Lipid-Modifying Enzymes in Human Tear Fluid and Corneal Epithelial Stress Response

Alexandra Robciuc,[1,2] Antti H. Rantamäki,[1] Matti Jauhiainen,[2] and Juha M. Holopainen[1]

[1]Helsinki Eye Lab, Department of Ophthalmology, University of Helsinki, Helsinki, Finland
[2]Public Health Genomics Research Unit - National Institute for Health and Welfare, Helsinki, Finland

Correspondence: Juha M. Holopainen, Department of Ophthalmology, University of Helsinki, P.O. Box 220, 00029 HUS, Finland; juha.holopainen@hus.fi.

Submitted: June 10, 2013
Accepted: November 17, 2013

Citation: Robciuc A, Rantamäki AH, Jauhiainen M, Holopainen JM. Lipid-modifying enzymes in human tear fluid and corneal epithelial stress response. *Invest Ophthalmol Vis Sci*. 2014;55:16–24. DOI: 10.1167/iovs.13-12577

Purpose. Since homeostasis at the ocular surface requires a delicate balance between numerous factors, and the external environment contributes as an unpredictable component, we aimed to understand the role that various lipids and their regulators have in the complex process that maintains a healthy corneal surface.

Methods. Through basic proteomics, we tested the presence of sphingolipid metabolism enzymes in normal human tears, and then used a cell culture model to study how the proteins are secreted and for what purpose.

Results. When studying healthy tears, we found that sphingolipid-specific enzymes, acid and neutral sphingomyelinases, and ceramidases can be detected. The role played by sphingolipid metabolism in stress provided the motivation for further studies concerning their secretion/leakage in the extracellular environment in a cell culture model of human corneal epithelial cells (HCE). Among the stress agents investigated (i.e., ultraviolet B [UV-B] radiation, hyperosmolarity [HO], and lipopolysaccharide [LPS]), UV-B and HO induced dose-dependent release/secretion of sphingomyelinases from the cells. In an attempt to identify the route of secretion or release of the enzyme, we discovered that the tested stress stimuli induced shedding of extracellular vesicles in the HCE-conditioned medium.

Conclusions. Extracellular stress affects tear fluid composition more profoundly than just secretion of proinflammatory mediators. Lipids at the ocular surface, either in tear fluid or within the corneal epithelial cells, can be modified by a relatively large array of lipases to modulate their functions. Moreover, extracellular vesicles in the tear fluid could represent a valuable noninvasive diagnosis tool for anterior segment diseases.

Keywords: tear fluid, phospholipases C, corneal epithelium, inflammation, stress response

Tear fluid is a semi-viscous, biomolecule-rich fluid that forms a thin film across the ocular surface and contributes in many ways to the function of the eye. The tear film has a poorly-defined trilaminar structure that is divided roughly into mucous, aqueous, and lipid layers. The overall homeostasis of the tear film is maintained by specific enzymes, which are produced by the lacrimal glands, and possibly the corneal and conjunctival epithelial cells as well. The proteins in the aqueous phase are responsible for fulfilling tasks that encompass stress response, immune and inflammatory responses, and wound healing.[1] The outermost lipid layer at the air–water interface comprises polar phospholipids, such as phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs), and nonpolar lipids, such as cholesterol esters, wax esters, and triglycerides.[2–4] The lipid layer decreases the surface tension and possibly increases the overall stability of the tear film.[5]

The lipid-interacting proteins lipocalin,[6,7] lipophilin,[8] phospholipid transfer protein (PLTP),[9] group II phospholipase A_2,[10] and acid sphingomyelinase (ASM)[11] have been discovered in tear fluid. These proteins can have very diverse functions in lipid interactions: lipid binding/transport proteins or hydrolytic enzymes. Lipids organize into several different forms of aggregates, such as micelles, vesicles, or membranes; therefore, proteins may interact with lipids in tear fluid at several differing aqueous–lipid interfaces (e.g., the tear fluid lipid layer, bacterial cell wall, plasma membranes of epithelial cells, or other liposomal aggregates present in the tear fluid).

The fact that acid sphingomyelinase, along with sphingomyelin and ceramide,[2] is present and highly active in tear fluid[11] raises the question of whether tear fluid also contains other enzymes linked to the sphingomyelin metabolism. The bioactive lipids in this pathway have been linked to many vital cell processes, such as stress response, apoptosis, angiogenesis, and proliferation.[12]

The ocular surface is susceptible to several threats from the surrounding environment, such as pathogens, ultraviolet (UV) radiation from the sun, and physical irritation or injury. The UV radiation and bacterial aggression as well as the hyperosmolarity (HO) associated with dry eye syndrome were found to induce the sphingomyelin (SM) cycle and ceramide production as part of the eye's stress response mechanism.[13–16] The ubiquitous nature of this pathway in stress response stems from its fast engagement and intensity-modulated feedback.[12,17] The relative amounts of lipid second messengers in this pathway are kept in balance by specific enzymes; therefore, the presence of these enzymes in the internal environment is of paramount importance and, thus, has been the target of numerous studies.[18–20]

As the ocular surface is a stress-prone interface where the tear film has a defensive role, the variety of mechanisms that maintain tear film homeostasis must be studied in more detail. The aim of our study was to reveal the presence and to measure the activity of enzymes producing components of the sphingolipid metabolism in human tear fluid. Because it was evident that these enzymes are present in tears, our endeavor was to clarify their function. Specifically, a human corneal epithelial cell culture model (HCE cells) was used to study stress-induced secretion of these enzymes under hyperosmolar conditions or UV-B radiation and in the presence of lipopolysaccharide (LPS). We concluded that extracellular stress stimulates dose-dependent secretion of sphingolipid enzymes from the HCE cells and that these enzymes seem to be associated with extracellular vesicles. Moreover, these stress-induced membrane vesicles display antiinflammatory properties towards nonstressed cells.

Methods

Tear Fluid Sample Collection

Tear samples were collected from a total of five subjects with no symptoms of external eye disease (3 males, mean age 30 ± 5 years) using 5 μL glass capillary micropipettes (Blaubrand Intramark GmbH, Wertheim, Germany). Cellular debris was eliminated by centrifugation, and the sample was stored at −70°C until analyzed. The total protein content in the tear fluid was analyzed using a commercial kit (Promega, Madison, WI). All experiments were performed in accordance with the guidelines of the Declaration of Helsinki and the Ethical Committee of the Helsinki-Uusimaa Hospital District. A written informed consent was obtained from each subject.

Corneal Epithelial Cell Culture

The SV-40–immortalized human corneal epithelial cells[21] (HCE cells) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 15% fetal bovine serum (FBS) and epidermal growth factor (EGF), 1 μg/mL of insulin, and 40 μg/mL of gentamicin (all supplied by Invitrogen, Carlsbad, CA). Before the experiments, the cells were serum starved for 6 hours. Then, NaCl and LPS were added to serum-free media, while for the UV-B experiments, the cells first were washed with warm PBS and then exposed to radiation in PBS. Protein secretion was investigated 18 hours after the initiation of the stress. The media were collected immediately and spun at 400*g* for 10 minutes to eliminate floating cells and debris. For Western blots, medium was concentrated 10-fold using a Nanosep 10K omega centrifugal device (Pall Life Sciences, Port Washington, NY). Cellular toxicity induced by the stimuli mentioned above was investigated using the cell viability test reagent alamarBlue (Invitrogen). The redox sensitive reagent is reduced by live cells to a fluorescent compound. Fluorescence was measured using excitation/emission wavelengths of 570/590 nm and is proportional with the number of viable cells.

Western Blot Analysis

Pooled tear fluid samples (using at least two different pools) were run on a 4% to 12% Bis-Tris NuPAGE gel (Life Technologies Ltd., Paisley, UK). Proteins of interest electro-transferred onto the nitrocellulose membrane were detected using specific antibodies and enhanced chemiluminescence detection (GE Healthcare – Amersham, Buckinghamshire, UK).

Determination of PC-Specific Phospholipase C (PC-PLC) and Sphingomyelinase (SMase) Activities

Tear samples from individual donors and pooled samples were used for specific enzymatic activity analyses. The PC-PLC activity was determined using the Amplex Red phosphatidylcholine-specific phospholipase C assay kit and the EnzChek direct phospholipase C assay kit; neutral and acid SMase activity was determined using the Amplex Red sphingomyelinase assay kit (all from Life Technologies Ltd.) according to manufacturer recommendations. Fluorescence was measured using a microplate reader (Wallac, Turku, Finland) with excitation at 535 nm and emission at 585 nm. The amount of enzymatic activity in the tear fluid samples was determined using standard curves and expressed as milliunits (mU) normalized to protein concentration.

IL-8 Secretion

In cell-conditioned media, IL-8 secretion was measured using DuoSet ELISA for human CXCL8/IL-8 (R&D Systems, Minneapolis, MN) as recommended by the manufacturer. For assays, samples were diluted 1:1 (vol/vol) with Reagent Diluent. Plates were analyzed using a Victor 2 Multilabel Counter micro-plate reader (Wallac) at 450 nm.

Vesicle Separation From Cell-Conditioned Media

Media collected from stimulated cells and controls were freed of floating cells and either subjected to ultracentrifugation (TLA-100.3 rotor for Optima TL Ultracentrifuge, 100,000*g*, 90 minutes, 4°C; Beckman Coulter, CA) or filtered through a 22-μm pore PVDF filter (Millipore, Billerica, MA) to eliminate apoptotic bodies and then centrifuged as above. The supernatant after centrifugation was labeled as vesicle-poor media (VPM) and the sedimented vesicles were prepared for immunoblotting. The total protein concentration was determined using a commercial kit (Promega).

Lipid Measurements

Total cholesterol (T-Chol) and choline-containing phospholipids (choline-PLs, such as PC or SM) were measured in sedimented pellets using colorimetric assays (DiaSys GmbH, Holzheim, Germany) following an adapted protocol. Briefly, for all measurements, a maximum of 1.5 μg of protein (40 μL of solubilized pellets) was used in the conditions recommended by the manufacturer.

Expression Analysis for IL-8

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted to cDNA. Consequently, 50 ng of cDNA were amplified by Q-PCR (SYBR Green/ROX FastStart Universal master mix; Roche, Basel, Switzerland) in an ABI Prism7000 thermo cycler (Life Technologies Corporation, Carlsbad, CA). Primers for the *IL-8* gene were F-5′ CACACTGCGCCAA CACAGAAA and R-5′ AGCCCTCTTCAAAA ACTTCTCCACA. We used human actin as a control gene, and relative expression was quantified by calculating ddCt and 2-ddCt, and the fold change was represented graphically.

Statistical Analysis

Statistical significance was determined by *t*-test on average values from three independent experiments. All experiments

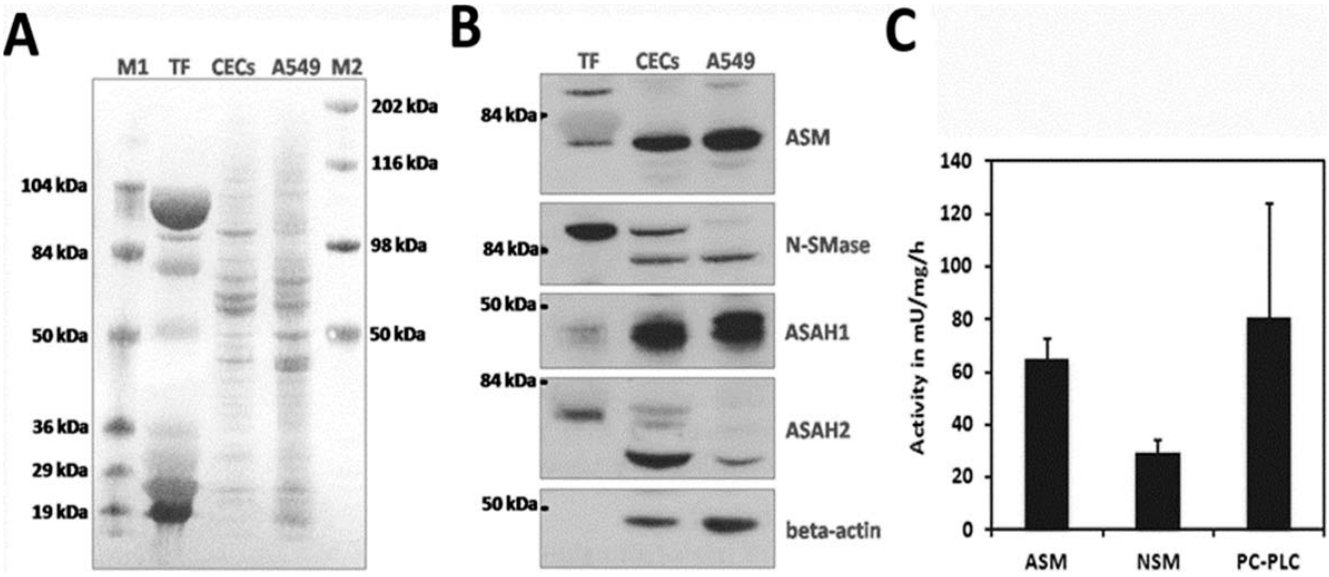

Figure 1. Lipid-modifying enzymes in human tear fluid. Western blot of (**A**) Ponceau red image of the transferred proteins and (**B**) specific antibody detection of the enzymes in the following samples: whole tears (TF), corneal epithelial cells from patients undergoing PRK (CECs), and pulmonary alveolar type II epithelial cells (A549). For NSMase and β-actin, the amount of protein per lane was 30 μg, while the amounts of total protein used for tear fluid enzyme detection and the cell lysates were 60 and 20 μg, respectively. M1, low molecular weight marker; M2, high molecular weight marker; N-SMase, neutral sphingomyelinase; ASAH1, acid ceramidase. (**C**) Enzymatic activity assay for sphingomyelinases (acid and neutral) and PC-PLC in human tear fluid. A 20-μL pooled sample was used in all reactions.

were performed at least twice. Data are reported as mean ± SD unless otherwise specified. Values of $P < 0.05$ were considered significant.

Results

Enzymes of the Sphingolipid Signaling Pathway Are Present in Human Tear Fluid

Using pooled tear fluid samples for immunoblotting, we were able to identify acid and neutral sphingomyelinases (which are products of the human genes *smpd1* and *smpd3*, respectively) as well as ceramidases (which are products of the *asah1* and *asah2* genes). Figure 1A shows a representative result of the specific identification of the SMPD1 (known as ASM), SMPD3 or NSMase 2, ASAH1, and ASAH2 proteins in normal tear fluid in parallel with their intracellular counterparts from human corneal epithelial cells and an A549 cell line. Based on these results, the most immunodetectable enzyme in the tear fluid appeared to be the neutral sphingomyelinase 2 enzyme (SMPD3 or NSM2). The enzyme in tear fluid displays a slightly higher apparent molecular weight than expected, and this atypical form of NSM2 has an intracellular counterpart in epithelial cells and cornea-derived cells (CECs), although it is more evident in the latter. Intracellular neutral ceramidase (ASAH2) usually is visualized as a double band,[22] as observed in the case of the CEC lysate (Fig. 1B). We identified lysosomal and secreted isoforms of the acid sphingomyelinase (ASM) all tear fluid samples (Fig. 1B).

The enzymatic activities of the sphingomyelinases were measured in pooled samples of tear fluid and compared to the PC-PLC, since the level of PC was shown to be at least 4-fold higher than that of SM in tear fluid.[2] Sphingomyelin hydrolysis was more pronounced under acidic conditions than at a neutral pH, although the Western blot analysis suggested otherwise (Figs. 1C, 1B, respectively).

Extracellular Stress Stimuli Induce SMase Activation and Secretion From HCE Cells

To determine whether corneal epithelial cells are a source for the enzymes found in tear fluid, we set up a cell culture model of immortalized HCEs. The HCE cells were exposed to different stress agents (i.e., UV-B, HO, and LPS) to determine whether the enzyme secretion/leakage in tear fluid is a consequence of extracellular stress. Mild stress (i.e., UV-B energy in the range 0.5–1 mJ/cm^2 or 50–70 mM of added NaCl) induced less than 10% cell detachment compared to the nonstressed control. Cell death increased significantly in the high-stress samples (Fig. 2A). However, LPS incubation induced minimal cell death despite the robust inflammatory response (Figs. 2A, 3C).

Cellular NSM2 was activated by UV-B radiation (Fig. 3A) as well as high osmolarity (Fig. 3B; the effects of adding NaCl and adding KCl were similar[13]) and LPS (Fig. 3C); however, the latency of the response was not always the same. Furthermore, the extent of the subsequent proinflammatory response was not equal across the stress agents (Figs. 3A–C).

We then investigated whether the sphingolipid enzymes were also present in the culture medium. Because identification of ceramidases in the tear fluid proved to be challenging, we sought to identify the sphingomyelinases in the HCE-conditioned medium (which was significantly more dilute than the tear fluid). The UV-B and HO stress induced a dose-dependent release of ASM and NSM2 (Figs. 4A, 4B). We could identify both forms of ASM in the medium, but only lysosomal ASM (L-ASM) seemed to be induced by stress (Fig. 4B). Increasing the concentration of LPS did not influence the release of these enzymes; however, both enzymes were recognized in the medium (Fig. 4C).

The Sphingolipid Pathway Enzymes Are Released From Stressed Cells as Vesicle Cargo

Because most of the enzymes in the sphingolipid pathway are membrane-associated or bound, we tested whether the

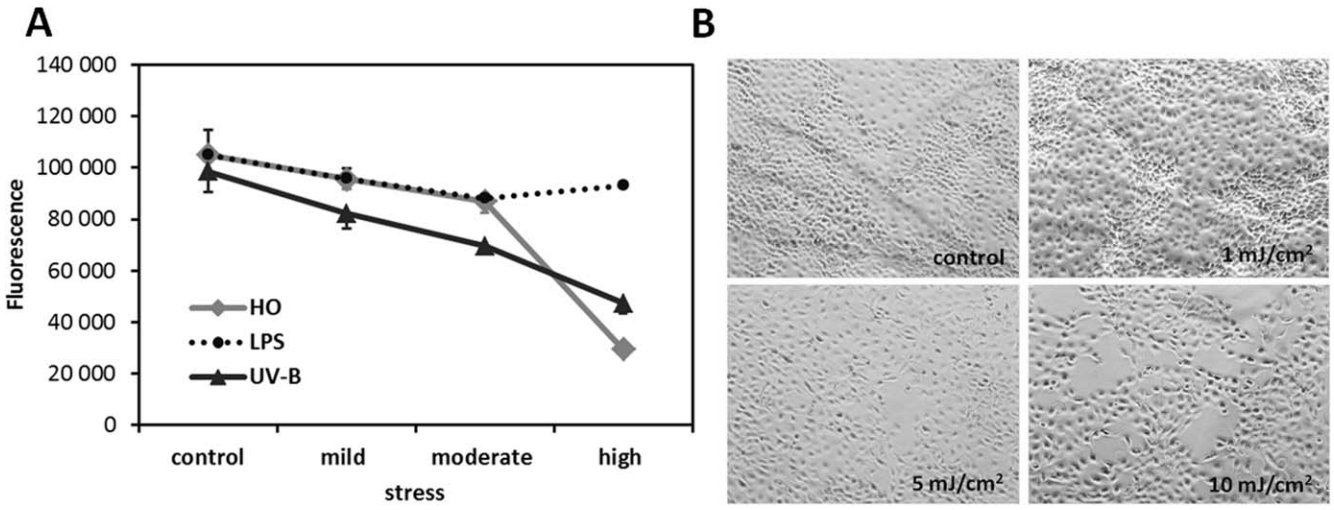

FIGURE 2. Cell survival after stress. (**A**) Cellular viability measurement of cells exposed to stress. Cells were incubated with the stimuli for 18 hours, after which the redox potential was assayed by incubating the cells with the alamarBlue reagent. (**B**) Light-microscopy image of the UV-B exposed cells (objective magnification ×10). It can be observed that, with increased energy of the UV-B radiation, there is proportional loss of cells from the culture substrate.

enzymes were released in membrane vesicle-like structures. For this purpose, vesicles from HCE-conditioned media were pelleted using ultracentrifugation and then characterized using specific markers,[23] including CD-63 (as an exosome marker), histone H3 (for apoptotic bodies), and integrin β1 (for microparticles, Fig. 5A). The HCE cells seemed to produce CD-63–enriched vesicles even in unstressed conditions, and all populations of vesicles (i.e., CD63-positive, integrin β1–positive, and H3-positive vesicles) were induced by UV-B and HO stress. Integrin β1 was not detectable in the media from LPS-incubated cells, and LPS did not affect CD63 vesicle secretion (Fig. 5A).

The protein/lipid (cholesterol and choline-phospholipids) rations indicated that the UV-B– and HO-induced vesicles were, yet again, noticeably different when compared to those generated by LPS stress (Fig. 5B). The UV-B pellets were more abundant in protein, most likely due to the increased proportion of apoptotic bodies. The HO stress induced the release of vesicles with a more membrane-like composition. The low amount of sample prevented reliable measurement of cholesterol from the LPS-induced vesicles, but the protein-phospholipid ratio seemed to be similar to that found in HO-induced vesicles.

Next, the partitioning of the SMases between vesicles and VPM was investigated. The ASM had a very strict division between these fractions, as the secreted ASM (S-ASM) was observed only in the VPM (data not shown), while the L-ASM sedimented with the vesicles (Fig. 5C). In contrast, NSM2 was enriched in the vesicle fraction, but there still were traces of the protein in the VPM, which could imply that the vesicle sedimentation procedure was not entirely efficient. Therefore, the vesicles potentially could carry both SMases.

Anti-Inflammatory Effects of Stress-Induced Vesicles on HCEs

All the stress stimuli tested were proinflammatory because they all induced secretion of IL-8 from HCE cells. Therefore, we were interested in determining what kind of messages these vesicles could convey to the corneal epithelium. Consequently, IL-8 secretion from naïve HCE cells (i.e., cells not exposed to any environmental stress) incubated with isolated stress-induced vesicles was measured (Fig. 6A). For this purpose HO-, UV-B–, and LPS-induced vesicles were isolated, resuspended in PBS, and added to the cell culture media of the naïve HCEs. After 18 hours of incubation, we observed that, independent of the amount of HO or UV-B stress to which the producing cells were exposed, the vesicles did not affect the proinflammatory profile of the resting (nonstimulated) HCEs. Conversely, the LPS-induced vesicles stimulated a vigorous response from the naïve HCEs (Fig. 6A, LPS). Most likely, this finding is due to residual LPS molecules attached to the isolated extracellular vesicles. For our experimental setup, this result was valuable because it showed that the isolated vesicles come in contact with the underlying cells and affect their expression pattern. With this in mind, in the following experiment, naïve HCE cells were incubated with either HO- (100 mM of NaCl) or UV-B– (5 mJ/cm^2) induced vesicles, while control cells were incubated in serum-free medium. After 18 hours, the medium was replaced with hyperosmolar medium (70–100 mM of added salt), and IL-8 secretion was measured after an additional 4 hours (Fig. 6B). Surprisingly, the cells incubated with extracellular vesicles induced under UV-B or HO stress secreted lower amounts of IL-8 (Fig. 6B, $P < 0.05$). Furthermore, after 18 hours of incubation with the stress-induced vesicles, IL-8 gene expression was reduced by 50% (Fig. 6C, $P < 0.05$) when compared to the cells without added vesicles. Decreased levels of mRNA for IL-8 also were observed after the 4 hours of salt stress, although the differences were not statistically significant. The vesicles did not affect the viability of the cells in any way (data not shown).

DISCUSSION

A normally functioning tear film is important for eye function; therefore, in past years, tear fluid composition was studied intensely to uncover the mechanisms behind pathology-driven alteration in protein composition[1,24–27] or lipid distribution.[2,28–31] Through the tear fluid, the corneal epithelium comes into direct contact with the environment and, thereby, is exposed to numerous environmental challenges; for this reason, we were interested in SM metabolism enzymes at the ocular surface. We have shown previously that HCEs express most of the SM pathway components.[13] Moreover, HO engages the cellular stress response through the activation of SMases

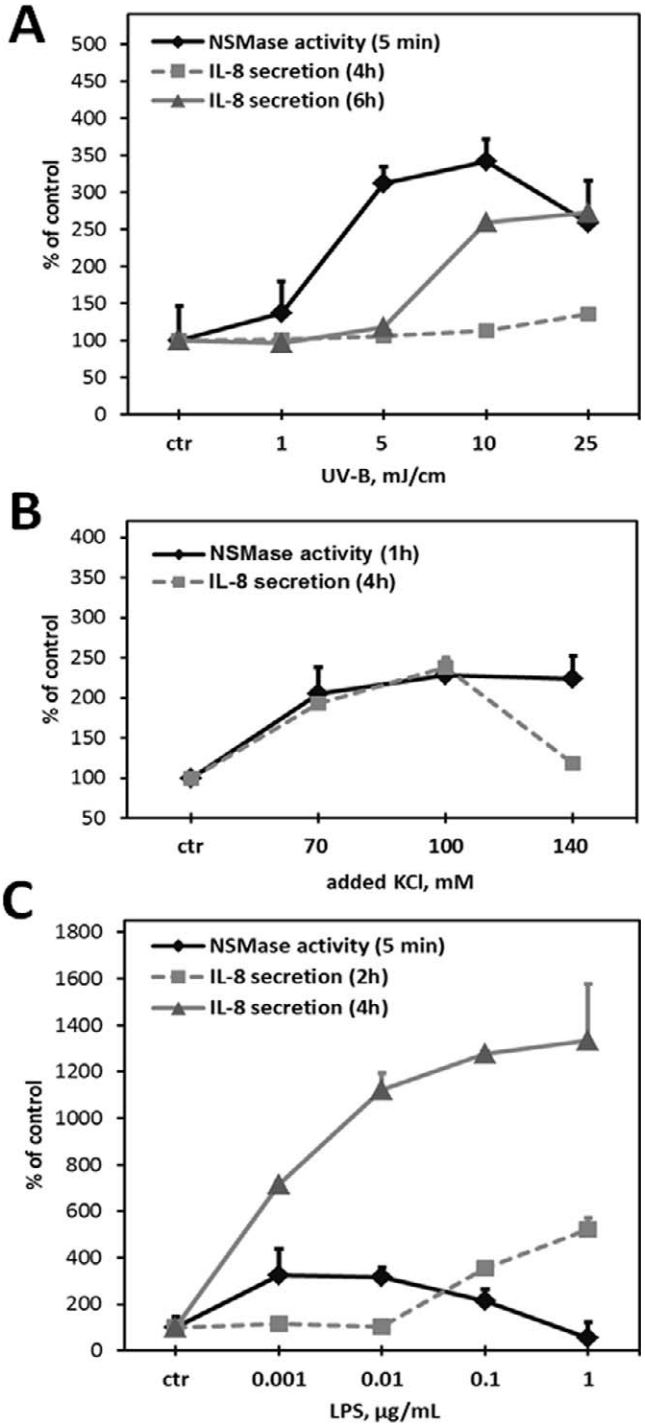

FIGURE 3. Neutral sphingomyelinase activation in stressed corneal epithelial cells. Cells were exposed to increasing UV-B energies (**A**), potassium chloride (KCl)-induced HO (**B**) or LPS (**C**). Sphingomyelinase activity and IL-8 secretion are presented as percentages normalized to control values (considered 100%).

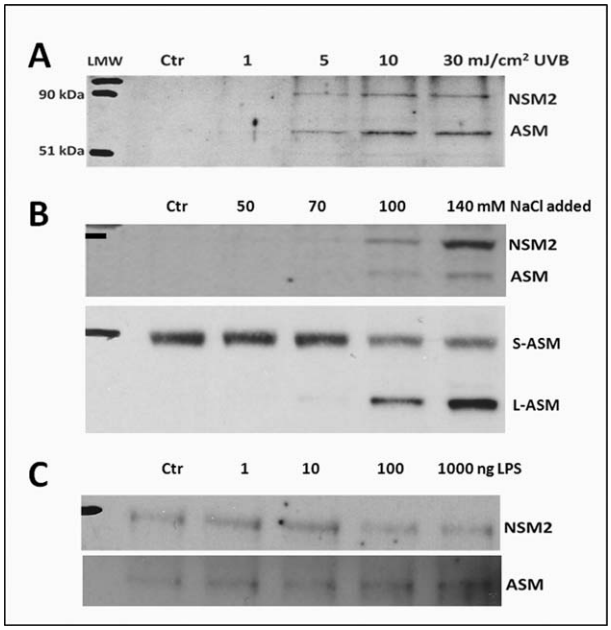

FIGURE 4. Stressed HCE cells release phospholipases in the medium. (**A**) The ASM and NSM2 present in media from UV-B-stimulated cells. (**B**) The SMase secretion induced by hyperosmolarity stress in HCE cells (50–140 mM of NaCl were added to the growth medium to increase osmolarity). (**C**) The SMase secretion in LPS-stimulated cells. Cells were stimulated with 70 to 140 mM added concentrations of a NaCl/KCl solution to produce hyperosmolar conditions, UV-B with radiation energy within 1 to 25 mJ/cm^2 in a UV crosslinker (Spectroline XL-1500; Spectroline, Westbury, NY), and LPS (E. coli O55:B5; Sigma-Aldrich, St. Louis, MO) in concentrations ranging from 1 ng to 1 lg of LPS/mL medium. Media (200 lL) from control and stressed cells were loaded per lane on a 4% to 12% Bis-Tris NuPAGE gel. LMW, low molecular weight marker.

and proinflammatory cytokine secretion,[13] but little is known about the presence of these enzymes in the tear fluid and their possible role in the extracellular environment.

Lipidomic and proteomic analyses established that SM, ceramides, ASM, and ASAH1 are present in human tears.[1,2,11] In our study, we confirm these findings, and additionally report that tear fluid samples also contain neutral SMase and ceramidase (NSM2 and ASAH2). The presence of ASM and ASAH1 in tears is not at all surprising, as most lysosomal enzymes are secreted as proenzymes. The *smpd1* gene generates two forms of the ASM: the lysosomal enzyme (70 kDa) and the secreted sphingomyelinase, with a higher apparent molecular weight.[32] In our study, both isoforms could be identified in basal tears. To our knowledge, this is the first time that NSM2 was identified in the extracellular space. The protein NSM2 is a palmitoylated protein that integrates into membranes; thus, it is difficult to envision a secretion route for this enzyme. The most plausible hypothesis is that the protein would travel in small lipid vesicles shed from the plasma membrane of the epithelial cells. Considering that all the mentioned proteins can localize at the plasma membrane upon stimulation, this vesicular organization could accommodate all enzymes. Romiti et al.[22] reported that endothelial cells can secrete ASAH2 and that the secreted enzyme is associated partially with caveolin (i.e., originates from the plasma membrane caveolae). Thus, these findings support the hypothesis that lipid vesicles may serve as a possible trafficking route. Several other research groups have found that lipid microvesicles produced by tumors and normal cells often harbor components of the sphingolipid metabolism.[33]

To advance with our studies, we used a corneal epithelial cell culture model to establish whether corneal cells secrete the enzymes in the tear fluid, and if so, whether their secretion is inducible and for what purpose. Using the HCEs, we showed that the SMases are found in culture medium conditioned by the cells. Additionally, we demonstrated that enzyme secretion

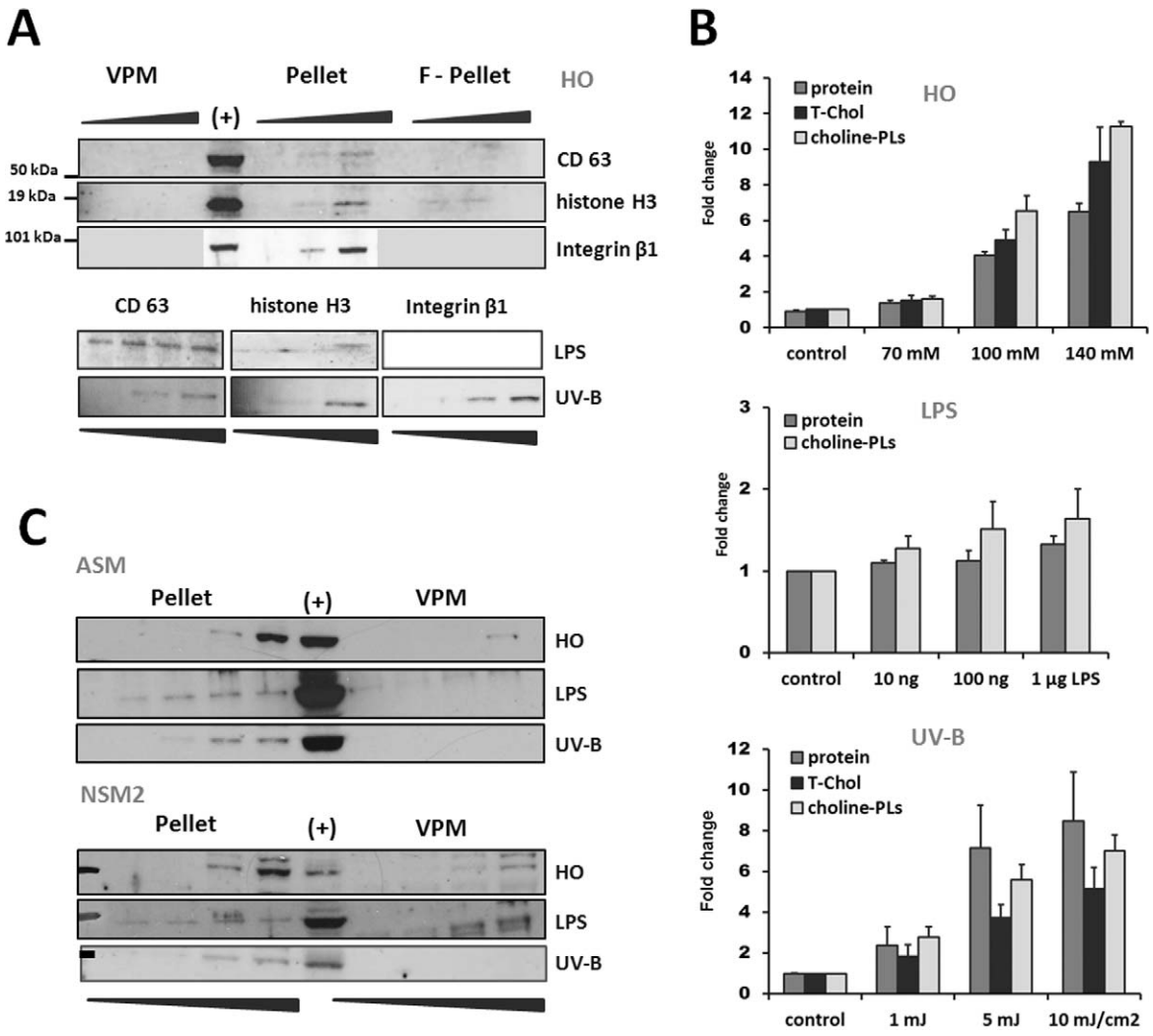

FIGURE 5. Vesicle sedimentation from cell conditioned media. (**A**) Vesicle characterization with CD63, H3, and integrin β1 antibodies. (**B**) Lipid/protein ratios in isolated vesicles. (**C**) The SMase distribution between vesicle pellets or supernatant after ultracentrifugation. *Black triangles* indicate increases in stress. Pellet, sedimented vesicles. F-Pellet, vesicles sedimented after the medium was filtrated through a 0.22-μm filter; (+), positive control represented by HCE cell lysate.

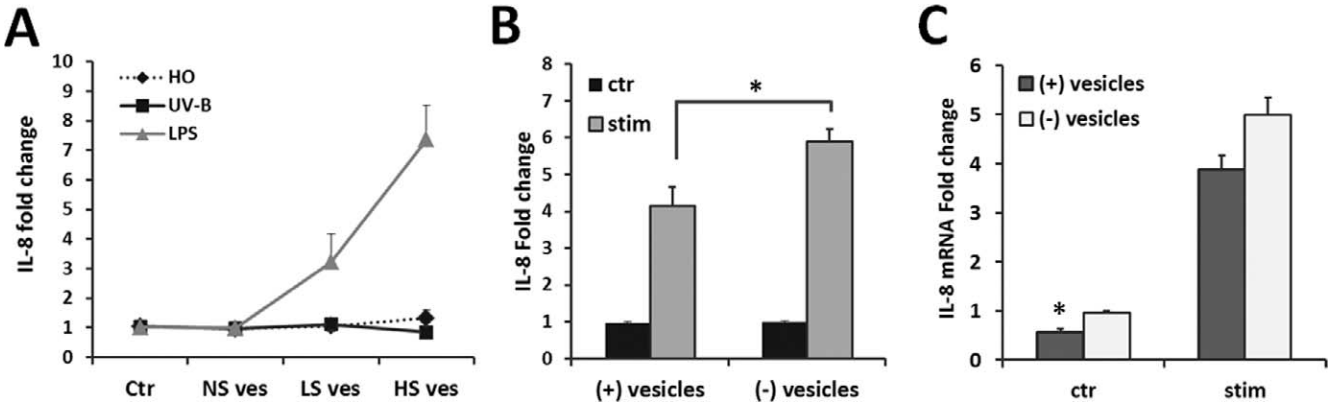

FIGURE 6. Isolated vesicle effects on HCE cells stress response. (**A**) Stress-induced vesicle effects on naïve HCE-cells' IL-8 secretion. NS ves, vesicles from nonstressed cells; LS ves, vesicles from low-stress cells (10 ng/mL LPS, 2 mJ/cm^2 of UV-B, or 70 mM NaCl); HS ves, vesicles from cells exposed to high stress (1 μg/mL LPS, 10 mJ/cm^2 of UV-B, or 140 mM NaCl). (**B**, **C**) Stress-vesicles' effects on the naïve HCEs' stress response. Vesicles were isolated from the medium of cells exposed to 5 mJ/cm^2 of UV-B or 100 mM NaCl and added to the serum-free media of naïve cells. After 18 hours of incubation, cells were stimulated with 70 mM of added NaCl, and IL-8 secretion (**B**) and gene expression (**C**) were measured. ctr, control cells; stim, cells stimulated with 70 mM NaCl. *Significantly different ($P < 0.05$) when compared to the (-) vesicle controls.

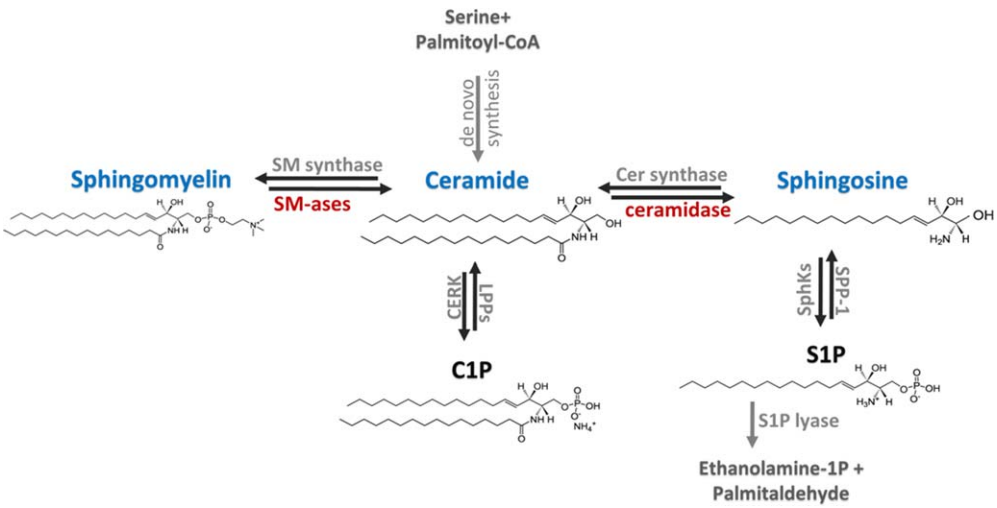

FIGURE 7. Schematic representation of the sphingolipid metabolic pathway. SMases produce ceramide from sphingomyelin, while ceramidases cleave-off a fatty acid to produce sphingosine. CERK, ceramide kinase; C1P, ceramide-1-phosphate; SK, sphingosine kinases; S1P, sphingosine-1-phosphate; LPP, lipid phosphate phosphatase; SPP-1, sphingosine phosphate phosphatase.

is induced by stress factors, and the amount of enzyme in the extracellular environment is proportional to the strength of the stimulus. As environmental stress factors, we used UV-B radiation, HO, and LPS.

The most challenging task was to define the role SMases have in the medium (and, by extension, in tears), if any. An in vitro assay demonstrated that the enzymes in tear fluid are active and also are accompanied by another C-type phospholipase, PC-PLC. Our first hypothesis was that these phospholipases might aid the secreted PLA_2 in its antibacterial activity[34]; however, SMases alone or together with the PC-PLC had no effect on bacterial growth (data not shown).

Secretion of ASM through prompt production of ceramide controlled the invasion of epithelial cells by pathogens, limiting cytokine production by acting on the epithelial cells.[35] Therefore, we considered that the secreted enzymes might have a role in stress response. All used stimuli activated the intracellular sphingolipid stress response pathway through the NSM2 enzyme and caused proinflammatory cytokine production at varying levels. The sphingolipid metabolic pathway (Fig. 7) represents a well-known signaling pathway in the cellular stress-response[36] and the extracellular presence of these enzymes can have important immunomodulatory consequences. For example, ceramide domains, through their interaction partners, promote cell death, growth arrest, and/or inflammation.

We found that NSM2 enzyme was present in the HCE medium along with both forms of ASM and proposed that extracellular vesicles may serve as secretory vehicles for these enzymes in tears. The HCEs produce most types of vesicles, including apoptotic bodies, microparticles, and exosomes. Moreover, these vesicles were also induced by stress stimuli (e.g., HO and UV-B). Extracellular vesicle production is not restricted to HCEs; rather, it is an inherent property of all cells.[37,38] The presence of some vesicles (exosomes in particular) in nonstimulated cells demonstrated that their secretion is constant. We also showed that the SMases are associated with these vesicles and that only the secreted ASM is soluble in the medium. Furthermore, the vesicles secreted by HCEs potentially can accommodate all components (e.g., proteins and lipid substrates) and simultaneously serve as excellent trafficking carriers. Extracellular vesicles could potentially convey messages across long distances in tissues, with the message contained in the lipid layer as packed receptors or ligands, enzymes, or the bioactive lipids themselves.[39–41] The ceramide contained in vesicles induces apoptosis in recruited immune cells and this is thought to be the mechanism behind vesicle-induced immunosuppression.[42,43] Furthermore, apoptotic bodies from differentiated epithelia were shown to induce differentiation in tissue-resident stem cells to replenish the lost cells.[44] Although numerous functions associated with extracellular vesicles are relevant for the tear fluid and cornea, here we tested whether the isolated vesicles from stress-exposed HCEs have any effect on nonstressed HCEs or on their response to the environment. The result was that incubation with stress-induced vesicles diminished the response of naïve HCEs to stress, which suggests that the presence of the extracellular vesicles and their cargo might have antiinflammatory properties that would help control the inflammation at the surface of the eye.

By using ocular surface-relevant stimuli (UV, LPS, or HO), we induced a stress-dependent release of sphingolipid enzymes from the HCEs in the medium. We further investigated the mechanism by which proteins are released from cells and isolated lipid vesicles from HCE-conditioned media. The in vitro data supported our hypothesis that SM cycle components are present in tears, indicating not only that the cells at the ocular surface secrete these enzymes (as soluble proteins or in extracellular vesicles), but also that levels of these proteins might represent a valid measure of the stress to which the cornea has been subjected.[45] There still is a lot to be learned about the role proteins or vesicles have in this context, but one promising lead was suggested by the potential anti-inflammatory effect of stress-induced vesicles.

Acknowledgments

Supported by grants from the Sigrid Juselius Foundation (JMH), the Finnish Academy (Grant 132 629 [MJ], Grant 128 128 [JMH]), the Helsinki University Central Hospital Research Foundation (JMH) and the Finnish Eye Foundation (JMH).

Disclosure: **A. Robciuc**, None; **A.H. Rantamäki**, None; **M. Jauhiainen**, None; **J.M. Holopainen**, Croma-Pharma GmbH Austria (C)

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Investigative Ophthalmology & Visual Science

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