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Evaluation of the effects of latanoprost and benzalkonium chloride on the cell viability and nonpolar lipid profile produced by human meibomian gland epithelial cells in culture

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Purpose: The purpose of this study was to explore the effects of a $PGF_{2\alpha}$ analog, latanoprost, and its preservative, benzalkonium chloride (BAK), on the cell viability and lipidomic expression of immortalized human meibomian gland epithelial cells (HMGECs).

Methods: Differentiated HMGECs were exposed to latanoprost (0.05 to 50 µg/ml), BAK (0.2 to 200 µg/ml), or combined latanoprost-BAK (0.05–0.2 to 50–200 µg/ml). EP- and FP-type receptors, the cognate receptors of PGE_2 and $PGF_{2\alpha}$, were inhibited, thereby sparing and isolating the function of each receptor to one condition. Cell viability was assessed by ATP quantitation, and lipid extracts were analyzed by ESI-$MSMS^{ALL}$ with a Triple TOF 5600 Mass Spectrometer (SCIEX, Framingham, MA) using SCIEX LipidView 1.3.

Results: Latanoprost and BAK were found to be lethal to HMGECs at the highest concentrations ($p < 0.001$ for both). The cytotoxicity of latanoprost was mediated through FP- and EP-independent mechanisms. Both latanoprost and BAK significantly modulated the lipidomic expression of several cholesteryl esters (8% and 30%, respectively) and triacylglycerols (10% and 12%, respectively). The combined latanoprost-BAK agent appeared to be no more toxic and to only negligibly alter the lipid profile relative to its individual components.

Conclusions: The use of latanoprost and BAK in glaucoma may alter the viability of the meibomian glands and their lipid expression in vivo. Sublethal concentrations of BAK appear to modulate meibum lipid expression, particularly in relation to sterol biosynthesis. Non-preserved latanoprost had less cytotoxicity at lower doses and fewer lipidomic effects compared to BAK, further strengthening the argument in favor of BAK-free pharmaceutical preparations.

Glaucoma is a leading cause of irreversible blindness throughout the world [1]. Though incurable, glaucoma can be managed medically or surgically by lowering intraocular pressure, the disease's only modifiable risk factor [2]. There are currently five available classes of anti-glaucoma eye drops, but prostaglandin $F_{2\alpha}$ analogs (PGAs) are the most common first-line agent owing to their convenient dosing schedule, favorable systemic side effect profile, and overall efficacy [3]. Chronic daily use of this medication class, however, can lead to alterations in the ocular surface, including meibomian gland dysfunction (MGD) [4]. Ocular surface disease has been associated with ocular burning, redness, dryness, and fluctuating vision, among other conditions, and it has the potential to limit treatment success in glaucoma patients by interfering with drop adherence, reliable data acquisition (e.g., routine imaging and threshold visual field assessment), and, arguably, treatment efficacy [4-7].

MGD is a diffuse alteration of the structure and/or function of the lipid-producing meibomian glands [8]. It can be classified as obstructive or hypersecretory. In obstructive MGD, lipid secretion, termed meibum, becomes altered, which often results in increased meibum viscosity, stagnation, and ultimately ductal obstruction. Over time, obstructed glands lead to intraglandular hypertension and eventually meibomian gland atrophy [8]. Mocan et al. found a high propensity for patients with PGA-treated glaucoma to develop obstructive MGD [9]. The exact mechanism behind PGAs' promotion of this pathway remains unknown, although some have hypothesized that PGAs may alter lipid secretion from the meibomian glands [10]. We support this assertion, considering our recent discovery that a human meibomian gland epithelial cell (HMGEC) line expresses the $PGF_{2\alpha}$ FP receptor, and that treatment with $PGF_{2\alpha}$ modifies the lipidomic profile produced by HMGECs [11].

The role of the preservative system, particularly benzalkonium chloride (BAK), in the development of ocular surface disease must also be acknowledged. BAK is a cationic surfactant that exhibits antimicrobial properties through a detergent mechanism that disrupts lipid cell membranes [12]. As a form of collateral damage, BAK also perturbs the host cell membranes of the cornea and conjunctiva [13] and functionally reduces tear stability [14]. Several authors have found that preservative-free PGAs lead to less inflammatory cell

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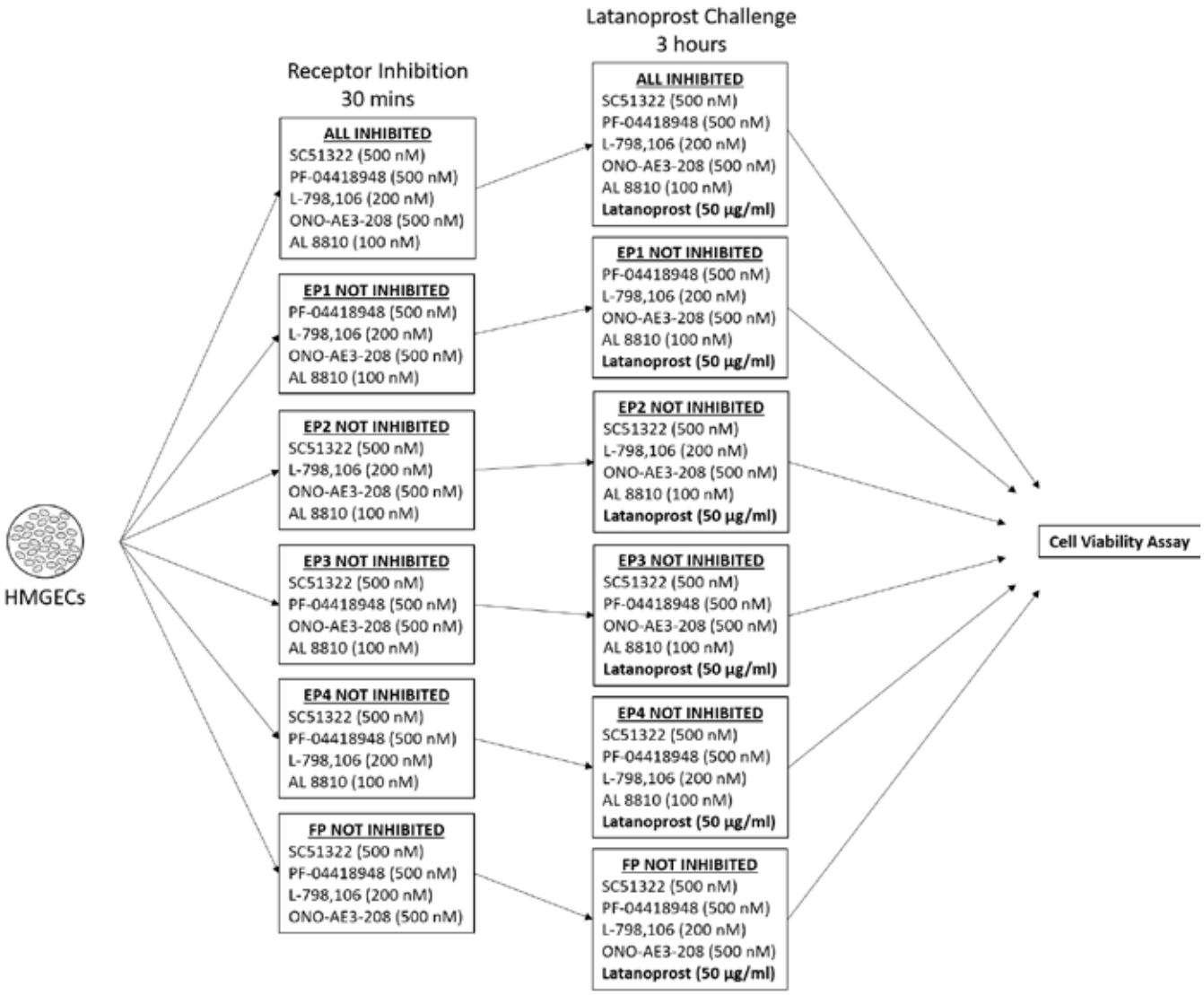

Figure 1. Study design for EP- and FP-type receptor inhibitor experiments. Each well was seeded with 30,000 HMGECs and allowed to differentiate for 48 h (see Methods). HMGECs were pre-treated with receptor inhibitors for 30 min before the introduction of latanoprost 50 µg/ml for 3 h. Receptor inhibition was maintained throughout the three-hour incubation with latanoprost. Following incubation, cell viability was assayed with the Cell Titer-Glo Luminescent Cell Viability Assay (see Methods). There were nine replicates per condition, which included SC51322 (EP1 inhibitor), PF-04418948 (EP2 inhibitor), L-798,106 (EP3 inhibitor), ONO-AE3–208 (EP4 inhibitor), and AL 8810 (FP inhibitor).

infiltration [15-19] and less apoptosis of the corneal epithelium [19] compared to BAK-preserved PGAs. Nevertheless, the exact contribution of PGAs and BAK to the underlying pathophysiology of MGD remains to be fully elucidated.

To address this question, we sought to explore the individual and combined effects of latanoprost (a common PGA) and BAK on cell viability and nonpolar lipid expression in an immortalized HMGEC line. We hypothesized that BAK exposure would decimate HMGECs at concentrations found in commercially available eye drops. We further predicted that treatment with latanoprost and BAK, both in isolation and in combination, would significantly alter the HMGEC lipidomic profile.

METHODS

Reagents and materials: Latanoprost and BAK were purchased from Sigma-Aldrich (St. Louis, MO). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI), as were all receptor antagonists, which included SC-51322 (EP1 inhibitor), PF-04418948 (EP2 inhibitor), L-798,106 (EP3

inhibitor), ONO-AE3–208 (EP4 inhibitor), and AL 8810 (FP inhibitor). Stock solutions of latanoprost, rosiglitazone, and all the receptor antagonists were made by dissolving each substance in dimethyl sulfoxide (DMSO; Hybri-Max™, Sigma-Aldrich). All stock solutions were purged with nitrogen, stored at −20 °C, and freshly added to the media just before experimentation. The DMSO concentration was maintained at 0.5% in all experiments, except for the cell viability experiments that used receptor antagonists, where the concentration was maintained at 0.8%. Clear-bottom, white-walled 96-well plates (ThermoFisher, Waltham, MA) were used for the luminescent cell viability assays, and during the lipidomics experiments, cell cultures were performed on glass petri dishes (Sigma-Aldrich). All experiments consisted of at least two independent experimental replicates and at least two technical replicates.

Immortalized HMGECs were applied in all steps of the experiment. The genotype of our HMGECs was independently verified through a short tandem repeat analysis to match the previously published reference profile [20]. Prior work has demonstrated that these immortalized cells produce lipids consistent with those from human meibomian glands and therefore exhibit the phenotype required to be considered a valid preclinical model for this study [21,22].

Cell viability: HMGECs were seeded at 30,000 cells per well in 96-well plates and differentiated in DMEM/F12 with 10 ng/ml EGF, 2% fetal bovine serum (FBS), and 50 µM rosiglitazone [21-24]. After 48 h of differentiation, the HMGECs were exposed to the same differentiation media containing latanoprost (0.05, 0.5, 5, or 50 µg/ml), BAK (0.2, 2, 20, or 200 µg/ml), or combined latanoprost-BAK (0.05–0.2, 0.5–2, 5–20, or 50–200 µg/ml) for 3 h. Following incubation, the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) was used to quantify ATP as a measure of cell viability, according to the manufacturer's instructions. Luminescence was quantified by the Wallac Perkin-Elmer 1420–041 Victor2 Multiplate Multifluorescent Reader (Mt. Waverly, Victoria, Australia) over an acquisition time of one second.

For the inhibition experiments, HMGECs were seeded in 96-well plates and differentiated with a methodology identical to the one just described. After 48 h of differentiation, the HMGECs were pre-treated with an antagonist cocktail for 30 min before being incubated with latanoprost 50 µg/ml for 3 h. The antagonist cocktail inhibited all the EP and FP receptors except one for each condition and was present during the three-hour challenge with latanoprost (Figure 1). Cell viability was assessed using the same Cell Titer-Glo assay.

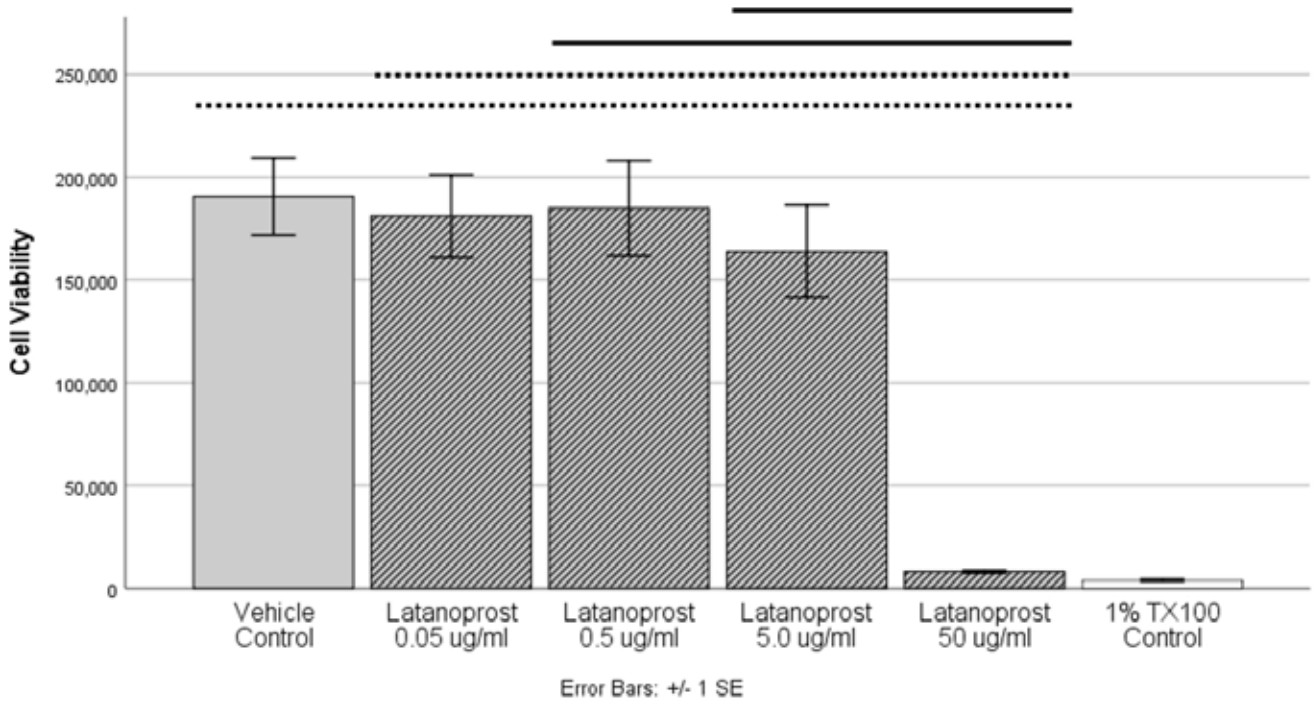

Figure 2. HMGECs were differentiated for 48 h before exposure to latanoprost for 3 h. At 50 µg/ml, latanoprost was lethal to the HMGECs. There were nine replicates per condition. TX100: Triton-X 100 positive control; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

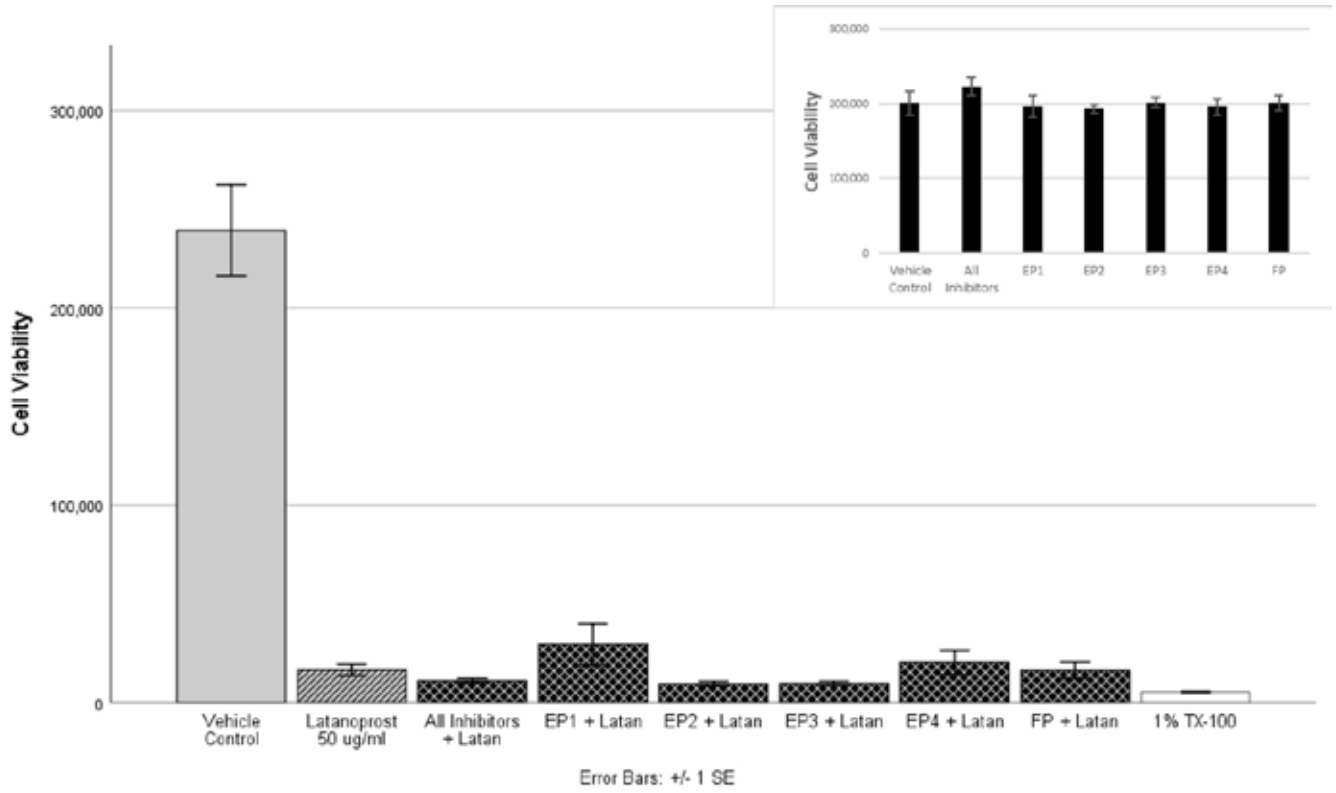

Figure 3. HMGECs were differentiated for 48 h, pre-treated with receptor inhibitors for 30 min, and exposed to latanoprost 50 µg/ml for 3 h, as depicted in Figure 2. Treatment with receptor inhibitors did not prevent latanoprost-induced cell death, suggesting that latanoprost's toxic effects on HMGECs at high concentrations are due to EP- and FP-independent mechanisms. There were nine replicates per condition. Inset: Receptor inhibition alone—in the absence of a latanoprost challenge—had no effect on cell viability, thereby confirming that latanoprost is the toxic agent. TX100: Triton-X 100 positive control; Latan: latanoprost.

Lipid extraction and analysis by mass spectrometry: HMGECs were seeded at one million cells per 6-cm glass petri dish and differentiated for 48 h, as described above. Following incubation, the HMGECs were exposed to latanoprost (0.05, 0.5, or 5 µg/ml), BAK (0.2 or 2 µg/ml), or combined latanoprost-BAK (0.05–0.2 or 0.5–2 µg/ml). Lipids were extracted as previously described [25], and the samples were stored at −80 °C until analysis. Glass, stainless steel, and polytetrafluoroethylene were used for all steps that involved organic solvents.

The lipid extracts were analyzed by direct infusion ESI-MSMS^{ALL} in positive mode ionization with a SCIEX Triple TOF 5600 Mass Spectrometer (SCIEX, Framingham, MA), as previously described [11,21]. All data were processed with SCIEX LipidView 1.3 software. Lipid identities were assigned by LipidView using a database of known ion fragments and confirmed by SCIEX PeakView 2.2 if further investigation was warranted. The mass tolerance window was set to 5 mDa, and only peaks with a signal-to-noise ratio greater than or equal to three were included in the analysis.

Data analysis and labeling convention: For all the lipidomic experiments reported here, cholesteryl esters (CEs), wax esters (WEs), and triacylglycerols (TAGs) were the primary focus. To be included in the data analysis, each lipid species had to be detectable in all replicates from all samples. For TAGs, an additional criterion was used, which required a threshold abundance of 0.1%. All data for both cell viability and lipidomics were analyzed by one-way ANOVA with Tukey post-hoc testing (Statistical Package for the Social Sciences [SPSS v26], IBM, Armonk, NY) when tests of normality (Kolmogorov–Smirnov) and homogeneity of variance (Levene's test) were satisfied. If the variance was not equal, then Games–Howell post-hoc testing was applied. If distributions deviated from normal, then the non-parametric Kruskal–Wallis test was performed. A p value of 0.05 was considered significant.

All CEs were labeled as n_c:db, where n_c and db represented the number of carbons and double bonds, respectively, in the fatty acyl chain. If a third number was present, it represented the number of oxygenations present on an oxidized CE

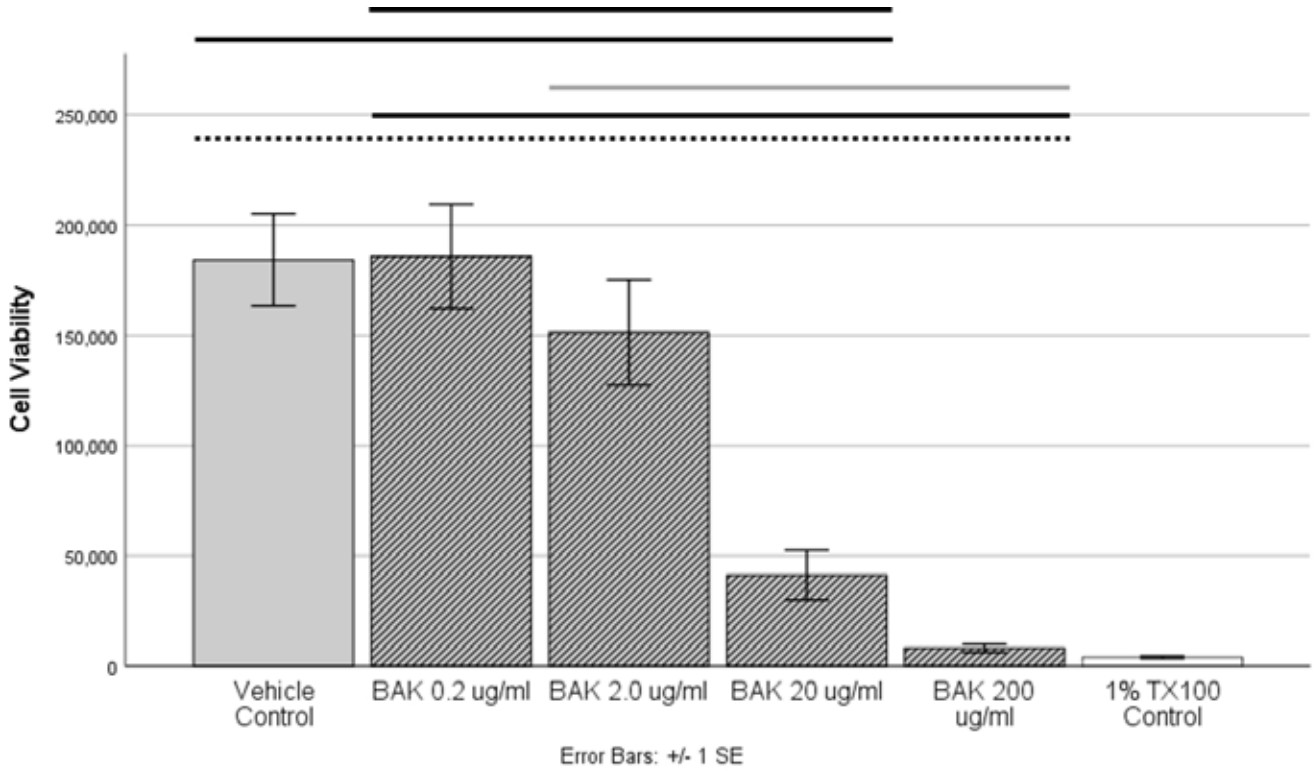

Figure 4. HMGECs were differentiated for 48 h before exposure to BAK for 3 h. At 20 and 200 µg/ml, BAK was lethal to the HMGECs. There were nine replicates per condition. BAK: benzalkonium chloride; TX100: Triton-X 100 positive control; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

(oxCE). TAGs followed a similar convention (n_c:db) to denote the total number of carbons (n_c) and double bonds (db) present in all three fatty acyl groups. TAGs were further labeled in parentheses with a product ion, either a fatty acid (FA) or another TAG, which also followed the same two-number labeling convention. This TAG notation showed that the LipidView software identified a neutral loss corresponding to the FA or TAG in parentheses. For example, TAG 54:3 (FA 18:1) described a TAG with 54 total carbons and three total double bonds among its three fatty acyl chains, with one of those chains consisting of 18 carbons and one double bond.

RESULTS

Influence of latanoprost and BAK on cell viability: To determine whether latanoprost affected HMGEC viability, ATP was quantitated from the differentiated HMGECs exposed to latanoprost (0.05, 0.5, 5, or 50 µg/ml) for 3 h. There was a statistically significant difference among the group means ($p < 0.001$) driven by latanoprost 50 µg/ml, which varied significantly from the vehicle control (95.8% reduction, $p < 0.001$) and all other latanoprost concentrations (mean 95.4% reduction, $p < 0.01$ for all, Figure 2).

In an attempt to determine whether an FP- or EP-type prostaglandin receptor mediated this latanoprost-induced cell death, the HMGECs were pre-treated with a cocktail of receptor antagonists for 30 min (Figure 1), leaving one receptor uninhibited for each condition before challenging with latanoprost 50 µg/ml. Receptor inhibition was maintained during the three-hour challenge. As already shown, latanoprost 50 µg/ml significantly reduced viability relative to the vehicle control by 93.1% ($p = 0.007$). However, inhibition of all the EP and FP receptors failed to prevent latanoprost-induced cell death (Figure 3). Furthermore, there were no significant differences between the samples treated only with latanoprost 50 µg/ml and any of the samples pre-treated with receptor antagonists.

To determine the extent of the toxicity of BAK, ATP was quantitated from differentiated HMGECs exposed to BAK (0.2, 2, 20, or 200 µg/ml) for 3 h. A dose-dependent effect was observed, whereby BAK reduced viability relative to the control by 17.9% (not significant), 77.6% ($p = 0.003$), and

95.6% (p = 0.001) for the 2, 20, and 200 µg/ml concentrations, respectively (Figure 4).

To assess whether the combination of latanoprost and BAK was more lethal than its individual components, differentiated HMGECs were exposed to both compounds for 3 h before quantitating ATP as a measure of cell viability. The toxicity of the combinations was equivalent to the most lethal component(s) across all concentrations tested (Figure 5).

Description of the CE and TAG profiles across all samples: While WEs were not detectable in any of the HMGEC samples, CEs and TAGs were detected. Specifically, 50 unique CEs were detected across all samples that met the inclusion criteria in the analysis (Figure 6). The chain length varied from 11 to 34, and the double-bond count varied from zero to five. Nine CEs were found to be oxidized. The most abundant CE was CE 18:1. Very long-chain (20 ≤ carbon number [n_c] ≤ 25) and ultra long-chain CEs (n_c ≥ 26) were present in all conditions and comprised 22.9% and 10.5% of the overall CE pool, respectively. Of the 50 CEs, nine were saturated, 22 were monounsaturated, and 19 were polyunsaturated. Monounsaturated CEs were the most abundant (50.9%), followed by polyunsaturated (27.9%) and saturated (21.2%).

There were 5,155 TAGs detected across all samples; however, only 121 met the criteria for inclusion in the analysis. The total carbon count from the three acyl chains, excluding the glycerol backbone, ranged from 46 to 60, with the majority (113/121, 93.4%) falling within the range of 46 to 56 (Figure 7A). All TAGs, except for one, consisted of an even carbon count (120/121, 99.2%). The number of double bonds in the acyl chains of the TAGs varied from zero to 10. Very few TAGs were fully saturated (5/121, 4.1%). The degree of unsaturation followed a bimodal distribution (Figure 7B), which was heavily weighted toward the lower end. The

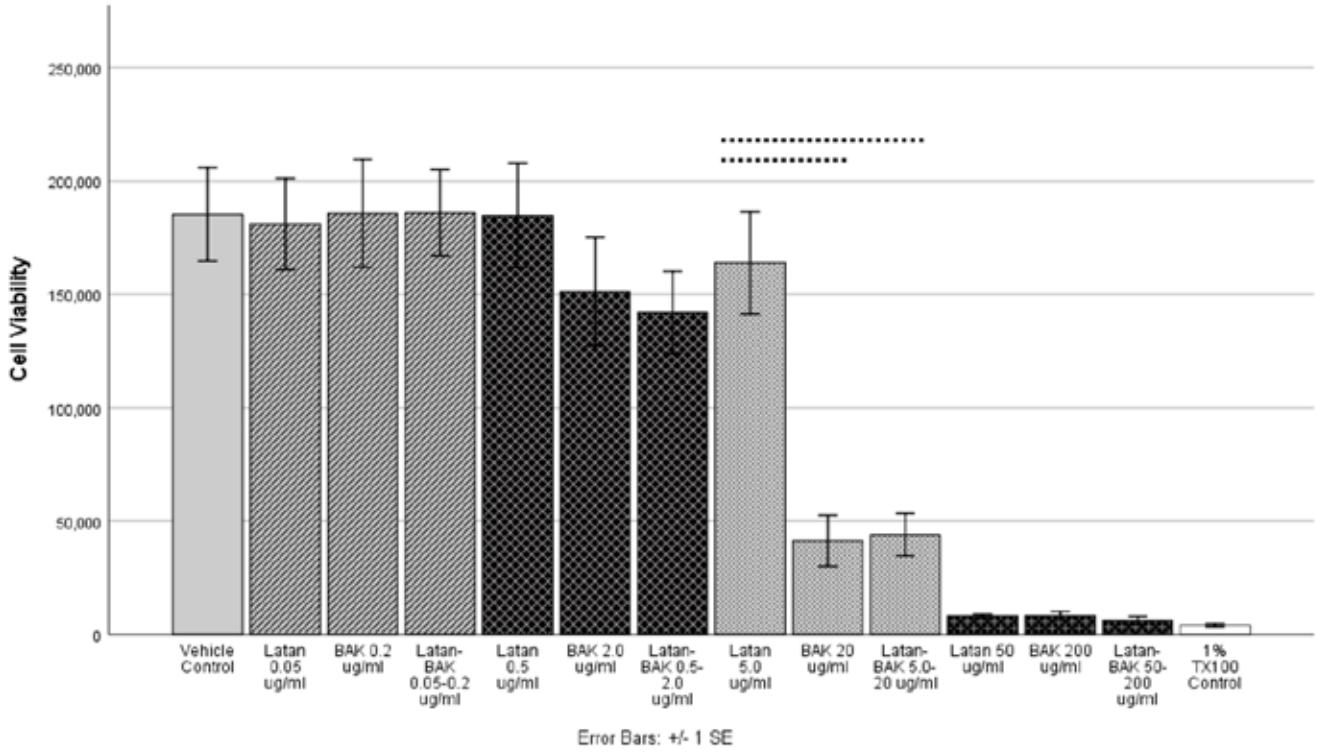

Figure 5. HMGECs were differentiated for 48 h before exposure to latanoprost-BAK for 3 h. The bars in the graph are grouped into "triads" by latanoprost-BAK concentration levels (0.05–0.20, 0.5–2.0, 5.0–20, and 50–200 ug/ml). At low concentrations (0.05–0.20), neither latanoprost, BAK, nor their combination affected cell viability. At both mid-level concentrations (0.5–2.0 and 5.0–20 ug/ml), the toxicity of combined latanoprost-BAK was similar to that of BAK alone. At high concentrations (50–200 ug/ml), both latanoprost and BAK—individually and in combination—were lethal to the cells. In summary, within each triad, the effect of combined latanoprost-BAK was equivalent to that of its more toxic component, indicating that latanoprost and BAK exhibit no additive effect on toxicity. Significance markers are shown only within each triad of concentrations. There were nine replicates per condition. Latan: latanoprost; BAK: benzalkonium chloride; TX100: Triton-X 100 positive control; Dashed bar: p ≤ 0.001.

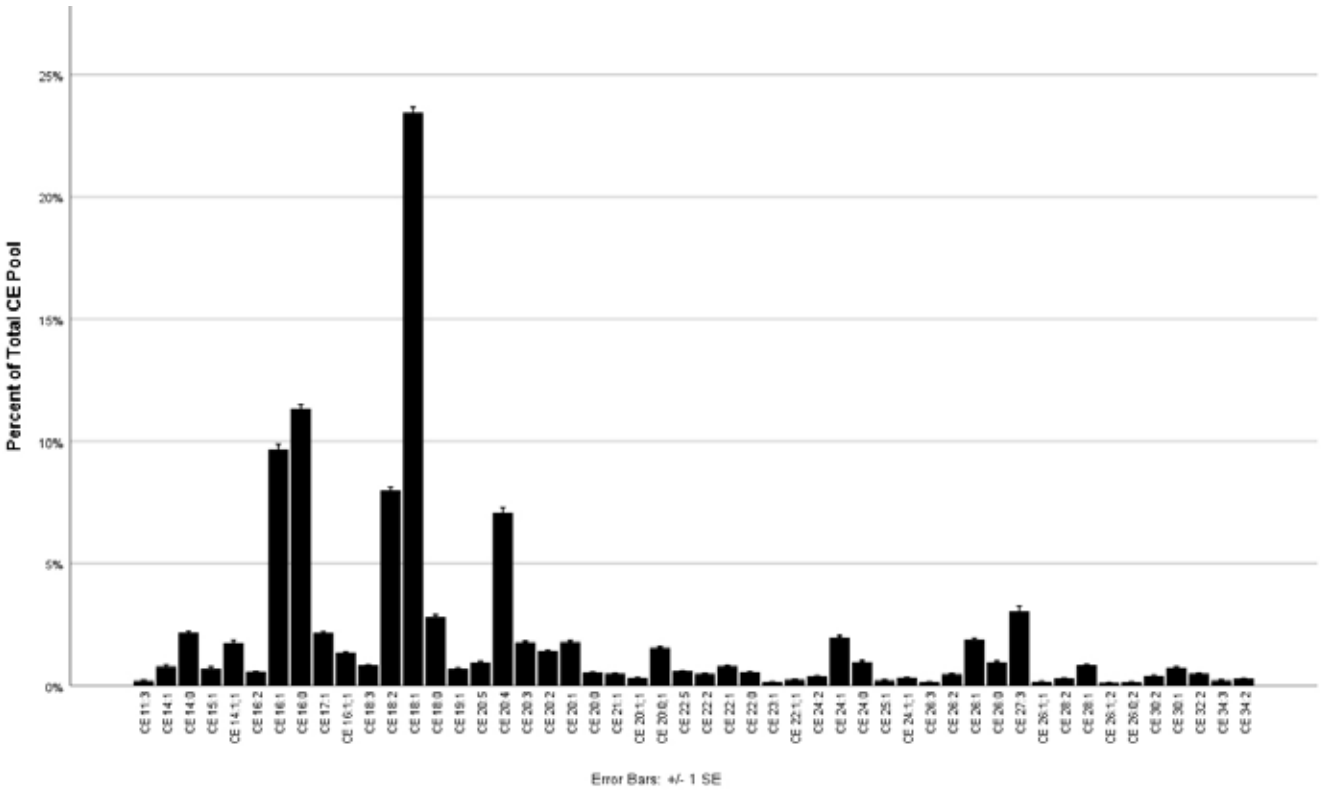

Figure 6. HMGECs expressed a diverse profile of cholesteryl esters (CEs) across all conditions. There were 80 replicates, of which CE 18:1 was the most abundant. CEs are labeled by carbon number and double-bond count, respectively. When a third number is present, it denotes an oxCE with the corresponding number of oxygenations. oxCE: oxidized cholesteryl ester.

TAGs were primarily of lower unsaturation (77/121, 63.6%, one to three double bonds) or higher unsaturation (39/121, 32.2%, seven to 10 double bonds). There were zero TAGs with a moderate degree of unsaturation (four to six double bonds). LipidView 1.3 software identified the neutral loss of 24 unique fatty acyl chains from the 121 TAGs (Figure 7C). Their individual carbon counts varied from 10 to 20, with double bonds ranging from zero to three. Although nearly all the parent TAGs consisted of an even carbon count, many of the fatty acyl chains comprised an odd number of carbons (27/121, 22.3%). The most frequently observed fatty acyl groups were oleic acid (FA 18:1) and palmitoleic acid (FA 16:1), both of which were present in 18 of the 121 TAGs (14.9%). The next most common was palmitic acid (FA 16:0), which was present in 16 (13.2%) TAGs. Relatively few TAGs (8/121, 6.6%) consisted of very long-chain fatty acids (i.e., at least 20 carbons).

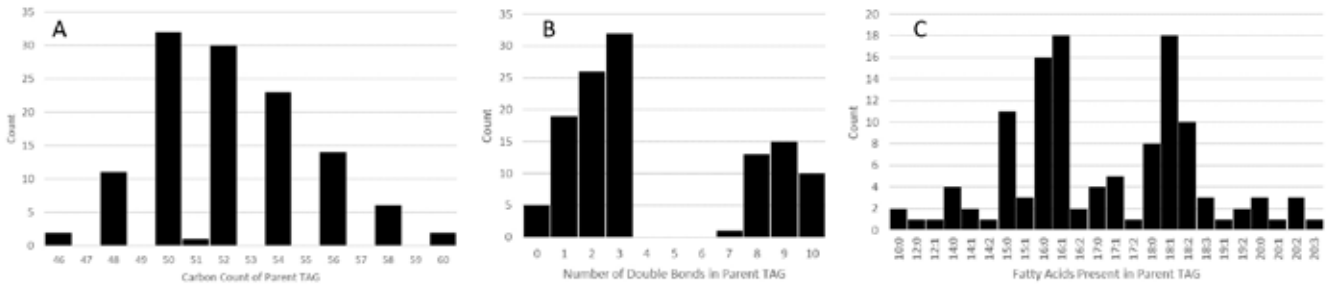

Figure 7. Across all conditions (80 replicates), HMGECs expressed a diverse TAG lipidome, with carbon counts ranging from 46 to 60 (A) and double-bond counts ranging from 0 to 10 (B). There were 24 unique fatty acyl chains identified from parent TAGs. TAG: triacylglycerol.

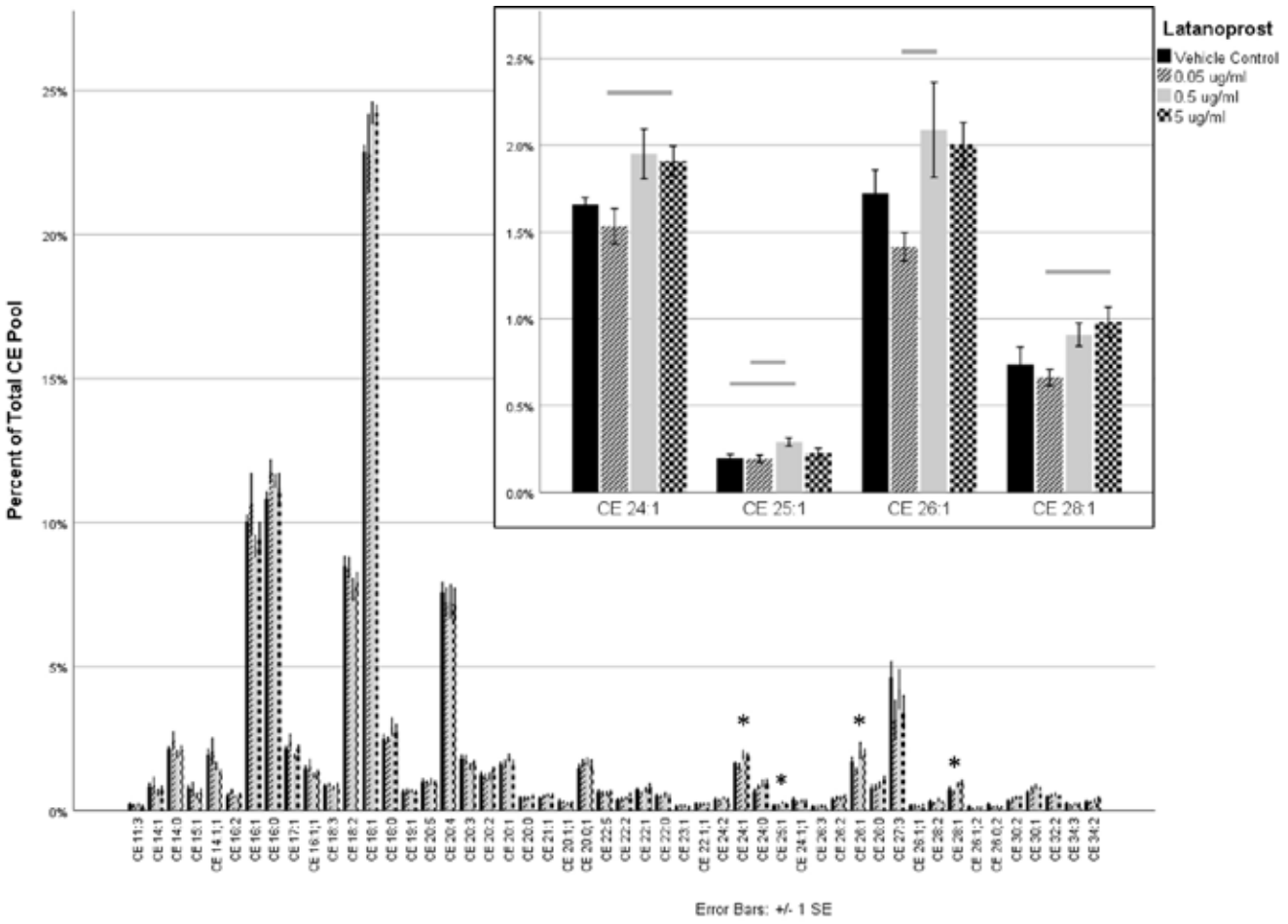

Figure 8. HMGECs were differentiated for 48 h before exposure to latanoprost for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). The inset depicts only the CEs that reached significance. Only four of the 50 CEs (8.0%) were significantly altered with latanoprost supplementation. CEs are labeled by carbon number and double-bond count, respectively. When a third number is present, it denotes an oxCE with the corresponding number of oxygenations. There were four replicates per condition. CE: cholesteryl ester; oxCE: oxidized cholesteryl ester; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

Influence of latanoprost on CE and TAG expression: To determine the effects of latanoprost on CE and TAG expression, lipid extracts from differentiated HMGECs exposed to 0.05, 0.5, or 5 µg/ml latanoprost were analyzed. Of the 50 analyzed CEs, only four (8.0%) showed a statistically significant change in expression, which were CE 24:1, CE 25:1, CE 26:1, and CE 28:1 (Figure 8). All four of these CEs demonstrated higher expression with higher concentrations of latanoprost, but only one concentration (0.5 µg/ml) for one CE (CE 25:1) reached significance against the vehicle control in pairwise comparisons (0.29 ± 0.02% versus 0.20 ± 0.06%, $p = 0.04$).

A similar portion of the TAGs, specifically 12 of 121 (9.9%), significantly varied in response to latanoprost supplementation (Figure 9). Most of the significant TAGs consisted of 50 (3/12, 25.0%), 52 (5/12, 41.7%), or 54 (2/12, 16.7%) carbons and one (4/12, 33.3%), two (5/12, 41.7%), or three (2/12, 16.7%) double bonds. TAGs with the following fatty acids increased with latanoprost supplementation: FA 16:1 (3/9, 33.3%), FA 18:1 (2/9, 22.2%), FA 18:2 (3/9, 33.3%), and FA 20:0 (1/9, 11.1%). Those that decreased with latanoprost supplementation consisted of FA 10:0 (1/2, 50.0%) and FA 15:0 (1/2, 50.0%).

Influence of BAK on CE and TAG expression: To determine the effects of BAK on CE and TAG expression, lipid extracts from differentiated HMGECs treated with 0.2 or 2 µg/ml BAK were analyzed. Of the 50 CEs detected, 15 (30.0%) showed a statistically significant difference among the group means (Figure 10). Five CEs were downregulated with both concentrations of BAK, and these included CE 16:0, CE 18:3,

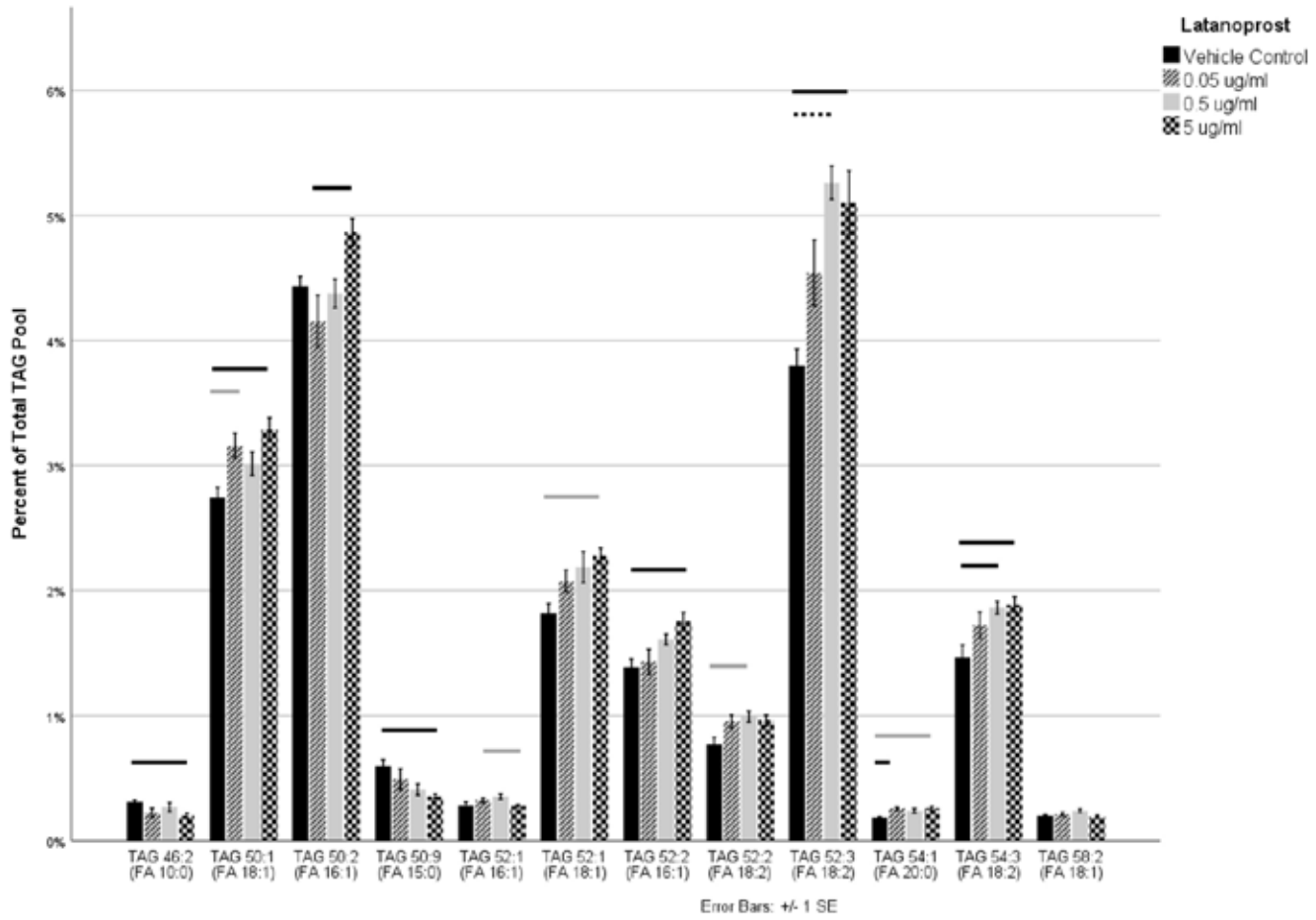

Figure 9. HMGECs were differentiated for 48 h before exposure to latanoprost for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). Only the TAGs that reached significance are shown. Only 12 of 121 TAGs (9.9%) were significantly altered with latanoprost supplementation. TAG 58:2 (FA 18:1) failed to reach significance in pairwise comparisons. TAGs are labeled by two numbers corresponding to the total number of carbons and the total number of double bonds, respectively. The FA in parentheses represents one of the three FAs of the parent TAG molecule. There were four replicates per condition. HMGEC: human meibomian gland epithelial cell; TAG: triacylglycerol; FA: fatty acid; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

CE 18:2, CE 20:4, and CE 20:0;1. Nine CEs were upregulated with both concentrations of BAK, which were CE 18:0, CE 20:0, CE 21:1, CE 20:1;1, CE 24:0, CE 25:1, CE 24:1;1, CE 26:0, and CE 26:1;1. One CE (CE 28:2) failed to reach significance in pairwise comparisons.

Of the 121 detected TAGs, 15 (12.4%) varied significantly with BAK supplementation. Most of the significant TAGs consisted of 50 (6/15, 40.0%) or 52 (3/15, 20.0%) carbons. The preferred saturation level for TAGs was less specific compared to latanoprost, as TAGs with zero, one, two, three, nine, or 10 double bonds were all affected, although a slight preference may have been observed for one (3/15, 20.0%) or two (4/15, 26.7%) double bonds. TAGs with 10 unique fatty acyl groups also varied significantly (Figure 11). Finally, significant TAGs with 16- or 18-carbon fatty acids more commonly increased than decreased.

Influence of combined latanoprost and BAK on CE and TAG expression: To determine whether the combination of latanoprost and BAK elicited changes that differed from those of the individual components, lipid extracts from differentiated HMGECs exposed to latanoprost-BAK 0.05–0.2 µg/ml and 0.5–2 µg/ml were analyzed. At the 0.05–0.2 µg/ml concentration, the effect of the combination mirrored those of its components for all CEs and TAGs, except for CE 18:2, CE 18:0, CE 24:1, CE 26:2, and CE 26:1 (Figure 12 and Figure 13). Similarly, at the 0.5–2 µg/ml concentration, the effect of the combination mirrored those of its components for all CEs and TAGs, except for TAG 50:2 (FA 18:1), TAG 52:0 (FA 16:0), and TAG 52:1 (FA 16:1; Figure 14 and Figure 15).

DISCUSSION

A growing body of evidence supports the association between MGD and the use of $PGF_{2\alpha}$ analogs to treat glaucoma [5,10]. We previously found that HMGECs do indeed express FP, the receptor for $PGF_{2\alpha}$, and that when exposed to $PGF_{2\alpha}$, HMGECs modify their lipidomic profiles [11]. Therefore, the effects of latanoprost and its preservative, BAK, on cell viability and lipidomic expression in HMGECs were explored. We report that both latanoprost and BAK are lethal to HMGECs at high concentrations equal to those found in commercially available eye drops. These concentrations, as further discussed below, are presumably higher than what penetrates into the meibomian glands. At sublethal concentrations, both compounds are capable of significantly shifting the lipidomic profile, specifically in CEs and triacylglycerols. When assessing the combination of latanoprost and BAK, we found no additional toxicity and only negligible changes in the lipidome relative to the individual components alone. These results suggest that, at the ocular surface, the instillation of latanoprost and BAK has the potential to change the chemical composition of the tear film lipid layer, thereby likely altering tear film dynamics and ocular surface lubrication.

Both latanoprost and BAK were found to be toxic at concentrations of 50 and 200 µg/ml, respectively, the same concentrations found in commercially available latanoprost. These results are unsurprising and consistent with our hypothesis for BAK, whose cytotoxic effects have been well documented in the literature [13,14,26-29]. The absolute

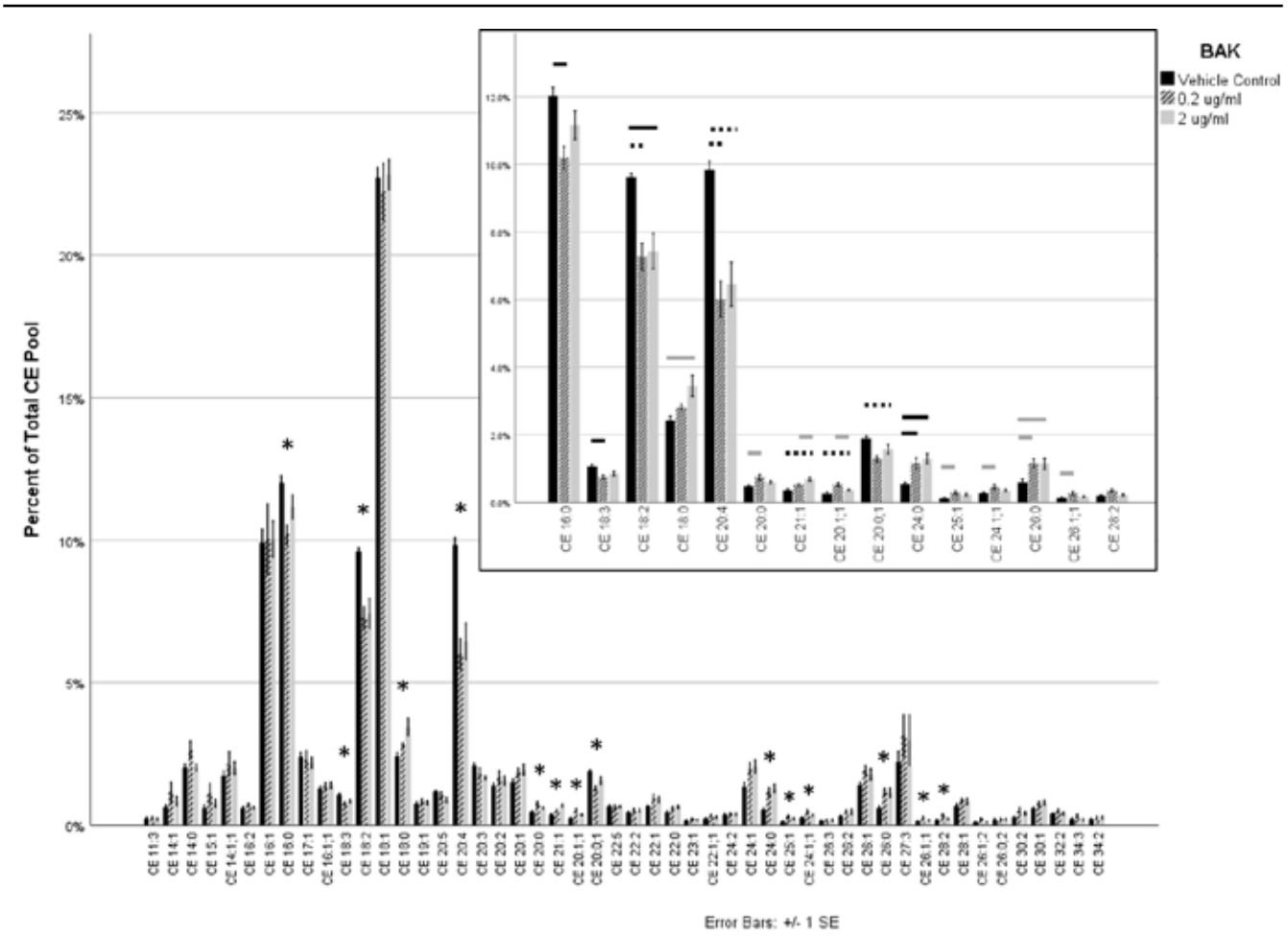

Figure 10. HMGECs were differentiated for 48 h before exposure to BAK for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). The inset depicts only the CEs that reached significance. Fifteen of the 50 CEs (30.0%) were significantly altered with BAK supplementation. CE 28:2 failed to reach significance in pairwise comparisons. CEs are labeled by carbon number and double-bond count, respectively. When a third number is present, it denotes an oxCE with the corresponding number of oxygenations. There were four replicates per condition. BAK: benzalkonium chloride; CE: cholesteryl ester; oxCE: oxidized cholesteryl ester; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

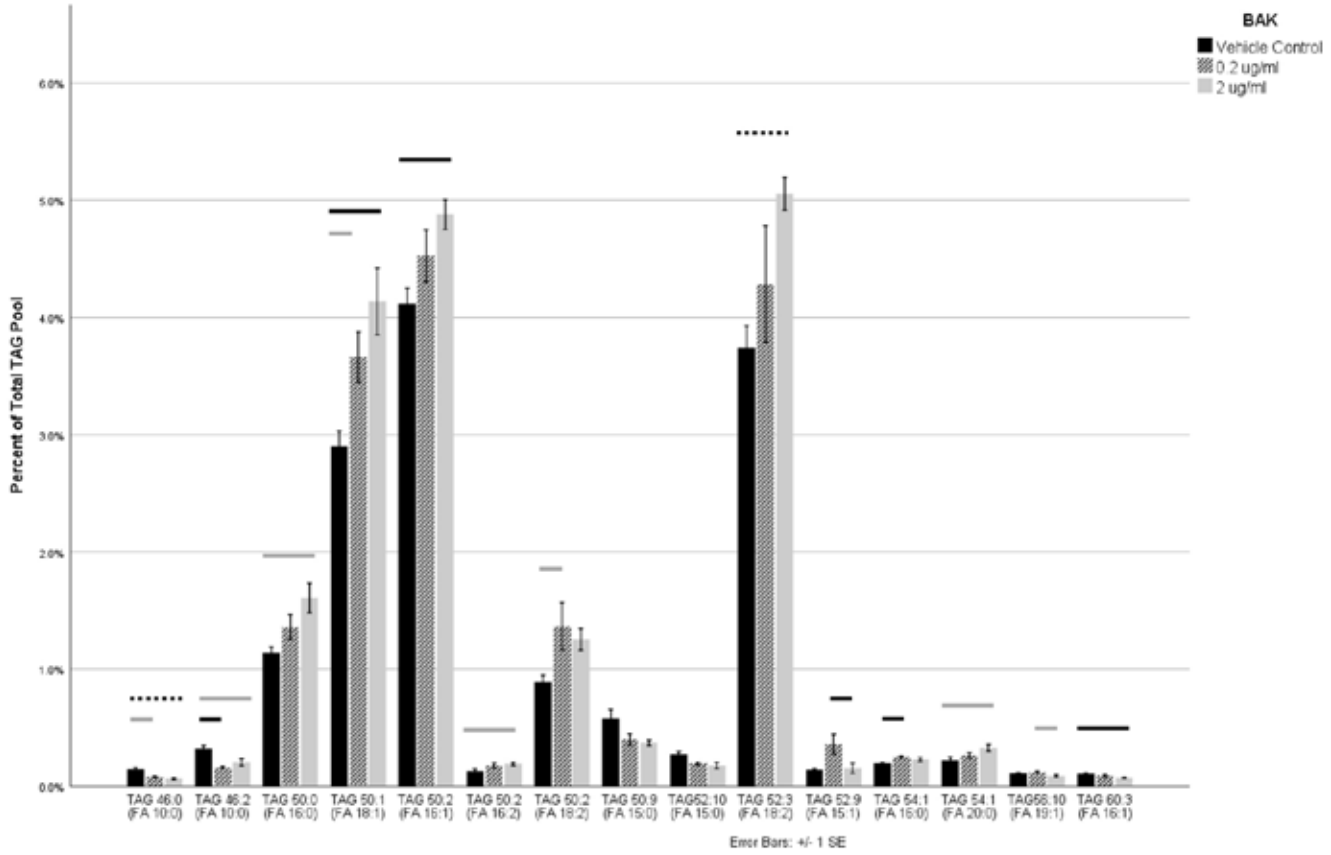

Figure 11. HMGECs were differentiated for 48 h before exposure to BAK for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). Only the TAGs that reached significance are shown. Only 15 of 121 TAGs (12.4%) were significantly altered with latanoprost supplementation. TAG 50:9 (FA 15:0) and TAG 52:10 (FA 15:0) failed to reach significance in pairwise comparisons. TAGs are labeled by two numbers corresponding to the total number of carbons and the total number of double bonds, respectively. The FA in parentheses represents one of the three FAs of the parent TAG molecule. There were four replicates per condition. HMGEC: human meibomian gland epithelial cell; TAG: triacylglycerol; FA: fatty acid; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

lethality associated with latanoprost, however, was greater than expected, although having some degree of toxicity is consistent with other reports. Other researchers have also detected decreased viability following latanoprost stimulation in HMGECs [26] and other cell types [27,30,31]. In one particular study, Kam et al. assessed a prostamide (a PGA-like compound) called bimatoprost and found that it significantly reduced the phosphorylation of protein kinase B, a surrogate marker of decreased cell survival [32]. However, whether latanoprost is toxic to the meibomian glands in vivo remains unknown, as the concentration that reaches the tarsal plate has yet to be elucidated. In the aqueous humor, however, the peak concentration is approximately 10^{-7} M (roughly equivalent to the lowest concentration used in this study) [33]. If similar tissue distribution dynamics exist throughout the eyelid, then latanoprost would be found at a sublethal concentration in the tarsal plate.

It should also be noted that latanoprost appears to induce its toxic effects through a mechanism other than signaling through the FP and EP receptors, which are the cognate receptors for $PGF_{2\alpha}$ and PGE_2. Even with a total FP- and EP-receptor blockade, the lethality of latanoprost 50 µg/ml was unchanged (Figure 3). These findings are in agreement with a recent report by Shen et al. [31], who evaluated the mechanism of cellular death attributed to latanoprost and found that both the extrinsic and intrinsic apoptotic pathways, which are mediated by death receptors on the cell surface and the mitochondria, respectively, are activated. While death receptors were once believed to send only pro-apoptotic signals, emerging data reveals that they do actually contribute to the regulation of cell proliferation, cell differentiation, inflammation, and chemokine production, among other processes [34]. The results of Shen et al.'s study, combined with our findings, highlight the possibility of alternate

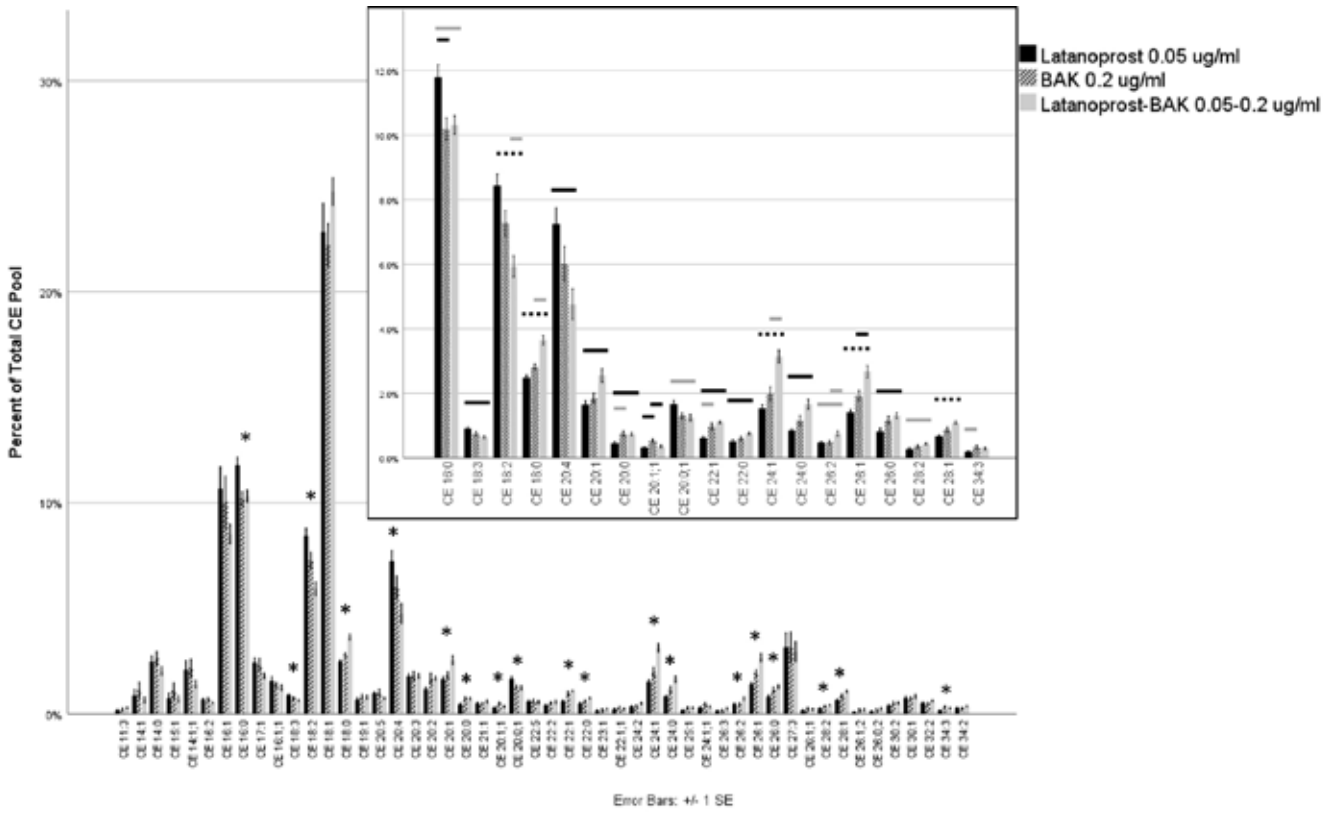

Figure 12. HMGECs were differentiated for 48 h before exposure to combined latanoprost-BAK for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). The inset depicts only the CEs that reached significance. Nineteen of the 50 CEs (38.0%) were significantly different between latanoprost alone, BAK alone, or combined latanoprost-BAK. However, only a few CEs (CE 18:2, CE 18:0, CE 24:1, CE 26:2, and CE 26:1) showed significant differences between the combined latanoprost-BAK and each component in isolation. CEs are labeled by carbon number and double-bond count, respectively. When a third number is present, it denotes an oxCE with the corresponding number of oxygenations. There were four replicates per condition. BAK: benzalkonium chloride; CE: cholesteryl ester; oxCE: oxidized cholesteryl ester; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

mechanisms beyond FP- and EP-receptor engagement that could explain the numerous local side effects observed clinically with PGA use.

Beyond their lethal effects at commercially available doses, both latanoprost and BAK demonstrated the ability to modulate lipid expression at sublethal concentrations. These findings are consistent with our own study [11] and others' previous work [35]. Though our experiments were not designed to identify the specific receptor pathways involved in latanoprost-induced lipid changes, these effects are presumed to be mediated through the FP receptor's downstream inhibition of the peroxisome proliferator activator receptor γ (PPARγ) [36], as previously discussed at length [11], and through BAK's regulation of the genes involved in sterol biosynthesis and liver X receptors [35]. An unexpected finding, however, was the appearance of a mostly positive influence on the expression of select CEs and triacylglycerols by both latanoprost and BAK. Latanoprost exhibited a concentration-dependent increase, albeit relatively weakly, on a few monounsaturated CEs that consisted of either very long- or ultra-long-chain fatty acyl groups—CEs that are considered to be "meibum-relevant" [21] and abundantly expressed in vivo [37]. At the same time, BAK downregulated select polyunsaturated CEs and upregulated some saturated and monounsaturated CEs, particularly those with very or ultra long-chain fatty acyl groups, thus emulating a profile that is similar to normal human meibum [21,37]. Furthermore, both latanoprost and BAK upregulated select TAGs, mostly bearing 16 or 18 carbons, which may potentially serve as surrogate markers of FA synthesis [21]. If these observed trends in the lipidomic profile are translated to the ocular surface, then it appears that latanoprost and BAK, paradoxically, could promote a more normal expression profile in the meibomian glands.

One possible explanation for this phenomenon is oxidative stress-induced FA synthesis. In this vein, Sedlak et al.

recently reported that latanoprost was associated with several elevated oxidative stress markers in human tears, and that these markers were further exacerbated by BAK [38]. There is also strong evidence for the role of oxidative stress in both MGD and dry eye disease [39-41]. Despite this association with ocular surface disease, recent papers have highlighted the link between oxidative stress, FA metabolism [42], and lipid droplet accumulation [43]. Specifically, the mouse model of MGD used by Bu et al. shows an association between oxidative stress and lipid accumulation [41]. Historically, in this line of research, researchers (ourselves included) have considered lipid accumulation to be a normal marker of meibomian gland health. However, these reports suggest that lipid accumulation may also be associated with oxidative stress, a known contributor to ocular surface disease. These observations emphasize the tissue-level complexity of MGD pathophysiology and highlight opportunities for further research.

Caution should be taken when extrapolating these findings to the ocular surface in vivo. Our experiments focused on lipidomic expression and cell viability in a meibomian gland epithelial cell line. Other mechanisms for MGD development in response to latanoprost and BAK could include hyperkeratinization [44], inflammatory cell infiltration [15-19], and physicochemical tear disruption [45], to name a few. Future research directions could therefore include a mechanistic interrogation into oxidative stress and FA synthesis in HMGECs.

In conclusion, we report that both latanoprost and BAK are lethal at therapeutic concentrations found in commercially available eye drops, an effect that appears to be independent of FP and EP receptors. The extent to which this toxicity is

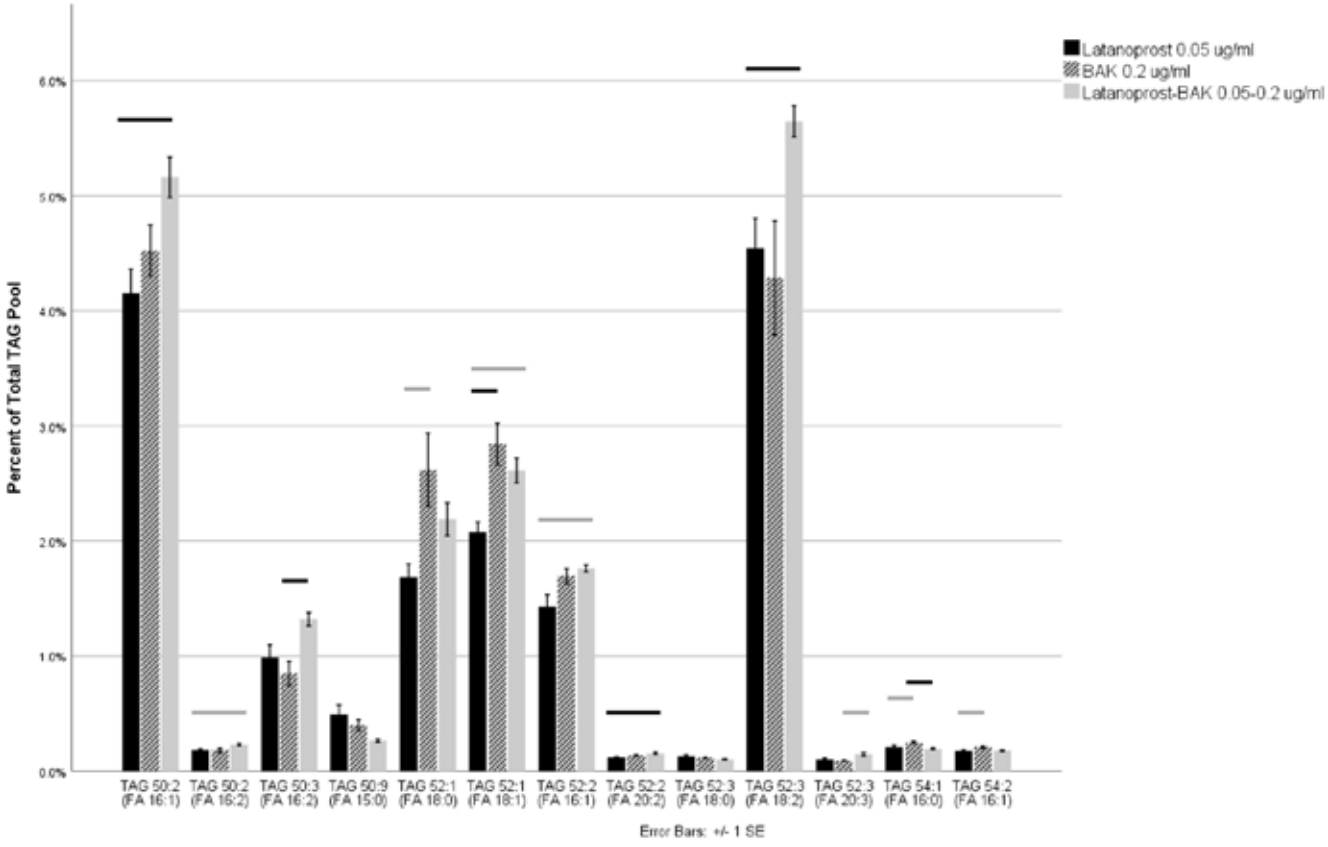

Figure 13. HMGECs were differentiated for 48 h before exposure to combined latanoprost-BAK for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). Only the TAGs that reached significance are shown. Thirteen of 121 TAGs (10.7%) were significantly different between latanoprost alone, BAK alone, and combined latanoprost-BAK. However, there were no TAGs that showed significant differences between the combined latanoprost-BAK and each component in isolation. TAGs are labeled by two numbers corresponding to the total number of carbons and the total number of double bonds, respectively. The fatty acid in parentheses represents one of the three fatty acids of the parent TAG molecule. There were four replicates per condition. BAK: benzalkonium chloride; CE: cholesteryl esters; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

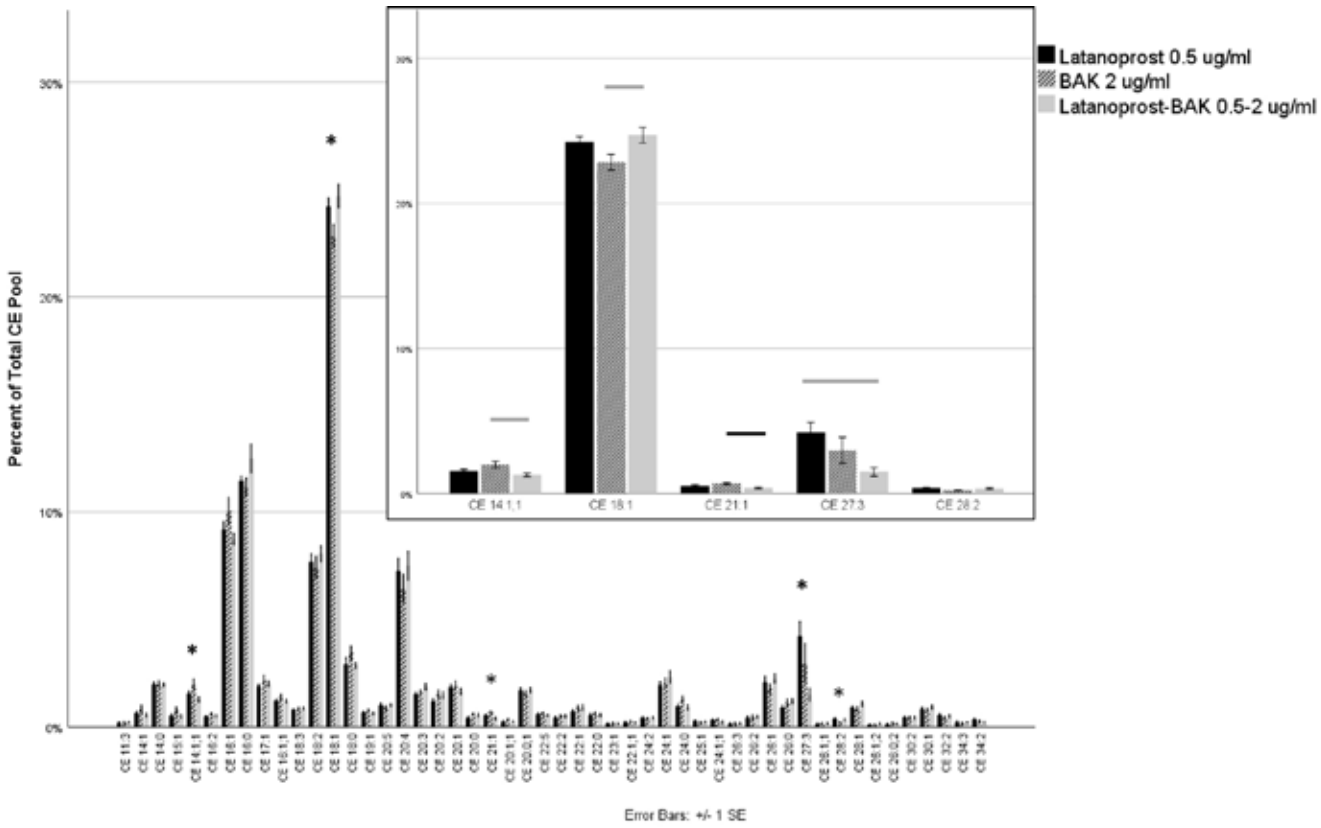

Figure 14. HMGECs were differentiated for 48 h before exposure to combined latanoprost-BAK for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). The inset depicts only the CEs that reached significance. Five of the 50 CEs (10.0%) were significantly different between latanoprost alone, BAK alone, or combined latanoprost-BAK. CE 28:2 failed to reach significance in pairwise comparisons. No CEs showed significant differences between the combined latanoprost-BAK and each component in isolation. CEs are labeled by carbon number and double-bond count, respectively. When a third number is present, it denotes an oxCE with the corresponding number of oxygenations. There were four replicates per condition. BAK: benzalkonium chloride; CE: cholesteryl ester; oxCE: oxidized cholesteryl ester; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$.

observed in vivo remains unknown and is likely contingent upon the respective degrees of latanoprost's and BAK's penetration into the tarsal plate. BAK appears to be more toxic than latanoprost at lower concentrations. We further report that while both compounds are capable of modulating the lipidomic profile of HMGECs, they do so, paradoxically, by promoting the increased expression of CEs and TAGs, which are largely believed to be associated with normal human meibum. As a conjecture, we presented the hypothesis that oxidative stress-induced FA synthesis could mediate these observations. Moving forward, clinicians involved in the care of glaucoma patients should consider the effects of PGAs and their preservative systems on the meibomian glands, evaluate the efficacy and safety profiles of all options before initiating treatment, and opt for BAK-free formulations when circumstances permit.

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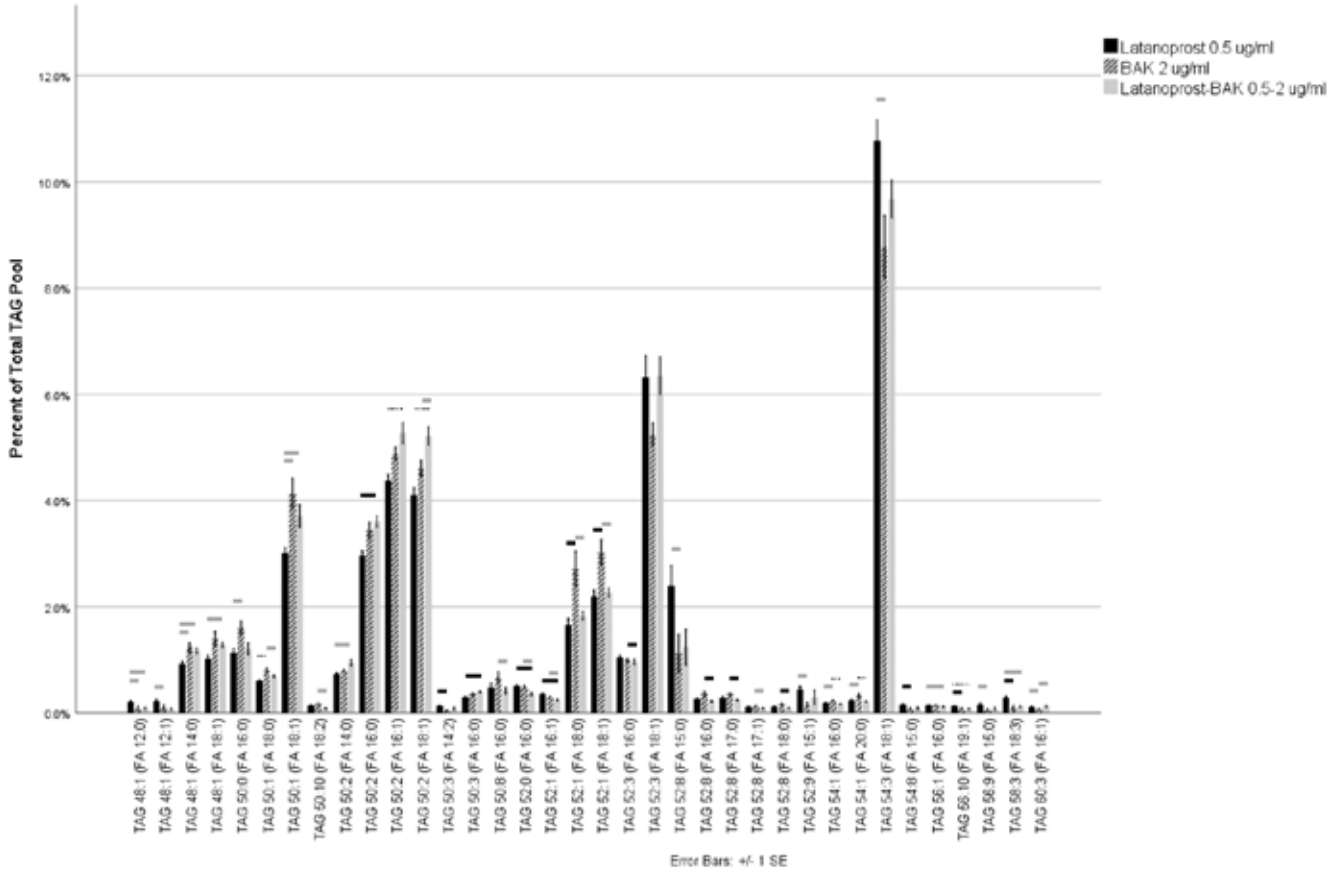

Figure 15. HMGECs were differentiated for 48 h before exposure to combined latanoprost-BAK for 3 h. Lipid extracts were analyzed by ESI-MSMS^{ALL} (see Methods). Only the TAGs that reached significance are shown. Thirty-six of the 121 TAGs (29.8%) were significantly different among latanoprost alone, BAK alone, or combined latanoprost-BAK. However, only three TAGs (TAG 50:2 [FA 18:1], TAG 52:0 [FA 16:0], and TAG 52:1 [FA 16:1]) showed significant differences between the combined latanoprost-BAK and each component in isolation. TAGs are labeled by two numbers corresponding to the total number of carbons and the total number of double bonds, respectively. The FA in parentheses represents one of the three fatty acids of the parent TAG molecule. There were four replicates per condition. BAK: benzalkonium chloride; CE: cholesteryl esters; Gray bar: $p \leq 0.05$; Black bar: $p \leq 0.01$; Dashed bar: $p \leq 0.001$.

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