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Platelet-activating factor reduces endothelial NO production - Role of acid sphingomyelinase

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Abstract

Platelet-activating factor (PAF) is a mediator of pulmonary edema in acute lung injury that increases vascular permeability within minutes partly through activation of acid sphingomyelinase (ASM). Since caveolae are rich in sphingomyelin and caveolin-1, that blocks eNOS by direct binding, we examined the relationship between ASM, caveolin-1 and eNOS activity in the regulation of vascular permeability by PAF. In caveolar fractions from pulmonary vascular endothelial cells (isolated from perfused rat lungs) the abundance of caveolin-1 and eNOS increased rapidly after PAF perfusion. PAF treatment decreased endothelial NO formation as assessed by *in situ* fluorescence microscopy. Restoration of endothelial NO levels with PAPA-NONOate mitigated the PAF-induced edema. PAF treatment increased the ASM activity in caveolar fractions and perfusion with ASM decreased endothelial NO production. Pharmacological inhibition of the ASM pathway with imipramine, D609 or dexamethasone blocked the PAF-induced increase of caveolin-1 and eNOS in caveolae, the decrease in endothelial NO production and edema formation. We conclude that PAF causes ASM-dependent enrichment of caveolin-1 in caveolae of endothelial cells, leading to decreased endothelial NO production which contributes to pulmonary edema formation. These findings suggest rapid reduction in endothelial NO production as a novel mechanism in the regulation of vascular permeability.

Keywords: pulmonary edema, vascular permeability, nitric oxide, caveolin, endothelial NO synthase, steroids

Introduction

PAF is an important regulator of vascular functions in the lungs [1]. The actions of PAF are mediated by lipid modifying enzymes: cyclooxygenase and lipoxygenase to produce thromboxane and leukotrienes that mediate vaso- and broncho–constriction [2], and cyclooxygenase and acid sphingomyelinase (ASM) to form PGE_2 and ceramide that increase vascular permeability [3,4]. The molecular consequences of the PAF-induced activation of ASM in the pulmonary endothelium remain to be identified. ASM is a critical enzyme in sphingolipid biochemistry that under conditions of inflammation or stress can rapidly be activated to convert sphingomyelin to ceramide [5]. One well described consequence of ASM activation is formation of ceramide-rich membrane microdomains that are involved in several cell functions such as stress signaling, apoptosis after death cell receptor stimulation and infection with various pathogens [6,5]. Caveolae, which are abundant in endothelial cells, represent a specialized form of microdomains that contain high levels of sphingomyelin as well as ASM activity [7].

Endothelial NO synthase (eNOS) is located inside caveolae and is kept in its inactive state by binding to caveolin-1 [8]. Both caveolin- and eNOS-deficient mice show increased vascular permeability at interendothelial junctions, suggesting that these molecules are critical for vascular barrier functions in the lungs [9,10,11]. The localisation of eNOS at cellular membranes and its interaction with sphingolipids is considered a key aspect of eNOS signalling and function [12]. Taken together, these findings suggest that an agent such as PAF that causes vascular permeability through activation of ASM may do so by regulating the balance between caveolin-1 and eNOS in pulmonary endothelial cells.

In the lungs, exogenous NO has reduced vascular leak formation in animal models of acute lung injury [13,14] and even in patients [15], although under certain conditions blockade of NO production may be beneficial as well [16]. These and other seemingly contradictory findings have not yet been completely reconciled [17], although it seems likely that vascular permeability is increased by both too little and too much NO. In response to PAF – at least in the mesenteric artery – the effect of NO on vascular permeability appears to be biphasic: exogenous NO inhibits the rapid (0-10 min) as well as the secondary (>15 min) neutrophil-dependent effect [18]. While the latter effect is explained by the direct effects of NO on neutrophils [19], the mechanism of the first phase has not yet been elucidated. This rapid protective effect of exogenous NO would be explained, if inflammatory mediators such as PAF caused a rapid drop in endothelial NO production. Here we tested this hypothesis and show that PAF rapidly decreases endothelial NO production by sphingomyelinase-dependent mechanisms inside caveolae.

Materials and Methods

A detailed material and method section is provided in the online supplement 1.

Animals

Male Sprague-Dawley rats (350 to 450g for *in situ* fluorescence imaging) and female Wistar rats (weight 220 to 250g for all other experiments) were kept on a standard laboratory chow and water *ad libitum*.

Preparation of isolated, ventilated and perfused rat lungs (IPL)

Rat lungs were prepared, perfused and ventilated essentially as described [20]. Briefly, lungs were perfused through the pulmonary artery at a constant hydrostatic pressure (12 cm H_2O) with Krebs-Henseleit-buffer. Perfusate buffer contained 2% albumin, 0.1% glucose and 0.3% HEPES. Edema formation was assessed by continuously measuring the weight gain of the lung. Imipramine (final concentration 10µM), D609 (300 µM), L-NAME (100 µM), dexamethasone (10 µM) and PAPAnonoate (100 µM) were added to the buffer reservoir 10 min prior to PAF administration. ASM (1 U/ml in perfusate buffer) was continuously infused for 30 min into venular capillaries of isolated lungs via a venous microcatheter. The lung filtration coefficient was measured by suddenly raising or lowering the perfusion pressure by 5 cm H_2O and analyzing the resulting weight transients by bi-exponential regression as described in detail before [21]. Fluid fluxes into and out of the alveolar space were quantified by a double-indicator dilution technique as previously reported [22].

Preparation of endothelial membrane fractions

In order to demonstrate that PAF increases the amount of Cav1 and eNOS inside caveolae from pulmonary endothelial cells *in situ*, we used perfusion with silica beads to tag the endothelial cells and separate their plasma membrane fractions in a sucrose gradient [23]. We have modified the published procedure and used a slightly different gradient (illustrated in Fig. 1) in order to expand the range where caveolae are found. Usually, when working with sucrose gradients caveolae are found in the fractions containing 10% to 20% sucrose [23]. After flow rate was reduced to 3 mL/min, perfusion with 1% cationic colloidal silica beads was started. The lungs were homogenized, mixed with an equal volume of 1.02 g/ml Nycodenz and layered over 0.5-0.7 g/mL Nycodenz containing 60 mM sucrose. After centrifugation at 20.000 rpm (30 min, 4°C) the pellet containing the silica-coated endothelial membranes fragments were resuspended with 1ml MBS and 10% Triton-X-100 (final concentration of 1%) was added to the membranes for 60 min at 4°C. Subsequently, the suspension was homogenized and 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 16-18h. Volumes of 3 x 150 µl were sampled from the top to the bottom and collected as five membrane fractions. The pellet was solubilized in 150 µl MBS (pellet fraction).

Gel electrophoresis and immunoblotting

Equal amounts of protein (5µg) were separated by SDS poly acrylamide gel electrophoresis (12% for caveolin-1, 8% for eNOS) and transferred to nitrocellulose. After transfer, nitrocellulose sheets were blotted with respective antibodies.

Acid sphingomyelinase activity was determined as described [4].

In situ fluorescence microscopy

In situ imaging of endothelial NO production was performed as previously described [24]. using membrane-permeant DAF-FM diacetate (5 µM/L). NO-sensitive DAF-FM was infused for 20 min into pulmonary capillaries via a venous microcatheter. Single venular capillaries were viewed at a focal plane corresponding to maximum diameter (17-28 µm). Fluorescence images obtained in 10s intervals were background-corrected and fluorescence intensity (F) was expressed relative to its individual baseline (F_0). Since the conversion of DAF-FM to the benzotriazole derivative is irreversible, NO production is reflected by changes of the ratio F/F_0 ($\Delta F/F_0$) over time and was determined in 5 min intervals.

Statistics. In case of heteroscedasticity data were transformed by the Box-Cox transformation prior to analysis. Data were analyzed by two-sided t-tests or by the Dunnett test (JMP 7). Fluorescence data were analyzed by the Kruskal-Wallis and Mann-Whitney U-test. If required, p-values were corrected for multiple comparisons according to the false-discovery rate procedure using the "R" statistical package (R Foundation for Statistical Computing, Vienna, Austria; 2005).

Results

PAF increases caveolin-1 in membrane fractions

Lungs were perfused for 50 min before perfusion with the colloidal silica beads was started to prepare endothelial cell membrane fractions. Immunoblotting of the resulting fractions showed a typical distribution for flotillin (not shown) and caveolin-1 (Fig. 1a); two bands are typical for rat caveolin-1 (cav1) representing cav1α and cav1β [25]. Angiotensin converting enzyme (ACE) (Fig. 1b) and the transferrin receptor (data not shown), markers of non-raft membrane fractions, were present only in the fractions with the highest density, confirming that the membrane fractions prepared by silica coating procedure were not contaminated by proteins from extra-caveolar/extra-raft membranes. To increase the amount of material available for subsequent analyses, we pooled the 17 fractions into 5 fractions labeled A-E. In line with previous work [23], the sucrose concentrations, the presence of Cav1 and the absent or weak signals for ACE, we consider fractions B & C to represent caveolae, while fraction D may represent a mixture of caveolar and non-caveolar structures. In most cases, the fractions from the different experiments were analyzed on separate gels; yet similar results were obtained when fractions were analyzed on the same gel (Supplement 2).
Compared to untreated lungs, PAF caused an increase in the amount of cav1, but not ACE, in several fractions from pulmonary endothelial cells (Fig 1). Also in the pooled fractions, the effect of PAF was clearly noticeable; the strongest increase in cav1 was noted in the fractions B, C and D (Fig. 1c). To demonstrate that caveolin-1 was recruited to the caveolae, we measured the abundance of cav1 in whole cell lysates, in the pooled fractions B/C representing the caveolae and in the remainder of the cells (fractions A,D,E and pellet) representing the non-caveolar fractions (Fig. 1d). Our findings show that the total amount of cav1 was unchanged in the total endothelial cell lysate, but was increased in the caveolar fractions and decreased in the remaining fractions.

Number and size of caveolae

Because cav1 is the signature molecule of caveolae we examined whether PAF-treatment increased the number of caveolae by analyzing electron microscopic images of microvascular endothelial cells from isolated perfused lungs (Fig. 2a,b). PAF did not increase the number of apical, cellular or basal caveolae (Fig. 2c), nor did it increase the size of caveolae: the maximal diameter at the centre of caveolae was 152±26 nm (n=103, from one lung) in control and 148±26 nm (n=106, from one lung) in PAF-treated lungs. These results are consistent with those of animals overexpressing endothelial cav1 that also did not show altered numbers of caveolae [26]. Furthermore, in PAF-treated lungs, we repeatedly observed loosening of the endothelial adherence junctions between cells in the capillaries (Fig. 2b).

Effect of PAF on caveolar eNOS and NO production

As cav1 binds and thereby blocks eNOS activitaction [8], we investigated the effect of PAF on the abundance of eNOS inside caveolae and on endothelial NO formation.
PAF increased the amount of eNOS inside the caveolar fractions B and C (Fig 3a). Imaging of DAF-FM loaded lung capillary endothelial cells *in situ* revealed stable and continuous NO production in control lungs (Fig 3b; representative images are shown below) that was completely blocked after addition of the NO synthase inhibitor L-NAME (Fig 3b). Addition of the NO-donor SNAP elicited a consistent rapid and marked increase of fluorescence at the end of all experiments (data not shown). These findings indicate adequate fluorophore labeling and confirm that the fluorescence yield was not affected by the pharmacological

interventions *per se*. PAF-treatment markedly reduced basal endothelial NO production for >30 min (Fig. 3b).
To test the effect of NO on PAF-induced edema formation, NO levels were increased (PAPA NONOate) or decreased (L-NAME) by pharmacological means. Given alone, both agents did not alter lung weight (Fog. 3c). Exogenous application of NO by perfusing lungs with PAPA NONOate attenuated PAF-induced edema formation, while inhibition of NO-synthase activity by L-NAME had no effect (Fig. 3c).
As NO has been involved in the regulation of the epithelial barrier [22], it was important to examine whether the intravascular application of PAF would alter alveolar fluid influx or absorption. Using a double indicator technique and assuming a two-compartmental distribution model [22], we found that PAF did not alter the alveolar barrier properties (Fig. 4a,b).
A tight alveolar barrier is also important for the gravimetric measurement of filtration coefficient (K_f). The K_f represents the hydraulic conductance of the pulmonary endothelium, and can only be determined, if the alveolar hydraulic conductance remains stable, because otherwise the measured value represents the sum of both conductances [21]; in addition, an isogravimetric state – a prerequisite for the measurement – is difficult to achieve in case of alveolar edema. Because PAF did not increase alveolar permeability or fluid absorption, we went on to measure the K_f. Fig. 4c illustrates a typical K_f measurement manoeuvre demonstrating that the weight transient induced by the pressure jump was reversible even after PAF, i.e. that another critical requisite for the Kf measurement was valid.
Given alone, the NO donor PAPA NONOate did not increase the K_f, whereas L-NAME increased the K_f to about half the value of PAF (Fig. 4d). Given 10 min before PAF, PAPA NONOate attenuated the PAF-induced K_f-increase, while L-NAME had no effect (Fig. 4d).

Role of ASM

10 min after addition of PAF, ASM activity was increased within the fractions A-C (Fig. 5a). Imipramine and the steroid dexamethasone – both prevent the PAF-induced activation of ASM and edema formation [4] – abolished the PAF-induced increase in ASM activity in the membrane fractions (Fig. 5a). Perfusion with ASM markedly decreased endothelial NO production (Fig. 5b).
Imipramine and D609 (two structurally different inhibitors of the ASM-pathway [5]), prevented the PAF-induced recruitment of Cav1 to caveolar membrane fractions (Fig. 6a,b). Furthermore, imipramine blocked the PAF-induced recruitment of eNOS (Fig. 6c). In line with previous findings [4], imipramine and D609 reduced the PAF-induced edema formation in these experiments (Fig. 6d). Finally, imipramine attenuated the PAF-induced decrease in endothelial NO-production (Fig. 7a,b). Imipramine alone (given ten minutes before PAF, t=-10 min) had no effect on basal NO production, as demonstrated by similar NO production rates at baseline (t=0 min).

Effects of dexamethasone

Because steroids provide an independent pharmacological approach to block ASM activity in this model (Fig. 5, [4]) and because the short-term anti-inflammatory mechanisms of steroids are still poorly understood, we studied the effects of dexamethasone on cav1- and eNOS recruitment, NO production and edema formation. Treatment of perfused rat lungs with dexamethasone reduced the abundance of cav1 in caveolar fractions in PAF-treated lungs (Fig. 8a). Moreover, dexamethasone attenuated the PAF-induced increase in caveolar eNOS expression (Fig.8b), the decrease in endothelial NO production (Fig. 7b) and lung edema formation (Fig. 8c).

Before, we had shown that quinine attenuates the PAF-induced edema formation [27] and speculated that the effect of quinine is somewhere along the ASM-pathway [1]. While quinine reduced the PAF-induced weight gain (Fig. 8c), it had no effect on the enhanced expression of Cav1 in the fractions B, C and D of PAF-stimulated lungs (Fig. 8a).

Discussion

The present work shows that a reduction in endothelial NO formation contributes to increased pulmonary vascular permeability under pathophysiological conditions mimicked by PAF. These findings are consistent with previous work showing increased vascular permeability in eNOS-deficient mice [10,11]. Our findings further indicate that PAF reduces endothelial NO levels by the ASM-dependent recruitment of cav1 to caveolae with subsequent inhibition of eNOS, providing the first mechanistic explanation for the role of ASM in the development of pulmonary edema [4]. Our findings may also provide a rationale for the treatment of pulmonary edema with exogenous NO or NO donors in order to restore normal endothelial NO levels. Interestingly, acute lung injury patients with higher urinary NO levels had reduced mortality, more ventilator-free days and more organ failure free days [28]. And finally, our observations raise the hypothesis that interference with ASM activity inside caveolae may explain some of the short term effects of steroids.

Role of NO in PAF-induced edema formation

The role of NO in the development of pulmonary edema remains controversial [17]. Likely reasons to explain conflicting reports showing either attenuation [16] or aggravation [29,30] of pulmonary edema by nitric oxide synthase inhibitors are (i) the different timing of the measurements, (ii) the numerous and diverse effects of NO on leukocytes and endothelial cells and (iii) the notion that both too much and too little NO can increase vascular permeability [11]. The model of the isolated blood-free perfused rat lung allows to circumvent most of these confounders: (i) *In vivo* the PAF-induced edema formation appears to consist of two phases; an early one (<10 min) caused by a direct effect on the vascular endothelium and a later one (>20 min) mediated by PAF-activated leukocytes [31]. The focus on the first 10 min and the absence of leukocytes in our model [32] allowed us to exclusively study the early effects of PAF on the pulmonary endothelium. (ii) NO affects neutrophils in various ways [19], so that in intact animals the effect of NO inhibitors is difficult to interpret. Again, blood-free perfusion allows to exclude such complications. (iii) It is well established that both too little or too much NO can aggravate pulmonary edema [33], which has been explained in terms of the different amounts of NO being produced by eNOS (low NO levels) and inducible NOS (high NO levels) [34]. Given the short time span of our experiments, a role of iNOS in our model appears highly unlikely. Another potential concern arises from the observation that NO is involved in the regulation of alveolar epithelial barrier functions, and thus that the effect of PAPA NONOate might be on the alveolar epithelium rather than on the endothelium. Therefore, it was important to show that PAF does not cause alveolar edema or alter alveolar fluid absorption in our model (Fig. 4). Our findings support the notion that the isolated perfused rat lung is a useful tool to study the effects of PAF on the pulmonary endothelium. We would also like to emphasize that PAF appears to increase vascular permeability by a very well regulated mechanism that leads to interstitial but not alveolar edema (see Fig. 4). Using this model we have shown for the first time that PAF rapidly attenuates endothelial NO production which in turn contributes to enhanced vascular permeability, as is illustrated by the effects of the L-NAME and PAPA NONOate on weight gain and the K_f (Fig. 3c,4d). This conclusion is concordant with altered endothelial junctional permeability in eNOS-deficient or L-NAME-treated mice [10,11] and with the finding that endothelial NO synthesis reduces the lung vascular filtration coefficient (K_f) in hydrostatic lung edema [35]. The view that a drop in endothelial NO production promotes vascular permeability is further supported by the finding that edema formation was prevented if the PAF-induced drop in endothelial NO levels was prevented by imipramine or dexamethasone. Others have reported that NO donors also reverse the PAF-induced protein clearance in the intestine [36].

It is important to note, however, that L-NAME alone had no effect on lung weight, despite the fact that it increased the filtration coefficient. This is similar to the observation in eNOS-deficient mice that also showed increased vascular permeability but no lung edema [11]. These findings suggest that the role of the PAF-induced cessation of NO-production is to amplify the increase in vascular permeability that is caused by another mechanism. Hence along the PAF-ASM-axis additional mechanisms are likely to come into play such as PGE_2 [3] or calcium [1].

The present data help to explain many previous findings on the role of NO in the regulation of vascular permeability during PAF-mediated inflammation. However, they contradict yet other studies on PAF, showing increased NO levels in bovine coronary venular endothelial cells [37] and rat blood-free perfused mesenteric venules [38], and protection against the increase in hydraulic conductivity by NOS-inhibition or interference with the cav1-eNOS interaction site [39,37]. And opposite to what we have found, it was observed that upon stimulation with PAF eNOS rapidly translocated to the cytosol [37] rather than to accumulate in the membrane. Obviously, neither leukocytes, timing nor species differences are likely to explain all these discrepant findings. One possible explanation is that this differential regulation of NO represents another example of the frequently contrarian regulatory mechanisms of the systemic and the pulmonary circulation, examples being high versus low arterial blood pressure or hypoxic dilatation versus hypoxic constriction. However, that would not explain why NO donors are protective against PAF-induced edema in both the lungs (Ref 16 and this work) and the intestine [36]. Alternatively, these differences might be explained in terms of the bell-shaped dose-response curve of NO where either too little or too much NO increases vascular permeability. Another important factor may be the basal NO tone, as endothelial NO production is highly dependent upon shear stress, so that studies in the absence of significant shear stress may be biased by low initial NO levels.

Regulation of Cav1 by PAF

Our study showed a rapid increase in the caveolar fractions of both cav1 and eNOS after stimulation with PAF. Indirect evidence for the recruitment of cav1 in response to PAF is provided by the fact that at the same time PAF decreased endothelial NO production. Most caveoli are expected in the 10-16% sucrose fraction [23] which roughly corresponds to our fractions B and D, that were largely devoid of angiotensin-converting enzyme. While also fraction A (8-10% sucrose) and fraction D (20%-30%) may contain some caveolae, our data do not exclude the possibility that cav1 was recruited to membrane fractions distinct from caveolae.

Our findings provide some indications as to the source of the cav1 that translocates to the caveolae in response to PAF. Fig. 1d shows that the total amount of cav1 remains unchanged and that the increase in the caveolae (fractions B/C) was matched by a decrease in the remainder of the cells. As this remainder consists of the entire cells except the caveolae, it is tempting to speculate that cav1 was recruited from inside the cell. In fact, recruitment of cav1 to the plasma membrane has previously been described for bovine aortic endothelial cells in response to prolonged laminar shear stress [40], and also in experimental models of coronary ischemia reperfusion [41], portal hypertension [42] and liver cirrhosis [43]. In the latter studies, elevated cav1-levels were associated with reduced NO-synthesis. However, these studies have not identified the responsible mediators, examined NO levels in endothelial cells or identified a molecular mechanism to explain the recruitment of cav1 to caveolae. Based on our findings it might be interesting to examine the role of sphingomyelinase in these and other clinically relevant situations [44,45] that are characterized by reduced NO production.

Our conclusion that the PAF-induced redistribution of Cav1 is mediated by ASM is supported by several findings: (i) Using pharmacological tools (imipramine, D609, steroids) and ASMdeficient mice we had previously shown that the PAF-induced pulmonary edema is mediated by ASM [4]. (ii) While before we had shown that PAF increases circulating ASM levels, here we demonstrate that PAF increases ASM activity inside endothelial caveolae. While the absolute increase in ASM activity may appear modest, the increase shown is enough to convert approximately 8% of the sphingomyelin in the plasma membrane to ceramide within the 10 min that the PAF response lasts (see supplement 2). In addition, it needs to be considered that some of the ASM is released into the buffer/serum so that the caveolar ASM activity will underestimate the extent of ASM activation/recruitment [4]. Since no reliable ASM antibodies are available we could not directly examine whether the increased activity is related to elevated amounts of enzyme. However, since recent studies have shown that receptor ligands can stimulate transfer of ASM from lysosomes to the plasma membrane [46], such a transfer appears possible. Interestingly, activation of ASM by fas ligand, prevented the (NO-dependent) bradykinin-induced vasodilation in coronary arteries [46], which is in line with the current findings. (iii) Measuring the ASM activity inside caveolar fractions we demonstrated that imipramine, which is not an enyzme inhibitor but accelerates the proteolytic degradation of ASM [5], reduced the ASM activity below baseline levels. (iv) Injection of ASM reduced endothelial NO production similar to PAF, providing direct evidence for the ability of ASM to affect endothelial NO synthesis. Unfortunately, the effect of ASM on Cav1 and edema formation could not be examined, because such measurements had to be performed in an isolated lung model with 100 mL of recirculating buffer, so that the required amounts of ASM were beyond feasibility. (v) The reduction of caveolar ASM activity by imipramine resulted in reduced caveolar levels of Cav1 and eNOS and finally in continued NO production despite the presence of PAF, and inhibition of edema formation. This is in keeping with findings in a model of coronary ischemia and reperfusion, where desipramine reduced the interaction between Cav1 and eNOS and improved cardiac functions [41]. The specificity of imipramine for ASM under these conditions is further supported by the finding that D609, an ASM-pathway inhibitor that is structurally completely unrelated to imipramine, also prevented the PAF-induced redistribution of Cav1 (Fig. 6). (vi) Finally, the same line of evidence as for imipramine could also be shown for dexamethasone. Dexamethasone was previously shown to prevent the PAF-induced increase in ceramide and edema formation [4]. Here we show that dexamathasone prevented the PAF-induced increase of ASM-activity in endothelial caveolae and all its sequelae such as cav1- and eNOS-recruitment to the plasma membrane, the loss of endothelial NO production and subsequent formation of lung edema. These findings concerning dexamethasone are not only of interest because they corroborate the conclusions of the present study, but also because they suggest a novel hypothesis on how to explain the poorly-understood short term effects of steroids that have already been linked to caveolae [47]. It seems possible that steroids attenuate the formation of ceramide-rich microdomains, which are required for many signalling events [5], by interfering with the activation of ASM.

The present findings indicate that PAF increases vascular permeability by the ASM-dependent enrichment of cav1 inside caveolae which in turn inhibits eNOS and blocks NO synthesis. Several steps in this process require further definition. Recent studies indicate that sphingomyelinase produces ceramide to stabilize membrane microdomains [48], yet it remains to be shown how this favours the redistribution of cav1 to caveolae. Further, it needs to be examined whether PAF, similar to fas ligand [46], causes ASM-recruitment from lysosomes to the plasma membrane.

Taken together our findings suggest that blockade of basal endothelial NO synthesis serves as a physiological mechanism to promote vascular permeability in the lungs. Activation of acid sphingomyelinase and subsequent recruitment of cav1 to the plasma membrane mediate this PAF-induced effect. These observations provide the first mechanistic clues about downstream targets of PAF-activated ASM metabolites and give a rationale for the substitution of endogenous endothelial NO levels or activation of downstream signals as potential therapeutic targets in permeability-type lung edema.

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Figure legends

Figure 1. ***Cav1 content of pulmonary endothelial cell plasma membrane fractions***. (**a**) Immunoblots showing the cav1 distribution in pulmonary endothelial cell plasma membrane fractions from untreated (control) and PAF-treated (10 min after bolus injection of 5 nmol PAF) isolated perfused rat lungs. (**b**) Immunoblots showing angiotensin converting enzyme (Ace) distribution in the same samples. (**c**) Pooled immunoblots showing Cav1 distributions from untreated (n=4) and PAF-treated (n=4) isolated perfused rat lungs according to the lettering in panel (a). (**d**) Immunoblots showing Cav1 in fractions from whole endothelial cells (n=3), from the pooled fractions B and C (n=3) and from the remainder of the endothelial cells without the fractions B and C (n=3). Please note latter two fractions (B/C and the remainder) were obtained from the same preparation. Mean+SD * $p<0.05$ vs control.

Figure 1

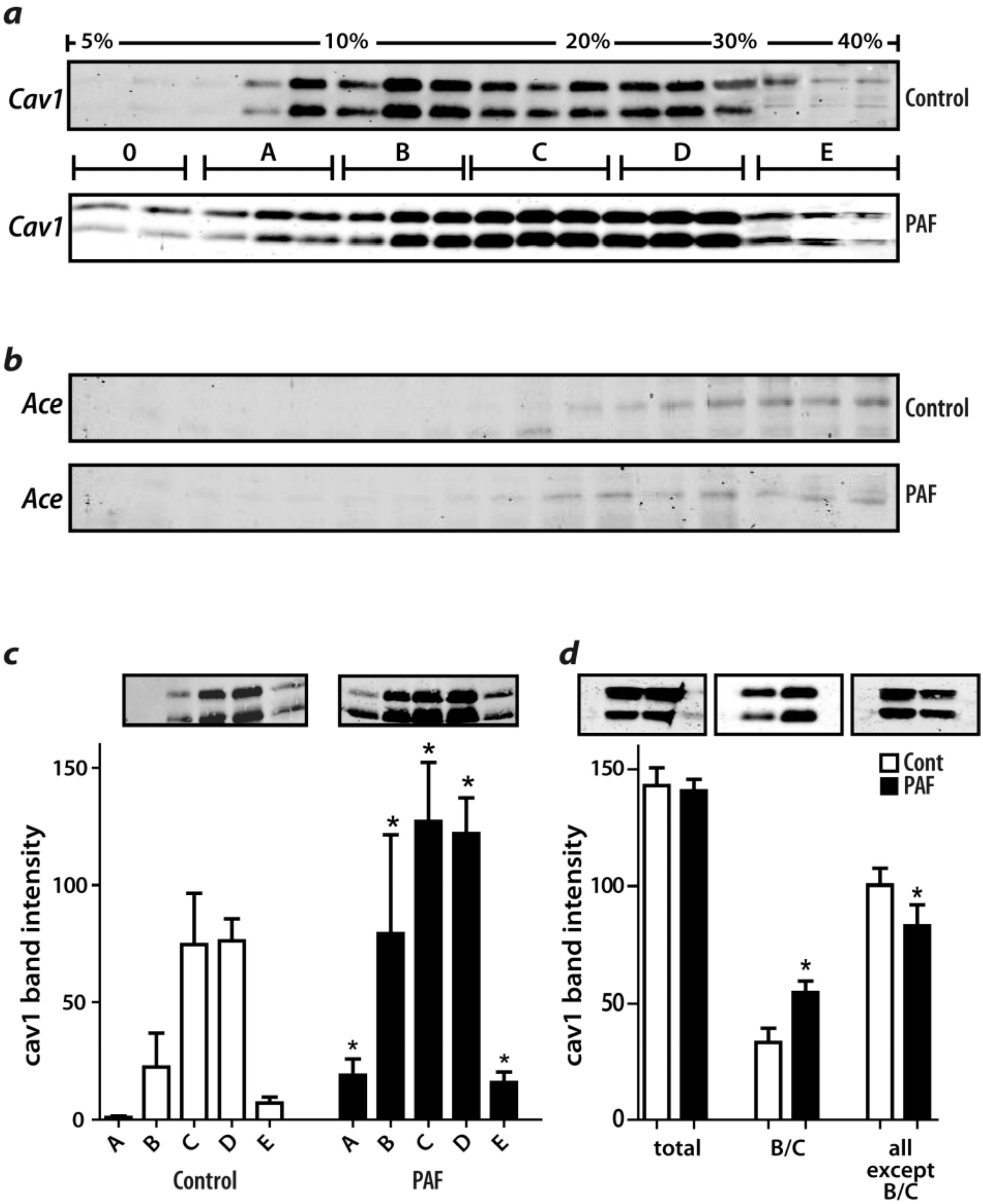
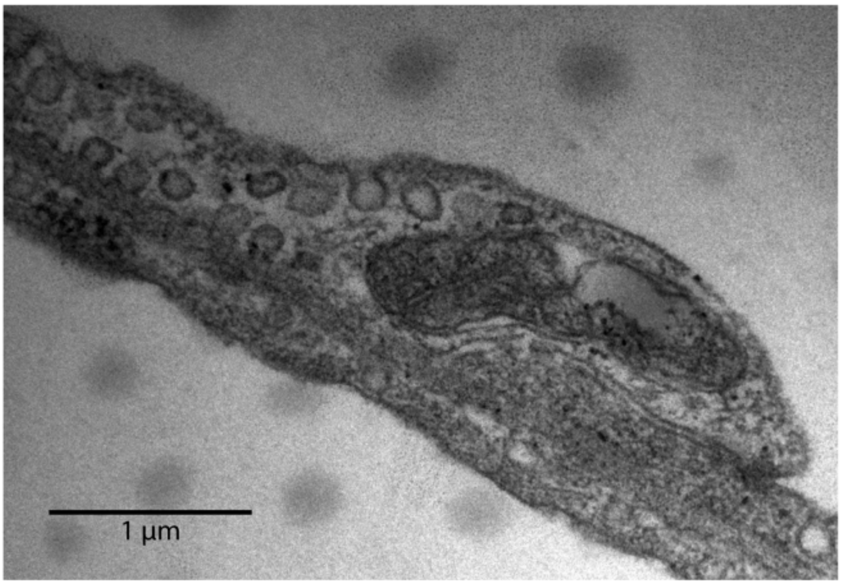


Figure 2. ***Number and size of caveolae***. Electron microscopic images of endothelial cells showing junctions and caveolae in control (**a**) and PAF-treated lungs (**b**). (**c**) Number of apical, basal and midcellular caveolae in endothelial cells from control and PAF treated lungs. Counted were 95-106 apical, basal and midcellular caveolae from one control and one PAF-treated lung.

Figure 2

a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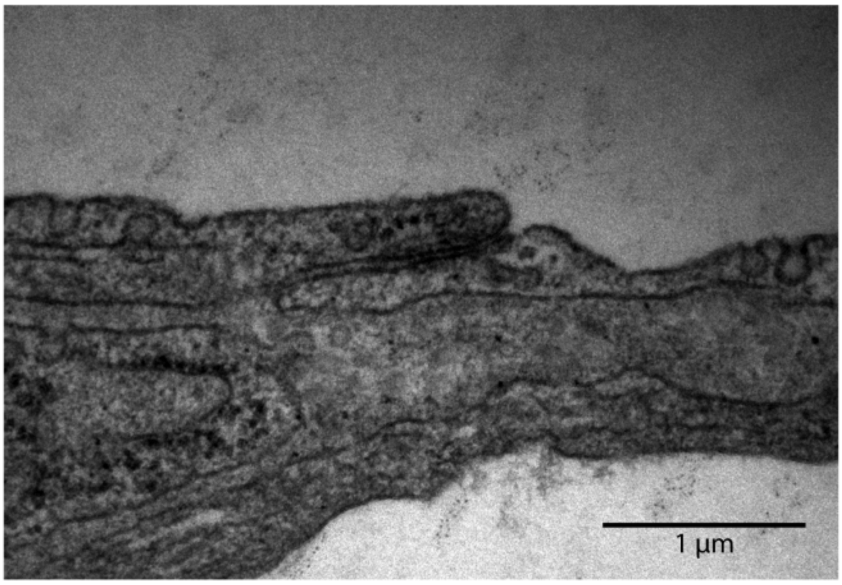
b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c

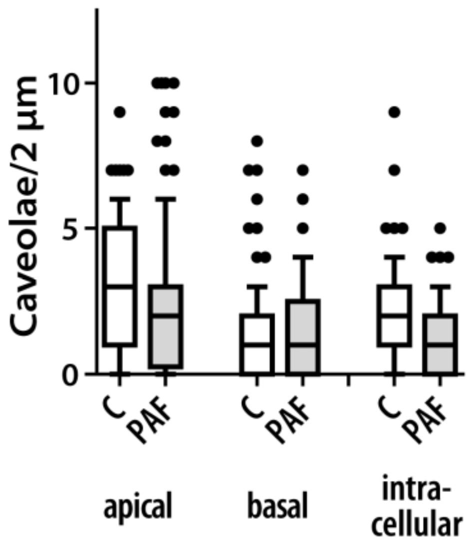
Caveolae/2 µm
0
5
10
C
PAF
C
PAF
C
PAF
apical
basal
intra-
cellular

Figure 3. ***Effect of PAF on caveolar eNOS and NO production***. (**a**) Immunoblots showing the eNOS distribution in pooled pulmonary endothelial cell plasma membrane fractions from untreated (control) and PAF-treated (10 min after bolus injection of 5 nmol PAF) isolated perfused rat lungs. * $p<0.05$ *vs* control, mean±SD, n=4. (**b**) Endothelial NO production as determined by *in situ* real-time fluorescence microscopy shown as 5 min averages in control, PAF (bolus injection of 5 nmol PAF after baseline recording) and L-NAME (250 µM) treated lungs. 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). * $p<0.05$ vs control, mean±SEM, n=5. (**c**) Weight gain in lungs perfused under control conditions (n=11), with PAF (5 nmol, n=12), NONOate/PAF (100µM NONOate, n=7), L-NAME/PAF (250µM L-NAME, n=8), NONOate (n=3) or L-NAME (n=3). The pharmacological agents were added after 30 min of perfusion and PAF after 40 min of perfusion. * $p<0.05$ vs control; #, vs PAF. mean±SD.

Fig 3: eNOS, NO and edema

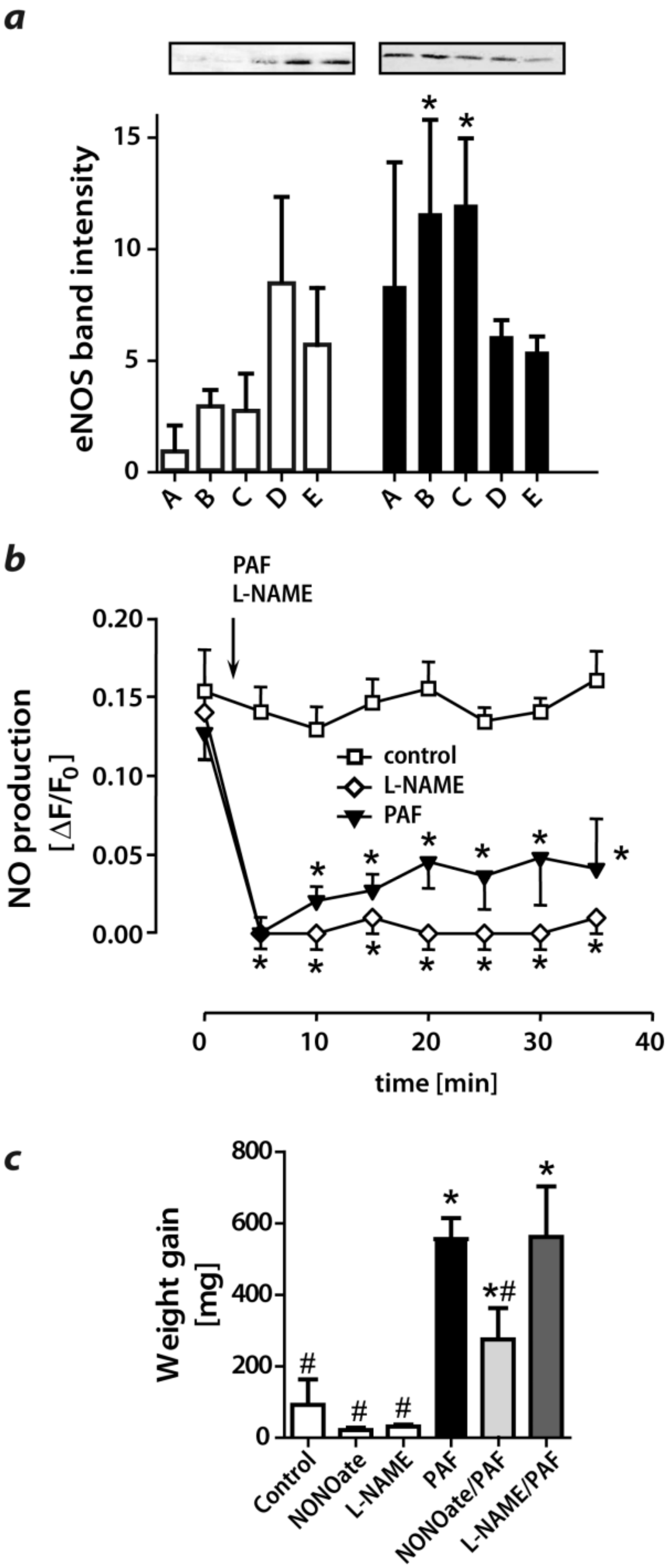

Figure 4. **Alveolar fluid influx, alveolar fluid absorption and filtration coefficient.** (**a**) alveolar fluid influx and (**b**) alveolar fluid absorption in control lungs, in PAF-treated lungs and in lungs exposed to 20 cm H_2O hydrostatic pressure for 60 min. (**c**) Weight gain in a typical experiment with administration of PAF and determination of the filtration coefficient (K_f). For the K_f-measurement perfusion pressure was raised by 5 cm H_2O for 15 min and reduced back to baseline. Both weight transients (up, down) were used to calculate the K_f. (**d**) K_f determined as the difference in K_f before and 10 min after injection of PAF. Lungs perfused under control conditions (n=3), with PAF (5 nmol, n=4), NONOate/PAF (100µM NONOate, n=3), L-NAME/PAF (250µM L-NAME, n=3), NONOate (n=3) or L-NAME (n=3). The pharmacological agents were added after 30 min of perfusion and PAF after 40 min of perfusion * $p<0.05$ vs control; # vs PAF. mean±SD.

Fig 4: Alveolar fluid, Kfc

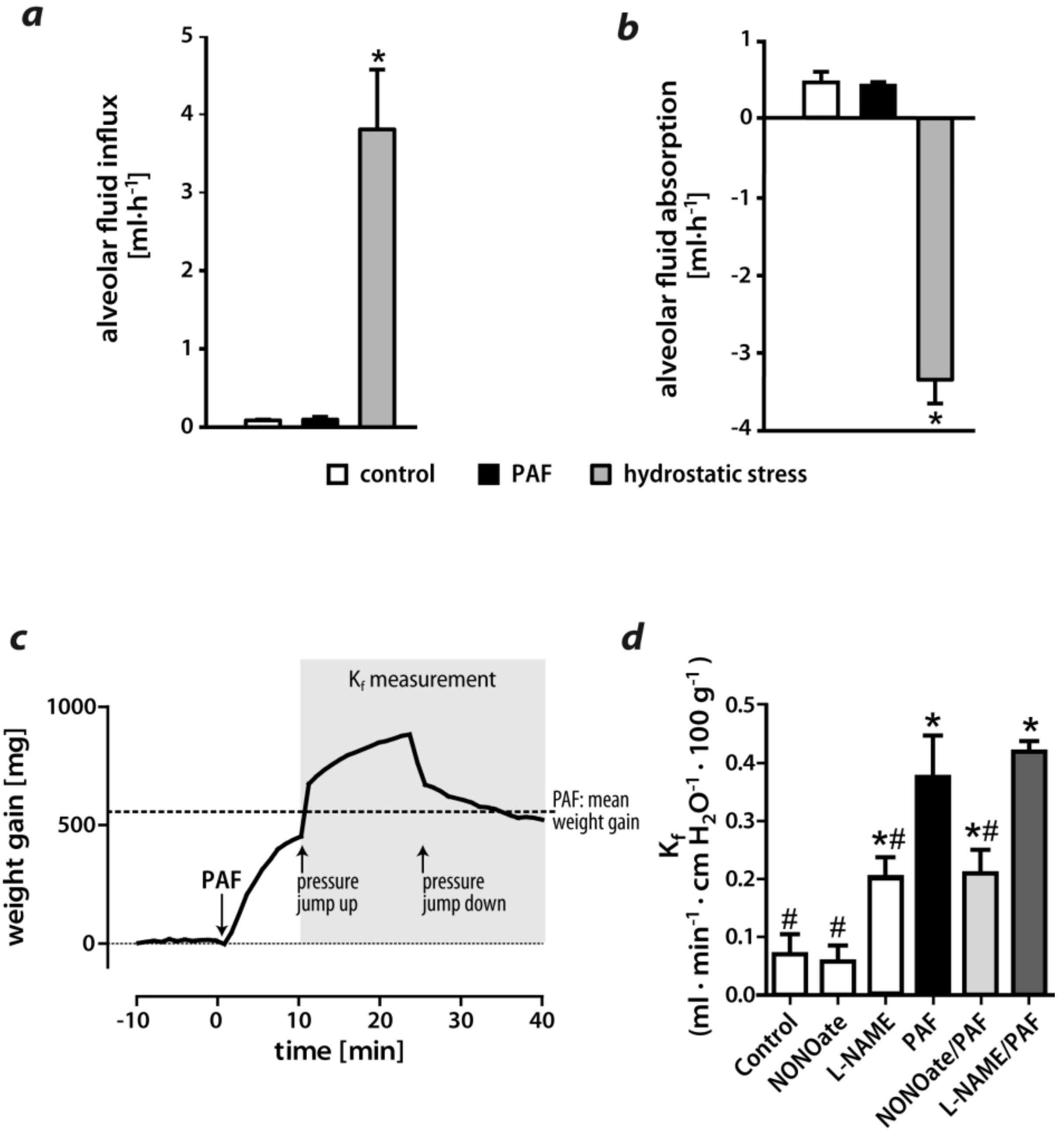

Figure 5. Role of ASM. (**a**) Acid sphingomyelinase (ASM) activity in pooled pulmonary endothelial cell plasma membrane fractions from untreated (control) and PAF-treated isolated perfused rat lungs. Shown are membrane fractions in lungs perfused for 50 min under control conditions, with PAF (5 nmol) alone, with dexamethasone (10 µM) and PAF (Dex/PAF) or with imipramine (100 µM) and PAF. Pharmacological agents were added after 30 min of perfusion and PAF after 40 min; the perfusion was stopped after 50 min and endothelial cell membrane fractions were prepared and analyzed for ASM enzymatic activity. Data are shown as mean±SD, n=4. * $p<0.05$, ** $p<0.01$ vs PAF. (**b**) Endothelial NO production as determined by *in situ* real-time fluorescence microscopy shown as 5 min averages in control and ASM (1 U/ml) treated lungs. 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). * $p<0.05$ vs control, mean±SEM, n=5.

Fig. 5: ASMase activity

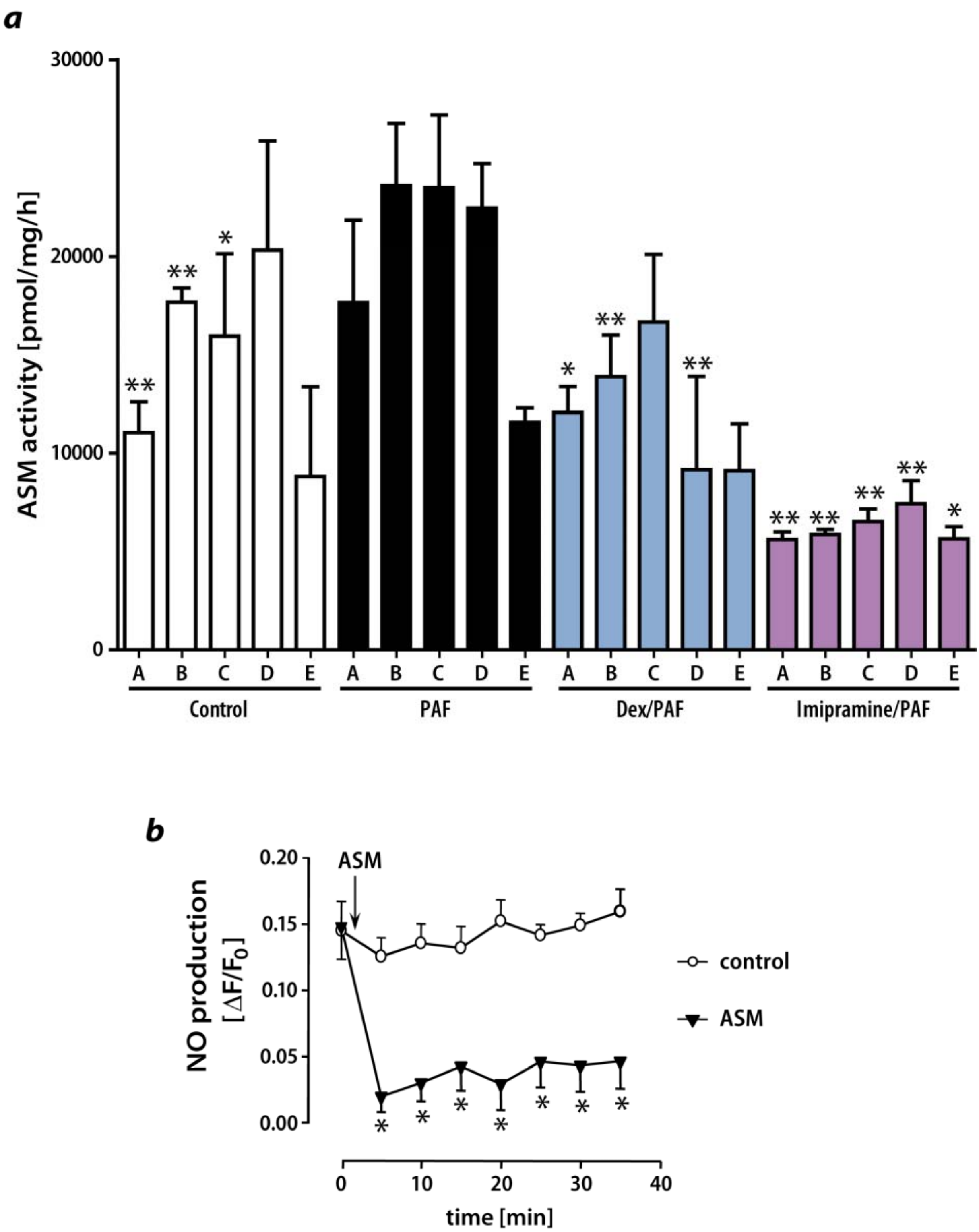

Figure 6. Effects of ASM pathway inhibitors on caveolar caveolin-1 and eNOS.
Immunoblots showing the Cav1 distribution in pooled pulmonary endothelial cell plasma

membrane fractions prepared from lungs perfused with PAF alone, with imipramine (100 µM) and PAF (**a**), or with D609 (300 µM) and PAF (**b**); panels a and b are from two independent experimental series. The pharmacological agents were added after 30 min of perfusion and PAF after 40 min; the perfusion was stopped after 50 min and endothelial cell membrane fractions were prepared and analyzed by immunoblotting. Panel (**c**) shows the eNOS distribution in the same samples shown in panel (a). Panel (**d**) shows the lung weight gain in the experiments shown in panels a-c. Data are shown as mean±SD, n=4. * $p<0.05$ vs PAF.

Fig. 6: Imipramine, D609

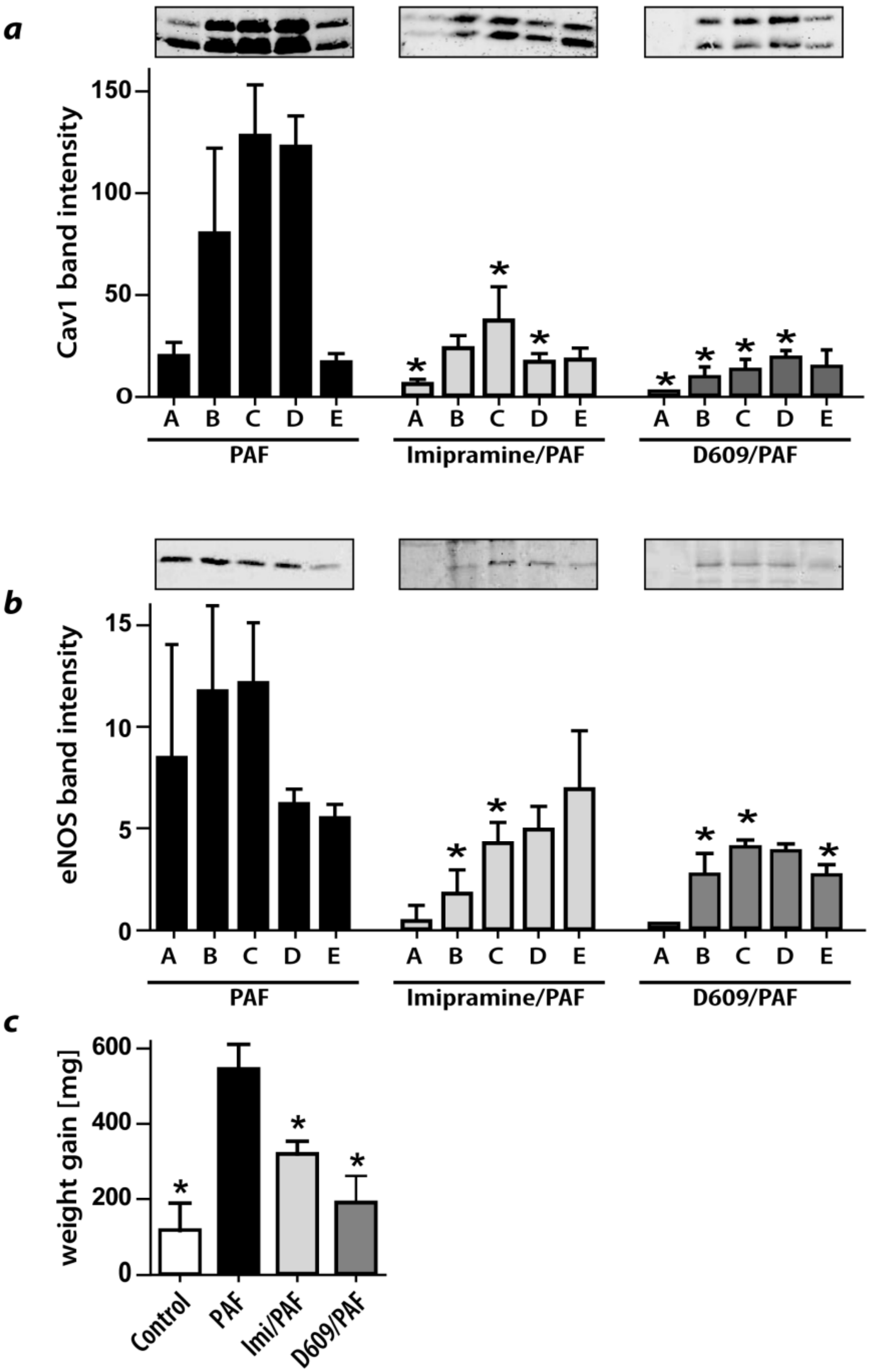

Figure 7. Effects of inhibitors on PAF-mediated decrease in endothelial NO.
(**a**) Representative fluorescence images of DAF-FM loaded lung venular capillaries in isolated perfused rat lungs. Images were obtained at baseline and 10 min after PAF (5 nmol) stimulation in control lungs, and in lungs perfused with either imipramine (100 µM) or dexamethasone (DEX; 10 µM). The pharmacological agents were added 10 min prior to PAF infusion. Note that due to the irreversible conversion of the fluorescent dye, absence of fluorescence increase as seen in PAF treated controls indicates lack of NO synthesis. (**b**) Endothelial NO production as determined by *in situ* real-time fluorescence microscopy shown as 5 min averages at baseline and after stimulation with PAF in control, imipramine or dexamethasone perfused lungs. 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). * $p<0.05$ vs PAF, mean±SEM, n=5.

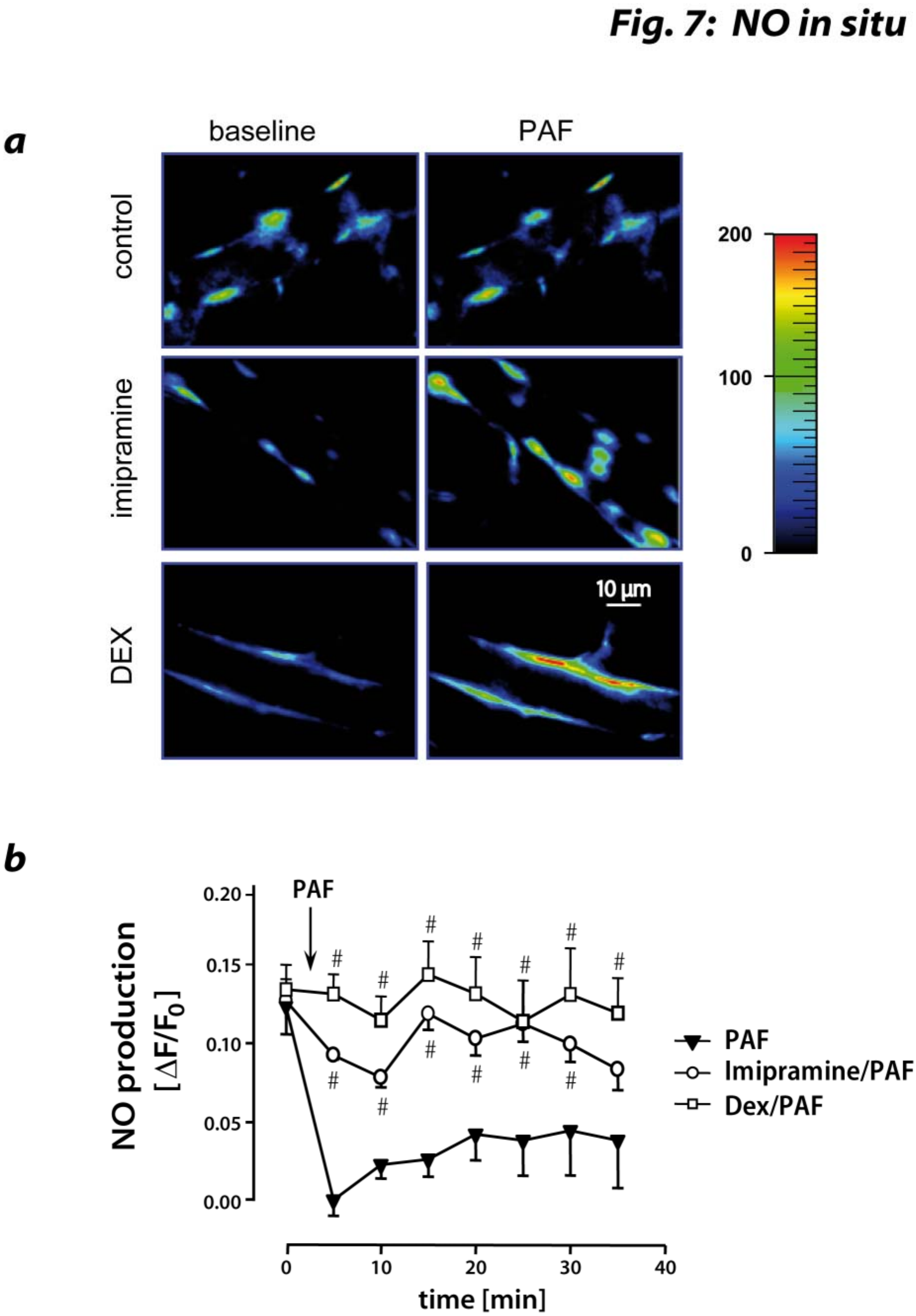

Figure 8. Effects of dexamethasone on caveolin-1, eNOS and weight gain. (**a**) Immunoblots showing the Cav1 distribution in pooled pulmonary endothelial cell plasma

membrane fractions prepared from lungs perfused under control conditions, with PAF (5 nmol) alone, with dexamethasone alone (100 μM), with PAF alone, with dexamethasone and PAF, or with quinine (100 μM) and PAF. The pharmacological agents were added after 30 min of perfusion and PAF after 40 min; the perfusion was stopped after 50 min and endothelial cell membrane fractions were prepared. Panel (**b**) shows the eNOS distribution in the same samples shown in panel (a). Panel (**c**) shows the weight gain in the experiments shown in panel a. Data are shown as mean±SD, n=4. * $p<0.05$ vs PAF.

Fig. 8: Dexamethasone