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PAF-mediated pulmonary edema: a new role for acid sphingomyelinase and ceramide

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Platelet-activating factor (PAF) induces pulmonary edema and has a key role in acute lung injury (ALI). Here we show that PAF induces pulmonary edema through two mechanisms: acid sphingomyelinase (ASM)-dependent production of ceramide, and activation of the cyclooxygenase pathway. Agents that interfere with PAF-induced ceramide synthesis, such as steroids or the xanthogenate D609, attenuate pulmonary edema formation induced by PAF, endotoxin or acid instillation. Our results identify acid sphingomyelinase and ceramide as possible therapeutic targets in acute lung injury.

Ceramide is a sphingolipid generated either by hydrolysis of sphingomyelin or by *de novo* synthesis. It regulates cellular responses to stress, cytokines, radiation and proapoptotic stimuli[1,2]. A potential role of ceramide in the regulation of vascular permeability and pulmonary edema has not yet been considered.

Noncardiogenic pulmonary edema is a hallmark of ALI, a major cause of death in intensive care units. In many experimental models of ALI (such as exposure to endotoxin, zymosan, acid aspiration or intestinal ischemia-reperfusion), edema formation is mediated by PAF[3–7]. In addition, application of recombinant PAF acetylhydrolase markedly decreases vascular leakage in animal models[8], and showed promising results in phase 2 clinical trials for severe sepsis[9].

The molecular mechanisms by which PAF triggers edema formation are only poorly understood, possibly because the study of PAF is complicated by several factors. *In vitro*, PAF does not alter the permeability of confluent endothelial cell layers[10,11]. The interpretation of *in vivo* experiments with PAF is confounded by its effects on local (hypertension) and systemic (hypotension) blood pressure[12]. One physiologically relevant model that can be used to address the mechanisms of PAF action is the isolated, perfused lung, in which edema formation can easily be monitored by measuring lung weight[13]. In this model, the confounding effects of pulmonary hypertension are excluded by perfusing the lungs with constant hydrostatic pressure, while perfusion with blood-free perfusate permits the elimination of blood-borne leukocytes.

Using this model, we recently showed that PAF triggers edema by increasing microvascular permeability through two independent mechanisms[5]. One pathway involves activation of EP-3-receptors by prostaglandin E_2, and can thus be blocked by cyclooxygenase inhibitors such as acetyl salicylic acid (ASA)[5,14]. The nature of the other pathway is unknown; the purpose of the present study is to elucidate its identity. Based on the observation that PAF stimulates ceramide formation in mouse $P388D_1$ macrophages[15], we hypothesized that ceramide is involved in PAF-induced edema formation. We investigated this hypothesis in isolated, perfused rat lungs and *in vivo* in mice. Our findings indicate an important role for ceramide and ASM in mediating PAF-induced pulmonary edema.

RESULTS

PAF mediates pulmonary edema through ASM and ceramide

To test the hypothesis that the ASM-ceramide pathway contributes to PAF-induced pulmonary edema, we treated ASM-deficient or wild-type control mice with PAF and assessed the development of pulmonary edema. Whereas normal mice developed pulmonary edema within 10 min of PAF administration, ASM deficiency reduced pulmonary edema by ~50%, as did pretreatment with ASA (**Fig. 1a**). Treatment of ASM-deficient mice with ASA completely eliminated PAF-induced edema (**Fig. 1a**), indicating that PAF-induced edema is mediated by the combined action of ASM and cyclooxygenase.

We next investigated whether PAF increases ASM activity. Compared with sham-treated mice, PAF treatment resulted in a 28% increase in serum ASM activity, but did not increase neutral sphingomyelinase (NSM) activity (**Fig. 1b**) or ceramide synthase activity (data not shown). The secretory form of ASM is Zn^{2+}-dependent[16,17], which distinguishes this ASM from intracellular, endolysosomal ASM. PAF-induced serum ASM activity was also strictly Zn^{2+}-dependent (data not shown), indicating that the enzyme we measured represents the secretory form of ASM and does not result from damaged cells. Western blot analysis confirmed that the increase in ASM activity was paralleled by an increase in ASM protein (**Fig. 1c**). To show that at least part of the increased ASM serum activity originated from the lungs and not from other organs or blood-derived leukocytes, we measured ASM in the venous effluent of isolated, blood-

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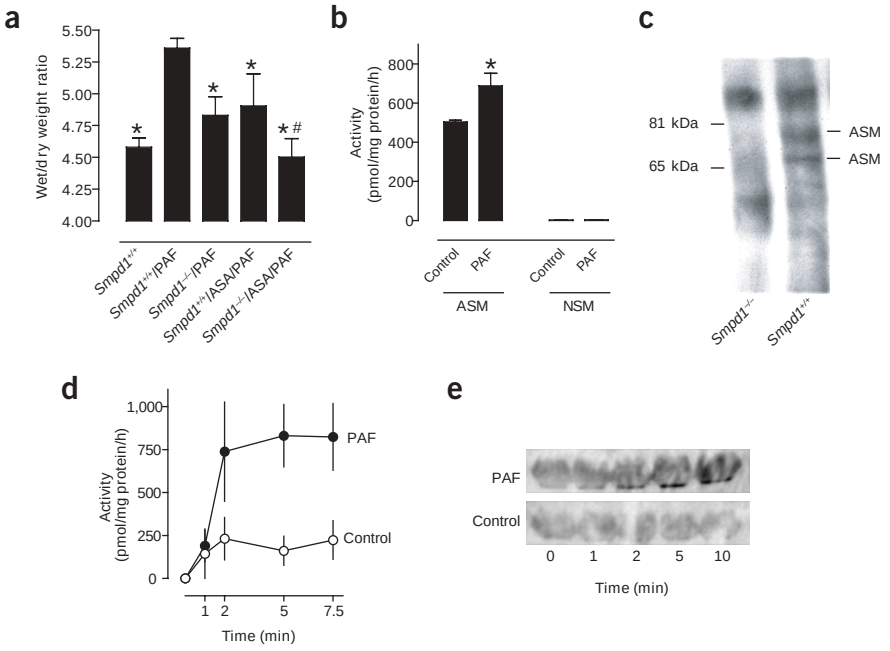

Figure 1 ASM mediates PAF-induced edema. (**a**) Edema formation in lungs of untreated wild-type mice (*Smpd1*$^{+/+}$; $n = 9$), PAF-treated wild-type mice (*Smpd1*$^{+/+}$/PAF; $n = 11$), PAF-treated ASM-deficient mice (*Smpd1*$^{-/-}$; $n = 6$), PAF-treated wild-type mice pretreated with ASA (*Smpd1*$^{+/+}$/ASA/PAF; $n = 5$) and PAF-treated ASM-deficient mice pretreated with ASA (*Smpd1*$^{-/-}$/ASA/PAF; $n = 6$). *, $P < 0.05$ compared with *Smpd1*$^{+/+}$/PAF. #, $P < 0.05$ for *Smpd1*$^{-/-}$ ASA/PAF compared with *Smpd1*$^{+/+}$/ASA/PAF. (**b**) Serum activity of ASM and NSM in untreated (control) and PAF-treated BALB/c mice ($n = 4$–6). (**c**) Representative immunoblot of sera from PAF-treated *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ mice using ASM-specific antibody, showing 72-kDa and 75-kDa bands (indicated by 'ASM'). (**d**,**e**) ASM activity (**d**; $n = 4$–5) and amount (**e**; immunoblot) in venous effluent of untreated and PAF-treated isolated, perfused rat lungs.

free, perfused rat lungs. Perfusion of rat lungs with PAF rapidly increased the activity and amount of extracellular ASM (**Fig. 1d**,**e**), demonstrating that PAF triggers ASM release from lungs.

Because ASM hydrolyzes sphingomyelin to ceramide, we determined the concentration of ceramide in the lungs of PAF-treated mice. In addition, to exclude an extrapulmonary origin of ceramide derived from invading leukocytes, some experiments were conducted in isolated, perfused rat lungs. Both *in vivo* (**Fig. 2a**,**b**) and in the isolated organ (**Fig. 2c**,**d**), PAF treatment increased the tissue concentration of long-chain ceramides by ~50%. In ASM-deficient mice, no increase in pulmonary ceramide content was observed; in wild-type mice, pretreatment with ASA had no effect on PAF-induced ceramide generation (**Fig. 2b**). We also observed an increase in diacylglycerol (DAG) content in PAF-stimulated lungs (**Fig. 2c**), which is consistent with the activation of phospholipase C isoenzymes.

Because long-chain ceramides (C16- or C18-ceramide) are insoluble in the perfusate buffer—some solvents such as ethanol or toluene that can be used in cell culture are toxic in isolated lungs—we were unable to directly investigate their effects, and instead used a short-chain analog, C2-ceramide. Perfusion of rat lungs with C2-ceramide (50 μM), but not C2-di-hydroceramide (50 μM), caused severe edema (**Fig. 3**), confirming that ceramides are able to generate lung edema. Taken together, these findings indicate a central role of ASM-derived ceramide in PAF-induced edema.

Therapeutic interventions attenuating edema

These and other findings[18,19] suggest that extracellular ASM mediates the release of ceramide, which is functionally active in the outer leaflet of the cell membrane. To neutralize surface ceramide, we investigated the effects of two recently described ceramide-specific antisera[20–22] on PAF-induced edema formation. Application of ceramide-specific antibodies to mice (5 mg/kg) or isolated, perfused rat lungs (50 μg/ml) reduced the extent of PAF-induced edema (**Fig. 4a**,**b**). The specificity of this inhibition was supported by the finding that the antisera prevented PAF-triggered pulmonary edema but had no effect on other PAF actions, such as the decreases in vascular and pulmonary conductance, which indicate vasoconstriction and bronchoconstriction (**Fig. 4c**,**d**), events that are predominantly mediated by thromboxane[23]. In addition, the IgG monoclonal antibody A6 (ref. 24), directed against lipid A (which did not cross-react with

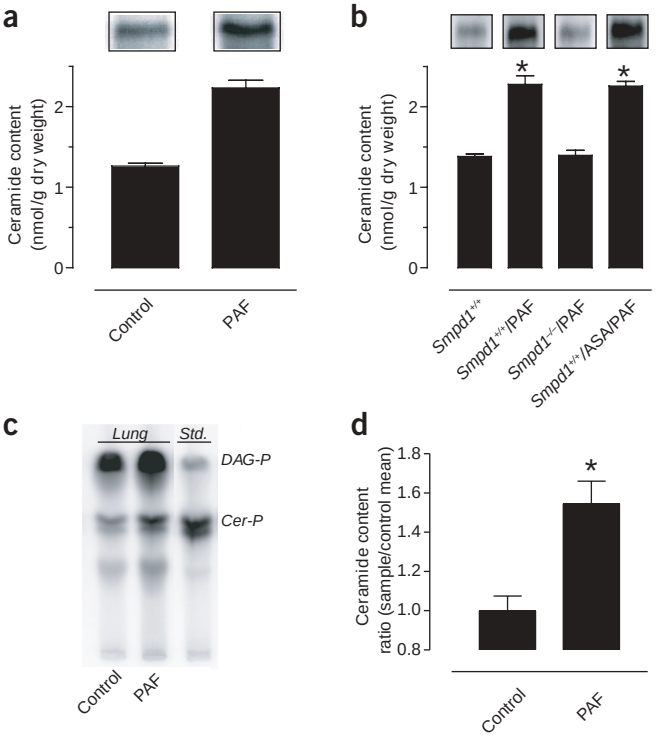

Figure 2 PAF increases pulmonary ceramide content. (**a**,**b**) Pulmonary ceramide content ($n = 4$–5), as determined by charring densitometry in lungs of untreated (control) and PAF-treated BALB/c mice (**a**) and wild-type or ASM-deficient mice treated as indicated with PAF and ASA (**b**). (**c**,**d**) Pulmonary ceramide content, as determined by DAG kinase assay in isolated lungs perfused with or without PAF for 10 min. **c**, Thin-layer chromatography. Std., ceramide standard (Sigma type III); DAG-P, diacylglycerol-phosphate; Cer-P, ceramide-phosphate. **d**, Ceramide content of PAF-treated ($n = 11$) or untreated (control) ($n = 6$) lungs, expressed as ratio of content of PAF-treated to control lungs on the same thin-layer plate. *, $P < 0.05$ compared with control.

ceramide (data not shown), although lipid A has been suggested to be structurally similar to ceramide[25]), did not alter PAF-induced pulmonary edema (**Fig. 4a**,**b**).

We next examined the effects of two different techniques for pharmacologically preventing ceramide formation. We interfered with ceramide biosynthesis by treatment with the xanthogenate D609 (refs. 26,27), and we used imipramine to induce proteolytic degradation of ASM[28,29]. To exclude any extrapulmonary effects caused by changes in blood pressure or by leukocytes, we used isolated, blood-free, perfused rat lungs. Pretreatment with either agent prevented the PAF-induced increase in pulmonary ceramide (**Fig. 5a**) and attenuated the development of PAF-induced edema (**Fig. 5b**). Fumonisin B1, an inhibitor of the *de novo* synthesis of ceramide, had no effect on PAF-induced edema (**Fig. 5b**), a finding that supports the role of ASM in ceramide release. Simultaneous treatment with D609 and ASA further reduced the development of edema in response to PAF (**Fig. 5b**). These results further corroborate the hypothesis that PAF triggers edema through at least two independent pathways. To extend these *in vitro* findings to whole, intact animals, we pretreated mice with D609 and then challenged them with PAF. Pretreatment of mice with D609 prevented PAF-induced edema *in vivo* (**Fig. 5c**).

Steroid treatment reduces pulmonary edema in many models, such as PAF-treated rats[30], by an unknown mechanism. Because steroids may lower ceramide synthesis[31], we investigated the effect of the glucocorticoid dexamethasone on PAF-induced ceramide and edema formation in mice *in vivo*. In this model, pretreatment of mice with dexamethasone prevented the increase in pulmonary ceramide and attenuated edema formation (**Fig. 5d**).

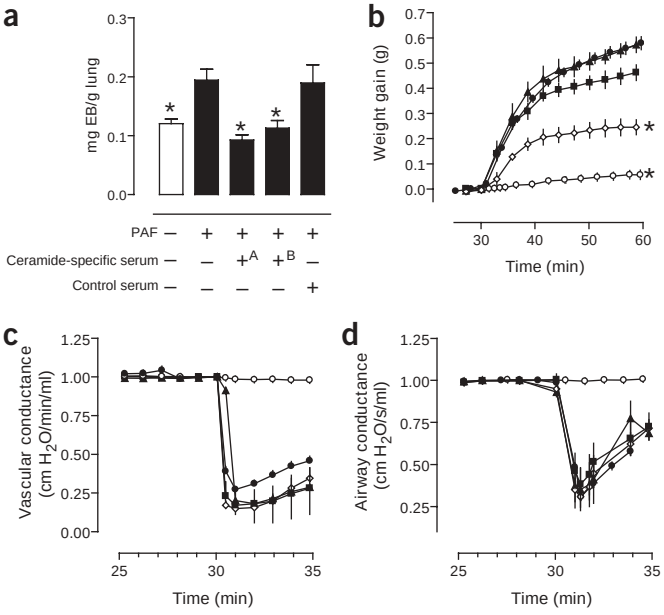

Figure 4 Ceramide-specific antisera selectively reduce PAF-induced edema formation. (**a**) PAF-induced edema formation in mice *in vivo*. Controls, $n = 11$; PAF, $n = 12$; ceramide-specific MAS0020 polyclonal antiserum (A) and PAF, $n = 10$; ceramide-specific MID15B4 monoclonal antibodies (B) and PAF, $n = 5$; lipid A–specific antibody (control) and PAF, $n = 8$. *, $P < 0.01$ compared with PAF alone. EB, Evans blue. (**b**–**d**) Weight gain (**b**), vascular conductance (**c**) and pulmonary conductance (**d**) in isolated, perfused rat lungs. Conductance was normalized to the time when PAF was added. Controls (○), $n = 9$; PAF (●), $n = 42$; 5 μg/ml antiserum A and PAF (■), $n = 3$; 50 μg/ml antiserum A and PAF (◇), $n = 3$; 50 μg/ml control antiserum and PAF (▲), $n = 3$. *, $P < 0.05$ compared with PAF alone.

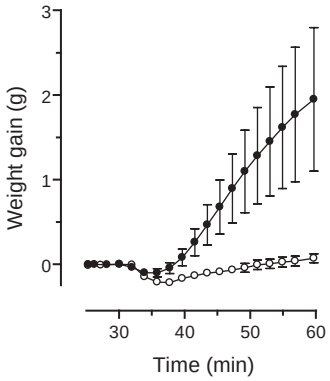

Figure 3 Ceramide causes pulmonary edema. Lungs perfused with 50 μM C2-ceramide (●; $n = 5$) experienced significantly ($P < 0.05$) greater weight gain than did those perfused with 50 μM C2-dihydroceramide (○; $n = 3$) or untreated control lungs (data not shown).

Finally, we examined whether D609 is also effective in PAF-dependent models of acute lung injury, such as endotoxin- or acid-induced pulmonary edema[3,5,6]. Pulmonary edema developed in the control animals, whereas D609-pretreated mice or rats were partly resistant to the edema-promoting effects of endotoxin (**Fig. 6a**) and acid instillation (**Fig. 6b**), respectively. In addition, D609 largely restored arterial oxygenation in the acid-instillation model (**Fig. 6c**), but had no effect on mean blood pressure (**Fig. 6d**).

DISCUSSION

The present study shows that PAF-induced pulmonary edema is partly mediated by ASM and ceramide. This conclusion is supported by the following findings: (i) PAF treatment increased extracellular ASM activity, leading to higher pulmonary ceramide levels; (ii) ASM deficiency attenuated lung edema in mice treated with PAF; (iii) PAF-induced edema formation was reduced in the presence of ceramide-specific antibodies; (iv) application of C2-ceramide caused edema formation; and (v) inhibition of ceramide formation by D609 or imipramine attenuated both ceramide production and edema formation. None of the approaches that interfered with the synthesis or action of ceramide completely prevented PAF-induced edema, however. Complete inhibition was only achieved by combining ASM deficiency (**Fig. 1a**) or D609 (**Fig. 5b**) with ASA, a finding that is consistent with the hypothesis that PAF-induced edema is mediated by two separate pathways[5,14]. Taken together, these findings indicate that PAF triggers pulmonary edema by simultaneous activation of cyclooyxgenase and ASM, followed by subsequent production of prostaglandin E_2 and ceramide.

Our findings suggest, but do not prove, that ceramide may be generated in the outer leaflet of the cell membrane by extracellularly active ASM. This conclusion is supported not only by the increased extracellular ASM protein level and activity observed after PAF treatment, but also by the protection against edema formation provided by ceramide-specific antisera (in all likelihood, the IgM-type antibodies are unable to penetrate the plasma membrane). The hypothesis that ceramide might be formed in the outer leaflet of the plasma membrane is also consistent with the localization of cellular sphingomyelin[32], the substrate of ASM, predominantly in the outer leaflet. Two extracellular forms of ASM have been described. Grassmé *et al.* showed that ASM is translocated onto the surface of sphingolipid-rich rafts upon CD95 triggering[18]. Tabas *et al.* recently discovered a secreted form of ASM, S-Smase, which is encoded by the *Smpd1* gene

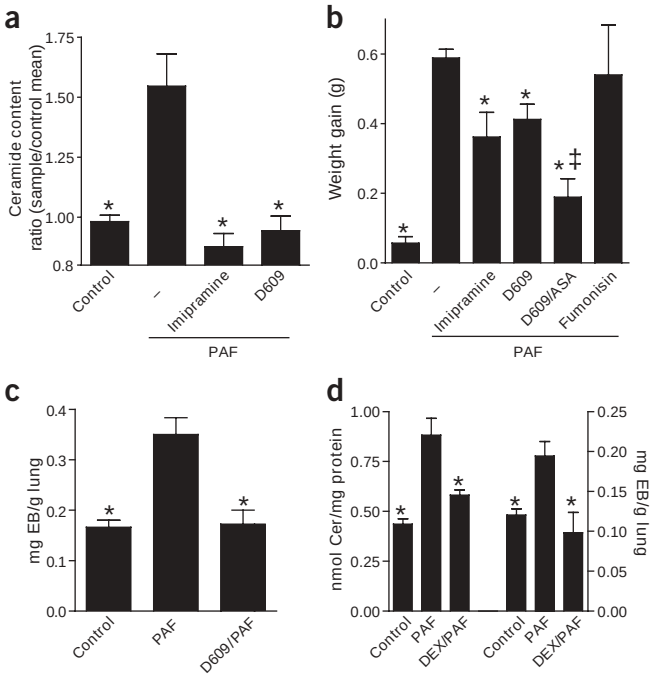

Figure 5 Attenuation of PAF-induced ceramide and edema formation. (**a**) Pulmonary ceramide content in isolated, perfused rat lungs. Controls, $n = 6$; PAF, $n = 11$; D609/PAF, $n = 3$; imipramine/PAF, $n = 3$. (**b**) PAF-induced weight gain in isolated, perfused rat lungs. Controls, $n = 9$; PAF, $n = 42$; D609/PAF, $n = 4$; imipramine/PAF, $n = 5$; D609/ASA/PAF, $n = 3$; fumonisin B1/PAF, $n = 3$. (**c**) Effect of D609 on PAF-induced edema formation in mice *in vivo*. Controls, $n = 28$; PAF, $n = 27$; D609/PAF, $n = 6$. EB, Evans blue. (**d**) Effect of dexamethasone (DEX) on PAF-induced ceramide (Cer; left three bars) and edema (right three bars) formation in mice *in vivo*. Control, $n = 11$; PAF, $n = 12$; dexamethasone/PAF, $n = 6$. *, $P < 0.05$ compared with PAF alone; ‡, $P < 0.05$ compared with PAF and D609.

and, under certain conditions, can function at neutral pH[16]. Endothelial cells, which are thought to regulate vascular permeability, are a particularly rich source of active S-SMase[16]. Taken together, the increased activity of ASM in the serum of endotoxin-treated[17] and PAF-treated mice (this study) indicates an important role for extracellularly active ASM in ceramide formation.

It is generally accepted that increased vascular permeability results from alterations in endothelial cells[33]. These cells are the probable targets for the two different ceramide-specific antisera, as they were given intravascularly. Therefore, and in line with previous observations in lipopolysaccharide (LPS)-induced lung injury[34], we believe that ceramide is formed in and acts on vascular endothelial cells. The importance of ceramide in PAF-induced edema formation was shown using pharmacological interventions and specific antibodies. D609 interferes with activation of ASM[26] and sphingomyelin synthase[27], while imipramine reduces ASM stability and triggers the degradation of ASM protein[29]. Although neither agent is absolutely specific, they are structurally unrelated and their common effect was the downregulation of ceramide production (**Fig. 5a**), suggesting ceramide release by ASM as a common target.

Both the ceramide measurements and the specificity of the antisera suggest that PAF-induced edema is mediated by long-chain ceramide species. Because of their insolubility, however, we were unable to examine long-chain ceramides. The potential of ceramide to induce pulmonary edema was shown by perfusion of rat lungs with C2-ceramide, although it is not clear to what extent C2-ceramide mimics long-chain ceramides[35,36]. Perfusion with ASM (up to 2 U/ml) through the pulmonary artery did not result in edema formation (data not shown). These negative findings could be explained by a number of mechanisms, such as: the high perfusate flow rate of 25–30 ml/min, which shortens the duration of contact between ASM and the endothelium; elimination of the perfused ASM before it reaches its potential target cells; failure of genetically engineered ASM to bind to endothelial cells *in situ*; or the possibility that ASM acts intracellularly. Although little is known about the signaling mechanisms that transmit signals initiated by ceramide[35,36], our experiments suggest that ceramide causes edema by increasing vascular permeability rather than by increasing hydrostatic pressure. First, the lungs were always perfused at a constant pressure, largely excluding the possibility that the edema resulted from hydrostatic pressure. Second, treatment with ceramide-specific antisera (**Fig. 4c,d**) or imipramine (not shown) attenuated PAF-induced edema but had no effect on PAF-induced vasoconstriction. These observations also show that these treatments did not interfere directly with PAF or the PAF receptor.

Several *in vivo* findings suggest that ceramide has clinical relevance as a mediator of pulmonary edema and acute lung injury. Similar to PAF, endotoxin treatment increases circulating ASM concentrations[17] and pulmonary ceramide levels[34], and pretreatment of mice with D609 attenuated PAF- and endotoxin-induced edema formation (**Figs. 5** and **6**). Of note, D609 prevents endotoxin-induced expression of adhesion molecules in the pulmonary vasculature, infiltration of leukocytes, and death[37], events to which PAF might contribute. Tumor necrosis factor has been thought to promote the production of

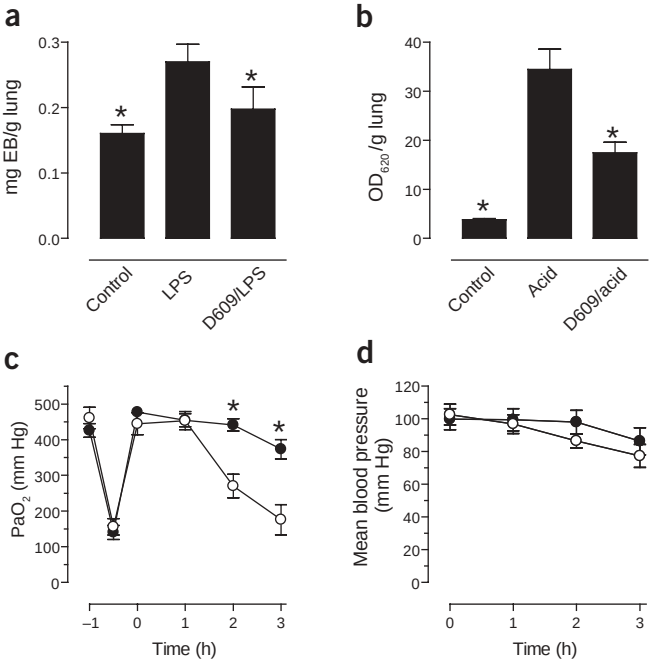

Figure 6 D609 attenuates edema formation in models of ALI. (**a**) Effect of D609 on endotoxin-induced edema formation. Controls, $n = 21$; endotoxin (LPS), $n = 15$; D609/LPS, $n = 5$. EB, Evans blue. (**b**–**d**) Effect of D609 on acid-induced lung injury in rats ($n = 6$). **b**, Pulmonary edema formation, as indicated by amount of Evans blue (OD_{620}, optical density at 620 nm) sequestered in lung tissue. **c**,**d**, Arterial oxygen pressure (PaO_2) and mean blood pressure. Acid instillation, ○; D609 and acid instillation, ●. *, $P < 0.05$ compared with LPS- or acid-treated animals.

ceramide in response to endotoxin. However, the wide range of proinflammatory actions of PAF, together with the present findings, suggest that some activities previously attributed to tumor necrosis factor might also be mediated by PAF through its capacity to trigger the production of ceramide. Acid instillation is another model of ALI in which a central role for PAF is clearly established[6]. The protective effect of D609 in this model therefore lends further support to the proposition that the ASM pathway is of clinical relevance in ALI, at least when PAF is involved. Finally, our finding that dexamethasone prevented the PAF-induced increase in ceramide provides a new explanation for the antiedematous effects of steroids.

Together, these findings suggest that inhibition of extracellular ASM and ceramide biosynthesis or action may serve as a new therapeutic strategy to treat pulmonary edema and other sequelae of inflammatory lung injuries.

METHODS

Materials. Female Wistar rats were obtained from Harlan Winkelman, female BALB/c mice from Charles River, and ASM-deficient (*Smpd1*$^{-/-}$) and wild-type mice from R. Kolesnick (Memorial Sloan-Kettering Cancer Center). All animal experiments were ethically approved by the local authorities (Ministerium für Umwelt, Landwirtschaft und Forsten des Landes Schleswig-Holstein). Antibodies to ceramide were obtained from Glycobiotech (MAS 0020 IgM-enriched polyclonal mouse serum) and Alexis Deutschland (MID 15B4 monoclonal IgM antibody).

Preparation of isolated, ventilated and perfused rat lungs. Rat lungs were prepared as described[13,38]. Lungs were perfused through the pulmonary artery at constant hydrostatic pressure (12 cm H_2O), with Krebs-Henseleit buffer containing 1% rat serum (except in ceramide perfusion experiments during which binding to serum factors was minimized by using 0.1% albumin), 0.1% glucose and 0.3% HEPES.

Experimental design of perfused lung studies. After 30 min of perfusion, 5 nmol PAF (final concentration, 50 nM) was injected as a bolus directly into the tubing leading to the pulmonary artery. All other agents, including ceramide and dihydroceramide (50 μM), were added to the buffer reservoir. Solutions of imipramine (final concentration, 10 μM), D609 (300 μM) or fumonisin B1 (1 μM) were prepared in perfusate buffer and added 10 min before addition of PAF. Antisera (5 or 50 μg/ml in RPMI 1640 medium) were added at the beginning of the experiments. For ceramide quantitation, lungs were shock-frozen and ground in liquid nitrogen 10 min after the addition of PAF.

Experimental design of *in vivo* studies. Edema was assessed by measuring the pulmonary concentration of Evans blue. Evans blue (20 mg/kg) was injected into the lateral tail vein either simultaneously with PAF (4 μg/kg)[5] or 6 h after endotoxin (10 mg/kg intraperitoneally). Seven minutes after injection of Evans blue, PAF- and the LPS-treated animals were killed by intravenous administration of pentobarbital (200 mg/kg) and heparin (100 U/mouse). The acid instillation model was carried out as described[39]. D609 (40 mg/kg intraperitoneally) and antibodies (5 mg/kg intravenously) were administered 30 min before injection of PAF or endotoxin. Because of its short half-life, animals received additional injections of D609 at 30, 60 and 120 min after challenge. After the animals were killed, their lungs were perfused free of blood with ice-cold PBS before the lung tissue was used to determine wet weight–to–dry weight ratio or Evans blue content[40]. In the acid instillation model, Evans blue was quantified by measuring optical density at 620 nm. Evans blue content was always referred to the dry weight of the lung tissue.

Measurement of ceramide levels. Lipids were extracted from freeze-dried lung tissue[41]. The amount of ceramide in the lung tissue from the *in vivo* experiments was determined by charring densitometry[42]; the ceramide content of isolated rat lungs was determined by diacylglycerol kinase assay[43]. As a standard, Sigma ceramide (type III, mostly composed of *N*-stearoylsphingosine) was run in parallel to the lung samples.

Measurement of ASM, NSM and ceramide synthase activity. Sphingomyelinase activity was determined by a modified micellar *in vitro* assay[44] using ^{14}C-labeled sphingomyelin as a substrate. Concentrated perfusate samples (200 μg protein) were assayed for ASM activity in the presence of 100 μM $ZnCl_2$, in a buffer containing 250 mM sodium acetate, 1 mM EDTA (pH 5.0) and 0.1% Triton X-100. Zn^{2+} dependency was assayed by replacing Zn^{2+} with 1 mM EDTA.

NSM activity was assayed in a buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na_3VO, 0.1 mM Na_2MoO_4, 30 mM *p*-nitrophenylphosphate, 10 mM β-glycerophosphate, 1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, 750 μM ATP and 0.2% NP-40. To start the reaction, we added a mixture of 0.5 μM sphingomyelin and 0.04 μCi *N*-methyl-[^{14}C]sphingomyelin and increased the temperature to 37 °C. The reaction was stopped after 1 h, and 750 μl $CHCl_3$:CH_3OH (2:1, vol/vol) was added to extract the phosphorylcholine. After adding 250 μl H_2O, scintillation counting of the aqueous phase was done to determine the amount of radioactive phosphorylcholine produced by hydrolysis of [^{14}C]sphingomyelin.

Ceramide synthase (sphinganine *N*-acyltransferase) activity was assayed in a reaction mixture (1.0 ml) containing 20 mM HEPES (pH 7.2), 2 mM $MgCl_2$, 20 μl BSA, 20 μM sphinganine, 70 μM unlabeled palmitoyl-coenzyme A, 3.6 μM (0.4 μCi) [1-^{14}C]palmitoyl-coenzyme A and 75 μg protein. After 1 h (37 °C), lipids were extracted and separated by thin-layer chromatography in $CHCl_3$, methanol and 3.5N NH_4OH (85:15:1, vol/vol/vol). Radiolabeled dihydroceramide was determined by two-dimensional laser scanning.

Statistics. Data were expressed as mean ± s.e.m., and were analyzed using unpaired Student *t*-tests. In the case of repeated measurements (such as those obtained during time courses), the maximum value of each curve was analyzed. The false discovery rate due to multiple comparisons was controlled by the Benjamini-Hochberg method[45]. $P < 0.05$ was considered to indicate statistical significance.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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