Cytochrome P450-Epoxygenated Fatty Acids Inhibit Müller Glial Inflammation

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Abstract

Background

Müller cell (MC) inflammation is an early pathogenic step in the vision-threatening disease diabetic retinopathy (DR). Recent evidence indicates that diabetes-induced free fatty acid (FFA) dysregulation can elicit the release of inflammatory cytokines from MC, promoting DR progression. Palmitic acid (PA) is elevated in the sera of diabetics and stimulates the production of the DR-relevant cytokines by MC, including interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-α (TNFα). IL-1β is elevated in DR and is purported to be a “master regulator” of cytokine-induced inflammation. IL-1β activates MC to produce itself (IL-1β auto-amplification) and other inflammatory cytokines in the retina. Consequently, a modality that blocks both FFA- and IL-1β-induced MC inflammation has therapeutic potential in DR. The CYP-derived epoxygenated fatty acids, epoxyeicosatrienoic acid (EET) and epoxydocosapentaenoic acid (EDP), offer one such therapeutic strategy. In this study we propose that experimental elevation of EET and EDP will reduce PA- and IL-1β-induced MC inflammation.

Methods

Human MC (hMC) were treated with vehicle, broad-spectrum CYP inhibitor SFK-525a, or PA or IL-1β in the presence or absence of 11,12-EET, 19,20-EDP or GSK2256294, a soluble epoxide hydrolase (sEH) inhibitor. Relative expression of TNFα, IL-1β, IL-6, and IL-8 was assayed via qRT-PCR and NFκB-dependent transcription was tested using a luciferase assay. Data were analyzed using ANOVA with Tukey’s multiple comparisons post-hoc test.

Results

CYP inhibition by SKF-525a increased hMC expression of inflammatory cytokines. Exogenous 11,12-EET and 19,20-EDP both significantly decreased PA- and IL-1β-induced hMC expression of IL-1β and IL-6. Both epoxygenated fatty acids significantly decreased IL-8 expression in IL-1β-induced hMC and TNFα in PA-induced hMC. Interestingly, 11,12-EET and 19,20-EDP significantly increased TNFα in IL-1β-treated hMC. GSK2256294 significantly reduced PA- and IL-1β-stimulated hMC cytokine expression, demonstrating efficacy over a range of concentrations. 11,12-EET and 19,20-EDP were also found to decrease PA- and IL-1β-induced NFκB-dependent transcriptional activity.

Conclusions

In summary, these data suggest that experimental elevation of 11,12-EET and 19,20-EDP decreases MC inflammation occurring under diabetes-relevant conditions and may represent a viable therapeutic strategy for inhibition of early retinal inflammation in DR. Our data suggest that these epoxygenated fatty acids exert their anti-inflammatory activities, at least in part, by blocking NFκB-dependent transcription.

Background

Diabetic retinopathy (DR) is the leading cause of irreversible vision loss among working age Americans, affecting ~35% of patients with diabetes mellitus (1). As the prevalence of worldwide diabetes increases, the number of people suffering from diabetes-induced vision loss increases as well (2). DR pathology is classified in two clinically distinct forms, non-proliferative (NPDR) and proliferative (PDR). NPDR is characterized by the appearance of microaneurysms, focal hemorrhaging, hard exudates beneath the retinal surface and retinal capillary death (3,4). The death of retinal capillaries in NPDR can result in vasoregression-promoted ischemia, causing retinal hypoxia that elicits the synthesis and release of vascular endothelial cell growth factor (VEGF) (5). Increased levels of retinal VEGF can trigger a vasoproliferative response, transitioning the retina to vision threatening PDR (5). Current DR therapies, such as laser photocoagulation or VEGF inhibition, target PDR after irreversible retinal damage has occurred. Therefore, there is an important unmet need to develop a therapy that intervenes prior to PDR onset to preserve retinal function.

DR progression is associated with systemic dyslipidemia, and circulating free fatty acids (FFAs) are known to initiate inflammatory cytokine release (6,7). Diabetic mice have over three times the retinal fatty acid content of healthy controls and palmitic acid (PA) is elevated above other free fatty acids in the circulation and tissues of diabetic patients and experimental models of diabetes (8–10). The detrimental effects of free fatty acids in the diabetic retina has been substantiated in two epidemiological human studies, ACCORD and FIELD, in which the lipid-lowering drug fenofibrate was shown to delay retinopathy progression (6,7). Müller cells (MC) are particularly responsive to PA and other FFA, and it is proposed that diabetes-related dysregulation of FFAs may damage MC, leading to their production of inflammatory retinal cytokines (8,12). These cytokines amplify through autocrine and paracrine mechanisms, reaching levels that promote chronic retinal inflammation (5). If these levels are sustained, retinal vascular pathology can ensue, promoting DR progression. In support of this notion, studies in human patients and animal models show that elevated levels of inflammatory cytokines in the vitreous and retina correlate with early DR progression (5,12–14). One such cytokine, interleukin 1β (IL-1β), is purported to be a “master regulator” of cytokine-induced inflammation (15,16). IL-1β is elevated in DR and is known to activate cytokine release in MC, with MC activation being one of the earliest observable changes in DR (8). The important roles of MC in retinal homeostasis, and their support of IL-1β-induced inflammatory cytokine amplification, suggest that much can be learned about the mechanisms of DR pathogenesis from investigations of MC behaviors under DR-relevant conditions.

There is abundant data suggesting that lipid mediators derived from ω-6 and ω-3 fatty acids regulate diabetes-induced retinal inflammation (5,18,19). Arachidonic acid (AA; ω-6) and docosahexaenoic acid (DHA; ω-3) are polyunsaturated fatty acids (PUFAs) highly abundant in retina, making their metabolites of particular importance to retinal vascular homeostasis (19–21). These PUFAs are metabolized through the cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 epoxygenase (CYP) pathways. Although there are exceptions, AA is metabolized by COX and LOX to yield oxygenated metabolites that are largely pro-inflammatory (5,23). For example, it has been shown that COX inhibitors such as aspirin and other NSAIDS reduce DR associated inflammation (23). Unlike AA, it has been reported that COX and LOX convert DHA into anti-inflammatory metabolites (22). COX converts DHA to hydroxyl DHA, and 15-

lipoxygenase (ALOX15) converts DHA to 17S-hydroperoxy-DHA that is further metabolized to yield the D-resolvins (24). Streptozotocin-induced diabetic rats that received intravitreal injections of resolvin D1 demonstrated reduced levels of retinal IL-1β and NFκB activity (25). There is growing interest in the epoxygenation of ω-6 and ω-3 fatty acids by cytochrome P450 epoxygenases (CYPs). CYPs are endoplasmic reticulum membrane-bound monooxygenases that metabolize fatty acids to epoxide derivatives that demonstrate potent anti-inflammatory activities in a variety of biological systems (3). CYP2C8, CYP2C9, and CYP2J2 are the most well-characterized human CYPs that epoxygenate AA and DHA to yield epoxyeicosatrienoic acids (EET) and epoxydocosapentaenoic acids (EDP), respectively (3,17). AA yields the four regioisomers 5,6-EET, 8,9-EET, 12-EET, and 14,15-EET, while DHA yields the six regioisomers 4,5-EDP, 7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP, and 19,20-EDP (3). We have previously shown that the administration of exogenous 11,12-EET reduces the expression of the leukocyte adhesion protein VCAM1 in human retinal microvascular endothelial cells (hRMEC) activated by TNFα (3). These data suggest that increasing EET/EDP levels may be an effective method to reduce DR-related inflammation.

Soluble epoxide hydrolase (sEH) hydrolyzes EET and EDP to their less biologically active diols, dihydroxyeicosatrienoic acid (DHET) and dihydroxydocosapentaenoic acid (DHDP) (3). By reducing the half-life of epoxides, sEH decreases their abundance in tissues and thus the potency of their anti-inflammatory activities. Therefore sEH inhibition presents a rational therapeutic method to elevate epoxide levels and reduce inflammation. sEH inhibitors (sEHi) have been tested in animal models of inflammatory disease to raise EET/EDP levels and mitigate inflammation (26). These successful studies have led to clinical trials testing sEH inhibition in diabetes-relevant pathologies, such as impaired glucose tolerance and insulin resistance (27,28). Furthermore, studies that use sEH inhibitors in combination with other pharmacologic strategies to raise EET/EDP levels prove more efficacious than sEHi's administered alone (3). For example, TNFα-induced leukocyte adhesion expression in human retinal endothelial cells was significantly reduced with the administration of sEHi and EET/EDP in combination, but not separately (3).

CYP levels are suppressed in diabetic conditions, and patients with NPDR and PDR have reduced levels of EETs observed in the vitreous (30,31). It was also found that soluble epoxide hydrolase activity is increased in response to diabetic conditions, contributing to lower epoxygenated fatty acids levels, and creating conditions permissive to inflammation (31,32). Thus, pharmacologic manipulations that elevate epoxygenated fatty acids might constitute a rational strategy to reduce retinal inflammation in DR. In this study, we tested the efficacy of increased epoxygenated fatty acid concentrations to mitigate PA- and IL-1β-induced expression of inflammatory cytokines in human Müller cells (hMC). The levels of 11,12-EET and 19,20-EDP were manipulated in hMC cultures via CYP inhibition, exogenous addition of epoxides, and the inhibition of epoxide hydrolysis.

Methods

Human Müller Cell Culture

Human Müller cells (hMC) were isolated from human donor tissue (NDRI, Philadelphia, PA, USA) within 24 hours post mortem. The retinas were dissected from the eyecups and dissociated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Carlsbad, CA) containing trypsin and collagenase (Worthington Biochemical Corp; Lakewood, NJ). Following incubation in dissociation medium, cells were grown in DMEM containing 10% fetal bovine serum (FBS) (R&D Systems; Minneapolis, MN) and 1X antibiotic/antimycotic solution (Thermo Fisher Scientific Asheville LLC; Asheville, NC). Cells were incubated at 37□C, 5% CO_2, 20.9% O_2, and 95% relative humidity. Collectively, these conditions favor the survival of MC over other retinal cell types (34). If needed, cultures were policed for removal of non-MC or colonies of pure MC were sub-cloned into a new dish. Final MC purity of cultures was >97% and was determined by immunohistochemistry IHC with antibodies against cellular retinaldehyde-binding protein (CRALBP), glutathione synthetase (GS), and glial fibrillary acidic protein (GFAP). Passages 4 to 6 were used for all experiments.

Human Müller Cell Treatment (SKF-525a, PA, IL-1β, 11,12-EET, 19,20-EDP, GSK2256294)

In preparation for treatment, hMC were seeded in 6-well dishes and grown to 70% confluence using 10% FBS-containing DMEM culture medium. Culture media were changed to serum-reduced conditions (2% FBS) for 12 hours before treatment. Cells were treated with SKF-525a (5.0μM; ENZO Life Science, Farmingdale, NY, USA) or vehicle for 24 hours. *Experiments using PA as a stimulus are described as follows.* Cells were treated for 24 hours in 2% FBS medium with BSA-bound palmitic acid (PA; 250μM; Sigma-Aldrich; St Louis, MO) or fatty acid-free BSA vehicle (100mg/ml in PBS; Sigma-Aldrich; St Louis, MO). BSA-bound PA was prepared by dissolving PA in EtOH at 200mM. This PA/EtOH solution was mixed for 2 hours at 37°C with 100mg/ml BSA in PBS to yield 5mM PA before dilution to the final concentration of 250μM in culture media. hMC treated with BSA-bound PA were subjected to one of the following co-treatments during the final 3 hours of the 24-hour PA treatment: 0.1nM, 1.0nM, or 10nM GSK2256294 (sEH inhibitor; Axon Medchem LLC; Reston, VA); 11,12-EET (0.5μM; Cayman Chemical; Ann Arbor, MI); or 19,20-EDP (0.5μM; Cayman Chemical; Ann Arbor, MI). *Experiments using IL1β as a stimulus are described as follows.* Cells were treated for 8 hours in 2% FBS-containing DMEM culture medium supplemented with 1.0ng/ml of human recombinant protein IL-1β (R&D Systems; Minneapolis, MN) and vehicle, 0.1nM, 1.0nM, or 10nM GSK2256294; 11,12-EET (0.5μM); or 19,20-EDP (0.5μM). In experiments using GSK2256294, cells were pretreated with corresponding concentrations for 2 hours before treatment with IL-1β. In all experiments, epoxygenated fatty acid concentrations (0.5 μM) were based on our previously published studies and literature precedents.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) of IL-1β, IL-6, IL-8 and TNFα mRNAs

After treatment, cells were washed twice with cold PBS, lysed with RNeasy Lysis Buffer (RLT; Qiagen; Germantown, MD), and total RNA was isolated using an RNeasy Mini kit (Qiagen; Germantown, MD). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems; Waltham, MA). qRT-PCR was performed in duplicate by co-amplification of cDNA vs. *18S* using gene-specific TaqMan Gene Expression Assays (Applied Biosystems). The delta Ct method was used to

determine relative expression of the targeted mRNA normalized to 18S levels. These commercial assays were performed according to the manufacturer's protocol.

NFκB Promoter Assay

hMC were seeded on 96-well black-walled, clear bottom plates. Each well was transfected with NFκB-luciferase promoter-reporter, negative control, or positive control constructs, from the Cignal NFκB Reporter Assay (Qiagen). Seventy-five µL of fresh 10% medium was added to each well 30 minutes prior to transfection. A transfection mixture was prepared in a separate PCR tube, consisting of 200ng of construct, 1.8µL of Targefect solution A (Targeting Systems; El Cajon, CA), and 3.6µL Virofect (Targeting Systems) in 50µL of Optimem (Life Technologies). Fifteen tube inversions were performed between the additions of each reagent, and the transfection mixture was incubated at 37°C for 25 minutes before use. Fifty µL of the transfection mixture was added per well of cultured hMC. Twelve hours after transfection, cells were washed and treated with fresh 10% medium for 12 hours. Twenty-four hours post-transfection, cells were treated with vehicle, IL-1β (1.0ng/ml) or PA-BSA (250µM) in the presence or absence of 11,12-EET (0.5µM) or 19,20-EDP (0.5µM) for 4 hours and 8 hours respectively. Luciferase was quantified using the Dual-Glo Luciferase Assay System (Promega; Madison, WI), according to the manufacturer's protocol. Data are reported as the relative ratio of firefly-to-renilla luciferase.

Statistical Analysis

Data were analyzed using Prism software (GraphPad; La Jolla, CA). T Test and ANOVA with Tukey's multiple comparisons post-hoc test was used to evaluate significant differences among treatment groups. Values of $p<0.05$ were considered statistically significant.

Results

The CYP epoxygenase inhibitor SKF-525a promotes inflammatory cytokine expression in hMC. hMC were treated with the CYP epoxygenase inhibitor SKF-525a or vehicle and inflammatory cytokine expression was assayed via qRT-PCR. SKF-525a increased expression of the DR-relevant cytokines *TNFα* (5.13 fold; p=0.057), *IL1β* (3.92 fold; p<0.0001), *IL6* (2.38 fold; p=0.0001), and *IL8* (2.90 fold; p=0.033). Only *TNFα* did not achieve statistical significance (Fig. 1).

11,12-EET, 19,20-EDP or the sEH inhibitor GSK2256294 reduces PA-stimulated inflammatory cytokine expression. The epoxygenated fatty acids 11,12-EET, 19,20-EDP or the sEH inhibitor, GSK2256294, was tested against PA-induced inflammatory cytokine expression in hMC. hMC were treated with PA in the presence or absence of 11,12-EET, 19,20-EDP or GSK2256294 and inflammatory cytokine expression was assessed by qRT-PCR. 11,12-EET significantly reduced PA-stimulated expression of *TNFα* by 84.67%, *IL1β* by 68.72% and *IL6* by 58.54% (Fig. 2A-C. p=0.0099, p<0.0001, p=0.0008, respectively). 19,20-EDP significantly reduced PA-stimulated expression of *TNFα* by 63.67%, *IL1β* by 56.76%, and *IL6* by 56.19% (Fig. 2A-C. p=0.0434, p=0.0001, p=0.0011, respectively). 11,12-EET and 19,20-EDP reduced PA-stimulated *IL8* expression by 26.70% and 28.59%, however, statistical significance was not achieved (Fig. 2D). A

range of GSK2256294 concentrations were tested (0.1nM, 1.0nM, and 10nM) and GSK2256294 significantly reduced PA-stimulated hMC cytokine expression at each concentration. At the lowest concentration tested, 0.1nM, GSK2256294 reduced PA-stimulated expression of *TNFα* by 90.94%, *IL1β* by 67.31%, *IL6* by 60.86%, and *IL8* by 47.02% in hMC (Fig. 3A-D. p=0.0017, p<0.0001, p=0.0003, p<0.0001, respectively). At 1nM, GSK2256294 reduced PA-stimulated expression of *TNFα* by 91.64%, *IL1β* by 70.39%, *IL6* by 62.13%, and *IL8* by 58.74% in hMC (Fig. 3A-D. p=0.0016, p<0.0001, p=0.0002, p<0.0001, respectively). At 10nM, GSK2256294 reduced PA-stimulated expression of *TNFα* by 94.65%, *IL1β* by 79.87%, *IL6* by 75.36%, and *IL8* by 58.83% in hMC (Fig. 3A-D. p=0.0012, p<0.0001, p<0.0001, p<0.0001, respectively).

11,12-EET, 19,20-EDP or the sEH inhibitor GSK2256294 reduces IL-1β-stimulated inflammatory cytokine expression. hMC were treated with IL-1β in the presence or absence of 11,12-EET, 19,20-EDP or GSK2256294 to test the effect of each on IL-1β-induced inflammatory cytokine expression. Total RNA was isolated and inflammatory cytokine expression was assessed by qRT-PCR. 11,12-EET significantly reduced IL-1β-stimulated expression of *IL1β* by 35.65%, *IL6* by 30.06%, and *IL8* by 27.26% in hMC (Fig. 4B-D. p=0.0036, p=0.0125, p=0.0184, respectively). 19,20-EDP significantly reduced IL-1β-stimulated expression of *IL1β* by 22.88%, *IL6* by 37.18%, and *IL8* by 24.10% in hMC (Fig. 4B-D. p=0.0414, p=0.0036, p=0.0342, respectively). *TNFα* expression, however, was significantly increased by both epoxygenated fatty acids (Fig. 4A. p=0.0026, p=0.0136). sEH inhibition was tested at a range of GSK2256294 concentrations (0.1nM, 1.0nM, and 10nM) and at each concentration IL-1β-stimulated cytokine expression was significantly reduced. At the lowest concentration tested, 0.1nM, GSK2256294 reduced IL-1β -stimulated expression of *TNFα* by 25.11%, *IL1β* by 40.78%, *IL6* by 29.05%, and *IL8* by 36.37% in hMC (Fig. 5A-D. p=0.0003, p<0.0001, p<0.0001, p<0.0001, respectively). At 1nM, GSK2256294 reduced IL-1β-stimulated expression of *TNFα* by 38.56%, *IL1β* by 48.33%, *IL6* by 42.56%, and *IL8* by 44.91% in hMC (Fig. 5A-D. p<0.0001, p<0.0001, p<0.0001, p<0.0001, respectively). At 10nM, GSK2256294 reduced IL-1β -stimulated expression of *TNFα* by 79.45%, *IL1β* by 79.96%, *IL6* by 62.26%, and *IL8* by 78.05% in hMC (Fig. 5A-D. p<0.0001, p<0.0001, p<0.0001, p<0.0001, respectively).

11,12-EET or 19,20-EDP reduces PA- and IL-1β-induced NFκB promoter activity. hMC were transfected with a NFκB-luciferase promoter-reporter construct and treated with PA or IL-1β in the presence or absence of 11,12-EET or 19,20-EDP. As shown in Figure 6, 11,12-EET and 19,20-EDP decreased both PA and IL-1β-induced NFκB-dependent luciferase activity. 11,12-EET and 19,20-EDP decreased PA-induced reporter activity by 49.2% and 57.3%, respectively (Fig. 6A. p<0.0001, p<0.0001) and IL-1β-induced reporter activity by 23.6% and 17.2%, respectively (Fig. 6B. p=0.0006, p=0.0116).

Discussion

EET or EDP in combination with sEHi were previously shown to mitigate several DR-model experimental endpoints including: the expression of leukocyte adhesion proteins by hRMEC, peripheral blood monocyte (PBMC) adhesion to hRMEC monolayers, and acute TNFα-induced leukostasis in mice (3). In these studies, epoxygenated fatty acids were determined to act through NFκB-dependent signaling (3). The

anti-inflammatory potency of these lipid mediators in hRMEC caused speculation of their efficacy in other retinal cell types such as glia. MC are potent propagators of preliminary inflammation and serve as a functional link between the neuronal and vascular compartments of the vertebrate retina (35). MC span the retina's entire thickness and control retinal homeostasis including recycling neurotransmitters, maintaining the inner blood-retinal barrier, and photoreceptor maintenance (35). MC function in innate immunity (36) and some believe that diabetes-induced inflammation causes MC dysfunction, leading them to become destructive and promote DR pathogenesis (35). Additionally, changes in MC have been observed prior to the appearance of overt vascular pathology in DR (35,37). Consequently, therapeutics that block diabetes-related MC inflammation could prevent or slow the onset and progression of early DR. 11,12-EET and 19,20-EDP were selected for these experiments because both demonstrated superior efficacy compared to the other regioisomers in our previous studies. 19,20-EDP is the most highly abundant regioisomer in the retina because CYP epoxygenases preferentially mono-oxygenate the terminal double bond of DHA, and sEH hydrolysis of 19,20-EDP is slower compared to the other regioisomers (17,38,39). Therefore, 19,20-EDP turnover is presumably lower, allowing it to be more stable and therefore more efficacious. 11,12-EET was similarly chosen for its relative abundance and efficacy potential. Of the four EET regioisomers, 11,12-EET was one of the most abundant and demonstrated the most potent anti-inflammatory activity in previous studies (38,40,41). Notably, compared to other regioisomers, 11,12-EET and 19,20-EDP were also found in higher concentrations in MC-conditioned medium as determined by our mass spectrometric analysis. (Supp. Fig. 1)

Before testing the effects of increasing epoxygenated fatty acids levels in hMC, we first investigated the effects of their depletion. CYP epoxygenase activity is responsible for converting AA and DHA to regioisomeric EETs and EDPs respectively (3). hMC were treated with the broad-spectrum CYP inhibitor SKF-525a to presumably reduce intracellular EET/EDP levels. In the presence of SKF-525a we observed significant increases in the expression levels of the DR-relevant inflammatory cytokines *TNFα*, *IL1β, IL6* and *IL8.* These observations support our hypothesis that EET/EDP depletion, such as that occurring in DR, promotes hMC inflammation (Fig. 1). Exogenous addition of 11,12-EET, 19,20-EDP, and the sEH inhibitor GSK2256294, demonstrated a potent capacity to reduce inflammatory cytokine expression in hMC activated by PA and IL-1β. While previously demonstrated in hRMEC, this is the first report of the anti-inflammatory potential of these agents in retinal glia. PA was used as the initial stimulus to mimic the stress of diabetic dyslipidemia on hMC, and IL-1β was used as a stimulus to mimic the cytokine-induced-cytokine amplification that occurs subsequent to preliminary FFA-induced MC damage. Our data demonstrate that exogenous administration of 11,12-EET and 19,20-EDP significantly decreased hMC cytokine expression induced by the two different inflammatory stimuli, PA and IL-1β. While both epoxygenated fatty acids decreased PA-induced *TNFα* expression, they exacerbated IL-1β-induced *TNFα* expression, suggesting a different mechanism of action in the two cases. The exact mechanism of action by which EET and EDP function has yet to be determined, though the results of our NFκB-luciferase experiments indicated that both epoxygenated fatty acids decrease cytokine expression, at least in part, by modulating pathways that converge on NFκB-dependent transcription. NFκB is a pro-inflammatory transcription factor that controls the expression of inflammatory cytokines, and it plays an important, well

recognized role in early DR pathogenesis (12). Similar findings were obtained in our previous studies using retinal microvascular endothelial cells, and there is ample precedent for this mechanism occurring in other cells and tissues (3,42). Saturated fatty acids activate toll-like receptors expressed by MC that are upstream of NFκB-dependent transcription (41,42). Additionally, the canonical IL-1β signaling pathway includes NFκB activation (5). Therefore, we speculate that EET and EDP decrease *IL1β*, *IL6*, *IL8* and PA-induced *TNFα* mRNAs in part by an NFκB-dependent mechanism, while another signaling mechanism becomes overriding in the case of IL-1β-induced effects on *TNFα* mRNA. We do not consider this observation a deterrent to this therapeutic approach because we have previously shown that EET and EDP decrease TNFα-induced leukocyte adhesion functions in hRMEC (3). Therefore, any potentially detrimental effects of MC-derived TNFα on the retinal endothelium would be mitigated downstream.

We tested the capacity of sEH inhibitor GSK2256294 to reduce inflammatory cytokines in PA- and IL-1β-treated hMC. GSK2256294 blocks the hydrolysis of endogenous EET/EDP, raising their endogenous cellular concentrations to therapeutic levels. The results of several studies indicate that sEH inhibition is a promising therapeutic modality in a wide variety of systems. In our studies, we observed a consistent reduction of cytokine mRNAs across all GSK2256294 concentrations tested (0.1nM, 1.0nM, and 10nM). Interestingly, while hMC responded to sEH inhibition alone, hRMEC do not, suggesting that hMC may be the main sight of bioactive sEH that affects paracrine EET/EDP. Similarly, others have shown that sEH is more highly expressed in MC compared to other retinal cells types (3,45). While GSK2256294 potently inhibits sEH activity in HMC, it is important to note that it can exert off-target effects related to the end points explored in this study. For instance, sEH inhibition has been correlated with increased concentrations of lipoxin A4, an anti-inflammatory compound that resolved vascular damage and inflammation (46). However, in the present study, this metabolite was not detected when queried in the conditioned medium of MC by mass spectrometric analysis. sEH is constitutively expressed in the retina and is elevated in diabetic murine retina, human retina and in human vitreous (3,45). sEH activity in diabetes is thought to be responsible for pericyte loss and endothelial barrier dysfunction by promoting the production of pro-inflammatory diol 19,20-DHDP, the hydrolysis product of 19,20-EDP (45). 19,20-DHDP alters the localization of cholesterol-binding proteins in the cell membrane, disrupting pericyte-endothelial cell junctions and inter-endothelial cell junctions (45). Like the expression of sEH, the accumulation of 19,20-DHDP is significantly increased in samples from patients with diabetic retinopathy (45). To ensure that potential activity from vicinal diols did not confound any of the cytokine measurements observed in our experiments, we treated hMC with 11,12-DHET and 19,20-DHDP. Neither lipid metabolite increased any of the inflammatory cytokines that were assayed in this study.

Mimicking a chronic, multifaceted disease like DR is a challenge *in vitro*, but *in vitro* experiments remain crucial tools to dissect the mechanisms of disease in a controlled, step-wise fashion. We used primary human Müller cells in order to maintain physiological relevance in our studies and to more easily translate our findings to future clinical trials in humans. Our proposed therapeutic strategy provides a unique advantage in translation to the clinic because it relies on manipulation of an endogenous system, allowing for protection throughout multiple stages of DR progression, as well as minimizing toxicity. Current mainstream therapies focus on mediating late-stage DR morbidities directly associated with

vision loss, while we here propose a strategy that would focus on early stage DR, inflammation, before irreversible damage has occurred. Our results confirm the anti-inflammatory effects of epoxide elevation in hMC, paving the way for directed *in vivo* studies. In future studies, we hope to confirm the therapeutic potential of systemically administered epoxides over longer time-spans of pathogenesis in *in vivo* models of DR. These studies will be enabled by using water-soluble analogues of the epoxygenated fatty acids, as they will overcome issues of hydrophobicity and turnover of the parent EET/EDPs, enhancing their systemic circulation and bioavailability (47).

Conclusions

In conclusion, our data indicate that therapeutic manipulations to increase retinal levels of epoxygenated fatty acids offer the potential to be potently efficacious in the treatment of DR.

Abbreviations

DR: diabetic retinopathy. NPDR: non-proliferative diabetic retinopathy. PDR: proliferative diabetic retinopathy. VEGF: vascular endothelial cell growth factor. FFA: free fatty acids. PA: Palmitic acid. MC: Müller cells. IL-1β: interleukin-1β. AA: arachidonic acid. DHA: docosahexaenoic acid. PUFAs: polyunsaturated fatty acids. COX: cyclooxygenase. LOX: lipoxygenase. CYP: cytochrome P450 epoxygenase. ALOX15: 15-lipoxygenase. EET: epoxyeicosatrienoic acid. EDP: epoxydocosapentaenoic acid. hRMEC: human retinal microvascular endothelial cells. sEH: soluble epoxide hydrolase. DHET: dihydroxyeicosatrienoic acid. DHDP: dihydroxydocosapentaenoic acid. sEHi: sEH inhibitor. hMC: human Müller cells. FBS: fetal bovine serum. CRALBP: cellular retinaldehyde-binding protein. GS: glutathione synthetase. GFAP: glial fibrillary acidic protein. qRT-PCR: real-time quantitative reverse transcription polymerase chain reaction. PBMC: peripheral blood monocyte.

Declarations

Authors' contributions

Experiments performed by MEC, MJK, GWM and CDO. Data graphed and analyzed by CDO. GWM and CDO wrote the manuscript. All authors edited and approved final manuscript.

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Availability of data and materials

Data and materials will be available upon request.

Ethics approval and consent to participate

N/A

Consent for publication

All authors consent for publication.

Competing interests

The authors declare no conflict of interest.

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Figures

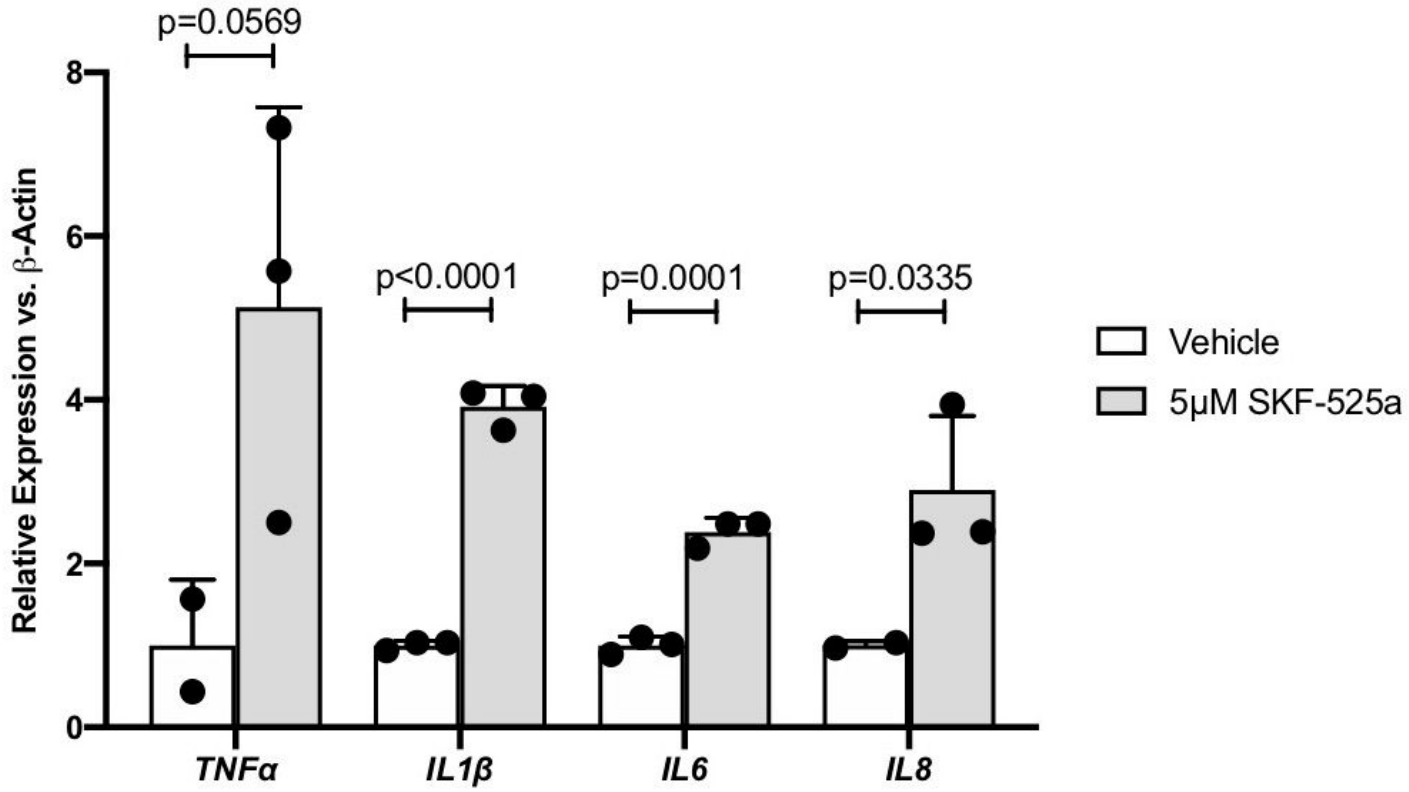

Figure 1

The effect of CYP inhibitor SKF-525a on Müller cell inflammatory cytokine expression. Human Müller cells were treated with SKF-525a (5.0μM) for 24 hours. After treatment, total RNA was isolated and inflammatory cytokine expression was assayed by qRT-PCR. TNFα, IL1β, IL6 and IL8 expression was increased by SKF-525a, though statistical significance was not achieved for TNFα. Data are displayed as mean ± SD (n=2 or 3).

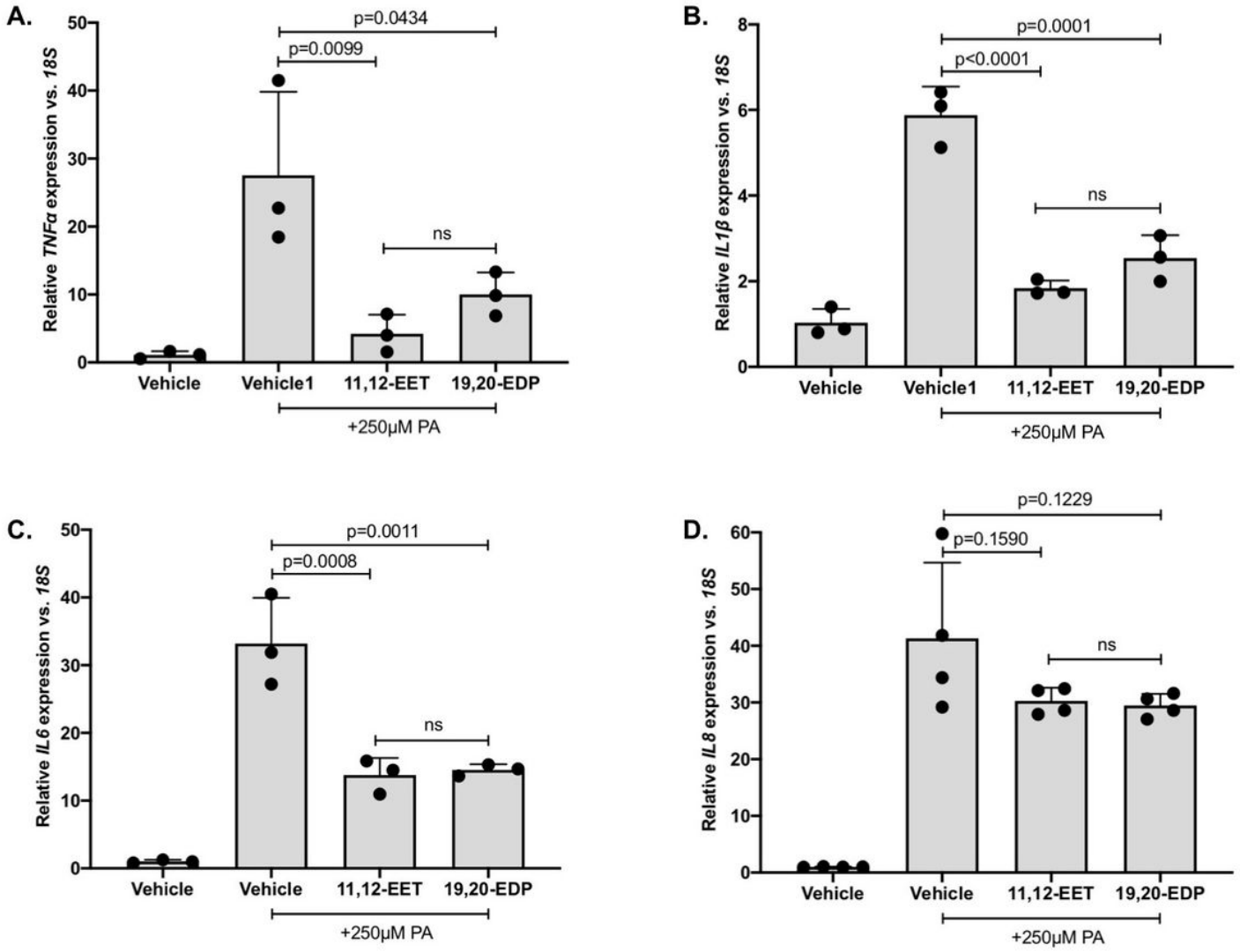

Figure 2

The effect of 11,12-EET and 19,20-EDP on PA-induced inflammatory mediator expression by Müller cells. Human Müller cells were treated with PA (250μM) for 24 hours. 11,12-EET (0.5μM) or 19,20-EDP (0.5μM) was added during the final 3 hours of treatment. After 24 hours, total RNA was isolated and expression was assayed by qRT-PCR. (A) TNFα (B) IL1β, and (C) IL6 expression was significantly decreased by both epoxygenated fatty acids. (D) IL8 expression was reduced but statistical significance was not achieved. Results depicted are representative of three separate experiments. Data are displayed as mean ± SD (n=3 or 4 for each experiment).

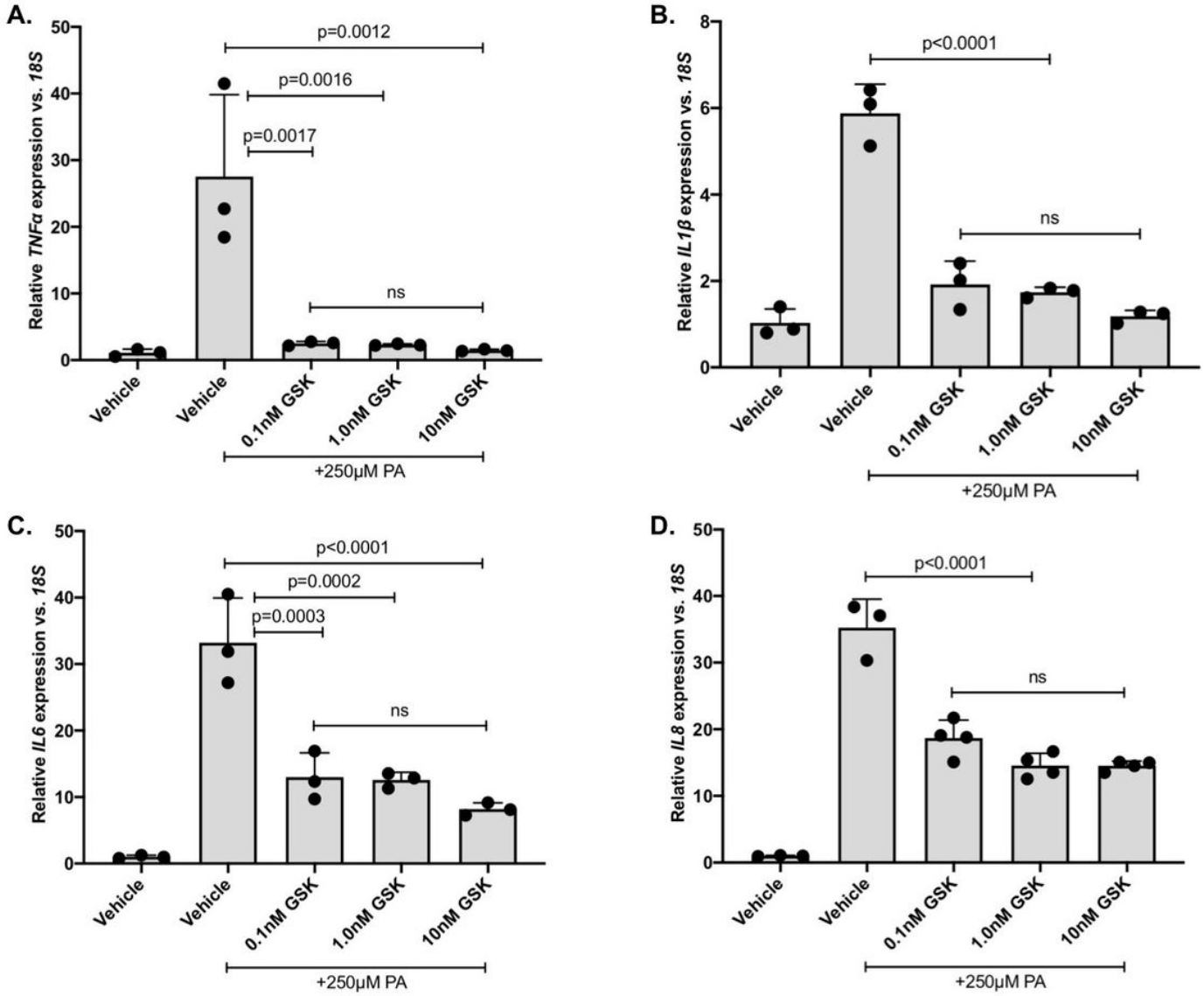

Figure 3

The effect of sEH inhibitor GSK2256294 on PA-induced inflammatory mediator expression by Müller cells. Human Müller cells were treated with PA (250μM) or PA plus 0.1nM, 1.0nM or 10nM GSK2256294 (sEH inhibitor). After 24 hours, total RNA was isolated, and expression was analyzed by qRT-PCR. (A) TNFα, (B) IL1β, (C) IL6, and (D) IL8 expression was significantly decreased by the addition of the sEH inhibitor at each of the concentrations tested. Results depicted are representative of three separate experiments. Data are displayed as mean ± SD (n=3 or 4 for each experiment).

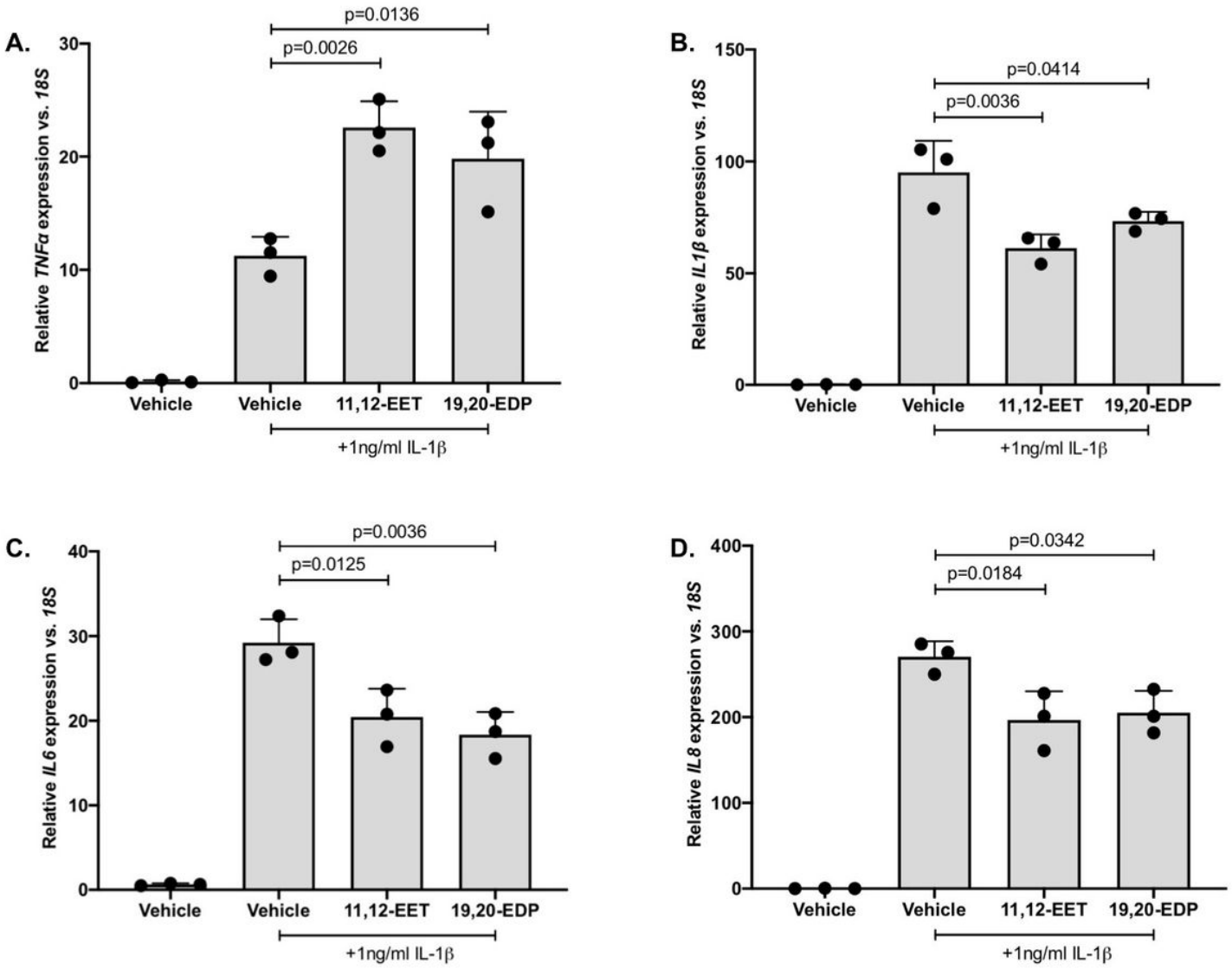

Figure 4

The effect of 11,12-EET and 19,20-EDP on IL-1β-induced inflammatory cytokine expression by Müller cells. Human Müller cells were treated with IL-1β (1.0ng/ml) or IL-1β plus 11,12-EET (0.5μM) or 19,20-EDP (0.5μM) for 8 hours. Total RNA was isolated and cytokine expression was assayed by qRT-PCR. (A) TNFα expression was significantly elevated while (B) IL1β, (C) IL6, and (D) IL8 expression was significantly decreased by the addition of both exogenous epoxygenated fatty acids. Results depicted are representative of three separate experiments. These data are normalized to induction levels illustrated in Figure 5. Data are displayed as mean ± SD (n=3).

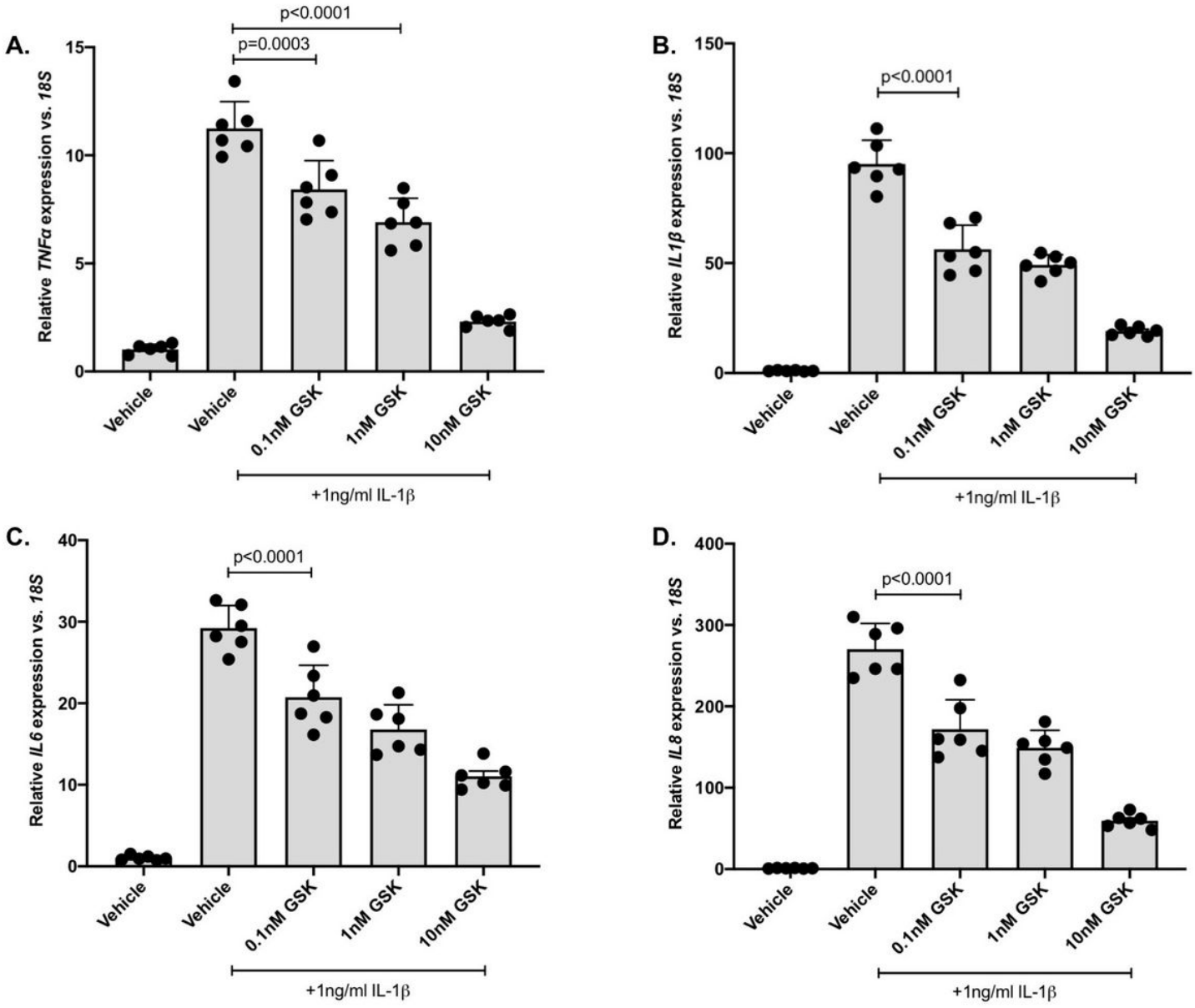

Figure 5

The effect of sEH inhibitor GSK2256294 on IL-1β-induced inflammatory mediator expression by Müller cells. Human Müller cells were treated with IL-1β (1.0ng/ml) or IL-1β plus 0.1nM, 1.0nM, or 10nM GSK2256294 (sEH inhibitor) for 8 hours. Total RNA was isolated and cytokine expression was assayed by qRT-PCR. (A) TNFα, (B) IL1β, (C) IL6, and (D) IL8 expression was significantly decreased at each sEH inhibitor concentration tested. Bars represent mean ± SD (n=6).

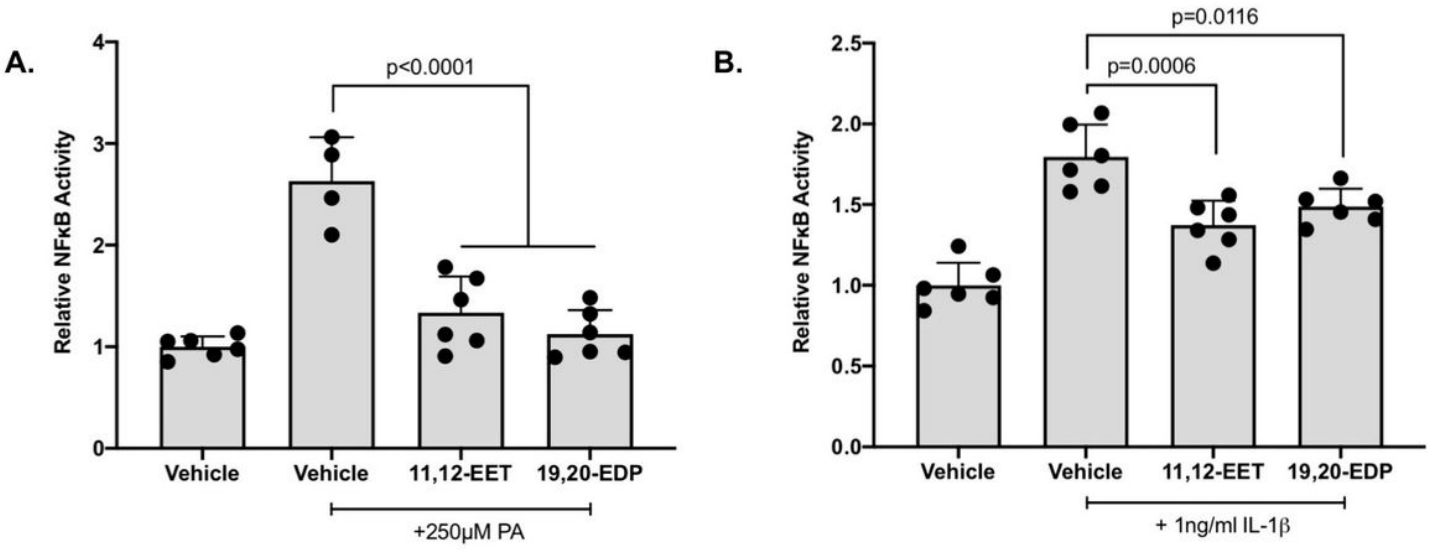

Figure 6

The effect of 11,12-EET or 19,20-EDP on PA or IL-1β-induced NFκB promoter activity. hMC were transfected with a NFκB-luciferase reporter construct and treated with (A) PA (B) IL-1β or in the presence or absence of 11,12-EET (0.5 μM) or 19,20-EDP (0.5 μM). NFκB activity was determined by measuring the ratio of firefly-to-renilla luciferase luminescence activity. Each bar represents the mean ± SD (n=4, 5 or 6).

Supplementary Files

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- SuppFig.jpg