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Vascular Barrier Regulation by PAF, Ceramide, Caveolae, and NO - an Intricate Signaling Network with Discrepant Effects in the Pulmonary and Systemic Vasculature

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Key Words

Permeability • Endothelium • Caveolae • Acid sphingomyelinase • Ceramide • Nitric oxide

Abstract

Increased endothelial permeability and vascular barrier failure are hallmarks of inflammatory responses in both the pulmonary and the systemic circulation. Platelet-activating factor (PAF) has been implicated as an important lipid mediator in the formation of pulmonary and extrapulmonary edema. Ostensibly, the PAF-induced signaling pathways in endothelial cells utilize similar structures and molecules including acid sphingomyelinase, ceramide, caveolae, endothelial nitric oxide synthase, and nitric oxide, in pulmonary and systemic microvessels. Yet, the constituents of these signaling pathways act and respond in distinctly different and frequently opposing ways in the lung versus organs of the systemic circulation. By confronting seemingly discrepant findings from the literature, we reconstruct the differential signaling pathways by which PAF regulates edema formation in the systemic and the pulmonary vascular bed, and trace this dichotomy from the level of myosin light chain kinase via the regulation of endothelial nitric oxide synthase and sphingomyelinase signaling to the level of caveolar trafficking. Here, we propose that PAF regulates vascular barrier function in individual organs by opposing signaling pathways that culminate in increased respectively decreased nitric oxide synthesis in the systemic and the pulmonary endothelium. The present review may provide a physiological explanation for the overall disappointing results of previous pharmacological strategies in conditions of generalized barrier failure such as sepsis, and instead advertises the development of organ-specific interventions by targeting the individual composition or trafficking of endothelial caveolae.

Vascular permeability

Blood vessels of the pulmonary and systemic circulation are outlined by a monolayer of endothelial cells forming a semi-permeable vascular barrier which controls the exchange of molecules and fluid between the blood and the interstitium [1]. The Starling equation [2] describes

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the relationship between fluid filtration rate (J_v) and transmural hydrostatic and oncotic pressure gradients as

$$J_v = L_p \cdot S\ [(P_c - P_i) - \sigma\ (\pi_c - \pi_i)]$$

where L_p reflects hydraulic conductivity of the vascular barrier, S the total vessel surface area, σ the reflection coefficient, and P and π the hydrostatic and oncotic pressure in the capillary (c) and interstitial (i) compartment, respectively. The product of L_p and S yields the vascular filtration coefficient K_f, a constant of proportionality that reflects vascular permeability and which can be measured in intact vascular beds of the pulmonary and systemic circulation using appropriate physiological techniques [3-5]. The Starling equation provides the basis for the classification into "hydrostatic" and "permeability" types of edema, i.e. edema formation as a result of altered hydrostatic ($P_c - P_i$) or oncotic ($\pi_c - \pi_i$) pressure gradients on the one hand [6], and due to changes in vascular permeability (K_f) on the other.

Permeability-type edema is a characteristic hallmark of allergic and inflammatory responses, and contributes to the morbidity and mortality in anaphylaxis, systemic inflammatory disorders such as sepsis or SIRS (systemic inflammatory response syndrome) and in pulmonary inflammation as seen in acute lung injury (ALI) and its most severe form, the adult respiratory distress syndrome (ARDS) [7-9]. The underlying increase in vascular permeability evolves from a dysregulation of endothelial barrier function brought about by inflammatory mediators and cells interacting with the endothelial layer. While various contributing signaling pathways have been identified in the past [1, 10, 11], the exact mechanisms mediating endothelial barrier failure in inflammatory conditions remain to be elucidated.

Relatively few mediators are capable of altering pulmonary vascular permeability within minutes, among them platelet-activating factor (PAF) and sphingolipids. Sphingosine-1-phosphate (S1P) enhances vascular barrier function by activation of its G-protein coupled receptor $S1P_1$ and subsequent downstream activation of small Rho GTPases, cytoskeletal reorganization, adherens junction and tight junction assembly, and focal adhesion formation [12]. Ceramide, on the other hand, increases vascular permeability by a mechanism that is not yet fully understood, but involves the regulation of both endothelial Ca^{2+} signaling and NO formation [13-16]. Here, we will focus on the role of caveolae - membrane rafts rich in sphingolipids [17] which play important roles in the regulation of endothelial Ca^{2+} signaling [18, 19] and NO synthesis [20] - as integrating signaling platforms in the pathogenesis of permeability-type edema. As we will discuss, the consideration of these intricate structures allows for new hypotheses pertaining to the frequently discrepant findings and divergent regulatory pathways of endothelial barrier failure in the pulmonary versus the systemic vasculature.

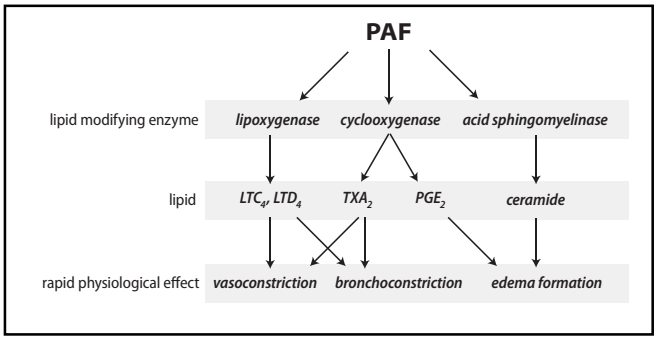

Fig. 1. PAF effects and signaling pathways in the lung. Within a few minutes PAF activates three lipid modifying enzymes leading to production of leukotriene C_4 and D_4 (LTC_4, LTD_4), thromboxane A_2 (TXA_2), prostaglandin E_2 (PGE_2) and ceramide. Key references in support of these conclusions are [13, 24, 25].

PAF in permeability-type edema

One important inflammatory mediator that plays a pivotal role in many experimental models of permeability-type lung edema is the phospholipid PAF [21]. The clinical relevance of this mechanism is highlighted by the fact that ARDS patients show an association between PAF-acetylhydrolase allelic variation, plasma activity, and outcome [22]. In the lungs, PAF contracts airway and vascular smooth muscle [21] and causes vascular gaps in capillaries, venules and veins [16, 23]. These effects are brought about by activation of other lipid modifying enzymes (Fig. 1), in particular cyclooxygenase and lipoxygenase which trigger smooth muscle contraction via their products thromboxane and leukotrienes C_4 and D_4 [24, 25], and cyclooxygenase and acid sphingomyelinase regulating lung vascular permeability via formation of prostaglandin E_2 (PGE_2) and ceramide [13, 21], respectively.

PAF likewise causes hyperpermeability in microvessels of the systemic circulation by generating interendothelial gaps [26, 27] that are large enough to allow for the extravasation of microspheres of 100 nm in diameter [28]. Notably, PAF-induced hyperpermeability in rat mesenteric venules appears to be independent of

actin-myosin contractions as indicated by the inability of the myosin light chain kinase (MLCK) inhibitor ML-7 (10 μM) to block the PAF-induced increase in L_p as measured by the modified Landis technique [26]. Consistently, PAF did not cause MLCK phosphorylation in human umbilical vein endothelial cells (HUVEC) [23]. In contrast, in the lungs ML-7 (35 μM) attenuated PAF-induced edema formation [14]. These conflicting findings allude to the possibility that the mechanisms underlying PAF-induced edema formation may differ between microvascular beds, namely that PAF increases lung microvascular permeability at least partly via an actin-myosin contractile mechanism, while paracellular gap formation in mesenteric venules is caused mainly by the untightening of endothelial-endothelial cell adhesion structures. The latter conclusion is supported by the finding that PAF increases mucosal permeability in the rat intestine and in mouse embryonic heart endothelial cells via tyrosine phosphorylation of E-cadherin [29, 30].

Regulation of vascular permeability by nitric oxide

The discrepant effects of PAF on lung and systemic microvessels (Fig. 2) may in part be attributed to its seemingly divergent effects on nitric oxide (NO) production in endothelial cells from the lung and other organs. Herein we focus on the instantaneous effects of PAF and NO on vascular permeability that are different from effects of PAF and NO on leukocyte activation that do contribute to PAF-induced edema at later time points [31-33].

In the isolated perfused lung preparation PAF caused a rapid cessation of endothelial NO synthesis in venular capillaries within less than 5 min [16] (Fig. 2D), as demonstrated by real-time imaging of endothelial cells which had been loaded *in situ* with the NO sensitive dye diaminofluorescein (DAF) by our recently reported techniques [34, 35]. Before, a similar almost instantaneous reduction in NO had only been described for PAF-treated human mesangial cells in culture [36]. Conversely, in the hamster cheek pouch (Fig. 2C) [37, 38], in perfused mesenteric venules [39], and in HUVECs [40] PAF induced a marked increase in endothelial NO formation.

In all of these instances, the regulation of endothelial NO synthesis appears to be critical for PAF-induced hyperpermeability. In the systemic microvasculature of the hamster cheek pouch, the increased leakage of fluorescein isothiocyanate (FITC) dextran in response to

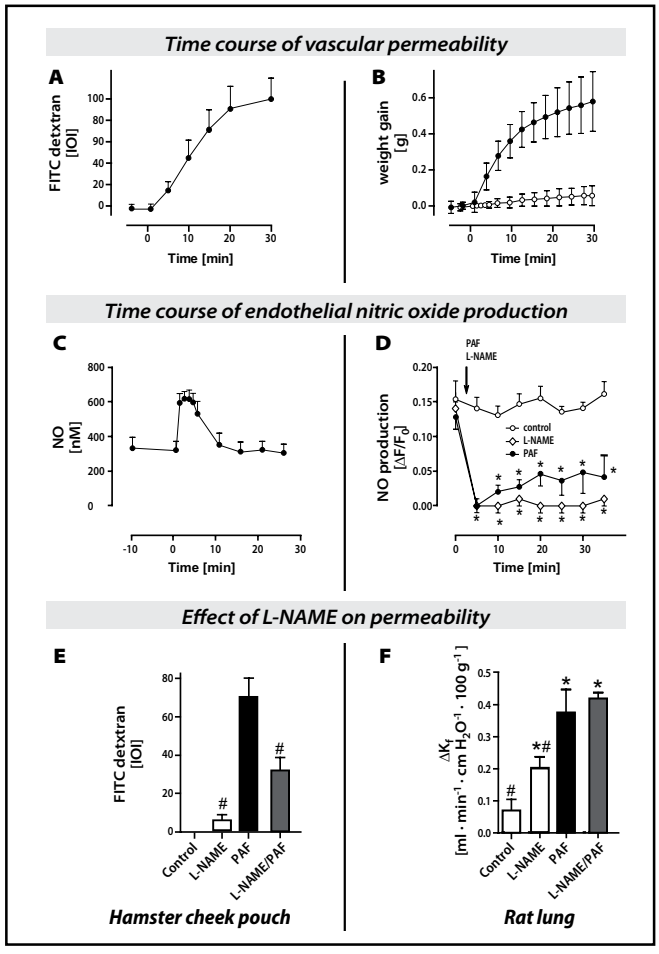

Fig. 2. Effects of PAF on vascular permeability and NO formation. Experiments were performed in the hamster cheek pouch following topical application of 100 nMol/L PAF (A, C, E) and in isolated perfused rat lungs challenged with 5 nMol PAF which after equilibration corresponds to 50 nMol/L (B, D, F). PAF was always added at t=0 or as indicated by the arrows. A. Hamster cheek pouch: Extravasation of FITC-dextran expressed as integrated optical intensity (IOI) [37]. B. Rat lung: weight gain in PAF-treated (filled circles) and control lungs (open circles). C. Hamster cheek pouch: nitric oxide levels measured with a NO-sensitive microelectrode [38]. D. Rat lung: endothelial nitric oxide production assessed by DAF-FM fluorescence microscopy in control lungs, lungs treated with PAF or 250 μM L-NAME as positive control for inhibited NO production [16]. E. Extravasation of FITC-dextran expressed as integrated optical intensity (IOI) in the hamster cheek pouch treated with PAF ± L-NAME (10 μMol/L) [37]. F. Rat lung: change in filtration coefficient (K_f) in lungs treated with PAF ± L-NAME (250 μMol/L) [16]. The data from the hamster cheek pouch are shown as mean±SEM, the data from the rat lung as mean±SD. *, $p<0.05$ vs. control, #, $p<0.05$ vs PAF. Data in panel D were analyzed by the Kruskal-Wallis test, data in panel F by two-sided t-tests and p values corrected for multiple comparisons according to the false-discovery rate procedure. Data in panel E were analyzed by the Student's-Newman-Keuls test.

PAF is inhibited by nitric oxide synthase (NOS) inhibitors (Fig. 2E) [37, 41], and this inhibition is overcome by concomitant administration of the exogenous NO donor *S*-nitroso-N-acetyl-penicillamine (SNAP) [41], indicating that NO plays an obligatory role in PAF-induced leakage of systemic microvessels. This notion is supported by studies in rat mesenteric microvessels [42], in the cremaster muscle of mice deficient in endothelial NO synthase (eNOS) [43] and *in vitro* in monolayers of coronary postcapillary venular endothelial cells in which PAF induces a characteristic hyperpermeability that is abolished after depletion of endogenous eNOS with small interfering RNA [44].

In contrast, recent studies from our own groups revealed that in isolated perfused lungs, PAF-induced edema formation is not mitigated by the NOS inhibitor L-N^G-Nitroarginine methyl ester (L-NAME) (Fig. 2F), but instead attenuated by the exogenous NO donor PAPA NONOate [16]. In rats *in vivo*, L-NAME even exacerbated the PAF-induced extravasation of albumin in the lungs, but interestingly also in other organs such as the duodenum and the skin, while it had no effect on PAF-induced hypotension [45].

The view that endothelial-derived NO is indeed barrier-protective in the pulmonary circulation is in line with our previous finding that eNOS inhibition aggravates lung vascular barrier failure under hydrostatic stress [46]. The fundamental role of NO in the regulation of pulmonary vascular permeability is further illustrated by the observations that lack of endothelial NO production following either L-NAME administration or eNOS ablation increases the K_f (Fig. 2F) as well as vascular permeability at interendothelial junctions [16, 47].

Thus, a pattern of two differential pathways emerges in that in the lungs PAF increases microvascular permeability by an MLCK-dependent mechanism that is facilitated by cessation of endothelial NO production, while PAF-induced hyperpermeability in many systemic microvessels is independent of MLCK, but instead requires stimulation of endothelial NO synthesis.

The coinstantaneous existence of these initially divergent, yet ultimately convergent pathways raises two critical questions which we will address next, i.e. first, what is the molecular basis for the differential regulation of endothelial NO synthesis in pulmonary versus systemic microvessels, and second, how NO may cause myofilament-independent, paracellular gap formation in extrapulmonary organs when it simultaneously attenuates MLCK-dependent hyperpermeability in the lung?

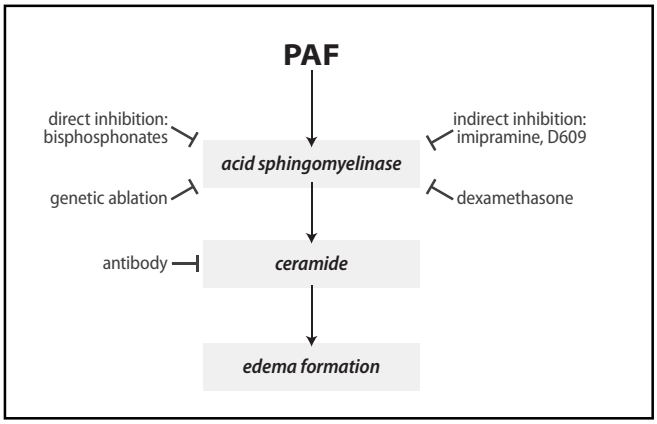

Fig. 3. Inhibitors of ASM and ceramide, respectively, and their effect on PAF-induced edema. Key references in support of the concept are [13, 50].

Regulation of endothelial NO synthesis by sphingomyelinase and ceramide

To illuminate the differential regulation of eNOS by PAF it is necessary to take a closer look at the underlying endothelial signaling events in both lung and systemic blood vessels. In endothelial cells of the rat mesentery, PAF increased cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_i$) more than 5-fold within 1-2 min, thus preceding the increase in endothelial NO production [39]. Removal of extracellular Ca^{2+} abolished the endothelial NO response to PAF, thus identifying PAF-induced Ca^{2+}-influx as an obligatory signal upstream of eNOS activation in systemic microvessels.

While PAF also induces endothelial Ca^{2+} signaling in the lung and contributes to barrier failure [14, 48], this second messenger response cannot account for the rapid cessation in NO formation following PAF, since an increase in endothelial $[Ca^{2+}]_i$ would be expected to stimulate rather than to inhibit eNOS [49]. Hence, in lung endothelial cells PAF must induce one or several dominant mechanisms to block eNOS almost instantaneously in spite of the simultaneous increase in endothelial $[Ca^{2+}]_i$.

Sphingolipid mediators may present such a PAF-dependent signaling pathway. We had previously shown that PAF causes lung edema by two mechanisms, activation of the cyclooxygenase pathway and acid sphingomyelinase (ASM)-dependent production of ceramide . Importantly, the ceramide-dependent increase in lung vascular permeability is not attributable to a PAF-dependent induction of endothelial apoptosis [21], but occurs as the result of ceramide-dependent signaling

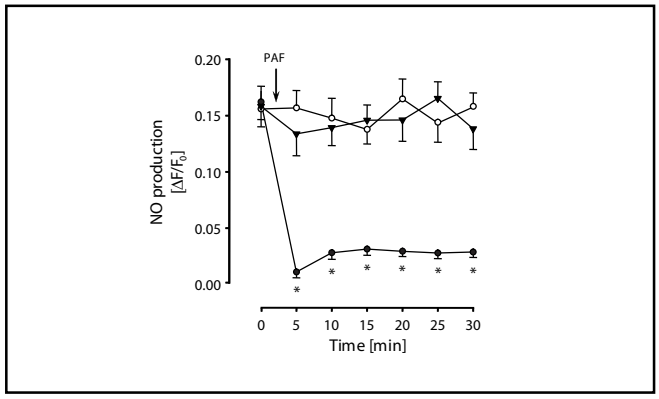

Fig. 4. Inhibition of endothelial NO production by PAF is absent in ASM-deficient (*Asm*$^{-/-}$) mice. Endothelial NO production was determined by *in situ* real-time fluorescence microscopy in isolated perfused mouse lungs as previously described [46]. Endothelial NO production is shown as 5 min averages in lungs of wild type (WT) control mice (open circles), and PAF (bolus injection of 5 nMol PAF after baseline recording) treated lungs of wild type (closed circles) and ASM$^{-/-}$ mice (closed triangles). NO production was quantified as increase in fluorescence of DAF-FM loaded endothelial cells over 5 min intervals relative to baseline Δ (F/F_0). PAF treatment caused a rapid cessation of endothelial NO production in lungs of WT, but not in lungs of ASM$^{-/-}$ mice. * $p<0.05$ vs WT control, mean±SEM, n=5. Data were analyzed by the Kruskal-Wallis test. These experiments were performed in collaboration with D. Adam (Kiel).

pathways that increase vascular permeability [13]. Accordingly, PAF-induced lung edema is attenuated by agents that interfere with ceramide or ceramide synthesis, such as the recently discovered ASM inhibitor ARC39 (structure 7c in ref. [50]) or related bisphosphonates [50], ASM pathway inhibitors such as the xanthogenate D609 or the tricyclic antidepressant imipramine, ceramide-specific antisera, steroids, or in mice deficient in ASM [13] (Fig 3). The pathophysiological relevance of ASM is further highlighted by the fact that several lung diseases are mitigated by genetic or pharmacological blockade of this enzyme [15]. In addition, elevated ASM activities are present in a variety of pathological conditions associated with increased vascular permeability such as models of acute lung injury [13] and sepsis [51] as well as in septic human patients [52].

Using real-time imaging of endothelial NO synthesis in the isolated perfused lung preparation [34, 35], we recently demonstrated that the rapid cessation of lung endothelial NO synthesis induced by PAF is mediated via the ASM-ceramide axis, in that it is blocked by imipramine, a pharmacological inhibitor of the ASM pathway, and replicated by lung perfusion with exogenous ASM [16]. This notion is further substantiated by previously unpublished data shown in Fig. 4 which demonstrate that the inhibition of endothelial NO formation by PAF is abrogated in isolated perfused lungs of mice deficient in ASM (*Asm*$^{-/-}$).

Once again the signaling events in endothelial cells of the systemic circulation are diametrically opposed to those in the lung, in that ceramide activates eNOS and stimulates endothelial NO formation in cultured bovine aortic endothelial cells (BAEC) [53, 54], as well as in eNOS expressing chinese hamster ovary (CHO) cells [55, 56]. Notably, the ceramide-induced stimulation of eNOS in systemic endothelial cells is independent of Ca^{2+}-regulated pathways since it is unaffected by the intracellular Ca^{2+} chelator 1,2-bis-o-aminophenoxyethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) and not accompanied by a simultaneous $[Ca^{2+}]_i$ signal [54], and likewise seems to be independent of the source of ceramide, as it is equally triggered by stimulation of both ASM and neutral sphingomyelinase (NSM) [57]. Taken together, sphingomyelinase-dependent ceramide formation causes divergent activation and inhibition of eNOS in systemic and pulmonary endothelial cells, which is likely to underlie the differential NO response and may contribute to the different forms of hyperpermeability induced by PAF in these cells. The question to be addressed next is the mechanism(s) underlying the differential regulation of eNOS by ceramide.

Role of caveolae in the endothelial response to PAF

Caveolae are caveolin positive lipid rafts that form small (50-100 nanometer) cup- or omega-shaped invaginations of the plasma membrane [58]. Like other lipid rafts, caveolar microdomains are enriched in cholesterol and sphingolipids such as sphingomyelin , the substrate for ceramide formation by sphingomyelinases. Importantly, caveolae have been recognized to play a fundamental role in the regulation of NO formation by eNOS [20]. In addition, the functioning of several transient receptor potential (TRP) channels appears to depend on their localization inside caveolae [19]. Several members of the canonical (TRPC) and vanilloid (TRPV) subfamilies of TRPs are expressed in vascular endothelial cells where they serve as Ca^{2+} entry channels and mediate endothelial hyperpermeability in response to inflammatory or mechanical stimuli [11, 19]. Over and above that, the PAF receptor itself was recently shown to localize within

lipid raft domains in close proximity to caveolin-1 (cav1), the major structural protein of caveolae, with potential interaction through a caveolin-1-binding sequence in the PAF receptor C terminus [60]. Hence, caveolae form an apt microenvironment for PAF to regulate endothelial permeability via ceramide and eNOS/NO signaling.

Notably, caveolae and cav1 regulate eNOS in a twofold and antagonistic manner. While the enrichment of eNOS in caveolae enables the compartmentation of the enzyme and thereby optimizes the process leading to its activation [61], direct interaction of eNOS with the so-called scaffolding domain of cav1 keeps the enzyme in its inactivated, dormant state in the absence of stimulation. The importance of this mechanism is illustrated by the observation that administration of a cell-permeable form of this scaffolding domain, prevented the PAF-induced increase in vascular permeability in rat mesenteric venules probably by interception of the eNOS-cav1 interaction in caveolae [42].

According to the conventional paradigm, increased $[Ca^{2+}]_i$ activates calmodulin which liberates the eNOS from cav1 and thus promotes NO production [62-64]. In line with this notion and the previous observation that PAF stimulates Ca^{2+}-entry in mesenteric endothelial cells [39], Sánchez and colleagues observed a PAF-induced translocation of eNOS away from caveolae into the cytosol in ECV304 cells - a cell line that was originally described as endothelial cell line but later turned out to be an epithelial cell line derived from bladder carcinoma [65] - transfected with an eNOS-green fluorescent protein construct [66]. In a subsequent study in coronary postcapillary venular endothelial cells, the same authors showed that eNOS translocation from caveolae to the cytosolic fraction was associated with increased NO production and a concomitant increase in endothelial permeability that was abrogated in cells depleted of eNOS with small interfering RNA [44]. In line with the view that caveolae may also serve as a vehicle for eNOS internalization and activation [67, 68], inhibition of caveolar scission by transfection of the dynamin dominant-negative mutant dyn2K44A likewise prevented the endothelial hyperpermeability in response to PAF [44]. The notion that anchoring of eNOS-containing caveolae to the plasma membrane may inhibit PAF-induced hyperpermeability by preventing NO delivery to subcellular targets was substantiated in a recent cell culture study in which dyn2K44A transfection eliminated NO production and blocked the permeability response to PAF, while a dominant negative mutant of cav1 (cav1Y14F) did not abrogate NO production in cells with caveolae bound to the plasma membrane, but was similarly effective in preventing the PAF-induced hyperpermeability [69].

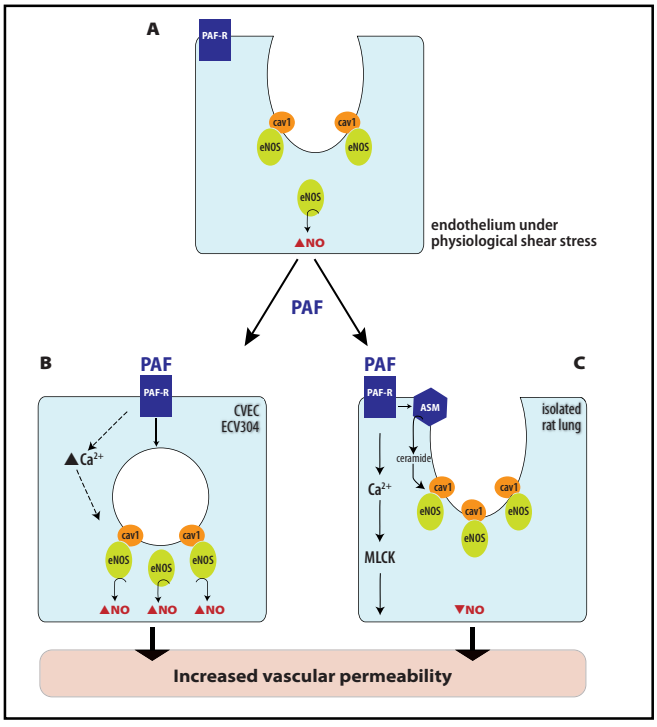

Fig. 5. Proposed schemes for the role of endothelial NO production in PAF-induced hyperpemeability. A. *In vivo*, shear stress stimulates continuous NO production in endothelial cells of both the pulmonary and systemic circulation. B. In bovine coronary postcapillary venular endothelial cells (CVEC) and in ECV304 cells PAF stimulates NO production that depends on internalization of caveolae [69]. The role of Ca^{2+} was not investigated in these cells, but seems likely in view of the well known role of Ca^{2+} in eNOS activation and from experiments in isolated rat mesenteric vessels [39]. C. In isolated rat lungs, PAF causes a cessation of NO production by stimulating the acid sphingomyelinase (ASM)-dependent translocation of eNOS to caveolae [16]. PAF also causes Ca^{2+}-dependent activation of the myosin light chain kinase (MLCK) that is thought to increase vascular permeability by endothelial cell contraction [14]. Our preliminary data suggest that the PAF-induced Ca^{2+} influx is also mediated by the ASM.

Taken together, these data suggest a critical role for caveolae in the PAF-induced endothelial hyperpermeability of the systemic circulation. Here PAF causes the internalization of eNOS-containing caveolae and the subsequent targeted delivery of NO biosynthesis to subcellular structures critical for the regulation of paracellular gap formation (Fig. 5). While this concept provides an intriguing model for the regulation of eNOS and vascular permeability by PAF, it also raises a number of questions pertinent to the role of conventional eNOS

regulators such as Ca^{2+} or PI3K-Akt signaling in this scenario [70]. First, since the density of caveolae is remarkably lower in cultured cells when compared with native endothelial cells [64], it remains to be determined whether caveolar internalization is an equally effective and relevant regulator of eNOS *in vivo*. Second, endothelial NO production in non-perfused tissues and in cell culture may differ from NO synthesis *in situ* [71]. Particularly pertinent problems in cell culture are unphysiologically high O_2-levels (O_2 is required for NO-production. Cells are commonly cultured at 21% O_2, hence at pO_2 levels considerably higher than those within the body) and loss of NO-inactivation in endothelial cell monolayers [71]. These factors would lead to supranatural NO synthesis in cell culture. Third, does the activation of eNOS following caveolar internalization require a Ca^{2+}-dependent release from cav1? NO production in response to caveolar internalization following activation of the albumin-binding glycoprotein gp60 has been shown to be independent of the intracellular Ca^{2+} chelator BAPTA [68], but whether the same holds true for the NO response to PAF remains to be determined. Fourth, how does caveolar localization affect the regulation of eNOS by Ser^{1177} phosphorylation? Phosphorylation at its serine residue 1177 constitutes a classic regulatory pathway by which serine/threonine specific protein kinases such as protein kinase A, Akt, AMP-activated protein kinase (AMPK), or calmodulin-dependent protein kinase II (CaMKII) activate eNOS in response to a variety of stimuli including vascular endothelial growth factor, estrogen, or shear stress [70, 72]. Unexpectedly, Ser^{1177}-eNOS phosphorylation by PAF stimulation is not mitigated in dyn2K44A transfected cells, although NO production is completely eliminated [69]. Even more surprisingly, cav1Y14F transfection attenuates the endothelial NO response to PAF only moderately, yet dephosphorylates eNOS at Ser^{1177}. These findings suggest that eNOS phosphorylation at Ser^{1177} and NO production may dissociate depending on caveolar localization and/or the interaction between caveolae and eNOS, a notion that deserves further exploration.

In view of the divergent regulation of vascular permeability and eNOS by PAF and sphingolipids in systemic versus lung microvessels outlined above, it may not come as a surprise that caveolar regulation of eNOS in PAF-stimulated pulmonary endothelial cells contrasts strikingly from the signaling pathway identified in coronary postcapillary venular endothelial cells and the ECV-304 cell line. Based on a procedure originally developed by Jan Schnitzer and colleagues [73], we recently established

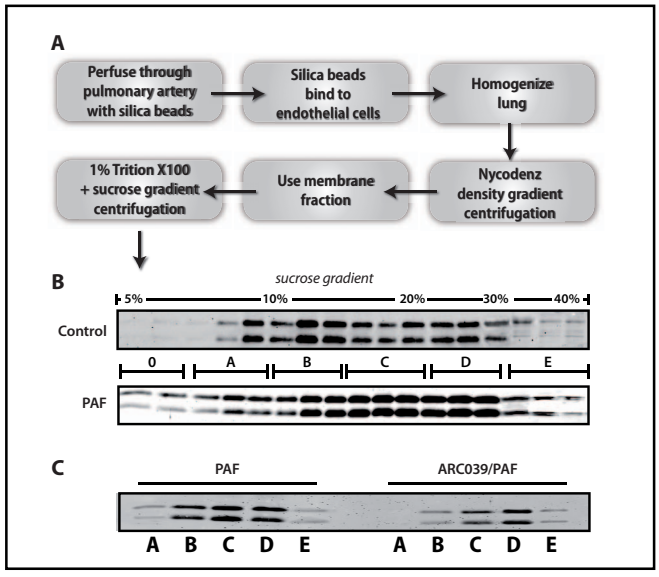

Fig. 6. Preparation of caveolae and PAF-induced caveolin-1 recruitment. A. Preparation of membrane fractions from pulmonary artery endothelial cells according to ref. [73]. These fractions were prepared 10 min after PAF administration. Briefly, isolated lungs were perfused with 1% cationic colloidal silica beads, and subsequently homogenized and filtrated. The suspension was mixed with 1.02 g/ml Nycodenz (containing 20 mM KCl), layered over 0.5-0.7 g/ml Nycodenz (containing 60 mM sucrose), centrifuged at 20.000 rpm for 30 min at 4°C and the pellet containing the silica-coated endothelial membranes fragments was resuspended in 1mL buffer. 10% cold Triton-X-100 (final concentration of 1%) was added to the membranes for 60 min at 4°C and subsequently mixed with 80% sucrose to achieve a 40% membrane-sucrose-solution. A 30-5% sucrose gradient was layered on top. Samples were centrifuged at 4°C and at 30.000 rpm for 16-18 h and volumes of 150 µl were sampled from the top to the bottom and collected as membrane fractions. The pellet was solubilized in 150 µl buffer and 5 µg of protein were analyzed on the gels. B. Immunostaining of 17 different density fractions obtained from sucrose gradient centrifugation with an antibody against caveolin-1. For experiments shown in C, the fractions were pooled into 0-E as indicated. The pooled fractions B-D are considered to contain caveolae (from ref. 16 with permission). C. Caveolin-1 staining of fractions prepared from a PAF-treated lung and one that was pretreated with the novel ASM inhibitor ARC39 (structure 7c in ref. [50]). These experiments were done in cooperation with C. Arenz (Berlin).

a method to isolate caveolae from isolated perfused rat lungs in that we perfuse lungs with silica beads to tag the endothelial cells and separate their plasma membrane fractions in a sucrose gradient (Fig. 6). Immunoblottings of the resulting fractions show a typical distribution for cav1 predominantly in the fractions B-D which are expected to contain the caveolae (Fig. 6B). This approach

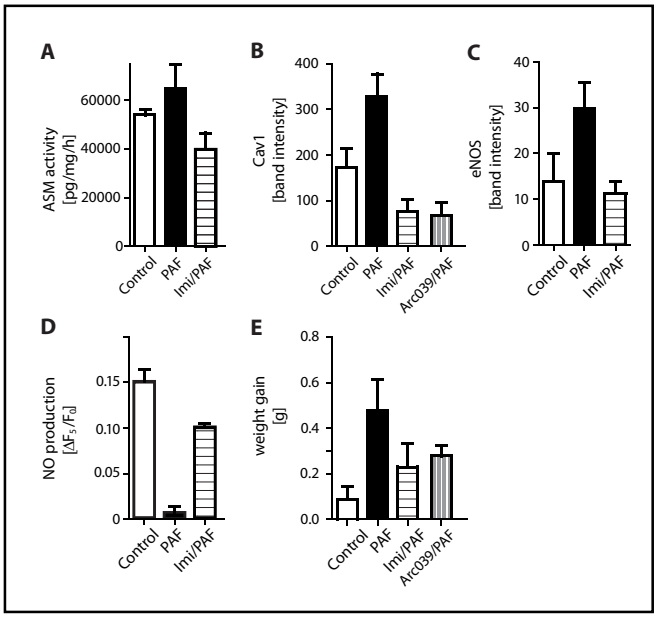

Fig. 7. PAF-induced changes in caveolae, endothelial NO production and edema formation. Panels A to C show measurements in caveolar fractions (sum of fractions B-D from Fig. 6) freshly isolated 10 min after treatment of isolated rat lungs with a bolus of 5 nMol PAF or in respective controls. A. Enzymatic activity of acid sphingomyelinase. B. Immunoblot against caveolin-1. C. Immunoblot against endothelial NO-synthase. D. *In situ* fluorescence microscopy to quantify NO production (fluorescence probe: DAF-FM) in endothelial cells determined 5 min after the addition of PAF and referenced to the NO production at baseline. E. Weight gain of isolated perfused rat lungs 10 min after the addition of PAF. Lungs were always infused with a bolus of 5 nMol PAF and pretreated 10 min before PAF administration with 10 μMol/L imipramine or 10 μMol/L Arc39 (structure 7c in ref. [50]). Data are shown as mean ± SD of at least 4 independent experiments. Most data represent a summary from ref. [50], the Arc39 data in panel E are from ref. and the Arc39 data in panel B have not been published before.

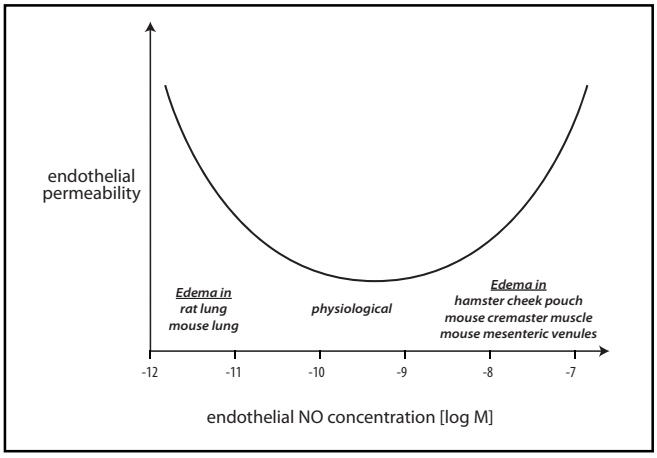

Fig. 8. Regulation of PAF-induced edema formation in different tissues. The abscissa shows putative NO concentrations derived from ref. [71]; the ordinate indicates increasing vascular permeability on an arbitrary scale. In rat and mouse lungs PAF leads to a decrease in NO formation that is related to edema formation (Fig. 2, 4, 7 and ref. [16]). In extrapulmonary tissues, such as the hamster cheek pouch (Fig. 2, [37, 41]), the mouse cremaster muscle [43] and the mouse mesenteric venules [39, 42, 43] PAF-induced edema is caused by increased endothelial NO production.

permitted us to study in detail how PAF treatment alters the composition and the functional properties of the caveolar compartment *in situ*.

In line with the regulation of eNOS by PAF and ASM/ceramide in the lung, PAF treatment induces a rapid (< 10 min) increase of ASM activity (Fig. 7A) in caveolar fractions in parallel with the recruitment of both cav1 and eNOS into caveolae in the pulmonary vasculature (Fig. 6B, 7B,C) [16]. Inhibition of ASM activity by ARC39 or imipramine blocked all these caveolar responses and prevented the PAF-induced cessation of NO production and the edema formation (Fig. 6C,7). These data show that the activation of ASM plays a critical role in the PAF-induced changes in caveolar composition and function, either by altering the biophysical properties of caveolae or by generation of a second messenger [15].

Our findings indicate a swift translocation of cav1 in response to PAF, as the increased abundance of the protein in caveolar fractions is paralleled by a reciprocal decrease in the non-caveolar fractions, while the total amount of cav1 in endothelial cell lysates remains unchanged [16]. Importantly and in contrast to the effects described in cultured coronary postcapillary venular endothelial cells, PAF-induced recruitment of cav1 in endothelial cells of the intact lung is not associated with an internalization of caveolae, as signified by the fact that the density of intracellular caveolae was not increased [16].

The apparent discrepancy in caveolar internalization may provide the mechanistic basis for two of the differential responses to PAF in endothelial cells of the systemic and pulmonary vasculature (Fig. 5): First, because PAF-stimulated NO production in systemic endothelia requires caveolar scission and trafficking (as illustrated by the lack of NO formation in dyn2K44A transfected cells [69]), the absence of caveolar internalization in pulmonary endothelial cells may explain why these cells do not increase NO synthesis in response

to PAF. Second, differences in caveolar trafficking may underlie the opposing roles of endothelial-derived NO formation in PAF-induced hyperpermeability. In coronary postcapillary venular endothelial cells, internalization of eNOS is considered to allow for targeted NO delivery to subcellular structures and the subsequent increase in endothelial permeability by promoting the internalization or inhibiting the arrival of endothelial junctional proteins [66, 69]. NO-driven *S*-nitrosylation of barrier-regulating proteins could play a major role in this scenario, since it has been shown to be associated with increased vascular permeability in pathological conditions such as cerebral ischemia and reperfusion [74]. The notion that eNOS internalization and targeted NO delivery are critical for PAF-induced barrier failure in systemic endothelial cells is supported by the fact that PAF-stimulated NO synthesis does not cause hyperpermeability in cav1Y14F transfected cells in which caveolae are anchored to the plasma membrane [69].

In contrast to the PAF-induced internalization of eNOS in coronary postcapillary venular endothelial cells, PAF recruits eNOS to caveolae in the plasma membrane in pulmonary endothelial cells (Fig. 7C). The fact that this effect coincides with a parallel recruitment of cav1 (Fig. 6, 7B), and a rapid cessation of endothelial NO synthesis (Fig. 2D) is in line with the well-established paradigm of eNOS inactivation by direct interaction with cav1 [75-77], and provides a mechanistic explanation for the inhibition of endothelial NO production by PAF in lung endothelial cells (Fig. 5).

The cessation of endothelial NO production in turn facilitates lung vascular leakage, indicating that basal NO synthesis constitutes a barrier-protective signal in the pulmonary circulation. The exact mechanisms underlying this stabilizing effect remain to be elucidated, but may involve the inhibition of endothelial TRP channels which are in close proximity to ASM and eNOS due to their localization in and/or recruitment to caveolar microdomains [19, 78]. Agonist-induced Ca^{2+} influx via TRP channels contributes critically to endothelial hyperpermeability, as is suggested by a series of findings: (i) increased expression of TRPC1 augments Ca^{2+} influx and permeability increase in thrombin-stimulated human dermal microvessel endothelial cells [79], (ii) $TRPC4^{-/-}$ mouse-lung endothelial cells show an attenuated $[Ca^{2+}]_i$ increase and lack of cell retraction in response to thrombin, and the characteristic increase in lung vascular permeability in response to protease-activated receptor-1 activation is absent in $TRPC4^{-/-}$ as compared to wild type mice [80], (iii) activation of TRPV4 by mechanical stress or its specific agonist 4α-phorbol 12,13-didecanoate (4αPDD) causes endothelial Ca^{2+} influx and increases microvascular permeability in isolated perfused lungs [46, 81, 82], while lung vascular barrier failure in response to increased hydrostatic stress or overventilation is attenuated in $TRPV4^{-/-}$ mice [46, 82, 83]. Importantly, members of both the canonical and vanilloid subfamilies of TRP channels have been shown to be negatively regulated by NO and cGMP [46, 84] which may provide one possible mechanism how basal NO production stabilizes the microvascular barrier in lung endothelial cells.

Summary

The pulmonary and the systemic circulation differ in many fundamental aspects such as the blood pressure (low versus high), the arterio-venous oxygen gradient (oxygenation versus deoxygenation), the response to hypoxia (vasoconstriction versus vasorelaxation [85]), and the endothelial permeability in response to histamine (no effect versus increased [86]), to name some of the most striking discrepancies. Another example of this dichotomy appears to be endothelial NO production in response to PAF: NO production decreases in lung endothelial cells and increases in the systemic circulation. Surprisingly, both responses have the same effect, in that they increase vascular permeability (Fig. 8). Therefore, we conclude that vascular barrier function in both the lung and extrapulmonary organs is only maintained if NO is kept at its physiological level. A clinical implication of this insight is that it will be enormously difficult to treat diseases with a wide-spread increase of vascular permeability, such as sepsis, by NOS-inhibitors or NO-donors, because these agents will be barrier-protective in one vascular bed, but detrimental in another.

It should be understood that our conclusions relate specifically to the effects of PAF in dissimilar vascular beds. Clearly, other mediators or pathophysiological situations may produce different scenarios. For instance, vascular permeability can also be increased by excessive NO production in the lungs [87] and conversely by lack of endothelial NO production in systemic vessels [47]. Thus, the permeability of any vascular bed is increased once endothelial NO levels do significantly deviate from the physiological norm. Remarkably, however, and focus of this review is the finding that PAF can cause deviations from this norm in either direction depending on the respective vascular bed.

On the basis of the discussed data, a scenario unfolds in which PAF equally causes barrier failure in systemic and pulmonary microvessels by signaling through an intricate network of apparently similar structures and molecules including ASM, ceramide, caveolae, eNOS and NO, yet in fact by distinctively different mechanisms (Fig. 4, Fig. 8). In pulmonary endothelial cells, PAF causes barrier failure by activation of both, cyclooxygenase and ASM resulting in the formation of PGE_2 and ceramide. The latter increases endothelial permeability partly through activation of MLCK by stimulation of Ca^{2+} signaling and a concomitant inhibition of basal NO synthesis which is brought about by the parallel recruitment of cav1 and eNOS to caveolae. Conversely, in endothelial cells of the systemic circulation PAF increases permeability by MLCK-independent formation of paracellular gaps. In these cells both PAF and possibly also ASM/ceramide increase endothelial NO synthesis. The PAF-induced stimulation of eNOS in the non-pulmonary endothelium is brought about by caveolar internalization, which simultaneously allows for targeted delivery of NO to subcellular targets which appears to constitute a prerequisite for the subsequent permeability increase. Taken together, the same constituents regulate endothelial permeability in the lung and extrapulmonary vascular beds in opposing manners. For reasons which remain to be elucidated, this divergence initiates at the level of caveolar trafficking. Molecules regulating caveolar composition and trafficking may thus present promising targets for interventional strategies specifically designed to prevent vascular barrier failure individually in the lung or in the systemic circulation.

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