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**Lipidomic Analysis of Human Corneal Epithelial Cells Exposed to Ocular Irritants Highlights
the Role of Phospholipid and Sphingolipid Metabolisms in Detergent Toxicity Mechanisms**

Romain Magny^{1,2*}, Nicolas Auzeil², Elodie Olivier², Karima Kessal^{1,3}, Anne Regazzetti², Melody Dutot^{2,4}, Stéphane Melik-Parsadaniantz¹, Patrice Rat², Christophe Baudouin^{1,3,5,6}, Olivier Laprèvote^{2,7},
Françoise Brignole-Baudouin^{1,2,3}

¹Sorbonne Université UM80, INSERM UMR 968, CNRS UMR 7210, Institut de la Vision, IHU Foresight, Paris, France

²UMR CNRS 8038 CiTCoM, Chimie Toxicologie Analytique et Cellulaire, Université de Paris, Faculté de Pharmacie, Paris, France

³Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts, Paris, France

⁴Recherche et Développement, Laboratoire d'Evaluation Physiologique, Yslab, 2 rue Félix le Dantec, 29000 Quimper, France

⁵Department of Ophthalmology, Hôpital Ambroise Paré, AP HP, 92100 Boulogne, France

⁶Université Versailles St Quentin en Yvelines, Paris Saclay, 78180 Montigny-Le-Bretonneux, France

⁷Hôpital Européen Georges Pompidou, AP-HP, Service de Biochimie, Paris, France.

Corresponding authors: romain.magny@inserm.fr

19 **Highlights**

- 20 • A lipidomic analysis of HCE cells incubated with ocular irritant was performed
- 21 • Ocular irritants induced significant change in lipidome of HCE cells
- 22 • BAK, SLS and TXT all increased the content of ceramide
- 23 • BAK, SLS and TXT similarly decreased the level of DHA-containing plasmalogen
- 24 • Some lipid modifications are specific to each irritant, especially for phospholipids

Abstract

Detergent chemicals, widely used in household products, in pharmaceutical, medical, cosmetic and industrial fields, have been linked to side effects and involved in several eye diseases. On the ocular surface, detergents can interfere with the corneal epithelium, the most superficial layer of the cornea, representing a line of defence against external aggression. Despite its major role in numerous biological functions, there is still little data regarding disruption of lipid homeostasis induced by ocular irritants. To this purpose, a lipidomic analysis using UPLC-HRMS/MS-ESI+/- was performed on human corneal epithelial (HCE) cells incubated with three widely known ocular irritants: benzalkonium chloride (BAK), sodium lauryl sulfate (SLS) and Triton X-100 (TXT). We found that these ocular irritants lead to a profound modification of the HCE cell lipidome. Indeed, the cell content of ceramide species increased widely while plasmalogens containing polyunsaturated fatty acid species, especially docosahexaenoic acids, decreased. Furthermore, these irritants upregulated the activity of phospholipase A₂. The present study demonstrates that BAK, SLS and TXT induced disruption of the cell lipid homeostasis, highlighting that lipids mediate inflammatory and cell death processes induced by detergents in the cornea. Lipidomics may thus be regarded as a valuable tool to investigate new markers of corneal damage.

Key words: ocular irritants, detergents, lipid homeostasis, PLA₂, cell toxicology

1. Introduction

Detergent chemicals have been widely used for numerous decades to meet people's need for hygiene and to fight against microorganisms. Their use is ubiquitous, not only in household products but also in the pharmaceutical, medical, cosmetic and industrial fields. However, detergent exposure has been linked to side effects, especially at dermatological, respiratory and ocular levels [1–3]. For example, chronic exposure may lead to irritant contact dermatitis, worsening asthma or corneal damage [1–3]. The most affected tissues are those directly exposed to external environments, as detergents mainly act by surface contact. Indeed, exposure to detergents induces remarkable stress at the cell membrane level, resulting in reorganization, cell activation and damage [4]. Regarding the ocular surface, detergents can profoundly interfere with the corneal epithelium, the most superficial layer of the cornea and the second line of defense against external aggression immediately under the tear film [5].

The main sources of chronic exposure of the cornea to chemicals with detergent properties come from eye drop medicine [3]. As recommended by Pharmacopoeias, ophthalmic preparations should contain a preservative agent when prepared in multidose bottles and dispensers. Benzalkonium chloride (BAK), a quaternary ammonium compound, is the preservative that is the most frequently used in multidose eye drops, and is thus widely administered for chronic disorders requiring mid- to long-term treatments, such as glaucoma, allergic reactions or dry eye diseases (DED) [3,6–8]. Since the 90's, numerous investigations based on *in vitro* and *in vivo* models as well as clinical observations have revealed that the chronic use of BAK is clearly responsible for induction or worsening of DED [9–11]. DED is a chronic inflammatory pathology of the ocular surface, which leads to disabling ocular symptoms, such as discomfort, foreign body sensation, irritations, burning and pain, or visual disturbance [12–15]. DED has also been previously related to chronic eye exposure to detergents due to occupational and domestic activities [16]. Indeed, changes in ocular surface tissues and tear film were reported in workers in the dry-cleaning industries. Besides, acute exposure leading to corneal damage has also been detected in children exposed to liquid laundry detergents pods [17].

To assess the ocular damage potency of chemicals and to understand the mechanisms underlying ocular irritation, the Draize eye test performed in rabbits has been widely used in the past decades [18]. Nevertheless, due to the increase in awareness of animal welfare and demands for non-animal-based test methods, *in vitro* models have progressively replaced those using animals [19]. These *in vitro* models are mainly based on corneal cells incubated with widely used ocular irritants, such as BAK, sodium dodecyl sulfate (SLS) or Triton X-100 (TXT) [19,20]. Using *in vitro* models, deleterious effects induced by detergents have been associated to numerous alterations of cell signaling pathways such as oxidative stress, mitochondrial damage or lipid peroxidation. Irritation processes also involve inflammation through the release of pro-inflammatory cytokines and cell death through apoptosis or necrosis pathways [21,22].

Inflammation and cell death processes are tightly linked to lipid homeostasis [23,24]. Indeed, lipids are not only molecules involved in energy storage, but they also play a critical role in cell signaling. For example, phospholipids (PL) constitute a pool of fatty acids (FA), as they can be hydrolyzed through the action of phospholipase A₂ (PLA₂) to generate free FA, which in turn are oxidized in oxylipins involved in inflammatory processes [23,25,26]. Sphingolipids (SL) are also well-known bioactive lipid species that induce the release of pro-inflammatory cytokines such as IL-1 β and activate caspase 3 resulting in cell apoptosis [27,28]. Perturbation of lipid metabolism has been previously described in corneal alteration [29–31]. Dysregulation of SL metabolism, especially ceramide species, play a key role in corneal injury due to nitrogen mustard exposure [32]. Moreover, it was reported that highly cytotoxic concentrations of ocular irritants in 3D-reconstructed cornea-like models alter the content of ceramide and the gene expression of enzymes involved in lipid metabolism [33]. Nevertheless, to our knowledge, little data is available regarding the effect of ocular irritants on cell lipid content and the potential consequences on inflammatory and cell death processes.

The aim of the present study was to investigate the mechanisms of three widely used detergents and to understand their effects on cell lipidome. To this purpose, human corneal epithelial (HCE) cells were incubated with BAK, SLS or TXT respectively, cationic, anionic and non-ionic detergents. We

95 first determined values corresponding to subcytotoxic concentrations respective to each ocular irritant.
96 Using an untargeted LC/MS/MS lipidomic analysis, we then comprehensively determined the
97 lipidome of HCE cells and characterized lipid alterations induced by BAK, SLS and TXT. Finally, the
98 mechanisms underlying this lipidome change were investigated.

2. Materials and methods

2.1. Cell line, culture conditions and incubation solutions

Human corneal epithelial cell line (HCE) was obtained from the RIKEN biobank (Tsukuba, Japan) [34]. HCE cells were grown in culture flasks using DMEM/F12 (1/1), 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin all from Gibco (Paisley, UK). At confluence, every 3 days, cells were harvested with trypsin-EDTA 0.05% in Dulbecco's phosphate-buffered saline (DPBS). The cells used in this study were from passages 2 to 10.

The 10⁻⁴% BAK solution was prepared with a stock solution of 0.1% (w/v) BAK (Sigma, Saint Quentin Fallavier, France). For the 5.10⁻³% and 3.10⁻³% solution of SLS and TXT respectively, a stock solution of 1% of SLS (w/v) or TXT (v/v) (Sigma, Saint Quentin Fallavier, France) was used. The stock solutions were prepared in water, prior to dilution in the culture medium for incubation. HCE cells were seeded in 96-well (20,000 cells/well) and 6-well (400,000 cells/well) plates to reach subconfluence in 24 hours and then incubated for 24 hours with 1.10⁻⁴% BAK, 5.10⁻³% SLS or 3.10⁻³% TXT containing 2% FBS. Cells were washed with DPBS prior to each assay.

2.2. Cell viability assay

The viability of HCE cells was assessed using the neutral red (NR) assay according to previously published data [35]. Briefly, a NR solution (50 µg/mL) prepared in culture media without serum was added to the cells after incubation with each ocular irritants. The NR solution was incubated for 3h at 37°C. Cells were washed with DPBS, lysed using a mixture solution containing water, ethanol, acetic acid (49.5/49.5/1, v/v/v) and finally homogenized at room temperature for 15 minutes on a stirring plate. Fluorescence intensity was measured using $\lambda_{\text{ex}} = 540 \text{ nm}$ and $\lambda_{\text{em}} = 600 \text{ nm}$ (Spark®, TECAN, Männedorf, Switzerland).

2.3. Reactive oxygen species production

ROS production was determined using the 2',7'-Dichlorohydrofluorescein diacetate (H₂DCFDA) assay. The H₂DCFDA 0.1 M stock solution in DMSO (Thermo Fisher Scientific, Les Ulis, France) was used to prepare a 20 µM solution in DPBS. HCE cells were incubated with the H₂DCFDA

working solution for 30 minutes at 37°C after incubation with each irritants and fluorescence intensity was measured using $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 535 \text{ nm}$ (Spark®, TECAN).

2.4. Caspase-1 activity

Caspase-1 activity was evaluated using the Caspase-Glo® 1 Inflammasome Assay (Promega, Madison, WI, USA). The assay was performed according to the manufacturer's instructions. Luminescence, resulting in luciferase activity, was quantified using the Spark® microplate reader (TECAN).

2.5. P2X7 activation

P2X7 activation was assessed with YO-PRO-1 probe as previously described [36]. The YO-PRO-1 1 mM stock solution in DMSO (Thermo Fisher Scientific) was used to prepare a 2 μM solution in DPBS. HCE cells were incubated with the YO-PRO-1 working solution for 10 minutes at room temperature after incubation with each irritants and fluorescence intensity was measured using $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 531 \text{ nm}$ (Spark®, TECAN).

2.6. Untargeted lipidomic analysis

2.6.1. Sample preparation

HCE cells were incubated with BAK ($10^{-4} \%$), SLS ($5 \cdot 10^{-3} \%$) or TXT ($3 \cdot 10^{-3} \%$) for 24 hours and were then washed with DPBS. Cells were harvested using trypsin-EDTA 0.05%, washed with DPBS, centrifuged at 2000 rpm for 10 min. Dry cell pellets were adjusted to 3 million cells and stored at -80°C until analytical process. After thawing, the cell pellets were resuspended in ultra-pure water (1 mL) and were sonicated for 5 minutes. The cell lysate was spiked with a mixture of internal standard (**Table S1**) prior to lipid extraction using a chloroform/methanol/water (5:5:2, v/v/v) mixture containing the antioxidant agent 3,5-di-tert-4-butylhydroxytoluene 0.01% (w/v). Samples were subsequently centrifuged at 3000 rpm for 10 min, organic phase was collected, and solvents were evaporated under reduced pressure at 45 °C. Dry residues were dissolved in 100 μL of a mixture

containing acetonitrile/isopropanol/chloroform/water (35:35:20:10, v/v/v/v) before injection into the UPLC-MS/MS system.

2.6.2.Data-dependent LC-ESI-HRMS/MS analysis

Liquid chromatography in both positive and negative electrospray ionization mass spectrometry analysis of lipid extracts was performed on a UPLC system (Waters, Manchester, UK) combined with a Synapt[®]G2 (Q-TOF) mass spectrometer (Waters). Chromatographic separation was achieved on an Acquity[®] CSH C18 column (100 mm × 2.1 mm; 1.7 μm). Elution was performed using a binary gradient system consisting in 10 mM ammonium acetate in acetonitrile/water mixture (40:60, v/v) as solvent A and 10 mM ammonium acetate in acetonitrile/isopropanol mixture (10:90, v/v) as solvent B. The eluent increased from 40% B to 100% B in 10 min, was held at 100% B for 2 min before returning to 40% B. The flow rate was kept at 0.4 mL.min⁻¹, the column oven was set at 50°C and the injection volume was 5 μL. The source parameters were as follows: capillary voltage 2,400 V, cone voltage 45 V, source temperature 120°C, desolvation temperature 550°C, cone gas flow 20 L.h⁻¹, and desolvation gas flow 1,000 L.h⁻¹. Leucine enkephalin (2 ng.mL⁻¹) was used as an external reference compound (Lock-Spray[™]) for mass correction. In a data-dependent acquisition mode (DDA), MS full scans were followed by 5 MS/MS scans performed on the most intense ions above an absolute threshold of 1,000 counts. Selected parent ions were fragmented at collision energy from 20 to 40 eV. Scan duration for both MS1 and MS2 was 0.2 s. In the full scan mode, the data were acquired between *m/z* 50 and 1200 using a resolution of 20,000 FWHM at *m/z* 500. Data acquisition was managed using Waters MassLynx[™] software (version 4.1; Waters MS Technologies). Sample injections order was orthogonalized to sample status into UPLC/MS/MS system to avoid any bias. A mixture of 65 standard lipids belonging to 9 of the main lipid classes : Fatty acid (FA), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), ceramide (Cer), sphingomyelin (SM), HexosylCeramide (HexCer), monoacylglycerol (MG), diacylglycerol (DG) and triacylglycerol (TG) at a final individual concentration of 1 μM was also periodically injected throughout the analytical batch (**Table S2**).

2.6.3.Data-preprocessing parameters

For both ion modes, raw data files were converted into universal open source mzXML file with MSConvert 3.0 and were then processed using MZmine 2.5 software. MS and MS/MS spectra were extracted with a mass detection noise level set at 1E2 and 0E0, respectively. Chromatograms were built with the ADAP algorithms [37] (minimum group size of 5 scans, a group intensity threshold of 1,000, and an m/z tolerance of 10 ppm). The ADAP wavelets chromatogram deconvolution algorithm was used with the following settings: signal to noise ratio = 10, coefficient/area ratio = 60, peak duration range = 0.05-0.5 min, retention time wavelet range = 0.0-0.1. Chromatograms were deisotoped using the isotopic peaks grouper algorithm using a m/z and t_R tolerance of respectively 10 ppm and 0.1 min. Peak alignment was performed through the join aligner method using the following parameters: m/z and t_R tolerance of respectively 10 ppm and 0.15 min. Each MS/MS scans were associated with the corresponding MS scans using a m/z and t_R tolerance of respectively 15 ppm and 0.15 min. The peak list was finally gap-filled using the so-called module "same RT and m/z range gap filler" with m/z tolerance of 5 ppm. Lipids were then annotated based on exact mass measurement and retention time with the "custom database" module using an in-house database. These preprocessing steps led to two matrixes, both in ESI+ and ESI- mode, listing for annotated lipid m/z , t_R and peak area values. These matrixes were normalized and filtered as previously described [38].

2.6.4.Lipid structure assignment

The structure assignment of lipid species was based on MS and MS/MS data, using a tolerance window of 5 and 15 ppm, respectively (**Table S3-S11**). Identification was supported by t_R values through comparison of experimental to expected values calculated using t_R prediction models. Indeed, the UPLC-ESI-MS analysis of the standard lipid mixture allowed determining for each lipid class (FA, PA, PE, PS, PC, PG, PI, Cer, SM, HexCer, MG, DG, TG) the linear relationship between retention time and equivalent carbon number (ECN) [38,39]. MS/MS data was used to fully determine phospholipid fatty acid composition (**Table S4-S10**). Fatty acyl sn₁ and sn₂ position was determined in

negative ion mode using the relative intensity of ions corresponding to carboxylates as previously described [40,41].

2.6.5. Estimation of lipid amounts

The cell lipid amounts, expressed as mol%, were estimated using internal standard lipid mixture spiked in cell suspension samples before extraction [42]. Lipid species intensities were individually normalized to the one of the corresponding internal standard chosen in the same subclass. The standard lipid mixture includes one lipid species representative of each investigated subclass (**Table S2**). Lipid species used as internal standard contain a fatty acid side chain with total odd carbon number or are deuterated derivatives and thus cannot overlap with endogenous lipid species present in HCE cells.

2.7. PLA₂ activity assay

The global PLA₂ activity was determined using Red/Green BODIPY based EnzCheck Phospholipase A₂ assay kit (Thermo Fisher Scientific) according to manufacturer's procedure. Briefly, cells were incubated for 24 hours with BAK or HO. Culture media was removed, and cells were washed with DPBS. Harvested cells were centrifugated, DPBS removed and the cell pellet was suspended in 100 µL of PLA₂ reaction buffer with protease inhibitor and finally sonicated for 10 sec. A volume of 50 µL of cell lysate was then transferred in a 96 well plate and mixed with liposomes prepared with the EnzChek Phospholipase A₂ substrate at a ratio of 1:1. Following a 30-minute incubation at 37°C, PLA₂ activity was determined as a FRET ratio ($\lambda_{\text{ex}} = 460 \text{ nm}$, $\lambda_{\text{em}} = 515/575 \text{ nm}$) using the Spark® spectrofluorometer.

2.8. Statistical analysis

Unsupervised multivariate analyses were performed for lipidomic data using SIMCA-P+ software version 13.0.3 (Umetrics, Umeå, Sweden) as previously described [38,43,44]. A pareto scaling was applied to the variables prior to unsupervised principal component analyses (PCA).

224 Each experiment was performed independently five times. Results are expressed in mol% or fold
225 change compared to control and are displayed as means $\pm$ standard deviation (SD). ANOVA, Dunnett
226 test and Student t-test were performed using GraphPad Prism 8 software (GraphPad Software, La
227 Jolla, CA) with a risk set at 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001 compared to control).

3. Results

3.1. Cytotoxic effects of ocular irritants

Human corneal epithelial (HCE) cells were incubated for 24 hours with BAK, SLS or TXT in concentrations ranging from 1.10^{-5} to $5.10^{-1}\%$. All the ocular irritants altered HCE cell viability in a concentration-dependent manner (Figure 1A). BAK $1.10^{-4}\%$, SLS $5.10^{-3}\%$ and TXT $3.10^{-3}\%$ induced a loss of cell viability of about 20% compared to control, indicative of a sub-cytotoxic effect (Figure 1B). The above concentrations were therefore used in the rest of this study.

BAK $1.10^{-4}\%$, SLS $5.10^{-3}\%$ and TXT $3.10^{-3}\%$ led to a 2.0-, 2.05- and 2.4-fold increase in ROS production, respectively (Figure 1C) and a 1.12-, 1.52- and 1.15-fold increase in caspase-1 activity (Figure 1D). SLS was the only irritant to trigger P2X7 receptor activation (Figure 1E). In contrast, BAK and TXT did not induce any P2X7 receptor activation.

3.2. Lipidome modifications induced by ocular irritants

To investigate the impact of incubation with ocular irritant on HCE cell lipidome, we performed a lipidomic analysis using liquid chromatography hyphenated to tandem mass spectrometry (UPLC-MS/MS). In positive ion mode, a typical chromatogram showed that lysophospholipids (LP) were first eluted before 3 min, phospholipids (PC, PE, PI, PG and PS) and sphingolipids (SM and Cer) were eluted between 5 and 8 min, and DG or TG were lastly eluted after 7 min. In negative ion mode, the elution was for FA before 6 min, and for Cer and phospholipids (PC, PE, PI, PG, and PS) between 6 and 9 min.

A PCA was performed on the data relative to control and treated cells in both positive and negative ion mode. Either in ESI+ and ESI- ion modes, the built PCA models showed good quality parameters and the score plots exhibited four clusters corresponding to BAK-, SLS- and TXT-treated and control cells (Figure 2A). These clustering indicate alterations of the HCE cell lipidome following incubation with ocular irritants. To identify the impacted lipids, the lipidome of HCE cells was first established thanks to an in-house database including lipid species representative of phospholipid (PL), glycerolipid (GL), sphingolipid (SL) and fatty acid (FA) classes. A total of 300 unique lipids,

belonging to 11 subclasses, was identified. Lipid species in HCE cells impacted by ocular irritant were then sought out.

The three investigated ocular irritants led to major changes in the lipidome of HCE cells. Indeed, BAK, SLS and TXT significantly modified the cell content of 104, 202 and 165 lipid species respectively, as shown on volcano plot (Figure 2C, 2D and 2E). Regarding the lipid classes, PL were significantly decreased in HCE cells incubated with BAK while a striking increase in GL was observed following incubation with SLS and TXT (Figure 2F). Moreover, SLS induced a decrease in SL level. Finally, no changes in FA class were displayed whatever the ocular irritant. Regarding the discriminant lipid species, 30 lipids were modified in the same way, whatever the ocular irritant, as displayed on the Venn diagram (Figure 2G). Indeed, 12 lipids increased while 18 decreased following incubation with BAK, SLS or TXT. These lipid species belonged to the PC, PE, DG, TG and Cer subclasses. Nine PC, including seven ether PC, decreased by about 40% (Figure 2H). LPE (18:0) content was increased by 2.2, 1.5 and 3.1-fold compared to control following incubation with BAK, SLS or TXT, respectively. PG (18:1/18:0) and PI (16:0/16:0) increased widely whatever the stressor. Two DG species exhibited a decrease while ten TG species increased. Finally, six ceramides, containing saturated FA, also increased.

3.3. Effect of ocular irritants on phospholipid content

Regarding effects at the subclass level, while the incubation with BAK and TXT led to a decrease in overall PC content, in contrast, SLS induced a significant increase in this subclass (Figure 3). No change in PE was observed following incubation with BAK or SLS while TXT induced a significant increase in this subclass (Figure 3). Regarding FA PL content, PL with saturated fatty acids (SFA) decreased following incubation with BAK but increased with SLS or TXT (Figure 3B). A decrease in mono-unsaturated fatty acid (MUFA) PL was induced by TXT and a decrease in poly-unsaturated fatty acid (PUFA) PL was displayed for SLS (Figure 3B and 3C).

While the overall ether PL (ePL) containing arachidonic acid (AA) displayed no change (Figure 4B), at the molecular species level, four AA-containing ePC at sn₂ position were impacted (Figure

2H). For example, PC (P-18:0/20:4) decreased by 35%, 65% and 45% following incubation with BAK, SLS or TXT, respectively (Figure 2H). In contrast, the overall content of ePL with docosahexaenoic acid (DHA) displayed a significant decrease of 55%, 95% and 45% in HCE cells incubated with BAK, SLS or TXT, respectively (Figure 4A). To investigate the consequences at the PL metabolism level, we determined PLA₂ activity. BAK, SLS and TXT significantly induced an increase in PLA₂ activity of 1.4-, 1.8- and 1.75-fold change compared to control, respectively (Figure 4C).

3.4. Effect of ocular irritants on sphingolipid content

The ocular irritants assessed in the present study significantly increased the overall Cer content in HCE cells following a 24-hour incubation (Figure 5A). In contrast, a significant decrease in SM was observed with SLS and TXT but not with BAK (Figure 5B). The SM/Cer ratio was calculated and it decreased substantially following incubation with SLS or TXT (Figure 5C). At the molecular species level, Cer (d18:1/16:0) increased while SM (d18:1/16:0) decreased because of SLS and TXT (Figure 5E). The increase in Cer at the expense of SM species was observed for 5 other sphingosine based-species (Figure 5E).

3.5. Effect of ocular irritants on glycerolipid content

Incubation with BAK, SLS or TXT decreased the overall HCE cell content of DG (Figure 6A). TG content displayed a striking increase following incubation with SLS and TXT while BAK induced no significant change (Figure 6B).

4. Discussion

Intensive use of detergents in household cleaning products, cosmetics and medical products for several decades has led to a chronical exposure of most of the population to these chemicals. Detergents are responsible for numerous deleterious effects, especially at the cutaneous and respiratory levels [1,2]. Detergents have also been involved in several eye diseases as they may interact with proteins and lipids at the ocular surface. One of the major ocular pathologies, namely dry eye disease, is characterized by irritation, itching, and/or burning sensations [12,15,45,46]. DED is favored by the used of preserved eye drops containing detergents such as BAK. To assess the irritant potential of surfactants, *in vitro* investigations of cytotoxicity and inflammatory cytokine production have been performed [20]. Quite amazingly, although lipids are known to play key roles in inflammatory processes and are targeted by detergents, to our knowledge, no studies have aimed at describing the effects of detergents on corneal cell lipids. Our purpose was therefore to investigate the change in lipidome of human corneal epithelial cells incubated with BAK, SLS or TXT, three chemicals known to induce ocular irritation.

To investigate these alterations, we first determined sub-cytotoxic concentrations, corresponding to a decrease in HCE cell viability of approximately 20%, to compare the three agents based on the same level of cytotoxicity. The use of sub-cytotoxic concentrations is highly valuable to investigate the effect of toxics on cell signaling pathways [36]. Sub-cytotoxic concentrations were observed at $1.10^{-4}\%$ BAK, $5.10^{-3}\%$ SLS and $3.10^{-3}\%$ TXT. In preserved eye drops, BAK concentrations, ranging from $1.10^{-2}\%$ to $5.10^{-3}\%$ [3], are between 50 to 200 times higher than the one used in the present study. In addition, it must be emphasized that BAK induced a similar decrease in cell viability at a concentration 30 to 50-fold lower than SLS or TXT did. This result is in accordance with previous published studies indicating that cationic detergents are more cytotoxic than other detergents [47]. To assess whether the ocular irritants induce alterations in HCE lipidome, we performed a comprehensive lipidomic analysis to characterize changes in cell lipid following incubation with BAK, SLS or TXT. We focused the analysis on PL, GL, SL and FA classes. Some of the lipid alterations are common to the three detergents: BAK, SLS and TXT similarly regulated the cell content of 30 lipid species,

which represent about 10% of total identified lipids. Among them, BAK, SLS and TXT induced a decrease in the abundance of few PL species, especially eight ether-PC. These lipid species contain an ether link in sn₁ position and are thus prone to oxidation [48,49]. Oxidative stress being a major mechanism of the deleterious effects induced by these irritants [50–52], the increase in ROS production following BAK, SLS and TXT incubation reinforced the aforementioned observations. Oxidative products of plasmalogens include both hydroxyaldehyde derivatives and lysophospholipids containing AA or DHA located in sn₂ position [49,53,54]. LPC containing AA or DHA were not detected in HCE cells using our methods, probably as they are produced in small amount and readily metabolized. Hydroxyaldehyde derivatives, such as hydroxy fatty aldehydes, may be regarded as interesting markers of plasmalogen degradation due to oxidative stress. Indeed, in systemic lupus erythematosus, a pathology for which oxidative stress also induced a decrease in plasmalogen species, an increase in hydroxyaldehyde derivatives has been previously reported [55]. Nevertheless, these compounds are highly reactive exhibiting very short half-life in cells and are therefore difficult to analyze [56].

Plasmalogens are known to be a major polyunsaturated fatty acids (PUFA) pool. Indeed, most ePL contain AA and DHA in sn₂ position [48,49,57]. To determine the nature and the position of FA side chain in PL, we used MS/MS data acquired in DDA negative ion mode as it represents a reliable and powerful tool [39]. At molecular level, the three investigated irritants strikingly reduced the total cell content of DHA-containing plasmalogens in sn₂ position. This result is in accordance with the increase in global PLA₂ activity we observed in HCE cells following incubation with irritants. The increase in PLA₂ activity obtained with the assay used in this study cannot unequivocally prejudge if the activity of the enzymatic isoform involved in the cleavage of the plasmalogens is actually increased. Indeed, previous studies indicated that plasmalogen species were substrates of Plasmalogen-Selective PLA₂ specific isoforms such as Pls-Etn PLA₂ [58–60]. The drop in DHA-containing plasmalogens may be however regarded as in agreement with the increase in overall PLA₂ activity mentioned in the present study. A specific assay targeting Pls-Etn PLA₂ activity was previously proposed and could be applied to selectively assess this isoform of PLA₂ [58]. PLA₂, the enzyme involved in sn₂ cleavage of PL,

leads to the release of free FA in the cytoplasm [23,61]. Free DHA can produce oxylipins through the lipo-oxygenase (LOX) and the cyclo-oxygenase (COX) pathways leading to the release of numerous lipid mediators such as resolvins, maresins or protectins [30,62]. These oxidized metabolites of DHA have been previously described as second messengers in the resolution of inflammation [30,63]. Our results thus suggest that the activation of PLA₂ decreases the total pool of DHA in plasmalogen species. Depletion in DHA content has been associated with pro-inflammatory profile [64–66], ocular irritants and especially detergents may thus partially exert their toxic effect in part through this pathway. A targeted LC-MS/MS analysis focusing on DHA-derived metabolites could be valuable to define which species are especially impacted, for further experiments.

Regarding sphingolipid metabolism, our study indicated a significant increase in the total ceramide abundance in HCE cells following incubation with BAK, SLS or TXT. Some species were increased by the three investigated stressors, it includes Cer (d18:1/14:0), Cer (d18:1/16:0), Cer (d18:1/18:0) and Cer (d18:1/24:1). Cer (d18:1/24:1) has been previously described as a targeted lipid of BAK, SLS and TXT in an *in vitro* 3D reconstructed cornea-like model [33]. In biological cell processes, ceramides play signaling roles promoting inflammation through IL-1 β release and inducing apoptosis through caspase 3 activation [27,67,68]. In addition, dysregulation of ceramide metabolism is now recognized as a key feature in many inflammatory diseases such as atherosclerosis, inflammatory bowel disease, non-alcoholic steatohepatitis, or multiple sclerosis [69–71]. In the frame of toxic mechanisms mediated by ocular irritants, ceramides may be regarded as important mediators. They could furthermore be suggested as relevant markers to identify ocular irritants, and they have previously been described as good candidates to determine ocular irritant potency in *in vitro* models [33,72].

Linked to the accumulation of Cer, our results reveal that SLS and TXT decreased the total content in SM of HCE cells. Indeed, as exemplified by Cer (d18:1/16:0) and SM (d18:1/16:0), ceramides increased while the corresponding SM decreased in the presence of SLS or TXT. Ceramides are produced by different biosynthetic pathways such as *de novo* or salvage pathways [25,27,73]. The latter involved the hydrolysis of SM in ceramide through the action of

sphingomyelinase (SMase), an enzyme located at the cell membrane as well as in the lysosomes [27]. The decrease in SM in favor of Cer species thus suggests that SLS and TXT increased SMase activity. This activation has been previously described in numerous pro-inflammatory processes such as those induced by TNF α or UV irradiation [74–76] and is also known to be involved in apoptosis and to contribute to cell death processes. Furthermore, SMase activation has been involved in endoplasmic reticulum stress, which is part of cell apoptosis [77]. It must be emphasized that SMase activation is required to induce the accumulation of TG species [78] and may thus be related to the increase in TG in HCE cells incubated with SLS or TXT. Nevertheless, in the case of the incubation with SLS or TXT, the exact role of SMase in inflammatory and cell death processes needs to be elucidated.

Beside lipid homeostasis alteration, BAK, SLS and TXT induced ROS overproduction and caspase 1 activation. P2X7 receptor activation, related to pore permeation, was also observed but only with SLS. Activation of P2X7 receptor were previously reported in ocular stresses [79]. P2X7 receptor activation and oxidative stress can trigger caspase-1 activation, leading to the release of pro-inflammatory cytokines such as IL-1 β [80]. Ceramide accumulation, especially through SMase, also leads to caspase-1 activation [81]. This suggests that, through caspase-1 activation, Cer accumulation leads to and thus contributes to inflammatory processes and cell death. In contrast to ceramides, other lipid species, especially DHA and DHA-derived oxylipins, are known to inhibit caspase-1. Indeed, DHA can counteract caspase-1 cleavage, an essential step to mediate its pro-inflammatory properties [82,83]. Interestingly, SLS induced the highest activation of caspase-1 and P2X7 receptor and the most important decrease in DHA-containing ePL. DHA released from plasmalogens may be therefore considered as a cell response to counteract the inflammatory processes mediated by BAK, SLS and TXT, through caspase-1 modulation. The crosstalk between SL and PL metabolisms, and caspase-1 activation nevertheless needs further investigations to be fully understood.

On the one hand, the three compounds shared some lipid alterations underlying similar mechanisms; on the other hand, other lipid alterations appeared to be specific and could discriminate cationic from anionic and non-ionic detergents (Figure 7). These alterations mainly concerned PL

species: BAK decreased the overall SFA-containing PL species, contrary to TXT that decreased MUFA-containing PL species in favor of those containing SFA. This result clearly supports a possible cell membrane stiffening induced by TXT [84]. The anionic agent SLS significantly decreased the PUFA-containing PL, mainly through the alteration of PI species. Interestingly, SLS is the only compound both activating P2X7 receptor and decreasing PI content. The phosphatidylinositol-3-kinase pathway, which is involved in P2X7 receptor activation [85], may thus be linked to the observed alteration of PI homeostasis. Further studies are needed to assess if the specific lipid changes observed for cationic, anionic and non-ionic surfactants may be extended to the entire classes of these three detergents.

5. Conclusions

This study highlights the role of lipid metabolism in corneal cells incubated with ocular irritants, especially detergents. The three investigated ocular irritants led to lipid homeostasis alteration, mainly involving SL and PL, two lipid subclasses related to inflammatory and cell death processes. PL metabolism perturbation, especially regarding species containing PUFA, is mainly mediated by PLA₂ activation. The exact role and the link between lipid alteration and inflammation induced by ocular irritants together with targeted signaling pathways need to be further explored. However, our results indicate that lipid markers may be helpful to determine and discriminate other well-known chemicals, including mild to moderate ocular irritants. The identification of lipid species at the fatty acid levels, including for PL and SL, is essential to understand the changes induced by stressors and to characterize toxicity mechanisms. Finally, the use of a lipidomic approach may thus be regarded as a valuable tool to investigate new markers of corneal damage.

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433 **Conflict of interest**

434 The authors declare that they have no conflicts of interest.

435 **Contribution**

- 436 Romain Magny: Conception, Collection of data, Analysis of data, Writing – original draft
- 437 Nicolas Auzeil: Conception, Collection of data, Analysis of data, Writing – original draft
- 438 Elodie Olivier: Conception, Collection of data, Analysis of data, Writing – original draft
- 439 Karima Kessal: Conception, analysis of data, Writing – original draft
- 440 Anne Regazzetti: Conception, analysis of data, Writing – original draft
- 441 Melody Dutot: Conception, analysis of data, Writing – original draft
- 442 Stéphane Melik-Parsadaniantz: Conception, Supervision, Review of the manuscript
- 443 Patrice Rat: Conception, Supervision, Review of the manuscript
- 444 Christophe Baudouin: Conception, Supervision, Review of the manuscript
- 445 Olivier Laprêvote: Conception, Supervision, Review of the manuscript
- 446 Françoise Brignole-Baudouin: Conception, Supervision, Analysis of data, Writing – original draft

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7. Figures

Figure 1. Cytotoxic effects induced by BAK, SLS or TXT on HCE cells. (A) Neutral Red cell viability assay: concentration curves. (B) Concentrations inducing a 20% viability decrease. (C) Reactive oxygen species (ROS) production, (D) Caspase-1 activity and (E) P2X7 receptor activation following incubation with BAK, SLS or TXT. Results are expressed as the mean $\pm$ SD fold change compared to control cells (ANOVA with Tukey's multiple comparison test. $n=5$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 2. Changes in lipidome of HCE cells induced by BAK, SLS or TXT on HCE cells. (A) Positive and (B) negative ionization mode of PCA score plots generated in UPLC-MS/MS. (C, D, E) Volcano plots of discriminant lipid species. (F) Content of HCE lipid classes. (G) Venn diagram of common and specific modulated lipid species (H) Heat-map representation of discriminant lipids impacted by the three ocular irritants. Results are expressed as fold changes compared to control. Blue and red color corresponds to, respectively, a decrease and an increase in the cell content compared to control. PL: Phospholipids, SL: Sphingolipids; GL: Glycerolipids; FA: Fatty acid.

Figure 3. Alteration of phospholipid metabolism induced by BAK, SLS or TXT on HCE cells. Content of the different phospholipid subclasses according to the incubation (A) Pie chart displaying the relative proportion of each phospholipid subclass in control HCE cells. Change in cell content of phospholipids containing (B) SFA, (C) MUFA, (D) PUFA following incubation of HCE cells with BAK, SLS or TXT. Results are expressed as mean mol% $\pm$ SD (ANOVA with Tukey's multiple comparison test. $n=5$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 4. Alteration of plasmalogen metabolism induced by BAK, SLS or TXT on HCE cells. Change in cell content of (A) ePL containing DHA, (B) ePL containing AA following incubation of HCE cells with BAK, SLS or TXT. Results are expressed as mean mol% $\pm$ SD (C) Change in PLA₂ activity induced by BAK, SLS or TXT. Results are expressed as fold change $\pm$ SD (ANOVA with Tukey's multiple comparison test. $n=5$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 5. Alteration of sphingolipid metabolism induced by BAK, SLS or TXT on HCE cells.

Change in cell content of (A) total Cer, (B) total SM, (C) SM/Cer ratio following incubation of HCE cells with BAK, SLS or TXT. (D) Metabolic pathway of Cer species. (E) Change in cell content of Cer and SM containing the same fatty acid chain following incubation with BAK, SLS or TXT. Results are expressed as mean mol% $\pm$ SD (ANOVA with Tukey's multiple comparison test. n=5. *p < 0.05; **p < 0.01; ***p < 0.001).

Figure 6. Alteration of glycerolipid metabolism induced by BAK, SLS or TXT on HCE cells.

Change in cell content of (A) total DG and (B) total TG species following incubation of HCE cells with BAK, SLS or TXT. Results are expressed as mean mol% $\pm$ SD (ANOVA with Tukey's multiple comparison test. n=5. *p < 0.05; **p < 0.01; ***p < 0.001).

Figure 7. Similar and specific lipid change induced by BAK, SLS or TXT. (A) Bar code

representation underlying specific and similar changes of the different lipid species. Red and blue color indicated respectively an increase and a decrease in the cell content of HCE cells. (B) Discriminating factors between the three irritants based on our results.