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Eosinophils synthesize trihydroxyoctadecenoic acids (TriHOMEs) via a 15-lipoxygenase dependent process

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ABSTRACT

Trihydroxyoctadecenoic acids (TriHOMEs) are linoleic acid-derived lipid mediators reported to be dysregulated in obstructive lung disease. In contrast to many other oxylipins, TriHOME biosynthesis in humans is still poorly understood. The association of TriHOMEs with inflammation prompted the current investigation into the ability of human granulocytes to synthesize the 16 different 9,10,13-TriHOME and 9,12,13-TriHOME isomers and of the TriHOME biosynthetic pathway. Following incubation with linoleic acid, eosinophils and (to a lesser extent) the mast cell line LAD2, but not neutrophils, formed TriHOMEs. Stereochemical analysis revealed that TriHOMEs produced by eosinophils predominantly evidenced the 13(S) configuration, suggesting 15-lipoxygenase (15-LOX)-mediated synthesis. TriHOME formation was blocked following incubation with the 15-LOX inhibitor BLX-3887 and was shown to be largely independent of soluble epoxide hydrolase and cytochrome P450 activities. TriHOME synthesis was abolished when linoleic acid was replaced with 13-HODE, but increased in incubations with 13-HpODE, indicating the intermediary role of epoxy alcohols in TriHOME formation. In contrast to eosinophils, LAD2 cells formed TriHOMEs having predominantly the 13(R) configuration, demonstrating that there are multiple synthetic routes for TriHOME formation. These findings provide for the first-time insight into the synthetic route of TriHOMEs in humans and expand our understanding of their formation in inflammatory diseases.

1. Introduction

Due to the widespread use of vegetable oils, linoleic acid is now the most highly consumed polyunsaturated fatty acid (PUFA) in the western diet [1–3]. The large increase in linoleic acid consumption over the last 50 years has raised questions concerning its potential impact upon human pathophysiology, particularly in regards to the effects on chronic inflammation [4–6]. The essential PUFA linoleic acid is converted to arachidonic acid via sequential fatty acid desaturase and elongase activity [7,8], which is in turn a precursor of pro-inflammatory lipid mediators such as leukotrienes and prostaglandins [9]. Multiple studies have suggested that linoleic acid exerts pro-inflammatory activities independent of its role as an arachidonic acid precursor [3]; however, recent work points to a more nuanced biological role [10,11]. A number of metabolites of linoleic acid have been reported to be potent lipid mediators, including 9(10)-EpOME (9,10-epoxy-12Z-octadecenoic acid, leukotoxin) and 9,10-DiHOME (9,10-dihydroxy-12Z-octadecenoic acid, leukotoxin-diol) [12], 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) [13], 10-hydroxy-*cis*-12-octadecenoic acid (HYA) [14] and the 11-hydroxy-epoxy-octadecenoates [15]. Recent work has in particular identified 12,13-DiHOME (isoleukotoxin-diol) to have links with asthma [16,17], serve as a mediator of thermal hyperalgesia during inflammatory pain [18], and to function as a lipokine to increase the uptake of skeletal muscle fatty acid [19] and promote fatty acid transport into brown adipose tissue [20]. The biosynthesis of these linoleic acid-derived oxylipins is relatively well characterized and has been shown to be controlled enzymatically by cyclooxygenases (COX), lipoxygenases (LOX),

Abbreviations: TriHOME, trihydroxyoctadecenoic acid; PMN, polymorphonuclear; LOX, lipoxygenase; COX, cyclooxygenase; CYP, cytochrome P450; sEH, soluble epoxide hydrolase; COPD, chronic obstructive pulmonary disease; TPPU, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl); FACS, fluorescence-activated cell sorting; RP, reversed phase; PPP, platelet poor plasma; PRP, platelet rich plasma; EE, enantiomeric excess; SEM, standard error of the mean

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cytochrome P450 (CYP) and soluble epoxide hydrolase (sEH) as well as non-enzymatically via reactive oxygen species (ROS).

Compared to other linoleic acid-derived oxylipins, trihydroxyoctadecenoic acids (TriHOMEs) are poorly characterized in humans. The most well-described regioisomers 9,10,13-TriHOME and 9,12,13-TriHOME were first identified by Graveland et al. in 1970, who isolated the TriHOMEs from suspensions of wheat flour [21]. Since then, TriHOMEs have been characterized in several plant systems including potato leaves [22,23], onion bulbs [24] and beetroot [25]. In mammalian systems, attempts have been made to characterize the synthesis of TriHOMEs in porcine leukocytes [26] as well as in fetal calf blood vessels [27]. More recently, TriHOMEs were suggested to be of physiological relevance for maintaining the water-skin barrier and 12(R)-LOX was identified to be involved in the biosynthesis of TriHOMEs in human skin [28,29]. However, aside from in the skin, the biosynthetic route of TriHOMEs in humans remains to be described.

Several recent studies have identified TriHOMEs to be dysregulated in respiratory disease including asthma and COPD [30–33], which has led to speculation about a possible pathophysiological relevance of TriHOMEs in chronic inflammatory disease. Polymorphonuclear cells (PMNs) are key effector cells in chronic inflammation, and neutrophils as well as eosinophils are directly involved in the pathogenesis of obstructive lung disease [34–36]. It has previously been suggested that TriHOME formation occurs in the lung in a neutrophil-dependent process [33]. We have therefore conducted for the first-time a thorough investigation of the biosynthesis of 16 TriHOME regio- and stereoisomers in human PMNs. Our findings demonstrate that eosinophils, but not neutrophils, are capable of synthesizing TriHOMEs. Furthermore, TriHOME formation in eosinophils was shown to proceed in a 15-LOX-dependent manner with no involvement of the sEH or CYP450.

2. Materials and methods

2.1. Reagents

Mass spectrometry-grade isopropanol, methanol, and acetic acid were purchased from Fisher Scientific (Waltham, MA). Acetonitrile, glycerol, DMSO, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dextran was purchased from Pharmacosmos A/S (Holbaek, Denmark) and calcium ionophore A23187 was obtained from Tocris Bioscience (Bristol, United Kingdom). Disodium phosphate, sodium chloride and citric acid monohydrate were obtained from Merck (Darmstadt, Germany). Sucrose was purchased from Duchefa Biochemie (Haarlem, Netherlands). Propidum iodide, StemPro-34 medium, StemPro-34 Nutrient Supplement, L-glutamine, penicillin and streptomycin were bought from Invitrogen/Gibco (Carlsbad, CA, USA) and recombinant human stem cell factor was obtained from Prepotech (Rocky Hill, NJ, USA). 9(*S*),10(*S*),13(*S*)-TriHOME, 9(*S*),12(*S*),13(*S*)-TriHOME, 13-HpODE (13-hydroperoxy-9Z,11E-octadecadienoic acid) and 13-HODE were kind gifts from Larodan (Solna, Sweden). Linoleic acid and the 12-LOX inhibitor ML-355 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). The Eosinophil isolation kit, MACSxpress Neutrophil Isolation Kit and human anti-CD16-FITC were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). Ficoll-Paque Premium was obtained from GE Healthcare (Uppsala, Sweden). The 15-LOX inhibitor BLX-3887 was a kind gift from Dr. Hans-Erik Claesson (Karolinska Institutet, Stockholm, Sweden). The sEH inhibitor 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) (TPPU) was kindly provided by Dr. Bruce Hammock (University of California Davis, CA, USA). The CYP inhibitor SKF 525A was purchased as its hydrochloride from Calbiochem (San Diego, CA, USA). Milli-Q ultrapure deionized water was used in all experiments (Millipore Corp., Billerica, MA, USA).

2.2. Isolation of PMNs from buffy coats

PMNs were isolated from buffy coats from healthy human donors (Karolinska Hospital Blood Bank) as previously described [37]. In brief, erythrocytes were sedimented by mixing equal volumes of buffy coat with 2% dextran (*w/v*) solution and removing the supernatant after 20 min. Afterwards, PMNs were separated by gradient centrifugation with Ficoll-Paque Premium (833 ×*g*, 20 min without brake) and residual erythrocytes were removed by hypotonic lysis.

2.3. Isolation of eosinophils

PMNs from buffy coats (obtained as described above) were further processed using an eosinophil isolation kit according to the manufacturer's instructions. Cell purity was determined by FITC-CD16-staining and subsequent FACS analysis (CD16 negative PMNs). Cell debris and dead cells were excluded from the FACS analysis based on scatter signals and propidium iodide staining.

2.4. Isolation of neutrophils

Neutrophils were isolated from human peripheral blood of healthy volunteers (Karolinska Hospital Blood Bank) using a MACSxpress Neutrophil Isolation Kit according to the manufacturer's instructions. For cell purity determination, neutrophils were stained using a fluorescently labelled FITC-CD16 antibody and analyzed by FACS (CD16 positive cells).

2.5. Preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP)

For preparations of PRP, human peripheral blood of healthy volunteers (Karolinska Hospital Blood Bank) was centrifuged for 10 min at 190 x*g* without brake. For PPP preparations, human peripheral blood was centrifuged for 10 min at 1600 x*g*. The upper 2/3 of the plasma phase (PRP or PPP) was collected for further processing.

2.6. Culturing and incubations of LAD2 cells

The human mast cell line LAD2 was maintained in StemPro-34 medium supplemented with 2.5% StemPro-34 nutrient supplement, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and recombinant human stem cell factor (100 ng/ml) at 37 °C, 5% CO_2. For the experiments, cells were washed and resuspended in PIPES buffer (9.2 mM Pipes, 140 mM sodium acetate, 5 mM potassium acetate, 0.6 mM calcium chloride dehydrate, 1.1 mM magnesium chloride). A total of 300,000 cells were incubated with 10 μg/ml linoleic acid for 30 min. For control incubations, cells without linoleic acid or PIPES buffer with 10 μg/ml linoleic acid were incubated in parallel. After the incubations, cells were centrifuged at 400 x*g* for 5 min and supernatant was stored at −80 °C.

2.7. Incubations of PMNs, neutrophils and eosinophils

Isolated cells were counted, re-dissolved in PBS and 500 μl of cell suspension was pre-incubated at 37 °C for 10 min with inhibitor, calcium ionophore A2318 or vehicle (DMSO). Substrate (10 μg/ml of linoleic acid, 13-HpODE or 13-HODE) was added and cells were incubated for 30 min. The reaction was stopped by addition of 1 ml of ice-cold methanol and incubation on ice for 20 min. The methanol-treated incubate was centrifuged at 10,600 ×*g* for 10 min and the supernatant was stored at −80 °C until further processing.

2.8. Extraction of oxylipins

Cell supernatants (800 μl) were diluted with 5200 μl of extraction

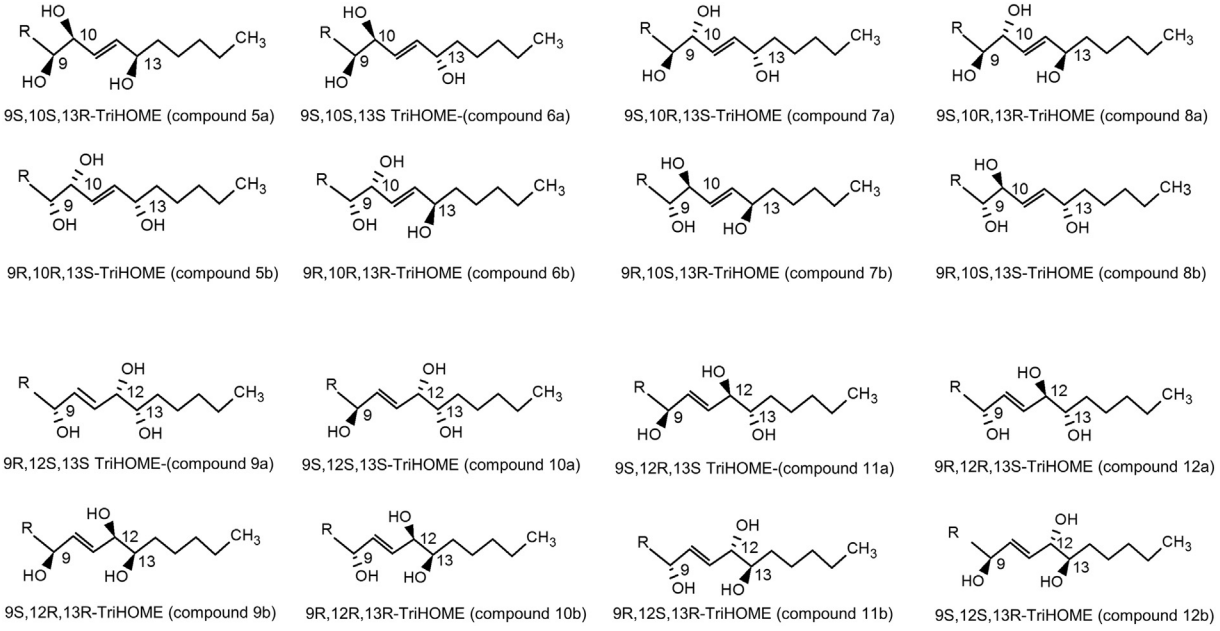

Fig. 1. Structures of the 16 different 9,10,13-TriHOME and 9,12,13-TriHOME isomers. The numbering of the compounds (5 a/b – 12 a/b) corresponds to the system proposed when their structures were originally reported [40]. R = $(CH_2)_7COOH$.

buffer (citric acid/NA_2HPO_4) and 10 µl of isotopically labelled internal standard mix was added. Solid phase extraction was performed using an automated Extrahera liquid handling system (Biotage, Uppsala, Sweden) and Evolute Express ABN SPE cartridges (Biotage) as previously described [38].

2.9. Quantification of TriHOME diastereomers by RP-UHPLC-MS/MS and analysis of TriHOME enantiomers by chiral HPLC-MS/MS

TriHOME diastereomer quantification and chiral analysis of TriHOME enantiomers was performed as previously described [39]. The enantiomeric excess (ee) was calculated using the formula: ee = (a − b) / (a + b) × 100 where a and b are the peak areas of the respective enantiomers. The numbering system for the TriHOME isomers is shown in Fig. 1 (isomers 5a/b – 12a/b) and corresponds to the system proposed when their structures were originally reported [40].

2.10. Quantification of other oxylipin species

Quantification of the additional oxylipin species, including the purported 15-LOX products as well as the linoleic acid-derived DiHOMEs was performed using a general oxylipin LC-MS/MS platform as described previously [38].

3. Results

3.1. PMNs synthesize TriHOME isomers from linoleic acid

To investigate whether PMNs are able to synthesize TriHOMEs, 2×10^6 PMNs were incubated with varying concentrations of linoleic acid and cell supernatants were analyzed by RP-UHPLC-MS/MS. PMNs were isolated by Ficoll centrifugation and therefore represent a mix of neutrophils (major fraction, ~95%) and eosinophils (minor fraction, ~5%, Fig. S1). As shown in Fig. 2, PMNs produced all four 9,10,13-TriHOME and all four 9,12,13-TriHOME diastereomers in a substrate concentration dependent fashion. As depicted in Fig. 2A, the 9,10,13-TriHOME diastereomers 7 a/b, 8 a/b were found in larger quantities than diastereomers 5 a/b and 6 a/b. For 9,12,13-TriHOMEs, the diastereomers 11 a/b and 12 a/b were produced in larger quantities compared to compounds, 9 a/b and 10 a/b.

3.2. Eosinophils, but not neutrophils, produce TriHOMEs

Given their abundance (~95% of PMNs are neutrophils), we reasoned that neutrophils are the most probable cell type responsible for the observed TriHOME production in PMNs. However, when we incubated linoleic acid with 2×10^6 neutrophils (isolated with a neutrophil isolation kit, neutrophil purity > 99%, residual eosinophils < 0.3%, determined by flow cytometry, Fig. S1), only very low amounts of TriHOMEs were observed (Fig. 2). We therefore investigated the potential of eosinophils (isolated with an eosinophil isolation kit, cell purity > 97%, Fig. S1) to synthesize TriHOMEs and found that as little as 3×10^5 eosinophils incubated with 10 µg/ml linoleic acid produced a larger amount of all analyzed TriHOMEs than 2×10^6 PMNs (Fig. 3). These data highlight that the eosinophilic and not the neutrophilic fraction is responsible for the observed production of TriHOMEs in PMNs. Furthermore, the isomer pattern of the measured TriHOMEs was similar to the isomer pattern observed in PMNs. Incubations with 10 µM of the calcium ionophore A23187 activated eosinophils did not lead to enhanced TriHOME production; however, this response is in accordance with the literature. The calcium dependent cytosolic phospholipase A_2 ($cPLA_2$) has high affinity for arachidonic acid [41], whereas the phospholipase with high affinity for linoleic acid is the Ca^{2+}-independent PLA_2 ($iPLA_2$) [42]. The levels of produced TriHOMEs were similar to other known 15-LOX products including 15-HETE and 17-HDoHE, but significantly less than the linoleic acid-derived 13-HODE and 9-HODE (Fig. S2).

3.3. Eosinophils predominantly produce TriHOMEs with the 13(S) configuration

In order to investigate whether the measured TriHOMEs were formed via a LOX-dependent process, we analyzed the cell supernatants by chiral HPLC-MS/MS (which can distinguish the a and b TriHOME enantiomers). While autoxidative processes generally lead to a racemic mixture of the (R) and (S) enantiomers, most lipoxygenases such as 15-LOX oxygenate their substrates in an (*S*)-enantiomer selective manner (with the notable exception of the 12(*R*)-LOX enzyme). As shown in

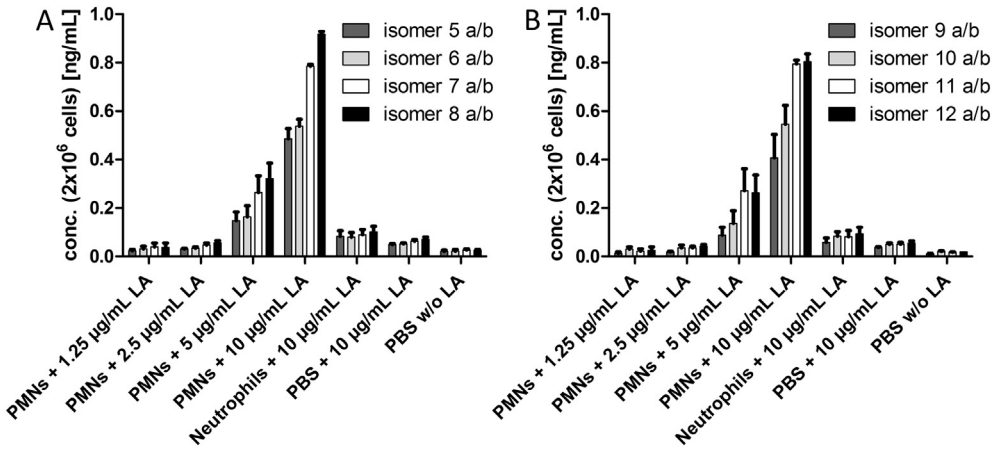

Fig. 2. Synthesis of (A) 9,10,13-TriHOME and (B) 9,12,13-TriHOME by polymorphonuclear neutrophils (PMNs, neutrophils plus eosinophils) and purified neutrophils. 2×10^6 cells were incubated for 30 min with 10 µg/ml linoleic acid (LA) as substrate. TriHOME formation was measured by RP-UHPLC-MS/MS. TriHOME isomer nomenclature is as shown in Fig. 1. Results are shown as mean values ± SEM ($n = 3$).

Fig. 4, TriHOME enantiomers with the 13(S) configuration were found in excess relative to TriHOMEs with the 13(R) configuration, suggesting that introduction of the hydroxyl group at position C-13 was to a large degree a LOX-dependent process. No ee pattern was found for the alcohol groups at positions C-9 and C-10 (for 9,10,13-TriHOMEs) or positions C-9 and C-12 (for 9,12,13-TriHOMEs), suggesting that this oxidation occurs via a non-enzymatic process.

3.4. TriHOMEs are formed via a 15-LOX dependent and sEH independent process

In order to analyze TriHOME synthesis in detail, eosinophils were pre-incubated with a range of enzyme inhibitors. Pre-incubation of eosinophils with the 15-LOX inhibitor BLX-3887 inhibited the formation of all measured TriHOMEs in a dose dependent fashion (Fig. 5A and B), demonstrating that TriHOME synthesis in eosinophils is a 15-LOX-dependent process.

We next investigated the involvement of sEH in TriHOME biosynthesis. Eosinophils were pre-incubated with the sEH inhibitor TPPU. The sEH converts epoxides (e.g., EpOMEs) into the corresponding vicinal diols (e.g., DiHOMEs). However, incubation with 0.1–1 µM TPPU did not inhibit the formation of isomers 5 a/b, 6 a/b, 9 a/b and 10 a/b, and gave only very moderate inhibition (< 25%) of compounds 7 a/b, 8 a/b, 11 a/b, and 12 a/b (Fig. 5C and D). At even higher concentrations of TPPU, the experimental setup appeared to become more unstable leading to large variations in measured TriHOMEs; however, no overall decrease of TriHOME formation was observed. To confirm that TPPU was effectively inhibiting sEH activity, we measured in the same experiments the amounts of formed DiHOMEs by RP-UHPLC-MS/MS. As shown in Fig. S3, 0.1 µM TPPU was sufficient to cause an almost

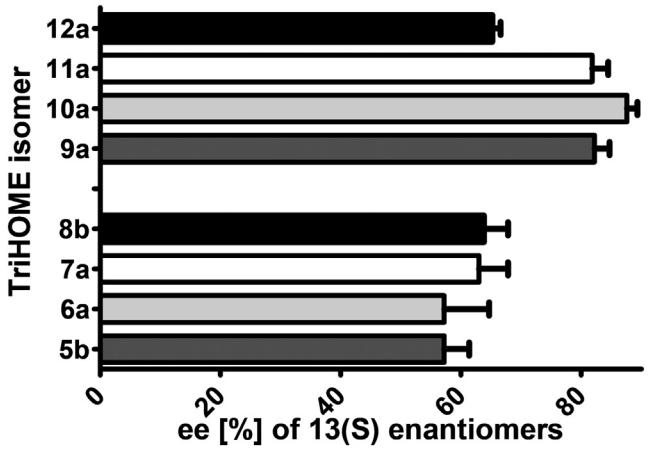

Fig. 4. The average enantiomeric excess (ee) of TriHOMEs with an (S) configuration at carbon 13 formed by 3×10^5 eosinophils incubated for 30 min with 10 µg/ml linoleic acid. Data represent the average ee ± SEM ($n = 5$). The ee was calculated using the formula: ee = (a − b) / (a + b) × 100, where a and b are the peak areas of the respective enantiomers. The nomenclature for the TriHOME enantiomers is as presented in Fig. 1. The synthetic route of these 8 TriHOME enantiomers (5b, 6a, 7a, 8b, 9a, 10a, 11a, 12a) is shown in Fig. 8.

complete inhibition of both 9,10-DiHOME and 12,13-DiHOME formation. These results suggest that different to the formation of DiHOMEs, which strongly depends on sEH activity, synthesis of TriHOMEs in eosinophils is a largely sEH independent process.

Furthermore, we investigated the inhibitory potential of SKF 525A, a general CYP inhibitor (Fig. 5E-F). No effect was observable at

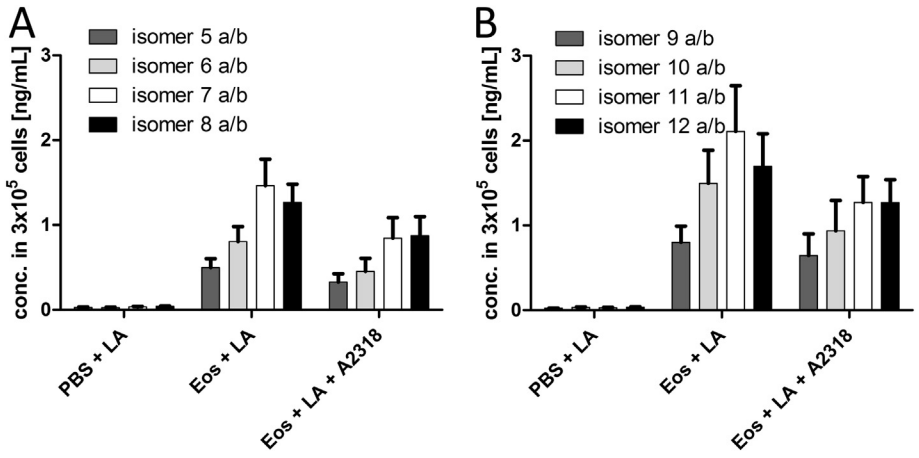

Fig. 3. Synthesis of (A) 9,10,13-TriHOMEs and (B) 9,12,13-TriHOMEs by eosinophils (Eos). Purified eosinophils (3×10^5 cells) were pre-incubated for 10 min with vehicle or 10 µM A2318 ionophore followed by incubation for 30 min with 10 µg/ml linoleic acid (LA) as substrate. The production of TriHOMEs was measured by RP-UHPLC-MS/MS. TriHOME isomer nomenclature is as shown in Fig. 1. Results are shown as mean values ± SEM ($n = 8$).

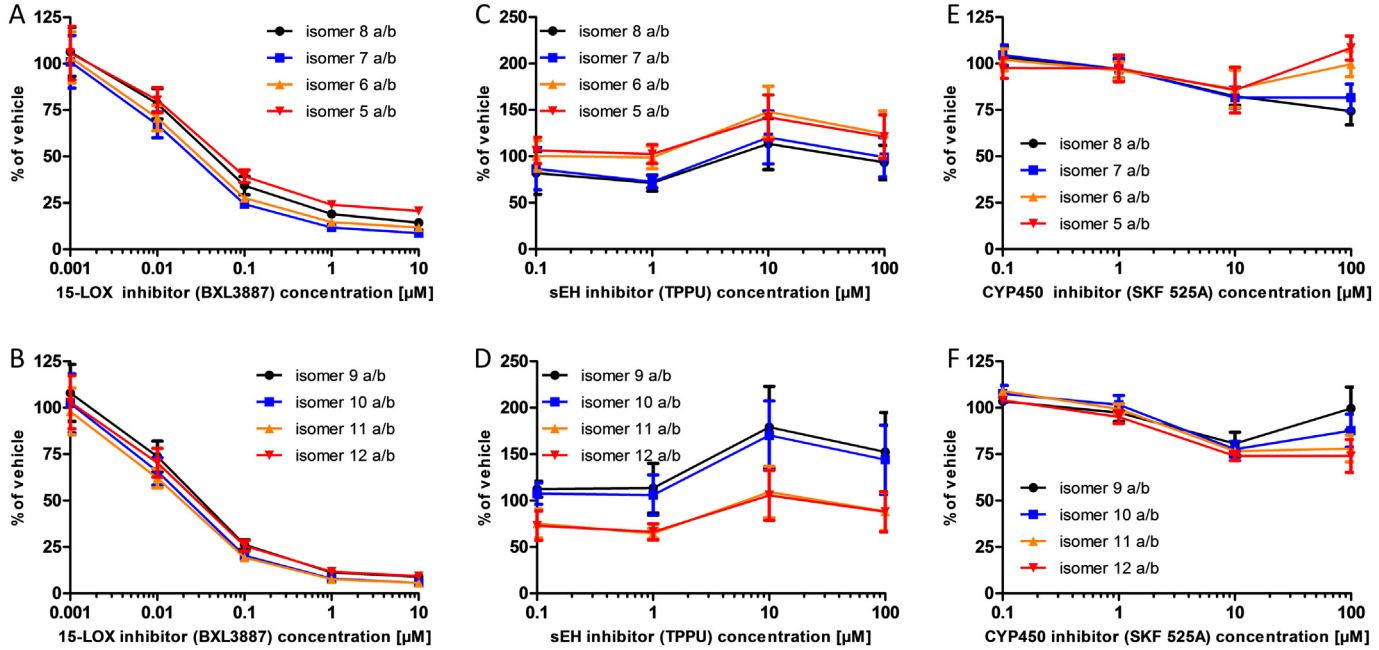

Fig. 5. Inhibition of TriHOME synthesis by (A, B) 15-LOX inhibitor BLX-3887, (C, D) sEH inhibitor TPPU and (E, F) CYP450 inhibitor SKF 525A. Eosinophils (3×10^5 cells) were pre-incubated for 10 min with vehicle or various concentrations of inhibitor before addition of 10 μg/ml linoleic acid. The reaction was stopped after 30 min and TriHOME formation was analyzed by RP-UHPLC-MS/MS. TriHOME isomer nomenclature is as shown in Fig. 1. Data represent average inhibition relative to vehicle incubations ± SEM (n = 3).

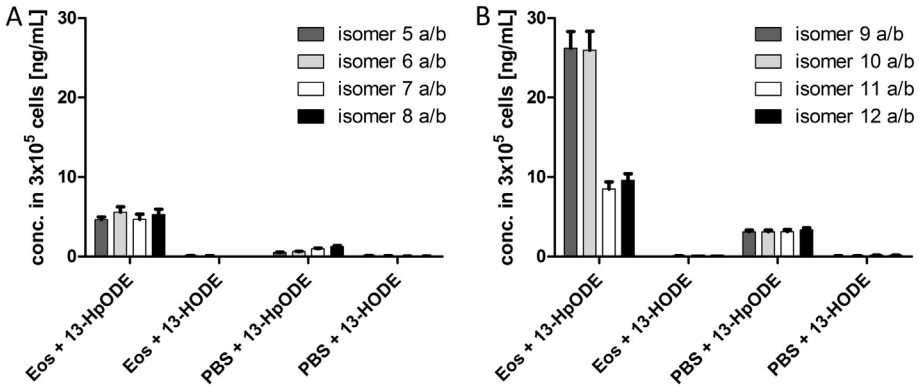

Fig. 6. Average amounts of (A) 9,10,13-TriHOME and (B) 9,12,13-TriHOME isomers produced by 3×10^5 eosinophils (Eos) incubated for 30 min with 10 μg/mL of 13-HpODE or 13-HODE. The formation of TriHOMEs was measured by RP-UHPLC-MS/MS. TriHOME isomer nomenclature is as shown in Fig. 1. Results are shown as mean values ± SEM (n = 3).

concentrations from 0.1–1 μM. At an inhibitor concentration of 10 μM, a slight inhibitory effect on TriHOME formation (~25% for all measured isomers) was observed. Further increase of the concentration to 100 μM did not lead to a stronger inhibition of TriHOME synthesis.

Even though linoleic acid compared to arachidonic acid is a relatively poor substrate for 12-LOX, we also investigated a potential role of 12-LOX in TriHOME formation by pre-incubating eosinophils with the 12-LOX inhibitor ML-355. At the tested concentration range of 0.01–10 μM, no inhibitory effect was detected (data not shown) indicating that 12-LOX is not involved in the biosynthesis of TriHOMEs in eosinophils. To examine the potential of platelets (which express 12-LOX in large amounts) [43] to synthesize TriHOMEs, we compared incubations of PRP and PPP with 10 μg/ml of linoleic acid. In both PRP and PPP, residual levels of all 9,10,13-TriHOMEs and 9,12,13-TriHOMEs were observed, but no concentration difference between PRP and PPP was observed (data not shown). The data indicate that platelets are unable to synthesize TriHOMEs in detectable amounts and that 12-LOX plays no major role in the synthesis of TriHOMEs.

3.5. 13-HpODE, but not 13-HODE, serves as a TriHOME precursor

Based upon the identified importance of 15-LOX activity in TriHOME formation in eosinophils, we analyzed the effect of exchanging linoleic acid with 13-HODE or 13-HpODE as substrate (Fig. 6). While eosinophil incubation with the hydroperoxide 13-HpODE led to large amounts of all measured TriHOMEs, no TriHOME biosynthesis was observed when eosinophils were incubated with 13-HODE, the reduced form of 13-HpODE. Notably, incubation with 13-HpODE appeared to favor formation of 9,12,13-TriHOME isomers over 9,10,13-TriHOMEs. Especially the 9,12,13-TriHOME isomers 9 a/b and 10 a/b were measured in large excess relative to other TriHOMEs. The ablation of TriHOME formation when 13-HODE was used as substrate strongly suggests that the two hydroperoxide oxygens of 13-HpODE are both required for TriHOME formation. As shown in Fig. 6, a considerable amount of 9,12,13-TriHOME isomers was also observed in the negative control where PBS was incubated with 13-HpODE (~10% of isomers 11 a/b and 12 a/b, and ~30% of 9 a/b and 10 a/b, relative to the cell incubations).

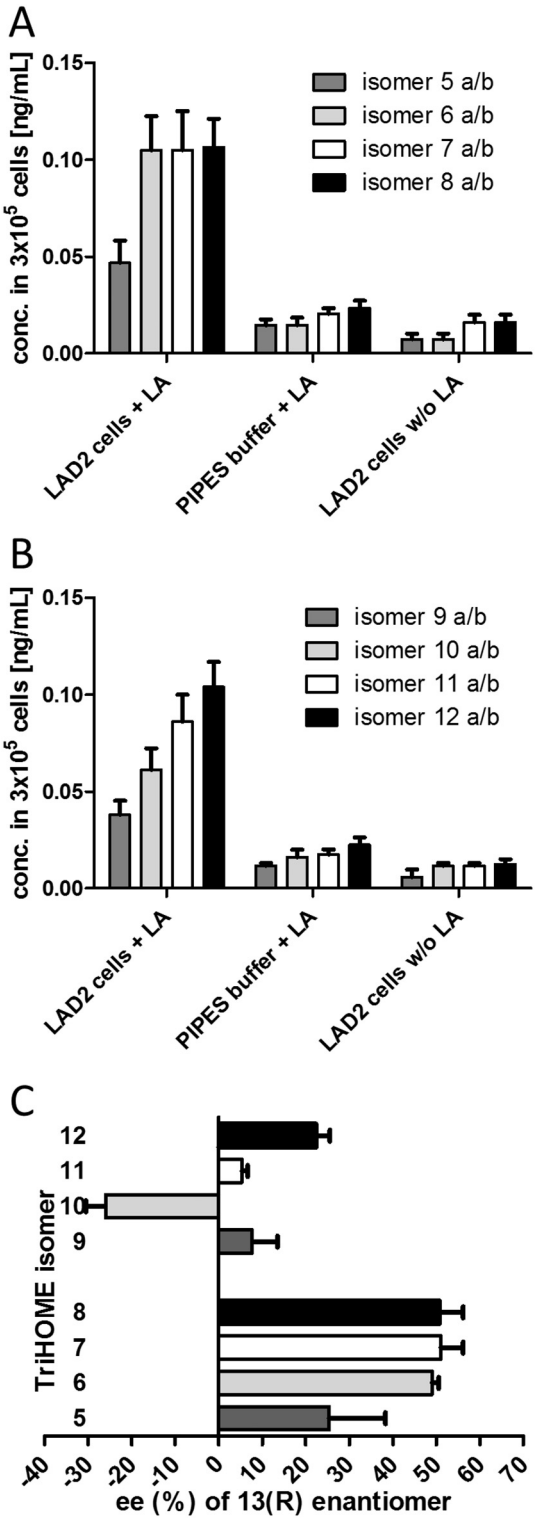

Fig. 7. Analysis of TriHOMEs formed by the mast cell line LAD2; (A) 9,10,13-TriHOME diastereomer concentrations. (B) 9,12,13-TriHOME diastereomer concentrations. (C) The enantiomeric excess (ee) of TriHOMEs with an (R) configuration at carbon 13. A total of 3 × 10^5 LAD2 cells were incubated for 30 min with 10 μg/ml linoleic acid as substrate. Quantification of TriHOME diastereomers was performed by RP-UHPLC-MS/MS. The ee was determined by chiral HPLC-MS/MS and was calculated using the formula: ee = (a − b) / (a + b) × 100, where a and b are the peak areas of the respective enantiomers. TriHOME isomer nomenclature is as shown in Fig. 1. Data represent average ± SEM (n = 3).

3.6. The mast cell line LAD2 produces TriHOMEs with a different stereochemical pattern than eosinophils

Given the importance of mast cells in chronic inflammation, we also investigated the potential of the mast cell line LAD2 to synthesize TriHOMEs. LAD2 cells incubated with linoleic acid were capable of synthesizing all measured TriHOMEs (Fig. 7A and B). However, under the same experimental conditions (incubation of 3 × 10^5 cells with 10 μg/ml of linoleic acid for 30 min), LAD2 cells produced TriHOMEs at much lower levels compared to eosinophils. In addition, LAD2 cell-derived 9,10,13-TriHOMEs exhibited a different stereochemical pattern compared to eosinophils (Fig. 7C), producing predominantly the 13(R) configuration. These findings suggest that TriHOME production in LAD2 cells occurs via a 15-LOX independent process. No clear pattern was observable for the 9,12,13-TriHOMEs. While for compound 12 the 13(R) configuration was in slight excess, the 13(S) isomers were in slight excess for compound 10. Compound 9 and 11 were essentially racemic.

4. Discussion

TriHOMEs are a class of oxylipins derived from linoleic acid that have recently been found to be dysregulated in respiratory disease [30–33]. They have also been reported to be enriched in atherosclerotic plaques [44], associated with lithium responsiveness in bipolar disorder [45], and suggested to be relevant to pain signaling and adaptation to chronic pain in pain circuits in the brain [46]. Their biosynthetic route in humans is however still largely unknown. Due to the importance of granulocytes in mediating inflammatory responses, we aimed to investigate the biosynthesis of TriHOMEs in human PMNs. Following isolation using Ficoll centrifugation, PMNs were incubated with linoleic acid and found to be capable of synthesizing both 9,12,13-TriHOMEs and 9,10,13-TriHOMEs in a substrate dependent manner (Fig. 2). PMNs obtained by Ficoll centrifugation are mainly composed of neutrophils (~95%) and eosinophils (~5%). Due to their high abundance, it was hypothesized that neutrophils were responsible for the measured TriHOME production, as has been previously suggested [33]. However, when neutrophils were isolated with a neutrophil isolation kit (which efficiently depletes eosinophils, Fig. S1), TriHOME production was almost completely abolished (Fig. 2). This finding suggested that the eosinophilic fraction accounted for the observed TriHOME production of PMNs. The ability of eosinophils to synthesize TriHOMEs was confirmed using cells isolated with an eosinophil isolation kit.

Most enzymatic reactions responsible for oxylipin production are enantioselective, while introduction of oxygen by ROS (autoxidation) results in a racemic product. Chiral analysis can therefore provide insight into the oxylipin synthetic route [47]. Recently, we developed a LC-MS/MS workflow that used chiral HPLC-MS/MS to differentiate all 16 TriHOME isomers [39]. Analysis of the eosinophil-derived TriHOME isomers by chiral chromatography found TriHOMEs with an (S) configuration at the C-13 position to be in enantiomeric excess (Fig. 4). 15-LOX can convert linoleic acid to 13(*S*)-HpODE, which is then readily reduced to 13-HODE [48], and eosinophils are known to express 15-LOX [49,50]. We accordingly hypothesized that TriHOME production in eosinophils involves 15-LOX activity. This hypothesis was investigated by pre-incubating eosinophils with the 15-LOX inhibitor BLX-3887 [51], which inhibited the formation of all measured TriHOME isomers (Fig. 5A and B). These experiments demonstrate that TriHOME production in eosinophils is a 15-LOX-dependent process. No enantiomeric pattern was observed for the alcohol groups at positions C-9 and C10 (for 9,10,13-TriHOMEs) or C-9 and C-12 (for 9,12,13-TriHOMEs), which argues against involvement of a lipoxygenase other than 15-LOX. The potential involvement of 12-LOX was ruled out by pre-incubating eosinophils with the 12-LOX inhibitor ML-355, which failed to inhibit TriHOME production (data not shown).

The 15-LOX product 13(*S*)-HpODE is a reactive compound that can

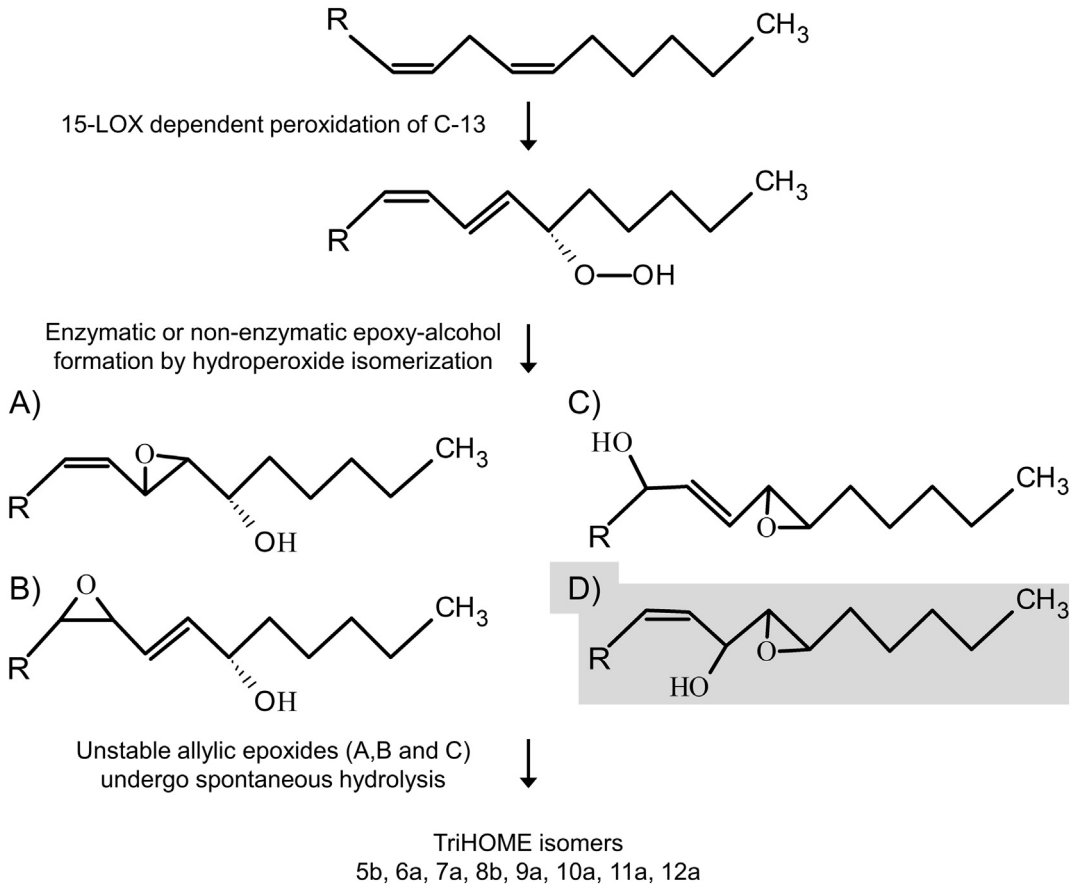

Fig. 8. Proposed biosynthetic pathway of TriHOMEs produced by eosinophils. Notably, compound D (indicated in grey) is a non-allylic and therefore a stable epoxy-alcohol. A major contribution of intermediate D to the overall formation of TriHOMEs is therefore unlikely. TriHOME isomer nomenclature is as shown in Fig. 1. R = $(CH_2)_7COOH$.

be readily converted to allylic epoxy-alcohols [52]. Such reactions to form epoxy-alcohols were described to involve cleavage of the O—O bond of the hydroperoxide followed by attack by the terminal hydroperoxide oxygen at a double bond leading to the formation of an epoxide ring (Fig. 8, compounds A and B). The so formed allylic epoxy-alcohols are labile compounds that readily undergo hydrolysis to form 9,10,13-TriHOMEs and 9,12,13-TriHOMEs [21]. In order to investigate if this conversion occurs in eosinophils, we substituted the substrate linoleic acid with either 13-HpODE or 13-HODE. We found that while eosinophil incubations with 13-HpODE led to TriHOME production, incubation with 13-HODE failed to form the measured TriHOMEs (Fig. 6). While it was not possible to directly measure allylic epoxy-alcohol intermediates (due to their instability), the ablation of TriHOME synthesis following use of 13-HODE as the substrate suggests that TriHOME synthesis in eosinophils involves oxygen transfer of a hydroperoxide oxygen that forms an epoxide ring at one of the double bonds. To further strengthen this hypothesis, future studies should include incubations with ^{18}O-labelled 13(*S*)-HpODE. Formation of epoxy-alcohols from 13-HpODE can also take place non-enzymatically in the presence of ferrous ion, hemoglobin or hematin [53,54] yielding compounds C and D (Fig. 8). A proposed biosynthetic pathway leading to the formation of TriHOMEs in eosinophils is illustrated in Fig. 8. It should be noted that glutathione peroxidase activity in freshly isolated eosinophils will rapidly reduce 13-HpODE to 13-HODE [55], shunting away the necessary substrate to form the reactive epoxides proposed in Fig. 8. This likely explains why the observed 13-HODE levels produced by eosinophils are 100-fold greater than the sums of both the 9,10,13-TriHOME isomers and 9,12,13-TriHOME isomers.

Autoxidation can also result in the formation of 9,10,13-TriHOMEs and 9,12,13-TriHOMEs [40,56,57]. However, the lack of TriHOME formation following eosinophil incubation with 13-HODE argues against a major role of ROS. It has been suggested that autoxidation can lead to significant TriHOME production under high levels of oxidative stress [39], such as experienced in COPD patients [58]. However, in the current study, ionophore activation (which leads to large amounts of ROS [59,60] did not lead to elevated TriHOME levels compared to non-activated eosinophils (Fig. 3). Therefore, these findings argue against a major contribution of autoxidative processes in the production of TriHOMEs in eosinophils.

In order to investigate alternative enzymatic routes of TriHOME formation in eosinophils, we also examined the role of the CYP450 inhibitor SKF 525A. CYP450 catalyzes the epoxidation of linoleic acid to form 9(10)-EpOME and 12(13)-EpOME (which are consequently metabolized by sEH into 9,10-DiHOME and 12,13-DiHOME) [61,62]. It has therefore previously been hypothesized that CYP450 dependent epoxidation of linoleic acid might be involved in the hydroxylation at positions C9 and C10 or C12 and C13, respectively [40]. Pre-incubation of eosinophils with SKF 525A did not lead to a significant decrease in TriHOME synthesis at the tested inhibitor concentration range (Fig. 5E and F). Furthermore, incubations of eosinophils with 13-HODE did not produce TriHOMEs, which further argues against an involvement of CYP450 driven epoxidation in the biosynthesis of TriHOMEs in eosinophils.

The sEH catalyzes the conversion of 9(10)-EpOME (leukotoxin) and 12(13)-EpOME (isoleukotoxin) into 9,10-DiHOME (leukotoxin-diol) and 12,13-DiHOME (isoleukotoxin-diol), respectively [12,63]. Studies on the physiological role of 9,10,13-TriHOME in maintaining the water-skin barrier identified sEH as crucial for the formation of

9(*R*),10(*S*),13(*R*)-TriHOME (compound 7b) in human skin [28]. We therefore hypothesized that sEH may also be involved in the formation of TriHOMEs in eosinophils. However, incubation of eosinophils with TPPU did not significantly affect TriHOME isomer production (Fig. 5C and D). This is in contrast to DiHOME formation, which was completely inhibited at the lowest tested TPPU concentration (Fig. S3). These findings argue against a major role of sEH in TriHOME production in eosinophils.

Mast cells are another important granulocyte that is involved in chronic inflammation and known to produce a wide range of oxylipins. We therefore investigated TriHOME production in the mast cell line LAD2, and indeed found them capable of synthesizing all measured 9,10,13-TriHOMEs and 9,12,13-TriHOMEs. However, the observed levels were approximately ten times lower compared to eosinophils. Importantly, the stereochemistry of TriHOMEs formed in LAD2 cells was different relative to eosinophils. LAD2 cells predominantly produced 9,10,13-TriHOMEs with an (R) configuration at the C-13 position. While the exact biosynthetic pathway of TriHOMEs in mast cells remains elusive, the results clearly suggest that TriHOME formation in LAD2 cells is a 15-LOX independent process. The most likely enzymatic source to produce the 13(R) stereochemistry is via CYP450 activity [64,65].

The last few decades have seen a shift in the western diet, with increases in unsaturated fat and in particular linoleic acid [1,2]. The potential effects upon the production of linoleic acid-derived lipid mediators and their associated bioactivity have not been well examined. Increased dietary linoleic acid has been demonstrated to increase circulating levels of the TriHOMEs and other linoleic acid-derived mediators [66]. While the physiological functions of many oxylipins are well established, little is yet known about the bioactivity of TriHOMEs. The current study demonstrates that TriHOMEs are readily formed by eosinophils, suggesting that inflammatory processes involving eosinophil recruitment represent a promising target for investigating TriHOME bioactivity. This is also potentially relevant to the hydroxy-epoxy-octadecenoates, which have been reported to exert bioactivity in pain and itch models [15] and are likely further metabolized to the TriHOMEs. While 9,10,13-TriHOME and 9,12,13-TriHOMEs represent by far the most well described TriHOME isomers, other structural isomers including 9,10,11-TriHOME and 11,12,13-TriHOMEs have also been reported [22,64]. Because their biosynthetic origin and physiological relevance are largely unknown, future research on TriHOMEs should also extend to the 9,10,11-TriHOMEs and 11,12,13-TriHOMEs isomers. In addition, it would be of interest to investigate the synthetic route of the alpha linolenic acid analogs of the TriHOMEs, the trihydroxyoctadecadienoic acids (TriHODEs). Taken together, these findings expand our knowledge of the linoleic acid cascade and demonstrate for the first time that TriHOMEs can be formed enzymatically in human granulocytes. Given the role of eosinophils and mast cells in the pathophysiology of lung disease, these data suggest that the linoleic acid pathway warrants further study.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2020.158611.

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