

Exogenous sphingomyelinase causes impaired intestinal epithelial barrier function

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Abstract

AIM: To test the hypothesis that hydrolysis of sphingomyelin to ceramide changes the composition of tight junctions (TJs) with increasing permeability of the intestinal epithelium.

METHODS: Monolayers of Caco-2 cells were used as an *in vitro* model for the intestinal barrier. Permeability was determined by quantification of transepithelial flux and transepithelial resistance. Sphingolipid-rich membrane microdomains were isolated by a discontinuous sucrose gradient and characterized by Western-blot. Lipid content of microdomains was analysed by tandem mass spectrometry. Ceramide was subcellularly localized by immunofluorescent staining.

RESULTS: Exogenous sphingomyelinase increased transepithelial permeability and decreased transepithelial resistance at concentrations as low as 0.01 U/mL. Lipid analysis showed rapid accumulation of ceramide in the membrane fractions containing occludin and claudin-4, representing TJs. In these fractions we observed a concomitant decrease of sphingomyelin and cholesterol with increasing concentrations of ceramide. Immunofluorescent staining confirmed clustering of ceramide at the sites of cell-cell contacts. Neutralization of surface ceramide prevented the permeability-increase induced by platelet activating factor.

CONCLUSION: Our findings indicate that changes in lipid composition of TJs impair epithelial barrier functions. Generation of ceramide by sphingomyelinases might contribute to disturbed barrier function seen in diseases such as inflammatory, infectious, toxic or radiogenic bowel disease.

INTRODUCTION

Abnormal mucosal permeability is a hallmark of inflammatory bowel disease (IBD)^[1,2]. The epithelial barrier function and its relevance in IBD pathophysiology has gained increasing attention in recent years^[2] as it may contribute to increased bacterial translocation and subsequent inflammatory responses in the mucosa.

Many signaling molecules involved in the pathogenesis of IBD such as TNF- α or IFN- γ may cause an alteration of the lipid composition in the cell membrane by activation of sphingomyelinases (SMases)^[3,4]. SMases are characterized by a specific optimal pH and accordingly are divided into acid, neutral and basic sphingomyelinase species. The acid sphingomyelinase (ASM) contributes to lysosomal sphingomyelin turnover and is also secreted upon cellular treatment with inflammatory stimuli^[5]. In contrast, neutral SMase is membrane-bound, alkaline SMase is found in the bile. Activation of SMases is followed by rapid hydrolysis of plasma membrane sphingomyelin to the second messenger ceramide^[6]. The sphingolipid ceramide is an important messenger involved in many signaling pathways with influences on cell differentiation, growth suppression and apoptosis. It is generated upon ligation of receptors like cluster of differentiation (CD) 40, CD95, IL-1 or TNF receptor^[7-10], as response to ionizing radiation^[11-13], ischemia-reperfusion injury^[14] and infections with bacteria like *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*^[15-17] or viruses like Sindbis- or Rhinovirus^[18,19]. Ceramide alters the composition of cholesterol- and sphingolipid-enriched membrane microdomains^[20] and thereby promotes transmembrane signaling^[7-9]. It also has the capacity to restructure the membrane to allow the release of vesicles^[21].

Grassme *et al*^[7,8] have demonstrated that cellular stimulation triggers a translocation of the acid sphingomyelinase from intracellular stores onto the extracellular leaflet of the cell membrane. Surface ASM initiates a release of ceramide which mediates clustering of sphingolipid-rich membrane domains, termed "lipid rafts". Lipid rafts are also described as detergent insoluble cholesterol- and glycosphingolipid-enriched membrane microdomains (DIGs) because the tight packing of the lipids renders rafts resistant to solubilization by non-ionic detergents at low temperatures^[22,23].

Intestinal permeability is influenced by the lipid content of epithelial cells. Dietary fatty acids are known to affect barrier function of the mucosa^[24,25]. Clinical studies showed that omega 3-fatty acids may be of beneficial effect on the course of Crohns disease^[26,27]. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight-junction (TJ). TJs have been identified as microdomains in the plasma membrane with similar characteristics as DIGs^[28,29]. Depletion of cholesterol from Caco-2 cell layers increases permeability^[30], suggesting that cholesterol is critical in maintaining the barrier function.

The proinflammatory cytokines TNF- α and IFN- γ , known to induce ceramide-generation, have recently been shown to disrupt the barrier function of epithelial cells independent from their apoptosis-inducing property^[31]. With regard to the finding that ceramide displaces cholesterol from sphingolipid-enriched microdomains^[20], we hypothesized that formation of ceramide may be an initial event leading to structural lipid-rearrangements of TJs with impaired barrier integrity.

To test this hypothesis we used the intestinal epithelial cell line Caco-2 to assess the effect of exogenous SMase on barrier function and the accompanying lipid composition of TJs.

MATERIALS AND METHODS

Cells and reagents

The human intestinal epithelial cell line Caco-2 was obtained from American Type Culture collection (HTB 37). Cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids 1% sodium pyruvate in an atmosphere containing 10% CO₂ at 37°C. Sphingomyelinase from staphylococcus aureus, platelet activating factor (PAF) and deoxycholic acid were purchased from Sigma-Aldrich, Germany.

Antibodies

The following antibodies were used for Western-blot analysis: Goat polyclonal anti-occludin (C-19) and goat polyclonal anti-claudin-4 (C-18) from Santa Cruz, CA, USA; mouse anti-E-cadherin (clone HECD-1) from Calbiochem, CA, USA; rabbit polyclonal anti p38 mitogen activated protein kinase (MAPK) from New England Biolabs, Beverly, MA, USA. The rabbit polyclonal anti-toll like receptor 4 (TLR4) Ab was a kind gift from Dr. Werner

Falk, Regensburg, Germany. For immunohistochemical staining of tight junctions we used the rabbit polyclonal anti-ZO-1 Ab from Zymed lab. Inc., CA, USA. For visualization and neutralization of ceramide, the monoclonal mouse IgM anti-Ceramide Ab (MID 15B4) from Alexis, Germany was used.

Measurement of transepithelial permeability and transepithelial resistance

For permeability assays, Caco-2 cells were seeded in 12 well plates with a growth area of 1.0 cm² and a pore size of 3 μ m (Transwell[®] permeable supports by Corning Incorporated, MA, USA) at a density of 2×10^5 cells/cm². Media was replaced every 3 or 4 d. Experiments were performed 13-15 d after cells reached confluency with a transepithelial electrical resistance (TEER) between 500-750 Ω -cm². Permeability was quantified by measuring the transepithelial flux of fluorescein-sulfonic acid (Molecular Probes Inc., Germany). After treatment of Caco-2 monolayers with the indicated substances, fluorescein-sulfonic acid was added to the apical side of the monolayers at a final concentration of 100 μ g/mL. After incubation for 4 h 100 μ L aliquots of medium were removed from the basolateral chambers, and fluorescence signal by fluorescein was measured using a fluorescence microplate reader. TEER of the Caco-2 monolayers was measured using a Millicell[®]-ERS epithelial voltohmmeter by Millipore with a pair of chopstick electrodes. Untreated monolayers were used as negative controls. All measurements were performed in duplicate.

Caspase-3/7 activity assay

Caco-2 cells were incubated with the indicated substances 6 h before measurement of caspase-activity. As a positive control for induction of apoptosis, cells were treated with deoxycholic acid (DCA) for 1 h. DCA was sonicated at 40°C for 20 min prior to the experiments. The colorimetric caspase-3/7-activity assay Apo-ONE[™] (Promega, WI, USA) was used according to the manufacturer's recommendations. Activity was quantified using a fluorescence microplate reader with appropriate wavelengths for excitation (485 nm) and emission (530 nm).

Fluorescence microscopy

For fluorescence studies Caco-2 cells were grown on chamber slides (Nunc, Germany) and stimulated with 0.25 U/mL SMase for 10 min. After stimulation the cells were washed with phosphate buffered saline (PBS) at 4°C and fixed for 15 min in 1% (w/v) paraformaldehyde in PBS at room temperature. Then cells were washed three times with PBS and blocked with Tween 20 0.05% in PBS. Cells were washed again and then incubated with anti-ceramide mAb 15B4 (1/50 dilution) and anti ZO-1 Ab for 30 min in washing buffer (PBS with 2% fetal calf serum, 0.01% Na₂S₂O₃) to block sites of non-specific binding. The anti-ceramide Ab was visualized with a PE-labeled anti-mouse IgM, the anti-ZO-1 Ab with a fluorescein-isothiocyanate (FITC)-anti rabbit Ab. Fluorescence staining was viewed with an Axiovert 100 fluorescence microscope (Zeiss, Germany).

Isolation of detergent resistant microdomains

Confluent layers of Caco-2 cells were stimulated in 75 cm² flasks in 5 mL of culture media. Stimulation was terminated by washing the cells with 5 mL ice-cold TNE (25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 5 mmol/L EDTA) on ice. Cells were washed twice with TNE at 4°C and lysed for 20 min in 1.5 mL of ice-cold TNE containing 1% of Triton X-100 and protease inhibitors (10 µg of aprotinin and leupeptin and 200 µmol/L phenylmethylsulfonyl fluoride (PMSF)). Cells were then further homogenized with 20 strokes in a Wheaton loose fitting Dounce-homogenizer. Nuclei and cellular debris were pelleted by centrifugation at 600 g for 5 min, 4°C. To isolate low-density, Triton X-100-insoluble complexes, the supernatant was adjusted to 40% sucrose, transferred to an ultracentrifugation tube, and overlaid with a 35% and 5% discontinuous sucrose gradient. Ultracentrifugation was performed at 29000 r/min (110000 × *g*) at 4°C for 16 h in a Beckman SW41 rotor. Gradient fractions (1 mL) were either collected from the top of the tube or from the 35%/5%-interface to compare the effect of cell stimulation on raft fractions. For mass spectrometry, fractions were prepared as described below. For Western blotting, proteins were precipitated using 400 µL of 10% trichloroacetic acid, neutralized and separated by 10% SDS-PAGE.

Immunoblotting

Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Hybond). Blots were blocked with 5% nonfat dry milk powder and incubated overnight at 4°C with the indicated primary antibodies. All antibodies were diluted 1:750 in Tris-buffered saline supplemented with 0.1% Tween 20. The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized using a commercial chemiluminescence detection kit (ECL plus, Amersham Biosciences).

Tandem mass spectrometry

Lipids were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode as described previously^[32-35]. Samples were quantified by direct flow injection analysis using the analytical setup and the data analysis algorithms described by Liebisch *et al.*^[34]. A parent ion scan of m/z 184 specific for phosphocholine-containing lipids was used for phosphatidylcholine, sphingomyelin^[33] and lysophosphatidylcholine^[33]. Neutral loss scans of m/z 141 and m/z 185 were used for phosphatidylethanolamine and phosphatidylserine, respectively. Ceramide was analyzed similar to a previously described method^[35] using N-heptanoyl-sphingosine as internal standard. Free cholesterol and cholesteryl esters were quantified using a fragment ion of m/z 369 after selective derivatization of free cholesterol^[36]. Quantification was achieved by calibration lines generated by addition of naturally occurring lipid species to cell homogenates^[32-36].

Statistical analysis

Data are shown using vertical scatter plots with Box-

Whisker plots (25% and 75% values), generated in the basic module of the program SigmaPlot. Statistical analysis was performed by Mann-Whitney *U*-test, with $P < 0.05$ considered statistically significant. Data are given as means ± SE (SD in case of lipid analysis).

RESULTS

Exogenous sphingomyelinase enhances permeability in Caco-2 epithelial cell layers

To study a potential regulation of intestinal permeability by sphingomyelinases, Caco-2 cell layers were exposed to different concentrations of exogenous SMase. Transepithelial permeability was determined by measurement of transepithelial flux of fluorescein-sulfonic acid across a monolayer grown on permeable supports. Incubation with SMase to the apical chamber induced a concentration-dependent increase of permeability which could be detected at concentrations as low as 0.01 U/mL SMase (181.6% ± 16.7%, $P < 0.01$) (Figure 1A). Using 0.05 U/mL SMase, permeability was increased by 201.1% ± 15.8% ($P < 0.01$) and by 224.0% ± 18.0% ($P < 0.01$) when 0.125 U/mL SMase were used. Increase of SMase-concentration to 0.25 U/mL did not further increase transepithelial flux (192.0% ± 15.3%, $P < 0.01$) (Figure 1A). In a different set of experiments with the same experimental conditions, PAF was used as a positive control. At a concentration of 5 µmol/L, PAF increased permeability by 162.8% ± 13.0% (Figure 2).

To gain insight into the mechanisms of ceramide-mediated permeability we measured the transepithelial electrical resistance (TEER). Exogenous SMase produced a significant decline in TEER at concentrations as low as 0.01 U/mL (17.5% ± 6.2%, $P < 0.05$) (Figure 1B). The fall in TEER with 0.05 U/mL was much higher (38.1% ± 6.0%, $P < 0.01$). Using 0.125 U/mL SMase or 0.25 U/mL SMase did not further decrease TEER (32.2% ± 7.3%, $P < 0.01$ and 33.2% ± 6.4%, $P < 0.01$, respectively).

To exclude apoptotic or necrotic cell death caused by SMase within the time frame of our experiments, caspase-3/7-activity and LDH release assays were performed. As shown in Figure 1C, 0.25 U/mL SMase induced no activation of caspase-3/7 within 6 h. Deoxycholic acid (500 µmol/L for 1 h) was used as a positive control. Release of LDH from Caco-2 monolayers by SMase was also not detectable (data not shown).

Neutralization of surface ceramide prevents permeability-increase induced by PAF

Next, we investigated whether the increased permeability induced by PAF might be linked to rearrangement of tight-junctional lipids. Incubation of the monolayers with 5 µmol/L PAF increased permeability by 162.8% ± 13.0% (Figure 2). To examine the role of ceramide in PAF-mediated permeability we co-incubated Caco-2 cell layers with ceramide-antiserum (1/100 dilution). Co-incubation of the Caco-2-monolayer with ceramide-antiserum prevented the increase of permeability induced by 5 µmol/L PAF (111.6% ± 9.86%, $P < 0.05$) (Figure 2), indicating a stabilization of tight-junctional complexes by the IgM-anti-ceramide Abs.

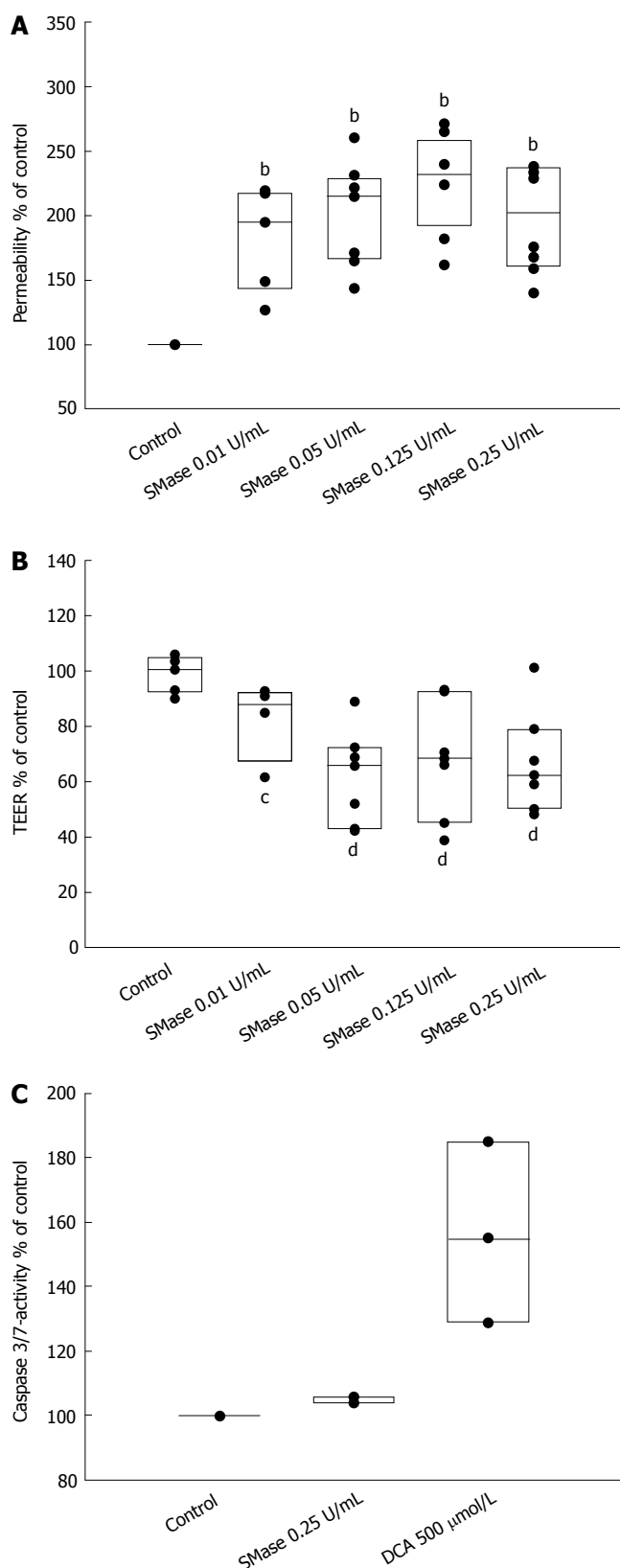


Figure 1 Exogenous sphingomyelinase increases permeability of Caco-2 epithelial monolayers. **A:** Caco-2 monolayers were incubated with different concentrations of exogenous SMase. ^b $P < 0.01$ between control and treated samples; **B:** Transepithelial electrical resistance (TEER) was measured across the cell monolayers 4 h after treatment of the monolayers with exogenous SMase. The data were compared with TEER before treatment and are expressed as % of untreated. ^c $P < 0.05$, ^d $P < 0.01$; **C:** Epithelial apoptosis was monitored 6 h after incubation with 0.25 U/mL SMase by measurement of caspase-3/7-activity with colorimetric assays. As a positive control, deoxycholic acid (DCA) was used for 1 h.

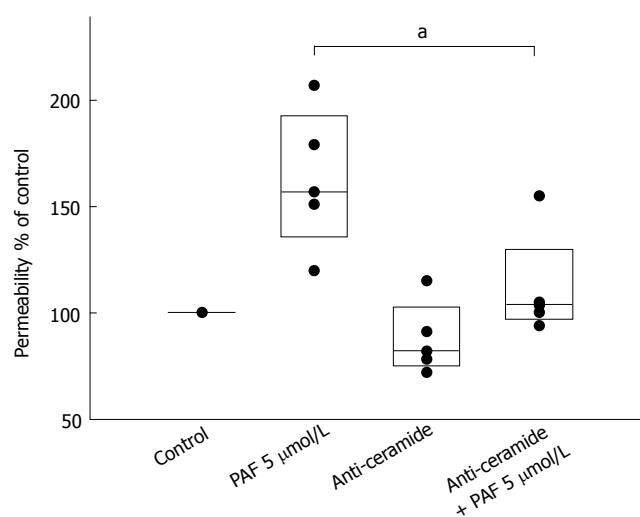


Figure 2 Neutralization of surface-ceramide prevents PAF-mediated increase of permeability. Caco-2 cell layers were incubated with the IgM ceramide-antiserum (15B4) 30 min prior to stimulation with PAF. Permeability was determined by measurement of transepithelial flux of fluorescein sulfonic acid from the upper chamber of the Transwell insert to the lower chamber across the Caco-2-monolayer after 4 h. The data are expressed as % of control. ^a $P < 0.05$ between PAF-treated samples.

Detergent insensitive glycosphingolipid-enriched domains (DIGs) contain major pools of tight junction proteins like occludin and claudin 4

To further test our hypothesis, DIGs were isolated using sucrose gradient techniques and analysed for their lipid- and protein-composition. Analysis of the lipid composition by tandem mass spectrometry revealed the presence of high amounts of sphingomyelin ($12.5\% \pm 1.4\%$), cholesterol ($44.9\% \pm 4.9\%$) and ceramide ($0.74\% \pm 0.18\%$) in DIGs with only $3.8\% \pm 0.6\%$, $19.2\% \pm 3.9\%$ and $0.19\% \pm 0.03\%$ in the total cell lysate (including DIGs), respectively (Figure 3A, values are given as % of analysed lipids \pm SD). This indicated a good separation of DIGs from other membrane components. To ascertain isolation of tight junctions within the Triton X-100 insoluble preparations, Western-blot experiments were performed to prove the presence of tight-junctional proteins. As shown in Figure 3B, major pools of the tight-junctional proteins occludin and claudin-4 were present in Triton X-100 insoluble preparations and to a lesser extent in cell lysate. The basolateral membrane protein E-cadherin was also present in DIGs but was not as prominent. To exclude contamination with proteins not associated with DIGs, Western-blot of TLR4 and p38MAPK were performed which could not be detected in preparations of DIGs (Figure 3B).

Ceramide accumulates in DIGs, whereas sphingomyelin and cholesterol decrease

Tandem mass spectrometry was used to analyze the effect of SMase on the lipid content of DIGs which overlap with tight junctions. Incubation of Caco-2 monolayers with 0.25 U/mL SMase resulted in rapid increase of ceramide in DIGs by $5.0\% \pm 2.4\%$ after 10 min ($P < 0.01$) and $7.3\% \pm 2.6\%$ after 30 min ($P < 0.01$), whereas the increase was

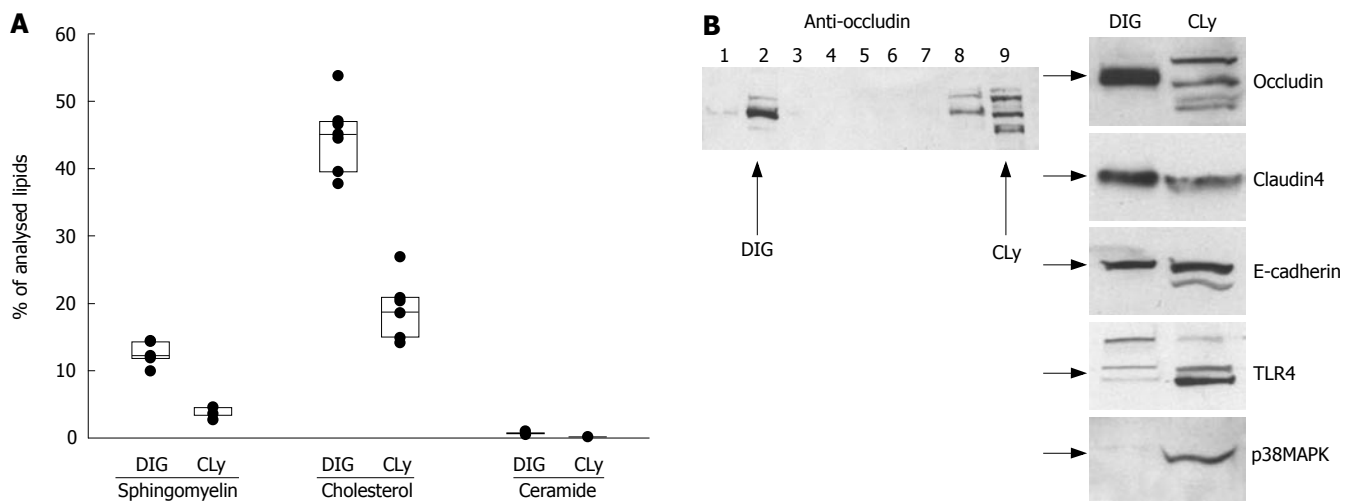


Figure 3 Preparations of detergent insensitive glycosphingolipid-enriched domains (DIGs) contain TJ-proteins. **A:** Lipid analysis of DIGs from non-stimulated Caco-2 cells by tandem mass spectrometry revealed the presence of high amounts of sphingomyelin, cholesterol and ceramide when compared to the total cell lysate; **B:** Gradient fractions were separated by 10% SDS-PAGE and analyzed by Western blot. Fraction No. 2 represents the raft-containing fraction at the 35%/5% interface (DIG); No. 9 represents the soluble fraction (cell lysate = CLy). The presence of high amounts of TJ-proteins occludin and claudin-4 and, to a lesser extent, E-cadherin show a clear association of TJ-proteins with DIGs. The simultaneous absence of other proteins like TLR4 and p38MAPK excludes significant contamination with non-raft fractions.

only $0.84\% \pm 0.14\%$ and $1.25\% \pm 0.23\%$ in the whole cell lysate (including DIGs), respectively (Figure 4A, data shown as % of analysed lipids). In the same preparations of DIGs the concentration of sphingomyelin decreased by $4.73\% \pm 1.4\%$ after 10 min ($P < 0.01$) and $7.6\% \pm 2.2\%$ after 30 min ($P < 0.01$) (Figure 4B). The concentration of cholesterol declined by $5.0\% \pm 3.2\%$ after 10 min and $4.8\% \pm 2.3\%$ after 30 min (Figure 4C). This reflects a decrease of the percentage of cholesterol by $10.9\% \pm 7.3\%$ after 10 min ($P < 0.05$) and $10.6\% \pm 4.8\%$ after 30 min ($P < 0.01$).

Clusters of ceramide colocalize with tight junctions

Fluorescence microscopy was performed to demonstrate the localization of ceramide accumulation. We induced generation of ceramide by incubation of Caco-2 cells with 0.25 U/mL SMase for 10 min. After stimulation, staining of the cells with anti-ceramide 15B4 antibodies revealed the formation of ceramide-clusters (Figure 5A and B) that were frequently located at the sites of cell-cell-contact. Costaining for the tight-junction protein ZO-1 confirmed colocalization of ceramide-clusters with the junctional complexes (Figure 5B).

These data indicate that the generation of ceramide by SMase leads to localized accumulation of ceramide at the sites of cell/cell-contact with increased intestinal permeability and suggest a central role of ceramide in the regulation of barrier integrity.

DISCUSSION

Our data indicate that hydrolysis of sphingomyelin to ceramide increases intestinal epithelial cell permeability in a well-established model of Caco-2 cell monolayers^[37]. To obtain naturally occurring long chain ceramides, formation of ceramide was induced by addition of bacterial sphingomyelinase (SMase). This approach was used because neutral as well as acid SMase are capable of generating ceramide in the outer leaflet of

the cell membrane by rapid hydrolysis of membrane sphingomyelin. Previous studies suggested that, upon cellular stimulation, the acid sphingomyelinase (ASM) translocates onto the outer surface of sphingolipid-rich membrane microdomains^[7,8]. The fact that ASM activity is also increased in the serum of mice treated with endotoxin^[38] or PAF^[39], indicates a possible role of extracellularly located ASM. Another advantage of using exogenous SMase is the enzymatic cleavage of sphingomyelin at the sites of sphingomyelin-enriched domains in the cell membrane, whereas the addition of long chain ceramides might lead to lateral assembly of ceramide to pre-existing DIGs (and/or TJs) without affecting the structure inside these microdomains. Apart from that, exogenous ceramides are likely to be rapidly metabolised, thus having a rather transient effect on lipid composition. Exogenous SMase was effective in terms of increased permeability and decrease of TEER, indicating a reduction of the paracellular barrier by hydrolysis of sphingomyelin. Other possible effects of SMase include an increase of transcellular transport of vesicles. Therefore, we measured intracellular fluorescence after exposure of the cells with SMase and fluorescein-sulfonic acid with colorimetric assays of the cell lysates and performed transepithelial flux experiments with simultaneous colchicine-treatment (data not shown). In both cases we did not observe any differences, making transcellular transport unlikely. To exclude damage of monolayers as a possible cause for increased permeability, activity of caspase-3/7 in the cell lysate and lactate dehydrogenase (LDH) in the supernatant were measured without significant results.

Lipid analysis of Triton X-100 insoluble preparations clearly demonstrated the separation of membrane portions which were enriched in cholesterol, sphingomyelin and ceramide. Western-blot experiments of DIGs revealed that these preparations contained major pools of the TJ-proteins occludin and claudin-4. Upon stimulation with

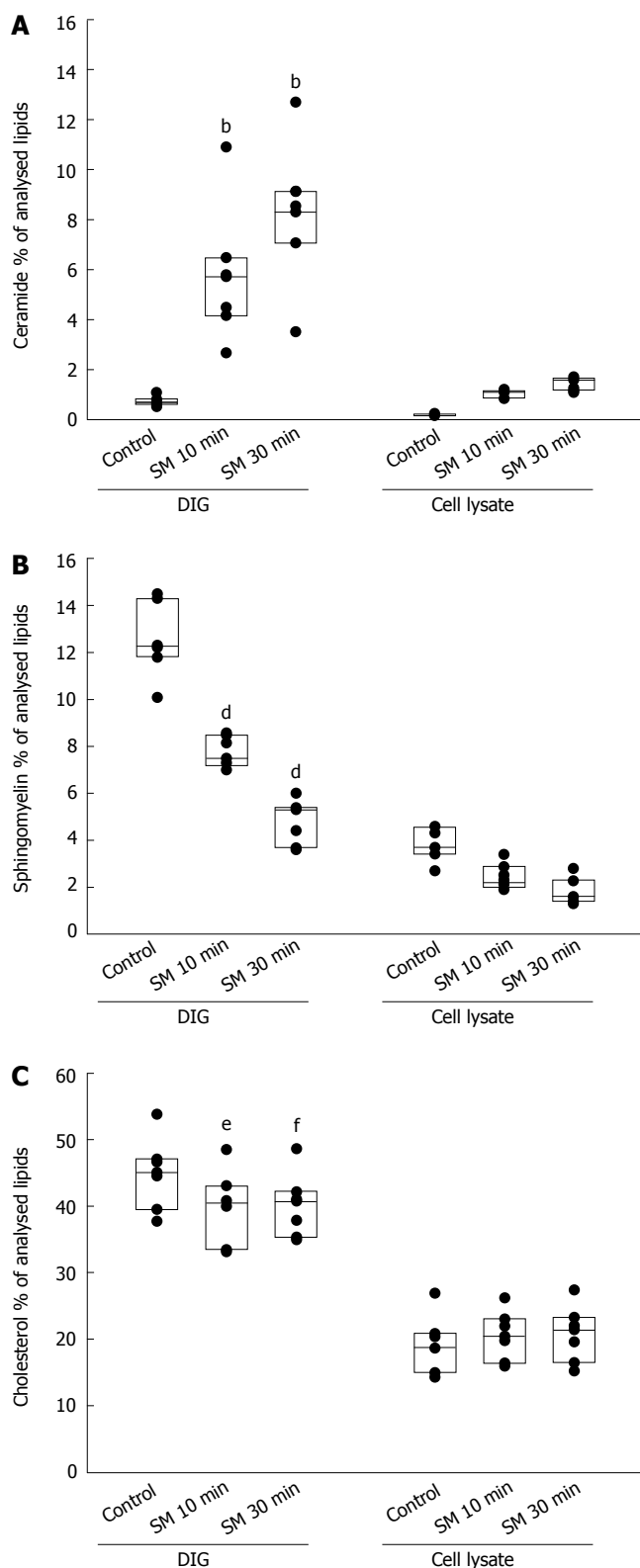


Figure 4 A: Lipid analysis by tandem mass spectrometry revealed a rapid accumulation of ceramide in preparations of DIGs after incubation with 0.25 U/mL SMase for 10 or 30 min whereas the increase of ceramide in the cell lysate was only small in comparison ($^bP < 0.01$); B: The content of sphingomyelin in DIGs decreased at the same time with a relative reduction comparable to the generation of ceramide ($^dP < 0.01$); C: Cholesterol also decreased in DIGs whereas the concentration in the cell lysate remained constant ($^eP < 0.05$ and $^fP < 0.01$).

SMase we detected a rapid accumulation of ceramide in the same membrane fractions, indicating selective

accumulation of ceramide in sphingolipid-enriched membrane microdomains, including junctional complexes.

Previous studies suggested displacement of cholesterol from DIGs by ceramide^[20,40,41]. To our knowledge, there is no data in the available literature about the modulation of ceramide and cholesterol in TJs by sphingomyelinases. Therefore we compared the content of cholesterol in DIGs which overlap with TJs. In accordance with these studies we also observed a decrease of cholesterol in these microdomains with increasing concentrations of ceramide, suggesting a rearrangement of tight junctional lipids by ceramide with impaired barrier function.

Lambert *et al*^[30] showed a decrease of TEER by 80%-90% after extraction of 40%-45% of cholesterol from Caco-2 monolayers. Compared to these results the decrease of tight-junctional cholesterol by ceramide is moderate. Nevertheless, these changes could explain early disturbances of tight-junctional integrity upon cellular stress, deriving from toxic, infectious or immunologic challenges of the intestinal epithelium. Therefore, we propose the following pathophysiologic model: Cellular stimulation leads to localized accumulation of ceramide with concomitant decrease of sphingomyelin and cholesterol leading to destabilization of tight junctional strands and loss of barrier integrity.

Long chain ceramides also have the unique property of fusing membrane domains and tend to form cluster, which was also observed in our studies. Mechanisms of ceramide to modify the structure of bilayers and their effect in promoting efflux and release have been studied in several models^[21,42,43]. Montes *et al*^[21] demonstrated release of molecules with a molecular mass up to 20 kDa upon treatment of unilamellar vesicles with sphingomyelinase. A possible mechanism for this release was suggested by Siskind and Colombini who detected formation of large stable channels by ceramide in planar bilayers through electrophysiological methods^[43]. The influence of ceramide on the architecture of TJs also seems to be important in the PAF-mediated increase of permeability. Pretreatment of Caco-2 cells with a monoclonal IgM ceramide-antiserum prevented the loss of barrier function induced by 5 $\mu\text{mol/L}$ PAF.

Together, these findings provide evidence for a new aspect of cellular ceramide generation. Lipid rearrangements triggered by enzymatically catalysed formation of ceramide upon cellular stimulation may be a frequent process for localized exposure of intraluminal antigens to the immune system. Repeated stimulation or extension of the stimulated area may lead to an imbalance of the lipid geometry producing a stronger leakiness of the affected junctional complexes resulting in initiation of an inflammatory process which may further perpetuate itself. Better knowledge of the relevant lipids which control this "lipid-barrier" and their modifying enzymes would be helpful to develop treatment strategies to strengthen this barrier. Lipid-enriched diets or inhibition of relevant lipid-modifying enzymes could be possible treatment modalities to control chronic inflammation of the intestine as seen in IBD^[44].

In summary, our data demonstrate that hydrolysis

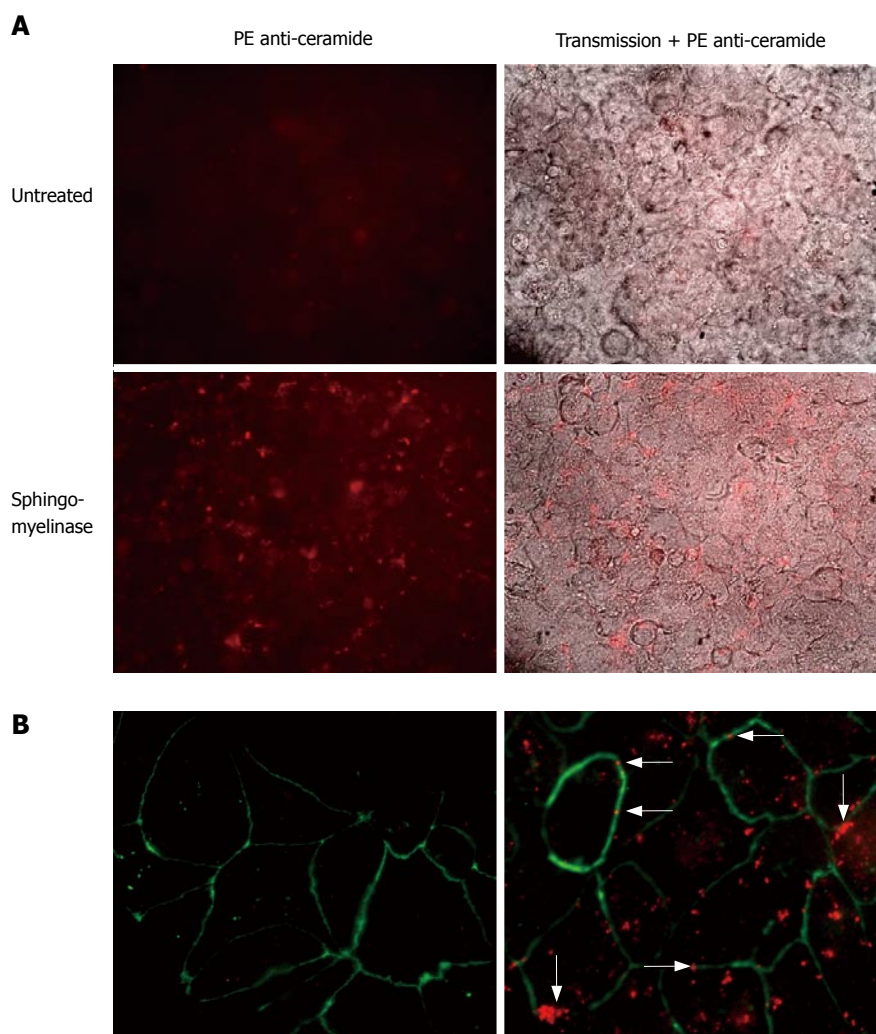


Figure 5 Ceramide clusters at the sites of cell-cell contacts. Caco-2 cell layers were incubated with 0.25 U/mL SMase for 10 min or left untreated. After fixation of the cells with paraformaldehyde, ceramide was visualized by staining of the cells with the monoclonal anti-ceramide 15B4 antibody and PE-coupled anti-mouse antibodies. **A:** Top, left: Non-stimulated cells displayed a certain level of background staining that was used to determine the time of exposure. Top, right: Overlay of fluorescence and transmission. Bottom, left: Incubation with SMase resulted in formation of multiple ceramide-clusters. Bottom, right: Overlay of fluorescence and transmission. Close inspection of the clusters revealed a frequent localization of the clusters at the sites of cell/cell-contact; **B:** Costaining of tight junctions with FITC-labeled anti ZO-1 Abs. Left: non-stimulated. Right: Predominant colocalization of ceramide-clusters with tight junctions upon stimulation with SMase (arrows indicate ceramide-clusters at the sites of cell-cell contact).

of sphingomyelin to ceramide affects transepithelial permeability and resistance. Long chain ceramides, generated by SMase, accumulate in cholesterol- and sphingolipid-enriched membrane microdomains which include TJs. These microdomains fuse to large, ceramide-enriched clusters, located on the surface of the cells and at the sites of cell-cell contact, explaining the effect of long-chain ceramides on barrier integrity. Finally, the effect of PAF on paracellular permeability is inhibited by neutralization of surface ceramide, suggesting a regulation of the paracellular permeability by the arrangement of membrane ceramide.

COMMENTS

Background

The sphingolipid ceramide, generated by signal-activated sphingomyelinases, has emerged as a second messenger of stimuli as diverse as ligation of various receptors, ionizing radiation, chemotherapy or infection with some bacteria and viruses. Upon stimulation of sphingomyelinases, ceramide is generated in distinct sphingolipid-enriched membrane microdomains of the cell membrane, termed "lipid rafts". Tight junctions (TJs) are structurally related to lipid rafts, and thus, we hypothesized that hydrolysis of sphingomyelin to ceramide changes the composition of TJs with increasing permeability of the intestinal epithelium.

Research frontiers

Intestinal permeability is influenced by the lipid content of epithelial cells and some lipids may be beneficial for the course of inflammatory bowel disease. Modification of the lipid

content of TJs may be a possible strategy to improve intestinal barrier functions.

Innovations and breakthroughs

Our data indicate that hydrolysis of sphingomyelin to ceramide by sphingomyelinase (SMase) increases intestinal epithelial cell permeability. Lipid analysis after stimulation with SMase demonstrated rapid accumulation of ceramide in the membrane fractions which contain the TJ-proteins occludin and claudin-4, while sphingomyelin and cholesterol decrease. Pretreatment of cells with a monoclonal IgM ceramide-antiserum prevented the loss of barrier function induced by platelet activating factor.

Applications

Better knowledge of the relevant lipids and their modifying enzymes that control this "lipid-barrier" of the intestine would be helpful to develop treatment strategies to strengthen this barrier. Lipid-enriched diets or inhibition of relevant lipid-modifying enzymes could be possible treatment modalities to control chronic inflammation of the intestine as seen in inflammatory bowel disease.

Terminology

Sphingomyelinase is an enzyme which hydrolyses sphingomyelin to ceramide upon various cellular stimuli. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight-junction (TJ). Platelet activating factor is a lipid messenger which increases paracellular permeability in intestinal epithelial cells.

Peer review

The authors set out to test the hypothesis that hydrolysis of sphingomyelin to ceramide changes the composition of tight junctions (TJs) with increasing permeability of the intestinal epithelium. Their findings suggested that changes in lipid composition of TJs impair epithelial barrier functions. Generation of ceramide

by sphingomyelinases might contribute to disturbed barrier function seen in diseases such as inflammatory, infectious, toxic or radiogenic bowel disease.

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