3.2 Molecular movement of eNOS, location of eNOS and regulation of microvascular permeability

Endothelial nitric oxide is found mainly in the plasma membrane and in the Golgi complex in EC, but it distributes itself also in the cytosol. Thus, it is plausible that subcellular location of the enzyme contributes to determine its microvascular functions.

The ability of eNOS to translocate from plasma membrane to subcellular compartments is well documented in cultured EC, in non-vascular cells transfected with eNOS and *in vivo* after agonist stimulation using fractionation or extraction techniques and confocal microscopy.^{46,54–59} The movement of eNOS from plasma membrane to subcellular locations has been classically associated with de-palmitoylation of eNOS.^{40,41} Studies to determine the functional significance of eNOS traffic or internalization via caveolae have only recently been undertaken.^{19,46,60}

Membrane-bound and cytosolic eNOS are able to release NO.^{51,61,62} Translocation of eNOS from the plasma membrane could be considered a mechanism to decrease the inhibitory association with caveolin-1 in order to activate the enzyme. However, elegant studies, using eNOS constructs designed to target it specific subcellular locations, have demonstrated that cell membrane-bound and Golgi-bound eNOS have the ability to release more basal NO than cytosolic eNOS.^{51,61,63} Cytosolic eNOS also appears to release less NO in response to agents that enhance inwardly directed movement of calcium, such as ionomycin.⁶⁴ However, the location of the enzyme when it releases NO may be more important than the amount of NO released for development of extracellular significant functions, such as transport of macromolecules across the endothelium. The concept that eNOS must translocate to specific subcellular compartments to determine the NO-stimulated function is puzzling as one would assume that a highly diffusible gas would not need translocation of its releasing enzyme for appropriate activity. However, the experimental evidence supports the concept that precise location of eNOS at subcellular compartments is necessary to achieve the specific functionally efficacious concentration of NO.^{19,54} The existence of at least two intracellular arginine pools in EC has recently been reported,⁶⁵ so it is conceivable that translocation may regulate access to substrates needed for NO production. In any case, this functional translocation may represent an adaptive and protective mechanism since NO is a highly reactive species and cells possess an abundance of scavengers of reactive species.

Agonists that increase permeability, such as VEGF, PAF, and bradykinin, cause eNOS movement to the cytosol.^{19,44,46,54} In contrast, ACh—a vasodilating agent—induces movement of eNOS preferentially to the Golgi *in vivo* and *in vitro* and does not promote hyperpermeability *in vivo* and *in vitro*.^{12,54,55} The difference in microvascular site of action and functional characteristics of these agents has provided a reasonable approach to correlate *in vitro* and *in vivo* experiments. The seminal *in vitro* studies on this topic were carried out in ECV-304 cells

transfected with eNOS-GFP (GFP, green fluorescent protein). ECV-304 cells, initially described as a HUVEC-derived cell line with lacks eNOS, were later found to be identical to T24/83 cells (urinary bladder carcinoma cell line) due to a cross-contamination of cultures.⁶⁶ However, these cells have remained a useful tool to study certain protein–protein interactions, localization, and function of transfected eNOS.^{54,67} The initial observations were confirmed and advanced subsequently in human umbilical vein EC and coronary post-capillary venular EC.¹⁹ Both PAF and ACh caused separation between eNOS and caveolin-1 as ascertained by discontinuous sucrose gradient fractionation. However, ACh promoted preferential movement of eNOS to the Golgi complex, whereas PAF promoted preferential movement of eNOS to the cytoplasm, as indicated by confocal microscopy.⁵⁴ Interestingly, the distribution of caveolin-1 is not significantly influenced either by PAF or ACh.^{54,55}

How does eNOS reach its target for development of hyperpermeability? The location of eNOS to the plasma membrane depends on N-myristoylation and palmitoylation reactions, which target it to the caveolae. In contrast, de-myristoylation and de-palmitoylation are processes associated with removal of eNOS from the membrane and its translocation to other subcellular compartments. Whether or not myristoylation and/or palmitoylation play a role in the translocation of eNOS was tested using a sophisticated, specific eNOS fusion protein (CD8-GFPeNOSmyr–) that transformed eNOS into a transmembrane protein with eNOS attached to the extracellular and transmembrane domain of the cell surface glycoprotein CD8⁵⁸, a feature that prevents translocation by de-palmitoylation. In addition, the construct includes a mutation at the G2A site of eNOS to prevent its myristoylation.⁵⁸ The underlying assumption in the approach is that because of this specific transmembrane fusion protein eNOS would stay in the plasma membrane, would not translocate to the cytosol, and not contribute to PAF-stimulated hyperpermeability. In marked contrast to the expectations, PAF elicited a remarkable increase in permeability to FITC-dextran-70 in ECV-304 cells transfected with the CD8-GFPeNOSmyr–construct.¹⁹ Experiments utilizing biotinylation of surface proteins revealed that PAF internalized the CD8-GFPeNOSmyr–.¹⁹ A reasonable explanation for these experimental findings is the conclusion that eNOS is internalized via caveolae, since CD8-GFPeNOSmyr– is a transmembrane protein. This concept has been tested elegantly using constructs based on mutations of dynamin 2 and of the phosphorylation site for caveolin-1⁴⁶. These mutations prevent caveolar scission from the plasma membrane and thus anchor caveolae and eNOS to the plasma membrane. The evidence confirmed that PAF and VEGF stimulate eNOS internalization (endocytosis) via caveolae and that this step is fundamental to activate the eNOS–NO signalling cascade leading to induced hyperpermeability.^{19,46} The basic proposal for eNOS internalization as a mechanistic process in increased endothelial and microvascular permeability is displayed in *Figure 2*.

3.3 The NO–cGMP axis

Soluble guanylyl cyclase (sGC) is the best described receptor of NO.^{68–72} Because of the low permeability of cyclic GMP through biological membranes, the assessment of the role of cGMP in the signalling cascade has been implemented using millimolar concentrations of its analog 8-Br-cGMP. Administration of 8-Br-cGMP increases permeability across EC monolayers (~50% increase in permeability to FITC-Dextran 70 kDa) as well as in single microvessels (~5-fold increase in hydraulic conductivity).^{25,73} The variation in reported

Putative steps in eNOS-derived NO stimulated hyperpermeability

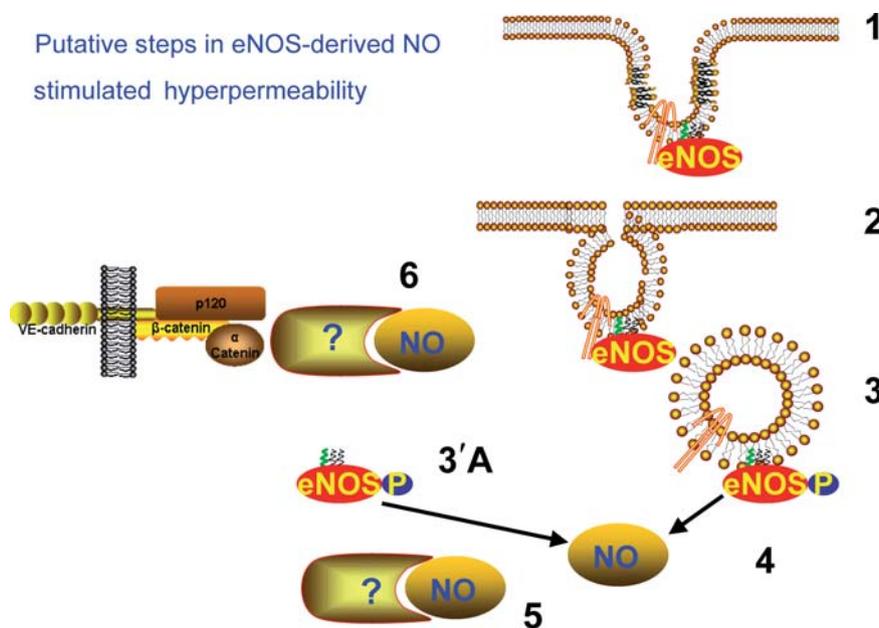


Figure 2 Putative mechanisms by which eNOS-derived NO causes increased microvascular permeability. A logical sequence supported by experimental data is proposed. (1) During a non-inflammatory state, eNOS is associated with caveolin-1 (depicted as a hairpin) and localized primarily in caveolae at the plasma membrane. (2) Activation of EC by pro-inflammatory signals causes caveolae to pinch off the plasma membrane. Scission of caveolae from the membrane leads to eNOS internalization into the cell. (3) Within the cell, eNOS dissociates from caveolin-1 and may undergo phosphorylation. (3'A) It is also possible that eNOS may dissociate from the caveolar membranes. (4) The combination of activation of eNOS by calcium–calmodulin, dissociation of eNOS from caveolin-1 and eNOS phosphorylation significantly increases the production of NO by eNOS. (5) NO then can bind intracellular target(s) that remain to be identified, which produce signals that (6) alter the junctional proteins between EC and increase microvascular permeability.

magnitudes is probably due to (i) differences in baseline permeability between the two models, and (ii) differences in parameter measurement.⁷⁴ The location of cGMP activity as downstream of eNOS-derived NO is supported by the observations that blocking sGC decreases the hyperpermeability induced by phorbol esters and by sodium nitroprusside.¹⁴ This concept is also corroborated by the observation that inhibition of PKG (cGMP-dependent protein kinase) causes a dramatic reduction in histamine-induced hyperpermeability in venules.¹⁴

The information in regards to the pathways downstream of cGMP–PKG leading to increases in permeability is very limited. PKG probably activates the ERK-1/2 MAP kinase pathway, inasmuch as pharmacological blockade of the ERK-1/2 MAP kinase pathway inhibits cGMP-induced hyperpermeability in HUVEC.²⁵ In addition, PKG-induced increases in endothelial permeability may be the result of interactions of PKG with the PKA pathway. It is possible that feedback regulation through phosphodiesterases plays a significant role in modulating the relative concentrations of cGMP and cAMP, thus controlling the ability of these cyclic nucleotides to affect their specific targets. cGMP may activate phosphodiesterase 2 and induce degradation of cAMP; in turn, degradation of cAMP may reduce the barrier properties of the microvascular EC, and thus increase permeability to macromolecules.^{69,70,75} How the cGMP or cAMP signals reach the target junctional proteins is unknown.

The *in vivo* and *in vitro* evidence indicates an important role for cGMP in the signalling leading to hyperpermeability to macromolecules. However, it is important to note that we are lacking experimental evidence that clearly documents an enhancement in cGMP

mass or activity in direct relationship to endothelial hyperpermeability. A recent PhD Thesis reported an increase in tissue cGMP in the hamster cheek pouch in association with PAF-induced hyperpermeability; however, the cellular source of cGMP was not identified.⁷⁶ Other indirect evidence comes from studies using HSP90 inhibitors. HSP90 serves as a chaperone to that binds to both eNOS and sGC and facilitates their interaction, stabilizing sGC and enhancing cGMP production. Application of HSP90 inhibitors to cultured EC monolayers caused an enhancement of barrier function and was protective against barrier dysfunction caused by various oedemagenic agents.^{77,78}

3.4 S-nitrosylation (nitrosation)

In addition to modulating the sGC–cGMP–PKG pathway, NO may influence biological processes by causing S-nitrosylation of proteins. The significance of S-nitrosylation as a post-translational modification that regulates enzymatic activity was reported in enzyme biology several years ago, but recognition of its contributions to the control of eNOS has emerged relatively recently.⁴⁴ There is evidence that S-nitrosylation inhibits eNOS activity; and this inhibition is independent of eNOS phosphorylation and may contribute to its return to basal state following enzyme activation.⁷⁹ S-nitrosylation also decreases NO-sensitivity of downstream sGC.^{71,80} However, the impact of S-nitrosylation of eNOS or sGC on endothelial permeability has not yet been investigated. The concept that S-nitrosylation of proteins depends more on the amount of NO produced by eNOS than on eNOS subcellular location has been advanced recently⁸¹ but requires further exploration as opposing views have been proposed.⁵³

3.5 Mechanisms that restore baseline microvascular permeability

Inflammatory processes are characterized by an increase in microvascular hyperpermeability to macromolecules. The regulation of this process involves factors controlling proteins that form intercellular adhesions. We have pointed out some of the mechanisms by which eNOS-derived NO contributes to the onset of hyperpermeability. Important remaining questions, addressed in depth elsewhere on this Spotlight issue, are: how does hyperpermeability return to baseline? Is this merely a passive phenomenon or does it involve regulation by cellular signalling? The notion that microvascular permeability is regulated by counterbalancing signalling mechanisms to increase or restore permeability is one that is simple and attractive.

It is well established that baseline permeability is regulated by a constellation of factors such that reduction of microvascular permeability below baseline has been rarely reported.^{14,25,82,83} Phospholipase C has been reported to impact baseline permeability to albumin in coronary venules,¹⁴ whereas inhibition of ERK-1/2 reduces baseline permeability to FITC-dextran 70 in human umbilical vein EC.²⁵ Activation of Epac (exchange protein activated by cAMP) leads to reduced baseline in endothelial permeability.⁶⁹ Plasmapheresis appears to be the only reported experimental intervention that causes a fall in baseline permeability *in vivo*.⁸² Blockade of PLC does not change basal permeability in the hamster cheek pouch.²⁷ The failure to recapitulate *in vivo* the results obtained under *ex vivo* conditions points to the need to reconcile the differences that exist in terms of environment, interactions among different cells, etc. when working in the better controlled *in vitro* or *ex vivo* situations relative to the more complex conditions that operate *in vivo*. However, recent communications confirm that stimulation of the Epac–Rap1 pathways contribute to enhance the microvascular endothelial barrier properties *in vivo*.^{84,85}

4. Final comments

The evidence in the literature demonstrates that (i) baseline permeability is not compromised by deletion of the gene encoding for eNOS; (ii) eNOS is an integral element of the signalling pathway for the microvascular hyperpermeability response to pro-inflammatory agonists, such as PAF and VEGF; (iii) eNOS-derived NO is essential for the onset of hyperpermeability inasmuch as NO produced by other NOS-isozymes in eNOS knockout mice does not restore the ability of striated muscle microvasculature to produce a robust hyperpermeability in response to PAF; (iv) eNOS translocates from the plasma membrane to cytosol via caveolae in response to pro-inflammatory stimuli; (v) location of eNOS in the cytosol is fundamental for hyperpermeability. In regards to eNOS internalization via caveolae, we speculate that (a) caveolae may possess a necessary target recognizing molecule that allows eNOS to efficaciously promote the appropriate protein–protein signalling interactions in the intracellular environment; (b) this traffic may serve to deliver the appropriate NO concentration to achieve the correct stimulation of sGC or of an unidentified protein that represents the link to the cell junctions.

It is expected that, by combining physiologic and molecular biology approaches, future advancements in the knowledge and understanding of the mechanisms that regulate microvascular transport will lead to

development of new pharmacologic agents and treatment modalities for conditions such as systemic inflammation, diabetes and ischaemia-reperfusion injury, in which management of the microvascular barrier is desirable for returning the afflicted tissues to an optimal functional state.

Conflict of interest: none declared.

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