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Ceramide alters endothelial cell permeability by a nonapoptotic mechanism

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1 Ceramide is a lipid second messenger that was recently identified as mediator of pulmonary edema *in vivo*. Here, we investigated the effect of ceramide on the permeability of confluent endothelial cell monolayers.

2 In monolayers of bovine pulmonary artery and human microvascular pulmonary endothelial cells, incubation with C6-ceramide for 3 h elevated permeability in a concentration-dependent manner, whereas dihydroceramide was without effect.

3 After 3 h of incubation with ceramide, we found no signs of necrosis (release of lactate dehydrogenase, loss of thiazylyl blue reduction) or apoptosis (ssDNA, caspase-8 activity).

4 The increased endothelial permeability in response to ceramide was attenuated by the Ser/Thr protein kinase inhibitors K252a, K252b and H-7, as well as by the phosphatidylinositol-specific phospholipase C inhibitor L108. Since in some systems sphingosine-1-phosphate (S1P) acts antagonistic to ceramide, the effect of S1P was studied. S1P transiently increased endothelial cell resistance, whether it was given together with ceramide or 90 min thereafter.

5 These data provide a novel example of the antagonism between S1P and ceramide. Our findings further suggest that ceramide alters vascular permeability by activation of pathways dependent on unidentified phospholipase C and Ser/Thr kinase isoenzymes.
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Abbreviations: C6-ceramide, *N*-hexanoylsphingosine; CPAE, calf pulmonary artery endothelium; dh-C6-ceramide, *N*-hexanoyl-dihydrosphingosine (dihydro-C6-ceramide); Edg, endothelial differentiation gene; FITC, fluorescein isothiocyanate; HMVEC-L, human microvascular endothelial cells from the lung; LDH, lactate dehydrogenase; MEM, minimum essential medium; PAF, platelet-activating factor; S1P, sphingosine-1-phosphate; TNF, tumor necrosis factor α; VEGF, vascular endothelial growth factor

Introduction

The sphingolipid ceramide is an important second messenger. So far, concerning endothelial cells most investigators focused on its role in apoptosis (Kolesnick & Fuks, 2003). Other functions of ceramide include regulation of differentiation, growth suppression and cell senescence (Kolesnick, 2002). Recently, we have identified a novel role for ceramide as a mediator of platelet-activating factor (PAF)-induced pulmonary edema (Göggel *et al*., 2004).

Noncardiogenic pulmonary edema is a hallmark of acute lung injury, which in conjunction with multiple organ failure is a major cause of death in intensive care units. Confluent endothelial cell monolayers are widely used as a model for the investigation of permeability changes induced by inflammatory agents such as thrombin (Laposata *et al*., 1983; Garcia *et al*., 1986) or TNF (Petrache *et al*., 2001; Tiruppathi *et al*., 2001). While the cellular mechanisms by which these mediators alter permeability have been investigated intensively, the role of sphingolipids in vascular permeability is just emerging. Interestingly, another sphingolipid, sphingosine 1-phosphate (S1P), was reported to promote endothelial cell barrier integrity by cytoskeletal rearrangement (Garcia *et al*., 2001; Schaphorst *et al*., 2003) and Rac1 activation (Vouret-Craviari *et al*., 2002), thereby attenuating the increased permeability induced by thrombin or vascular endothelial growth factor (VEGF) (Sanchez *et al*., 2003). The effect of S1P on ceramide-induced alterations in vascular permeability is unknown.

To extend our *in vivo* findings (Göggel *et al*., 2004) and to further study the role of ceramide as a mediator of permeability edema, here we characterized the effects of ceramide on endothelial cells with respect to endothelial cell permeability, apoptosis and necrosis. Various antagonists and S1P were used to examine possible signaling mechanisms and to identify strategies to prevent the permeability-enhancing effects of ceramide.

Methods

Cell culture

Calf pulmonary artery endothelial (CPAE) cells were obtained from the American Type Culture Collection (Catalog No. CCL-209, Manssas, VA, U.S.A.) and used at the 3rd to 10th passage after thawing. Cells were grown in minimal essential

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medium (MEM) with Earle's salts modified to contain 1.0 mM sodium pyruvate and 0.1 mM nonessential amino acids supplemented with 20% fetal calf serum (FCS) and penicillin–streptomycin (1 ×). Media, supplements and trypsin/EDTA were obtained from PAA Laboratories (Cölbe, Germany). Primary endothelial cells from human microvascular lung tissue (HMVEC-L) prepared by Clonetics (San Diego, CA, U.S.A.) were obtained from Cambrex Bio Science (Catalog No. CC-2725, Verviers, Belgium) and used between the 3rd to 8th passage after thawing. HMVEC-L cells were grown in EGM-2-MV complete growth medium and passaged with cell-specific reagents obtained from Cambrex Bio Science (Verviers, Belgium).

Endothelial monolayer permeability measurements

Endothelial cells were seeded on Costar Transwell® membrane inserts (12 mm diameter, 0.4 μm pore size, polycarbonate membrane, obtained from Corning Inc., New York, U.S.A.) at a density of 30,000 cells per cm^2. Medium was changed daily; cells were cultured for 3 days to obtain confluent monolayers. At 4 h before the start of the experiments, the medium was changed to low serum medium containing MEM supplemented with 2% heat-inactivated FCS. Endothelial permeability was determined by two different methods, that is, fluorescein isothiocyanate (FITC)–albumin diffusion and transendothelial electrical resistance.

FITC–albumin diffusion A measure of 1 mg ml^{-1} FITC-labeled bovine albumin (obtained from Sigma, Taufkirchen, Germany) was added to the upper chamber of the Transwell insert. After a diffusion time of 90 min, diffused unbound FITC–albumin was analyzed in the lower chamber by fluorescence measurement (485 nm excitation, 530 nm emission) essentially as described before (Garcia *et al*., 1986). The FITC–albumin diffusion rate across membranes without cells was defined as 100% permeability, and the diffusion rate across an untreated cell monolayer (control) was defined as 0% permeability. These two values were determined on each experimental day and used for calculation of the permeability index. All data were measured in triplicate. For statistical evaluation, we used the mean of the triplicate; $n = 3$ refers to three independent experiments performed in triplicate.

Transendothelial electrical resistance The transendothelial electrical resistance measurement system of World Precision Instruments (obtained from H. Fein, Berlin, Germany) was used. Transwell inserts with the endothelial monolayer were put in an Endohm-12 adaptor, which was preincubated with low serum medium. The electrical resistance of the monolayer at different time points after treatment was determined with the EVOMX Epithelial Tissue Volt/Ohmmeter. Resistance of an empty membrane and of an untreated monolayer were determined as references on every experimental day.

Phase-contrast microscopy

In 24-well plates, CPAE cells were grown to confluency and kept at this density for 2 days. Cell layers were incubated in low serum medium (MEM, 2% heat-inactivated FCS) for 4 h, and then treated with ceramide, dihydroceramide or ethanol (vehicle) for 3 h. Cells were photographed with a phase-contrast microscope (Leica, DM IRB). In phase-contrast microscopy, the space between cells appears bright. To focus on the endothelial gaps, we inverted the gray pattern of the images, so intercellular space and endothelial gaps appeared dark.

Cell viability and apoptosis tests

Cell viability was assessed by the MTT (thiazolyl blue) test (Mosmann, 1983) or by measuring the lactate dehydrogenase (LDH) activity in the supernatant and the cell pellet (Legrand *et al*., 1992) after 4 h incubation. Summing up the activity of LDH in supernatant and pellet yields the total amount of LDH (LDH_{total}). Since LDH is a cytosolic enzyme, the percentage of LDH released into the supernatant (LDH_{sup}/LDH_{total}) is an indicator of necrotic cell death. Single-stranded DNA (ssDNA) apoptosis ELISA was performed according to Frankfurt & Krishan (2001). The ssDNA ELISA kit was obtained from Chemicon International (Temecula, CA, U.S.A.). Caspase-8 activity was measured with the ApoAlert Caspase-8 colorimetric assay kit (BD Biosciences Clontech, Heidelberg, Germany).

Pharmacological intervention

Endothelial cells were seeded and grown as described above. On the day of the experiment, cells were incubated in low serum medium for 4 h, incubated with the pharmacological inhibitor for 30 min and then treated with C6-ceramide for 3 h in triplicate. K252a, K252b, H-7, L108, ML-7 and ML-9 were obtained from Biomol (Hamburg, Germany), genistein, phallacidin and cytochalasin B from Sigma (Taufkirchen, Germany).

The inhibitors used are characterized as follows: L-108 inhibits the phospholipase C with an inhibitory concentration (IC_{50}) of 10 μM (Powis *et al*., 1992); K252a inhibits calmodulin kinase II with an IC_{50} of 2 nM (Hashimoto *et al*., 1991) and protein kinase A (PKA), protein kinase C (PKC) and myosin light-chain kinase (MLCK) with an IC_{50} of 20 nM (Kase *et al*., 1987; Yamada *et al*., 1987). K252b inhibits PKC with an IC_{50} of 20 nM and PKA, PKG and MLCK with an IC_{50} around 100 nM (Kase *et al*., 1987) and was used at a maximum of 1 μM. ML-7 (IC_{50} 0.3 μM) and ML-9 (IC_{50} 3.8 μM) are fairly specific inhibitors of MLCK (Saitoh *et al*., 1987; Bain *et al*., 2003) and were used at a maximum of 30 μM. Phallacidin is an actin-stabilizing agent and inhibits the thrombin-induced permeability in endothelial cells at 0.3 μM (Phillips *et al*., 1989) and was used here at a maximum of 1 μM. Genistein is a tyrosine kinase inhibitor and has been shown to protect against thrombin-induced monolayer permeability at 5 μM (Liu *et al*., 2005) and was used here at a maximum of 30 μM. H7 inhibits PKA, PKG and PKC with an IC_{50} of about 5 μM and MLCK with an IC_{50} of 97 μM (Hidaka *et al*., 1984) and was used at a maximum of 10 μM.

Data and statistics

Unless stated otherwise all data are described as mean ± s.e.m. Median effective (EC_{50}) and median IC_{50} were calculated using Graphpad Prism version 4 for Windows (Graphpad Software San Diego, CA, U.S.A.). Statistical significance was calculated

using Dunnett's test (one-sided against control). $P<0.05$ were considered statistically significant.

Results

Ceramide increases permeability of endothelial monolayers

Incubation of confluent endothelial cell monolayers with 30 μM C6-ceramide led to a gradual loss of electrical resistance across the monolayer, which was maximal (50–60%) after 180 min in both CPAE and HMVEC-L cells and plateaued thereafter (Figure 1a and b). In both cell types, dihydro-C6-ceramide had no significant effect under the same conditions.

Increased endothelial cell permeability was also assessed by using FITC-labeled albumin diffusion across the cell monolayer (Figure 1c and d). After incubation for 180 min, C6- but not dh-C6-ceramide, concentration dependently increased endothelial cell permeability in both cell types. At 30 μM, C6-ceramide was about equally potent in both cell lines (30–40% loss of barrier function), but at higher concentrations HMVEC-L cells were more sensitive and almost completely lost their barrier function when 300 μM of ceramide were applied. The different EC_{50} values for the two cell types may be explained either by the fact that CPAE cells represent an immortalized cell line, while HMVECs are primary lung cells, by the species differences or by their different anatomical origin. However, at a dose of 30 μM, there was almost no difference in the absolute permeability increase between the two cell types. Therefore, for further experiments, we chose this concentration.

In C6-ceramide-treated monolayers, the spaces between cells became darker and wider, suggesting that cell–cell connections loosened throughout the whole monolayer (Figure 2). However, we observed no extensive gap formation as was shown in the case of thrombin (Laposata *et al*., 1983; Garcia *et al*., 1986; Rabiet *et al*., 1996).

Ceramide does not cause necrosis or apoptosis within 4 h

To exclude the possibility of necrotic cell death caused by the amphiphilic character of the C6-ceramide molecule, we performed LDH release assays with both CPAE and HMVEC-L cells. LDH-release of C6-ceramide (30 μM)-treated cells was not different from control conditions, while high concentrations of H_2O_2 led to severe cell disruption (Figure 3a and b). Similar results were obtained with the MTT (thiazolyl blue) turnover test that measures mitochondrial activity (Figure 3c).

C6-ceramide has been reported to cause endothelial cell apoptosis after 12–18 h of incubation (Slowik *et al*., 1996; Escargueil-Blanc *et al*., 1998); C2-ceramide was found to induce apoptosis at a rate of approximately 20% in umbilical

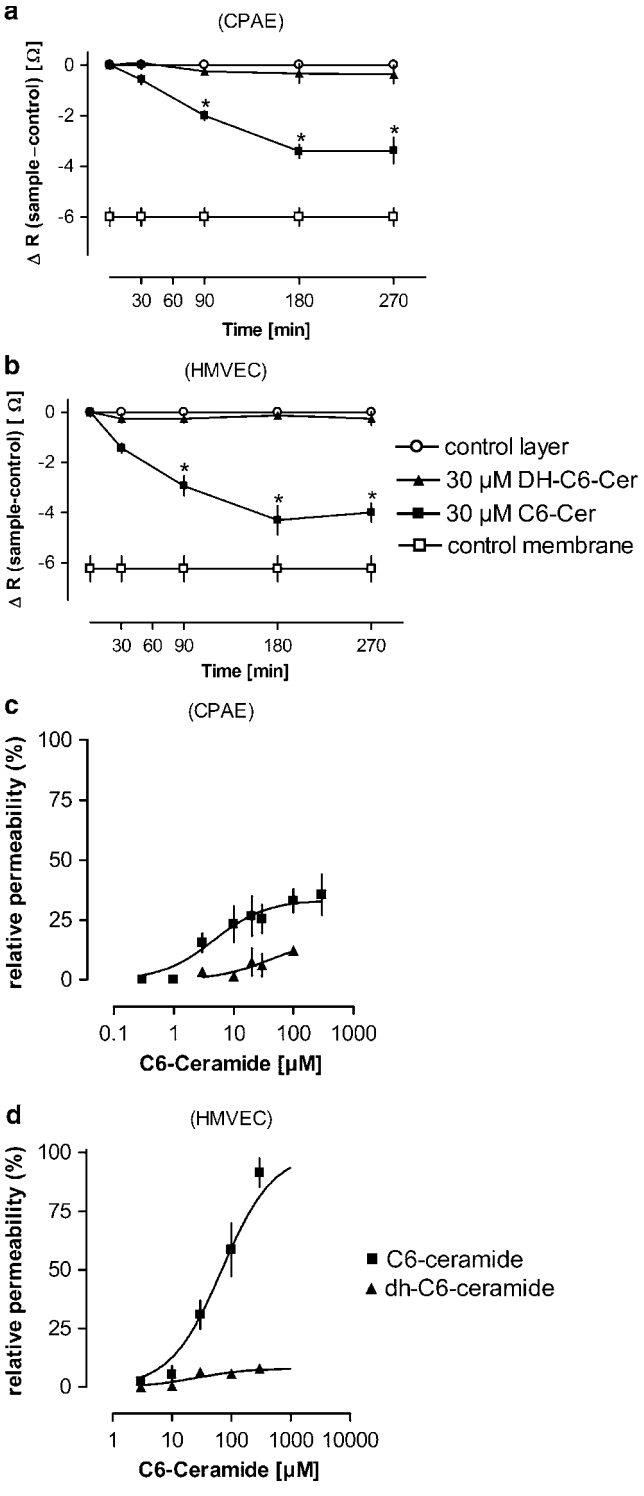

Figure 1 C6-ceramide increases endothelial permeability in CPAE and HMVEC-L cells. (a and b) Electrical resistance was measured across the cell monolayers 30, 90, 180 and 270 min after treatment with 30 μM C6-ceramide or dh-C6-ceramide. $^*P<0.01$ for C6-ceramide-treated compared to untreated monolayers, $n=4$–8. Vehicle (1 μl ethanol in 500 μl medium) treated monolayers were used as controls ($\Delta R=0$, upper line); the maximum permeability ($\Delta R=$ max., lowest line) was obtained from membranes without cells. (c and d) Concentration dependency of permeability changes in monolayers from both cell types. Cells were incubated for 3 h with C6-ceramide or dh-C6-ceramide ($n\geqslant 3$). Permeability was detected by FITC–albumin transmigration from the upper chamber of the Transwell insert system to the lower chamber across the cell monolayer. FITC–albumin concentrations in the lower chamber of vehicle treated membranes correspond to zero, FITC–albumin concentrations in the lower chamber of empty membranes correspond to 100% permeability index. The EC_{50} value for CPAE cells was 4.7 μM and for HMVEC-L cells 69 μM.

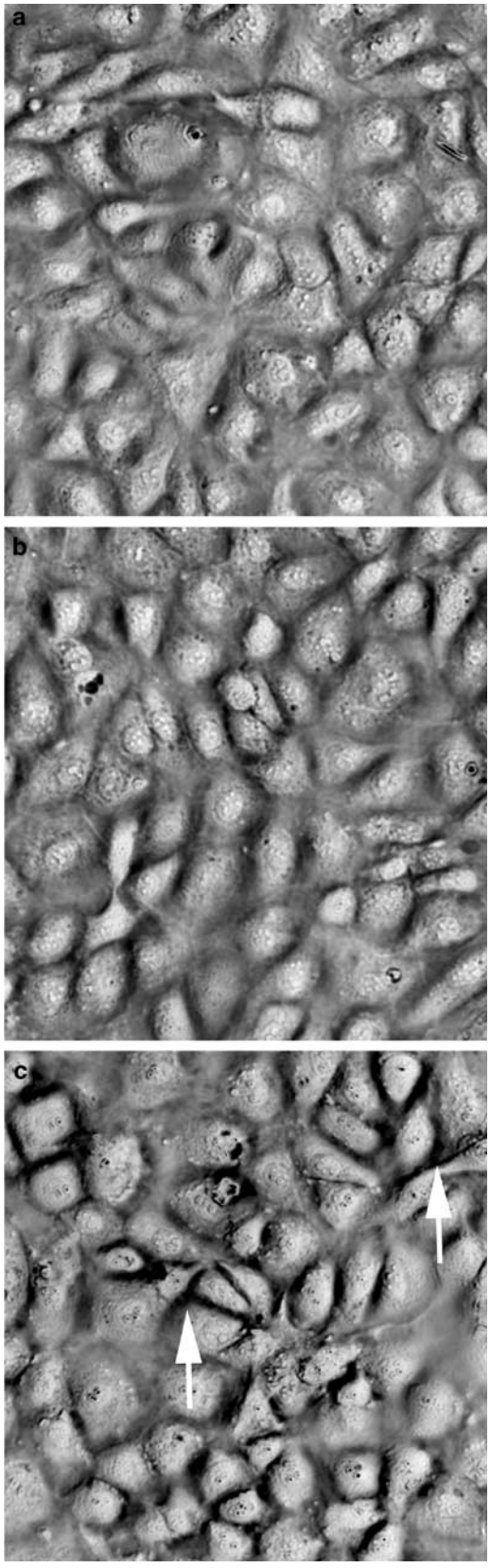

Figure 2 Microscopic images of endothelial CPAE cell monolayers (1 : 200). The images shown are representative for three independent experiments. Phase-contrast images are inverted, so dark areas show intercellular spaces while cells appear bright. Cells were incubated for 4 h with vehicle (a), 30 μM dh-C6-ceramide (b) or 30 μM C6-ceramide (c). Arrows mark loosened cell–cell connections.

vein endothelial cells after 4 h (Hisano *et al*., 1999; Madge *et al*., 1999). To investigate whether programmed cell death might contribute to increased permeability, we checked apoptosis by three different methods. First, we looked for nuclear condensation within 4 h after ceramide administration using the Hoechst 33342 fluorochrome to stain DNA. There was no difference in nucleus appearance between treated and untreated cells (data no shown). Secondly, we measured caspase-8 activity as an early sign of apoptosis. Caspase-8 activity was increased by cycloheximide and TNF, but not by C6-ceramide (Figure 3d). Finally, we assessed apoptosis by measuring ssDNA with an ELISA that uses formamide to denature DNA in apoptotic, but neither in necrotic nor healthy cells (Frankfurt & Krishan, 2001). Also, with this test, we did not detect any signs of apoptosis in ceramide-treated cells, while as a positive control the combination of TNF-α and cycloheximide induced apoptosis (Figure 3e and f).

Pharmacological interventions

To investigate the mechanisms by which ceramide increases permeability, we screened a number of pharmacological inhibitors aimed at a variety of signaling pathways. Pretreatment with the Ser/Thr kinase inhibitor K252a (1 μM) largely reduced the increase in endothelial cells' monolayer permeability induced by C6-ceramide in both cell types (Figure 4a and b). The structurally similar agent K252b (1 μM) also showed a strong inhibitory effect in CPAE cells, while being somewhat less potent than K252a in HMVEC-L cells (Figure 4a and b).

L108 inhibits the phosphatidylinositol-specific phospholipase C, and may thus affect the production of inositoltrisphosphate. Treatment of the monolayers with L108 largely reduced the ceramide-induced permeability in both CPAE and HMVEC-L cells (Figure 4a and b). Treatment with H-7 had a small nonsignificant effect on the ceramide-induced increase in monolayer permeability.

Inhibiting cytoskeletal arrangement with phallacidin, the MLCK with ML-7 or ML-9 and tyrosine kinases with genistein had no influence on ceramide-mediated permeability in endothelial cells (Figure 4c).

Next, we examined the concentration dependency of the two most potent inhibitors L108 and K252a on the permeability increase induced by 30 μM C6-ceramide in CPAE cell monolayers. L108 showed a maximum inhibitory effect of 80% at a concentration of 18 μM; the IC_{50} value was 11 μM. At higher concentrations, the substance became toxic and thus raised permeability indices by itself (data not shown). K252a completely abolished permeability alterations at a concentration of 100 nM with an IC_{50} value of 22 nM (Figure 5).

Antagonism of S1P

S1P was reported to promote endothelial cell barrier integrity (Garcia *et al*., 2001; Vouret-Craviari *et al*., 2002; Schaphorst *et al*., 2003), as exemplified for permeability changes caused by thrombin or VEGF (Sanchez *et al*., 2003). The maximum effective concentration of S1P in these models is 1 μM (Garcia *et al*., 2001). Here, we tested its effect on the increased permeability induced by C6-ceramide. First, we confirmed the barrier-stabilizing property of S1P (1 μM) in our model, as demonstrated by an increase in electrical resistance (Figure 6a).

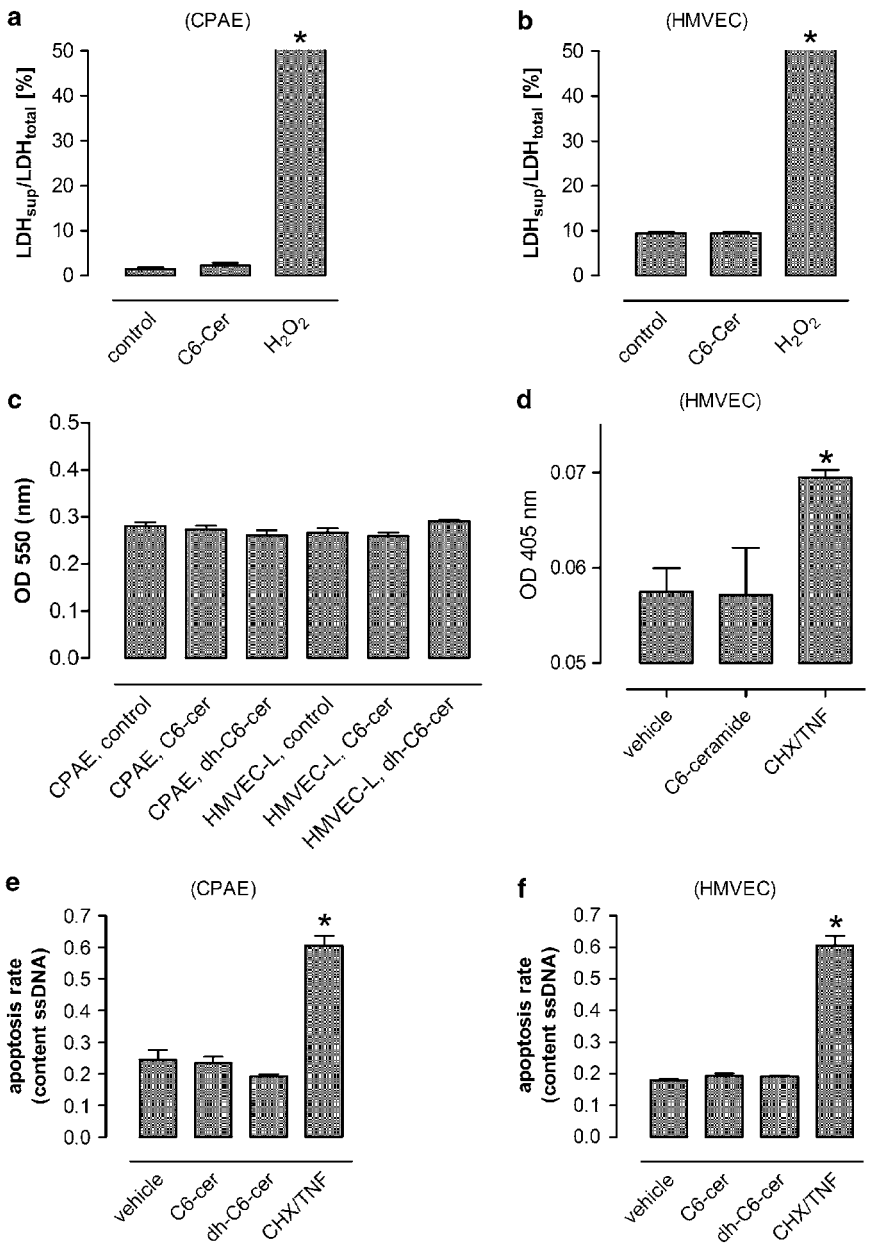

Figure 3 Necrosis and apoptosis. Cells were treated for 3 h with the various agents: 30 μM C6-ceramide, 30 μM dh-C6-ceramide, 10 mM H_2O_2, 100 ng ml^{-1} TNF, 100 μM cycloheximide (CHX) and vehicle (1 μl ethanol in 500 μl medium). (a and b) LDH activity was measured in supernatant and cell pellet in the same volume after 4 h in CPAE (a) and HMVEC (b) cells. The data are expressed as the percentage of total LDH released from the cells. *Higher than control, $P<0.01$, $n=6$. (c) MTT (thiazlyl blue) reaction of treated and control cells after 4 h. Only vital cells metabolize MTT to a blue dye ($n=3$). (d) Caspase-8 activity in HMVEC-L cells ($n=3$). (e and f) Apoptosis detection using altered DNA melting conditions of apoptotic cells in CPAE (d) and HMVEC (e) cells. ssDNA was measured with a peroxidase-conjugated antibody using ABTS as a substrate for peroxidase reaction, *Significantly ($P<0.05$) higher than vehicle; $n=9$ for control and C6-ceramide, $n=3$ for dh-C6-ceramide and CHX/TNF.

Secondly, we applied S1P simultaneously with C6-ceramide (30 μM). This reduced the S1P-induced increase in electrical resistance (Figure 6a). When we applied 1 μM S1P 90 min after incubation with 30 μM C6-ceramide, S1P transiently increased transendothelial resistance (Figure 6b).

Discussion

This study shows that C6-ceramide increases endothelial cell monolayer permeability. These findings raise the possibility that this model may be used to investigate the hitherto unknown molecular mechanisms by which ceramide increases pulmonary vascular permeability *in vivo* and in the whole intact organ (Göggel *et al*., 2004).

The naturally occurring long chain ceramides (C16 and C18) are almost insoluble in aqueous solutions, so that we could only use C6-ceramide. However, even C6-ceramide is rather hydrophobic and might therefore increase vascular permeability simply by its biophysical properties. However, several findings suggest that ceramide acted by specific pathways. First, dh-ceramide had no effect similar to that of ceramide,

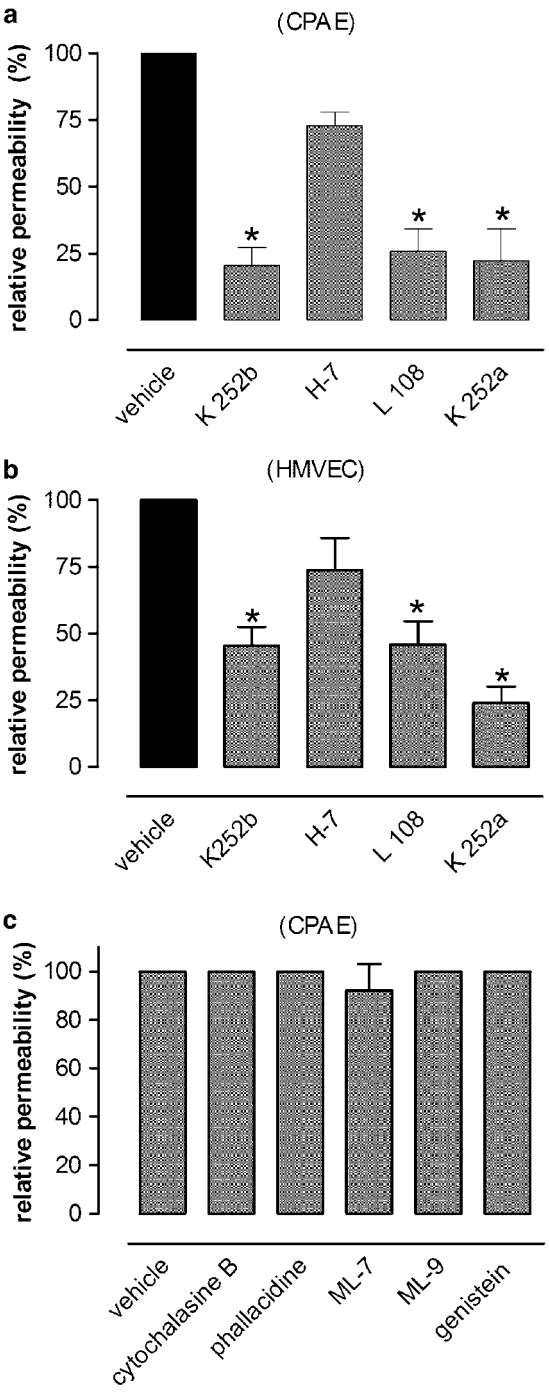

Figure 4 Pharmacological inhibition of ceramide-induced alterations of endothelial permeability. At 4 h of preincubation in low serum medium and 30 min incubation with the inhibitors was followed by 3 h incubation with 30 μM C6-ceramide. Relative permeability refers to ceramide-induced increase of permeability defined as 100% and permeability of untreated layers defined as 0%. (a and b) Permeability changes in CPAE (a) and HMVEC (b) cells. Inhibitor concentrations were: 30 μM L108, 10 μM H-7 and 1 μM K252a and K252b ($n=4$ for each inhibitor); *Significantly ($P<0.01$) reduced compared to ceramide alone, $n=4$. (c) Agents that had no inhibitory effect on ceramide-induced permeability alterations in CPEA cells: 1 μM cytochalasin B, 1 μM phallacidin, 30 μM ML-7, 30 μM ML-9 and 30 μM genistein.

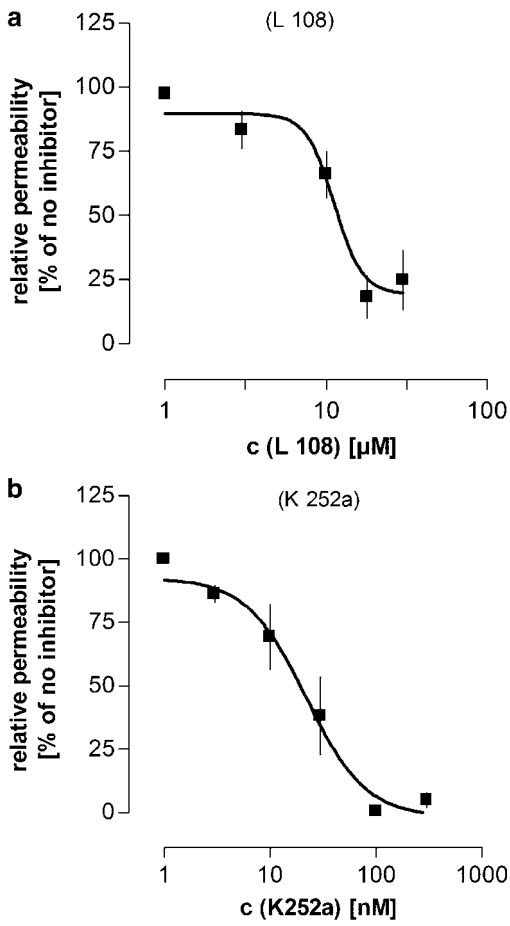

Figure 5 Dose dependency of the inhibitory effect of L108 (a) and K252a (b) on CPAE cells ($n\geqslant 3$). Cells were preincubated with low serum medium for 4 h, incubated for 30 min with different concentrations of the inhibitors and then treated with 30 μM C6-ceramide for 3 h. Relative permeability refers to ceramide-induced increase of permeability as 100% and permeability of untreated layers as 0%. The IC_{50} value for L108 was 11 μM (the curve was fitted with the constraint that the Hill slope be <5) and for K252a 22 nM.

recapitulating our findings in intact lungs (Göggel *et al*., 2004). Second, ceramide did not induce gross alterations in the plasma membrane, leading to cellular necrosis. And third, a variety of antagonists were able to prevent the effect of ceramide, implicating the existence of specific signaling pathways.

One possibility of how ceramide might act is by increasing transcellular albumin transport. However, as loss in transendothelial electrical resistance (50–60%, 30 μM ceramide) was higher than the increase in FITC–albumin transmigration (30–40%, 30 μM ceramide), this appears highly unlikely. Hence, we conclude that the altered permeability is the result of a leaky cell layer rather than due to an increase in transcellular albumin transport. Of note, this appears to be different from the effect of α-thrombin on microvascular endothelial cells, where we observed a high increase of FITC–albumin transmigration compared to a relatively moderate loss of electrical resistance within the first 3 h, which can only be explained by active albumin transport (data not shown). Ceramide treatment led to loosened cell–cell contacts with widened intercellular spaces, but we never observed gap formation as described for the effect of thrombin on

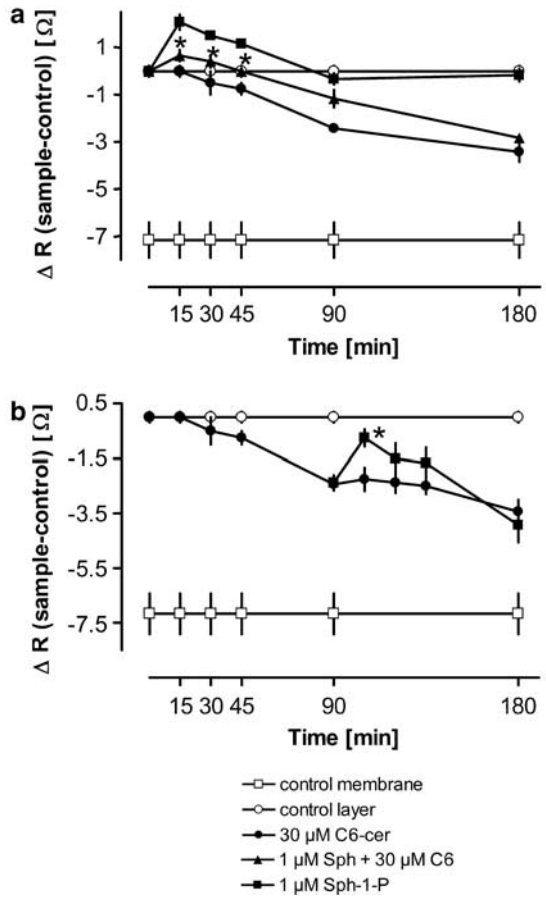

Figure 6 Antagonistic effects of ceramide and S1P. (a) Electrical resistance of CPAE cell monolayers treated with 1 μM S1P alone, C6-ceramide alone or S1P together with 30 μM C6-ceramide. Also shown are the results for control cell layers and for empty membranes. *$P<0.05$ (lower) compared to S1P-treated cells, $n=6$. (b) Electrical resistance of CPAE monolayers exposed to 30 μM C6-ceramide with the addition of 1 μM S1P after 90 min. *$P<0.05$ (greater) compared to ceramide-treated cells, $n=6$.

endothelial cell layers (Laposata *et al*., 1983; Garcia *et al*., 1986).

Ceramide is an important mediator of apoptosis in many cell types including endothelial cells. Therefore, it was important to exclude the possibility that endothelial permeability was simply caused by apoptotic cell death. Previously, apoptosis was shown to take place after C2- or C6-ceramide treatment of endothelial cells (Hisano *et al*., 1999), but at time points later than 3 h (Escargueil-Blanc *et al*., 1998) and at low rates (Madge *et al*., 1999). Within our 3 h study period, we did not observe any signs of increased apoptosis such as caspase-8 activation, nuclear condensation, membrane blebbing or DNA destabilization. In addition, none of the inhibitors that attenuated the effects of ceramide on vascular permeability is known to block apoptosis. Taken together, we exclude the possibility that the increase in permeability was simply the result of apoptosis.

The action of ceramide on endothelial cells was attenuated or abolished by several inhibitors of intracellular signaling pathways. K252a, K252b and H-7 are alkaloids that inhibit the Ser/Thr kinases PKA, PKB, PKC and MLCK (Kase *et al*., 1987; Yamada *et al*., 1987). The IC_{50} of 22 nM found for K252a in our model meets well the known specificity of this compound for PKC and PKA (20 nM). However, K252a is 10 times more specific for the calcium/calmodulin-dependent kinase than for the other kinases (Hashimoto *et al*., 1991; Howe *et al*., 2002). Before, K252a was reported to inhibit the effect of C2-ceramide on retinal ganglion cell survival (Ito, 2003). K252a is also known to induce rapid cell adhesion and spreading with concomitant formation of actin stress fibers in nonadherent colon adenocarcinoma Colo201 cells (Mohri *et al*., 1998). Whether a similar mechanisms applies in our model remains to be shown. L108 inhibits phosphatidylinositol-specific PLC (Powis *et al*., 1992), an enzyme that liberates inositol 1,4,5-trisphosphate (IP3) that subsequently may induce calcium release from intracellular stores (Streb *et al*., 1983). The IC_{50} value (11 μM) obtained for inhibition of ceramide-induced monolayer permeability agrees well with the known IC_{50} value of PLC inhibition (10 μM). Since L108 and K252a interfere at different sites of the same signaling pathway, it appears possible that ceramide increases vascular permeability *via* IP3-dependent calcium release.

As referred to before, the mechanisms by which ceramide alters vascular permeability clearly differ from that of thrombin. Ceramide did not cause transcellular transport and intercellular formation of big gaps. In line with the absence of sizeable gaps, we found no effect by inhibitors of MLCK or the cytoskeleton, the protective effects of which are well described for endothelial barrier dysfunction induced by thrombin (Garcia *et al*., 1986; Dudek & Garcia, 2001). Of note, an increase in vascular permeability independent of MLCK was also observed with TNF (Petrache *et al*., 2001). Since TNF may lead to generation of ceramide (Haimovitz-Friedman *et al*., 1997; Mallampalli *et al*., 1999), ceramide may be the intracellular signaling messenger for TNF in this biological response.

S1P is known to strengthen endothelial barrier integrity (Garcia *et al*., 2001; Schaphorst *et al*., 2003). This response to S1P is mediated by Edg-1 and Edg-3 receptors that are expressed on endothelial cells (Garcia *et al*., 2001). These receptors couple to $G_{i\alpha}$-receptors and signal through RhoA, Src and Rac1 to induce actin filament rearrangement (Garcia *et al*., 2001; Vouret-Craviari *et al*., 2002). In the present study, S1P induced a transient increase in cellular resistance, but did not inhibit C6-ceramide-induced increase in permeability at later time points. This would suggest that the effects of S1P and C6-ceramide are independent phenomena. Nevertheless, this is interesting information, since S1P and ceramide are structurally closely related, such that sphingosine is the product of ceramide hydrolysis by ceramidases (Huwiler *et al*., 2000). Recently, in another study S1P antagonized another effect of ceramide, that is, apoptosis induced by ceramide in mouse fibroblasts (Castillo & Teegarden, 2003). Thus, it seems possible that ceramide and S1P form a pair of chemically similar, but functionally antithetic lipid mediators, akin to the pair thromboxane and prostacyclin.

In summary, we have shown that endogenously applied C6-ceramide increases transendothelial permeability by a nonapoptotic mechanism, which is independent of cytoskeletal rearrangement and which is mediated by unidentified phospholipase C and Ser/Thr kinase isoenzymes.

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