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COX-1 dependent biosynthesis of 15-hydroxyeicosatetraenoic acid in human mast cells

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ABSTRACT

15-hydroxyeicosatetraenoic acid (15-HETE) is an arachidonic acid derived lipid mediator which can originate both from 15-lipoxygenase (15-LOX) activity and cyclooxygenase (COX) activity. The enzymatic source determines the enantiomeric profile of the 15-HETE formed. 15-HETE is the most abundant arachidonic acid metabolite in the human lung and has been suggested to influence the pathophysiology of asthma. Mast cells are central effectors in asthma, but there are contradictory reports on whether 15-HETE originates from 15-LOX or COX in human mast cells. This prompted the current study where the pathway of 15-HETE biosynthesis was examined in three human mast cell models; the cell line LAD2, cord blood derived mast cells (CBMC) and tissue isolated human lung mast cells (HLMC). Levels and enantiomeric profiles of 15-HETE and levels of the downstream metabolite 15-KETE, were analyzed by UPLC-MS/MS after stimulation with anti-IgE or calcium ionophore A23187 in the presence and absence of inhibitors of COX isoenzymes. We found that 15-HETE was produced by COX-1 in human mast cells under these experimental conditions. Unexpectedly, chiral analysis showed that the 15(*R*) isomer was predominant and gradually accumulated, whereas the 15(*S*) isomer was metabolized by the 15-hydroxyprostaglandin dehydrogenase. We conclude that during physiological conditions, i.e., without addition of exogenous arachidonic acid, both enantiomers of 15-HETE are produced by COX-1 in human mast cells but that the 15(*S*) isomer is selectively depleted by undergoing further metabolism. The study highlights that 15-HETE cannot be used as an indicator of 15-LOX activity for cellular studies, unless chirality and sensitivity to pharmacologic inhibition is determined.

1. Introduction

15(*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15(*S*)-HETE) is the major oxidative metabolite of arachidonic acid (AA) in asthmatic and normal lung tissue [1–3] and asthmatic airways [2]. The main source of 15-HETE in human airways is from 15-lipoxygenase (15-LOX) activity of the epithelium [4–6]. The levels of 15-HETE in airways have been found higher in asthmatics [1,7–9] and its release can be enhanced by antigen challenge [10]. In human bronchial tissue, 15-HETE is measured at similar levels as the major mast cell mediator PGD_2 after antigen challenge [11]. The view that 15(*S*)-HETE has a role in the pathogenesis of asthma is consistent with the upregulation of the 15-LOX that follows exposure to the type 2 cytokine IL-4 [12]. In line with this association, 15-HETE which is also biosynthesized in eosinophils, is increased five-fold from this source in severe asthmatics compared to mild asthmatics or healthy volunteers [7,8]. We have previously reported that 15-HETE was recovered at significant levels after challenge of human mast cells with hyperosmolar mannitol to

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mimic the reaction that causes exercise-induced bronchoconstriction [13].

Whereas 15-HETE undoubtedly is biosynthesized in contexts with relevance to asthma, the biological role of 15-HETE itself remains unclear. The literature contains a range of reported activities including potential pro- or anti-asthmatic functions, but as a whole, most observed effects of 15-HETE itself are fairly modest. 15-HETE has rather been perceived as a marker of 15-LOX activity which is involved in several different biochemical pathways leading to biosynthesis of bioactive molecules such as lipoxins [14], eoxins [15], and Specialised Pro-resolving Mediators (SPMs) [16] generated by oxygenation of AA as well as other membrane-stored Poly-Unsaturated Fatty Acids (PUFAs). Accordingly, detection of 15-HETE is generally regarded as evidence of the presence of 15-LOX activity in that particular system.

This communication is the result of unexpected findings when profiling the pattern of lipid mediator biosynthesis in human lung mast cells. First, we found significant levels of 15-HETE, but the formation was blocked by cyclo-oxygenase (COX) inhibitors. It is known that the COX reactions as by-products generate 11-HETE, 15(*S*)-HETE and 15(*R*)-HETE [17,18], which seemed to explain the finding. However, when performing UPLC-MS/MS using chromatographic separation by chirality, the next surprise was that essentially only the 15(*R*)-enantiomer was identified. Previous studies have consistently found that catalysis by COX results in 70% of the *S*-enantiomer and 30% of *R* [17].

We therefore performed this in-depth study to resolve why activation of human mast cells resulted in the accumulation of 15(*R*)-HETE. Another reason for the study was that the few previous studies of 15-HETE formation in human mast cells had produced conflicting data using different types of human mast cell lines and different means of activation [10,13,19]. The investigation therefore included three different human mast cell models; cord blood derived mast cells (CBMC), the mast cell leukemia cell line LAD2, and isolated human lung mast cells (HLMC), and cells were stimulated both in an IgE-dependent and independent fashion. The results explain how COX-1-dependent biosynthesis of 15-HETE in human mast cells results in the appearance of 15(*R*)-HETE.

2. Materials and methods

Cell culture and preparation.

LAD2 cells (kindly provided by Dr. Kirshenbaum and Dr. Metcalfe, NIH) were cultured in Stem-PRO medium (2.5% Stem-pro complement, 2 mM L-Glutamine, 1% penicillin-streptomycin and 100 ng/mL stem cell factor ((SCF) kindly provided by SOBI, Stockholm, Sweden). Cell culture medium was hemidepleted every week.

CBMC were developed from $CD34^+$ progenitors isolated from cord blood using CD34 MicroBead kit (Miltenyi Biotec) as previously described [20]. Mast cell maturity and purity was evaluated by staining for the mast cell-specific protease tryptase, of which >90% was defined as mature and ready for experiments.

With permission of the regional ethical review board in Stockholm (reference no. 2018/1819–31/1), macroscopically healthy human lung tissue was obtained by consent from patients undergoing lobectomy and HLMC isolated as in [21]. In short, tissue was cut to small pieces which were washed in PBS to remove loosely associated cells, the remaining tissue was digested with collagenase type 2 and DNase and the tissue passed through a syringe. Suspension was cleared by percoll centrifugation and red blood cells removed by ACK lysis. The remaining cells were cultured overnight (without SCF) to prevent clogging in the next step and the cells were further purified using a CD117 Micro Bead kit and MACS column (Miltenyi Biotec) to enrich for mast cells. The purified cells were recovered in RPMI-1640 medium (Gibco) supplemented with 100 ng/mL SCF 10% fetal bovine serum, 0.01 M HEPES, 0.5× non-essential amino acids, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/ml streptomycin for 4–7 days before the experiment. Bronchi were prepared as in [22].

Cell assays.

CBMC were primed with IL-4 (10 ng/mL, Peprotech) for 4 days before experiment and exposed one day pior to IgE (1 μg/mL, Millipore). HLMC were only preincubated with IgE 1 μg/mL O/N. Before the assay, cells were washed with PBS and resuspended in PIPES-BSA (9.2 mM PIPES, 139.7 mM NaAc, 0.6 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 0.2% BSA, Sigma-Aldrich). Cells were used at 200,000 per sample. Before stimulation by IgE-receptor cross-linking or calcium ionophore A23187, cells were incubated 15 min in the absence or presence of inhibitors, which included FR122047 (selective COX-1 inhibitor, 1 μM, Cayman Chemicals), etoricoxib (selective COX-2 inhibitor, 1 μM, Merck Research Laboratories), indomethacin 10 μM, (nonselective COX inhibitor, 10 μM, Sigma-Aldrich) 15-PGDH inhibitor Cay10397 (10 μM, Cayman Chemicals). Inhibitor concentrations were chosen based on our previous experience and data from the literature. LAD2 cells were stimulated by the addition of 1 μM A23187 for 30 min. CBMC and HLMC were washed and stimulated by addition of 2 μg/mL of anti-IgE (Sigma Aldrich) for 30 min. For exogenous addition of 15-HETE and 15-KETE (Cayman Chemicals) the lipid mediator was added to the cells at 40 ng/mL. After the assay, cells were spun down and supernatant stored at -80 °C until solid phase extraction (SPE) and analysis by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) which was done as in [11]. Bronchi were dissected immediately before the experiment and put directly in PIPES-BSA with or without 15-LOX inhibitor (3 μM BLX3887 or COX-1/2 inhibitor Indomethacin 10 μM). Supernatant was frozen after 30 min of incubation at 37 °C, 5% CO_2.

Western blot.

Cells were lysed in Laemmli sample buffer (Bio-Rad) containing β-mercapto ethanol and $1.5*10^5$ cells of dispersed lung (cells remaining after ACK lysis, see above) incubated for 22 h with or without 100 ng/mL LPS and $6.7*10^4$ HLMC and CBMC and $2*10^5$ LAD2 cells lysed and loaded/lane. Protein was separated on a 12% TGX™ Precast gels (Bio-Rad) and then transferred to a PVDF membrane. Primary antibodies were all diluted 1:200 (St Cruz biotechnology) in 5% skim milk in TBST and incubated for 1 h at room temperature or overnight at 4 °C. For detection a secondary anti mouse-IgGκ BP-HRP antibody (St Cruz biotechnology) was used diluted 1:1000 and incubated for 45 min at room temperature after which protein bands were visualized with enhanced chemiluminescence detection (Thermo Fisher).

Statistics

For single time points statistical analysis was done using one-way ANOVA with Dunnets correction for multiple comparisons and for time series 2-way ANOVA was used with Tukey correction for multiple comparisons. Graphs were prepared and statistical analysis was performed in the GraphPad Prism software v8.3.

3. Results and discussion

15-HETE release by human mast cells.

Mast cells were stimulated by anti-IgE or A23187 to assess the biosynthesis and release of 15-HETE. Since there are contradictory results in the literature, it was considered important to present data from more than one human mast cell model. HLMC and CBMC were stimulated by IgE-receptor crosslinking which mimics the allergen response (Fig. 1A-C; G and H respectively) and LAD2 by ionophore A23187 (Fig. 1D-F) to evaluate a different stimulus for cell activation. In addition, the LAD2 cells have a low response to IgE activation [23] which is another reason why ionophore was used in these cells to achieve higher levels of lipid mediator release. Release of 15-HETE and 15-KETE in the absence of added arachidonic acid was determined by UPLC-MS/MS. All three cell types responded by releasing 15-HETE (Fig. 1A, D and G) with a corresponding increase in the downstream metabolite 15-KETE (Fig. 1B, E and H). Levels of 15-HETE in LAD2 cells were low, with a relatively poor reduction after COX-1 inhibition compared to CBMC and HLMC, which suggests low levels of 15-HETE also being produced non-enzymatically in the LAD2 cells.

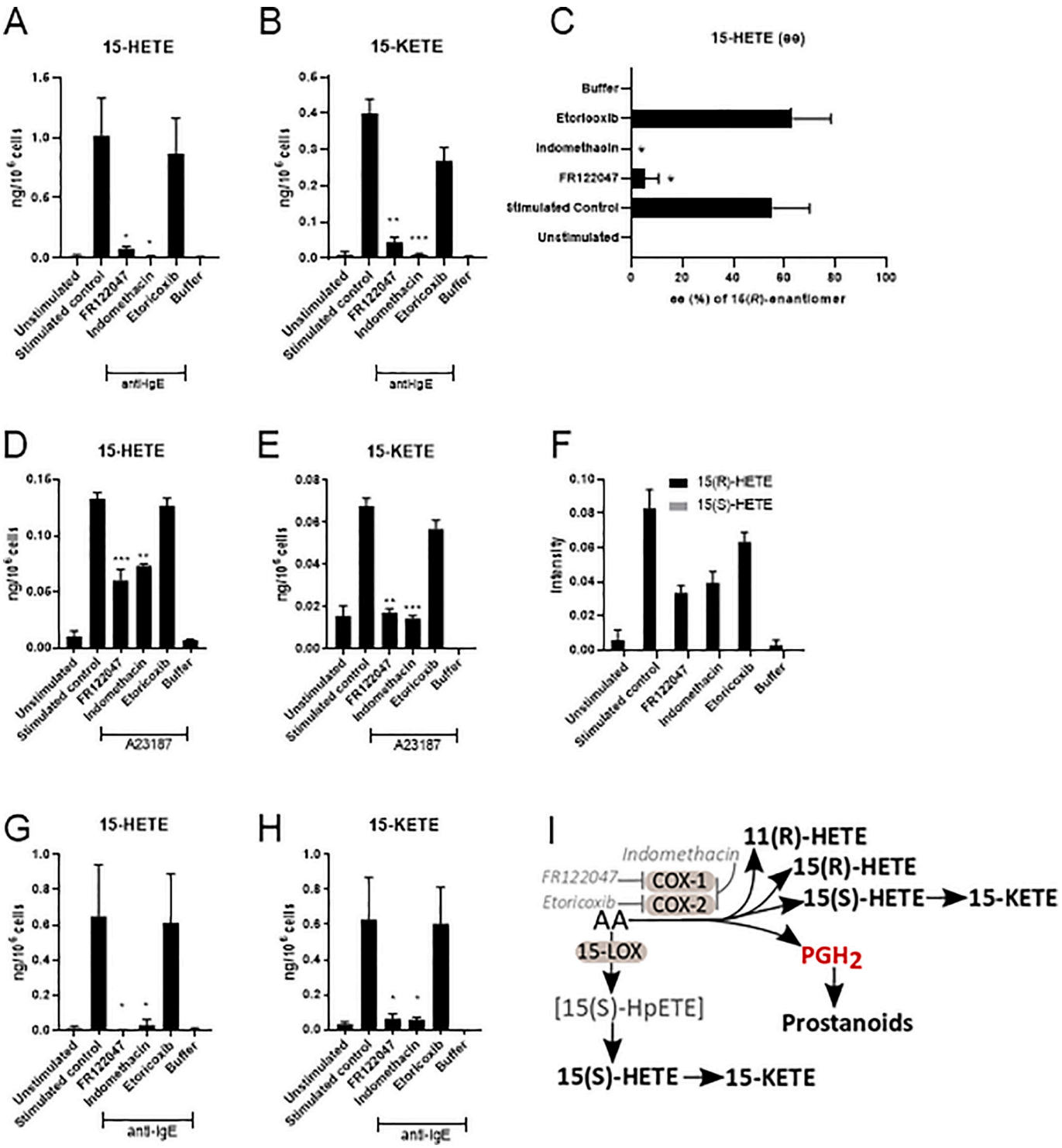

Fig. 1. 15-HETE is produced by COX-1 in human mast cells. Mast cells were stimulated with or without COX inhibition and levels of 15-HETE and its downstream metabolite 15-KETE measured by UPLC-MS/MS. Levels of 15-HETE (A), 15-KETE (B) and enantiomeric excess (ee) of 15*(R)*-HETE (C) released by HLMC after IgE-receptor cross-linking. Levels of 15-HETE (D), 15-KETE (E) and enantiomers of 15-HETE (F) released by LAD2 after 1 μM of A23187 stimulation. Levels of 15-HETE (G) and 15-KETE (H) released by CBMC after IgE-receptor cross-linking. Data from three experiments (two experiments in F). Values are expressed as mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significance is relative to stimulated control. Schematic of pathways for 15-HETE production (I), displaying analytes of interest for this study. Abbreviations: arachidonic acid (AA), cyclooxygenase (COX), 15-lipoxygenase (15-LOX), Prostaglandin H_2 (PGH_2), 15-hydroperoxyeicosatetraenoic (15-HpETE), 15-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15-HETE), 15-keto-*5,8,11-cis-13-trans-eicosatetraenoic acid* (15-KETE, 11-Hydroxy-5,8,14-*cis*-12-*trans*-eicosatetraenoic acid (11-HETE). For comparison, levels of the main prostanoids are shown in Table 1 for each of the mast cell types, documenting that the level of 15-HETE in stimulated cells consistently was higher than those of PGE_2.

Table 1
Levels of 15-HETE, 15-KETE and main prostaniods. The values are expressed in pg/10^6 cells ± SEM, $n = 3$.

	HLMC		LAD2		CBMC	
	Unstimulated	Stimulated (IgE-XL)	Unstimulated	Stimulated (A23187)	Unstimulated	Stimulated (IgE-XL)
15-HETE	18 ± 9	1016 ± 311	10 ± 5	133 ± 5	13 ± 16	649 ± 292
15-KETE	10 ± 8	400 ± 40	16 ± 5	68 ± 4	32 ± 16	630 ± 242
PGD_2	241 ± 120	58,937 ± 11,005	13 ± 6	5345 ± 130	2687 ± 2083	66,344 ± 15,063
PGE_2	204 ± 127	452 ± 282	6 ± 1	14 ± 4	39 ± 21	178 ± 26
TXB_2	58 ± 11	4824 ± 1767	0 ± 0	0 ± 0	478 ± 297	2320 ± 797

COX inhibitors were then used to delineate the possible enzymatic pathways for 15-HETE formation, see schematics in Fig. 1I. 15-HETE release was almost completely abolished by the COX-1 inhibitor FR122047 (1 μM) and the dual COX-1 and 2 inhibitor indomethacin (10 μM), whereas the COX-2 inhibitor etoricoxib (1 μM) had no effect (Fig. 1A, D and G). This contrasts to the previous finding that stimulation with exogenous AA or mannitol resulted in the formation of 15-HETE and 15-KETE despite pretreatment with indomethacin [13]. To study the enantiomeric profile of the 15-HETE release, UPLC-MS/MS utilizing chiral separation was used. Surprisingly, we found an enantiomeric excess of 15(*R*)-HETE being released by all three cell types, HLMC shown in (Fig. 1C) and LAD2 in (Fig. 1F; relative intensity is shown since only the *R*-enantiomer was detected). This agrees with the first interpretation of results from the experiments with pharmacologic inhibitors and confirms that 15-LOX could not be the enzyme responsible for the 15-HETE detected here, since 15-LOX produces only 15(*S*)-HETE and is not blocked by COX inhibitors. Nevertheless, the result of the analysis was intriguing. Aspirin- treated (acetylated) COX-2 solely makes 15(*R*)-HETE, but native COX-1 and COX-2 are known to make 70% (*S*) and 30% (*R*) during the oxygenation reaction [17,24,25]. The pharmacologic inhibitors unquestionably documented that 15-HETE was generated in a COX-1 catalysed reaction.

To compare the 15-HETE enantiomeric profile with that of 15-HETE known to be derived from 15-LOX activity, we also examined 15-HETE release by freshly isolated intact human bronchi. The bronchi spontaneously released 0.2 ± 0.1 ng/mL (mean ± SEM) 15-HETE after 30 min of incubation with 69.6 ± 10.4% enantiomeric excess of 15(*S*)-HETE. The release was 15-LOX dependent; since the 15-LOX inhibitor BLX3887 [26] (3 μM) gave a 50 ± 7.9% inhibition and there was no effect of COX-1/2 inhibition by indomethacin.

Origin of 15(*R*)-HETE excess.

Next, we wanted to find out why we detected 15(*R*)-HETE to be in excess. To examine whether the excess of 15(*R*)-HETE was produced from the downstream metabolite 15-KETE which lacks chirality, 40 ng/mL exogenous 15-KETE was added to LAD2 (Fig. 2A-C) and HLMC (data not shown). This indeed led to some conversion into 15-HETE that only took place in the presence of cells. The chirality of the 15-HETE formed showed that it was the *S*- and not the *R*-enantiomer being formed. Hence, 15-KETE conversion is not the cause for 15(*R*)-being in excess due to a reversed metabolic process.

As an explanation, we hypothesized that rate of metabolization was different for the two enantiomers. To test this hypothesis, a racemic mixture of 15-HETE was added to incubations with and without CBMC for the indicated time points (Fig. 3A-C). Here a time dependent decrease of 15-HETE was seen (Fig. 3A), as well as a corresponding increase in 15-KETE (Fig. 3B). Enantiomerically, it was only 15(*S*)-HETE which decreased, indicating that selective metabolization of 15(*S*)-HETE (the major COX-1 generated enantiomer) was the cause for the 15-HETE enantiomeric profile seen in our samples. The metabolization of 15(*S*)-HETE and stability of 15(*R*)-HETE was the same whether the 15-HETE enantiomers was added together (Fig. 3A-C) or separately (data not shown).

We hypothesized that 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) was responsible for the 15(*S*)-HETE conversion, because this has been observed in human monocytes. Indeed, when 15-PGDH was inhibited by 10 μM of Cay10397, exogenous 15-HETE was metabolized less rapidly with reduced levels of 15-KETE being formed by LAD2 cells (Fig. 4A and B). In HLMC a reduction of 15-KETE production was seen at 30 min (Fig. 4C and D), which was the only time point examined. Inhibition of 15-PGDH also led to reduced formation of 15-KETE in CBMC

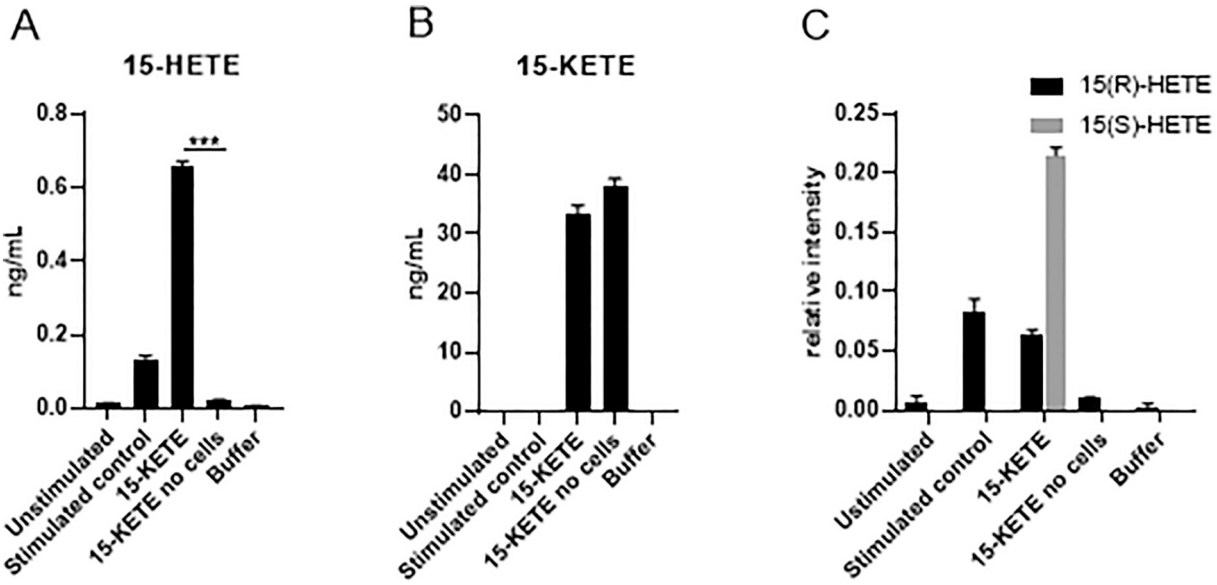

Fig. 2. 15-KETE metabolism measured in LAD2 Cells. 40 ng/mL 15-KETE was added to cells for 30 min and levels of 15-HETE (A), 15-KETE (B), and enantiomeric profile of 15-HETE (C) was analyzed. Data from two experiments presented as mean ± range.

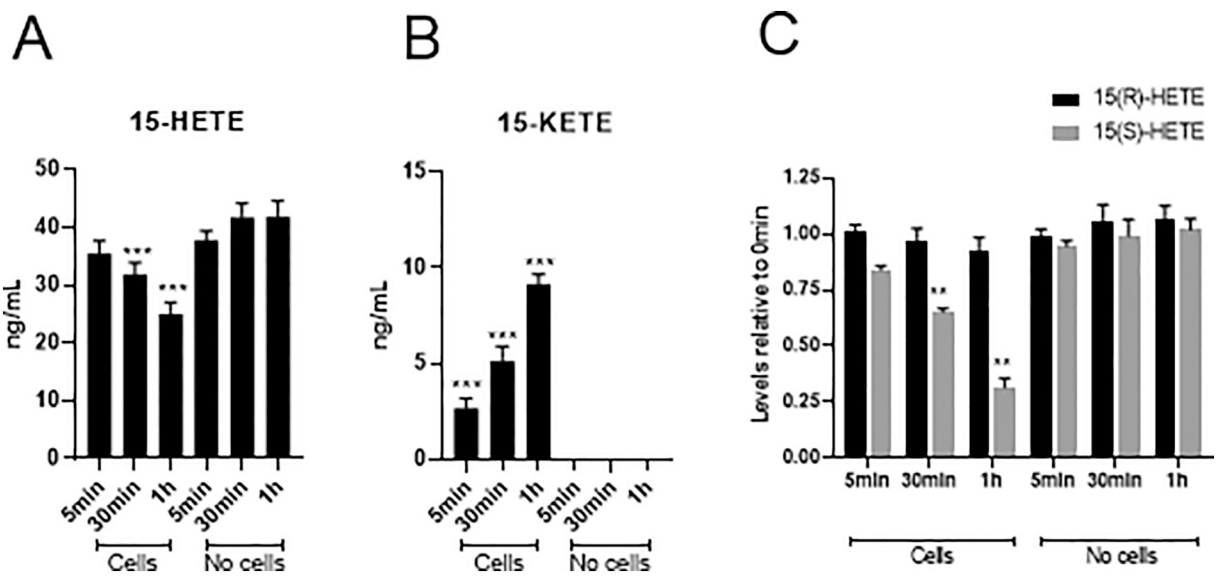

Fig. 3. 15(*S*)-HETE is metabolized to 15-KETE. (A-C), CBMC were exposed to 40 ng/mL exogenous 15(*R/S*)-HETE for the indicated time points. Samples were analyzed by UPLC-MS/MS. Levels of total 15-HETE (A) and levels of 15-KETE (B) is shown. Chiral separation was used to distinguish between enantiomers of 15-HETE (C). Significance is relative to respective 'no cells control', data from four donors is presented as mean ± SEM.

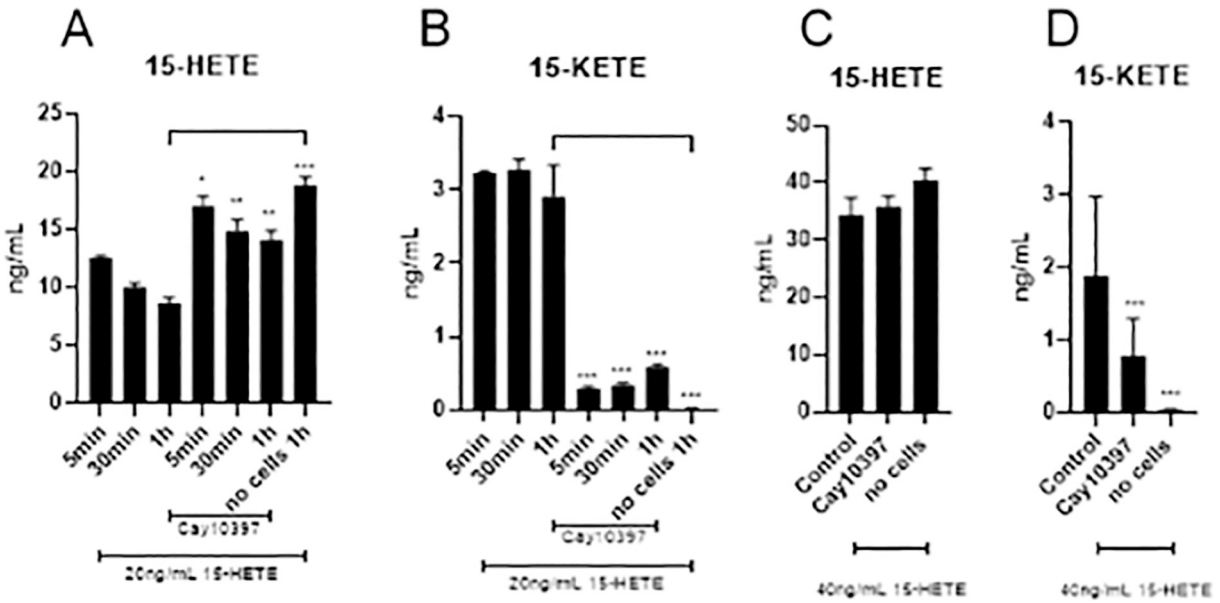

Fig. 4. Inhibition of 15-PGDH prevents 15(*S*)-HETE conversion. Exogenous 15-HETE was added at a concentration of 20 ng/mL to LAD2 for the indicated times in (A) and (B), and at 40 ng/mL to HLMC for 30 min in (C) and (D) with or without preincubation of the 15-PGDH inhibitor Cay10397 (10 μM). Levels of 15-HETE shown in (A and C) and 15-KETE (B and D). Data from three experiments (A, B) and three donors (C, D) and presented as mean ± SEM. Statistical significance is relative respective time control, in D statistics was calculated on normalized data, while the absolute values are shown in the graph.

(data not shown). This indicates that cells expressing 15-PGDH could efficiently convert 15(*S*)-HETE to 15-KETE which is in line with the stereospecific effects of 15-PGDH [27].

This study was performed in cell models where human mast cells are either cultured for a longer time (LAD2 and CBMC) or extracted from tissue and then recovered in culture over a few days (HLMC). A possible limitation is that this could impact basal levels of 15-LOX expression and therefore give an overestimation of the COX dependence impacted by culturing of the cells. In order to further substantiate the findings, the enzyme expression of the cells included in this communication are shown in a western blot (Fig. 5). We observed a high expression of COX-1 in all three types of mast cells, with HLMC showing the strongest expression. COX-2 was only observed in the positive control, which was a suspension of lung cells incubated with LPS. 15-PGDH was detected in all of the mast cells, with highest levels in LAD2 and lowest in HLMC, which is consistent with the observation of 15-PGDH dependent metabolization of 15-HETE in Fig. 4. Rather surprisingly HLMC had high levels of 15-LOX, while in CBMC only low levels of 15-LOX were detected in one case out of three and no 15-LOX was detected in LAD2. Our interpretation is therefore that human mast cells do not metabolise AA in 15-LOX catalysed reactions in response to activation by IgE or Ionophore, regardless of the presence of 15-LOX. We have previously published that addition of arachidonic acid to CBMC gives rise to 15-LOX activity [15]. It has also been shown that endogenous hydroxyeicosatetraenoic acids stimulate the human polymorphonuclear leukocyte 15-lipoxygenase pathway [28]. However, it remains to be

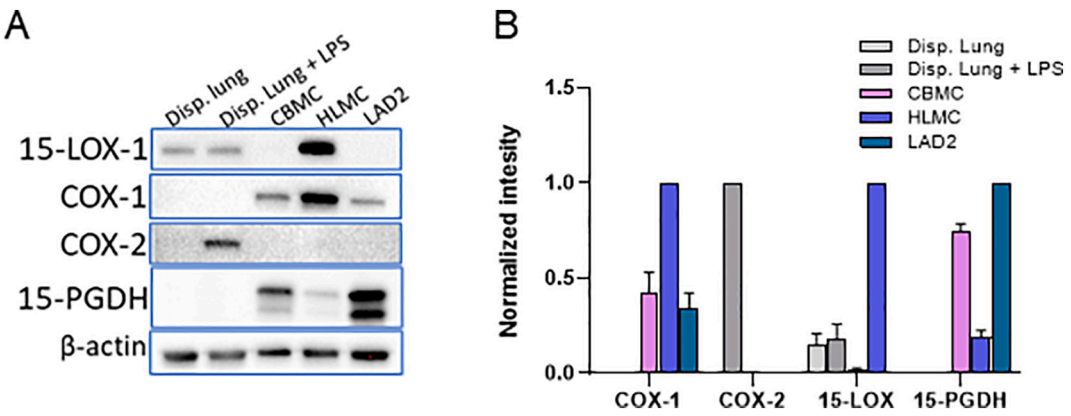

Fig. 5. Western blot of the enzymes of interest. In (A) representative blot showing the indicated enzymes as well as β-actin for loading control. Lane 1 and 2, lysate of dispersed lung (normally used for mast cell isolation) was incubated 22 h with or without 100 ng/mL LPS as indicated, as a control for COX-2 expression; lane 3 CBMC kept for 4d with IL-4, as was routine before each experiment; lane 4, HLMC (92% pure population); lane 5 LAD2 cells. In (B) Densitomitry analysis of 2–3 blots, bars represent SEM, values are adjusted for loading according to β-actin and normalized to highest value for each protein.

determined under what types of endogenous stimuli the 15-LOX in mast cells is active.

The physiological or pathological relevance of COX-1 derived 15-HETE in mast cells is not known. The metabolite 15-KETE is reported to have a role in the pathogenesis of atherosclerosis [29], and possibly also to protect against remodeling during pulmonary arterial hypertension [30] and to reduce macrophage derived cytokine expression by inhibiting NFκB [31]. Also worth mentioning is that 15(*R*)-HETE can be converted to pro-resolving 15-*epi*-lipoxin A_4, which may be responsible for parts of the anti-inflammatory effects seen by aspirin [32]. The data presented here suggests that inhibition of COX-1 could then block the formation of 15-*epi*-lipoxin A_4. There is still a lot to investigate about the functions of 15-HETE and downstream metabolites in inflammatory diseases. There are a range of reported activities arguing for both positive and negative effects being exerted by 15-HETE, partly relating to its downstream metabolites. 15-HETE is a weak contractile agonist in isolated human bronchi [33], however clinical data suggest that 15(*S*)-HETE has no direct bronchoconstrictive activity during inhalation [34]. It has been reported that it may inhibit bronchoconstriction in response to histamine and methacholine [34], and enhance the bronchoconstriction in response to allergen challenge [35], but the effects were small and have not been replicated. A potential role for 15-HETE in asthma could be in remodeling, as 15-HETE was found to recapitulate the effects IL-13 in airway epithelial cells [36]. There are also reports that 15-HETE is a potent stimulus for mucous secretion in human airways [36]. Recent work suggests a general function in the regulation of ferroptosis and cell death [37], returning to earlier observations suggesting a role for 15-HETE in the local control of membrane trafficking [38].

4. Conclusions

This study shows the importance of applying interventions with selective pharmacologic tools and using chiral analysis when assessing eicosanoid pathways. Although 15-LOX is known to be the main enzymatic pathway for the generation of 15-HETE in airway epithelium [4–6], this was not the case in the three different human mast cells upon IgE-receptor activation or calcium ionophore stimulation. In contrast, COX-1 was the enzymatic source of 15-HETE in the three mast cell preparations of this study. This demonstration, which agrees with findings in the mast cell line HMC-1 [19], has a general relevance, i.e. measuring 15-HETE without pharmacologic interventions and in the absence of chiral analysis does not prove that appearance of 15-HETE is an indicator of 15-LOX activity. This is particularly relevant if the measurement of 15-HETE has been made with an 15(*S*)-HETE selective immunoassay since the presence of *S*-isomer does not exclude the presence of *R*-isomer. The results also raise the possibility that some therapeutic effects of NSAIDs might relate to reduce levels of 15-HETE and 15-KETE in addition to the established effects on prostaglandin biosynthesis. Another important implication of this study is that the appearance of a particular compound should not only be related to upstream effects but also to downstream metabolism. We thus could explain that the excess of 15(*R*)-HETE was because the 15-PGDH catalysed stereoselective metabolism of 15(*S*)-HETE camouflaged that the two stereoisomers in fact were formed in parallel. Finally, some of the authors have previously published clear evidence that human CBMC when activated by exogenous arachidonic acid or mannitol indeed release 15-HETE in 15-LOX catalysed reactions [13]. This is not a contradiction of the current results but rather underpin how stimulus-dependent biosynthesis of eicosanoids is. Exogenous arachidonic acid thus being a particularly effective stimulus for activation of the 15-LOX [15,39]. It is interesting to note that the same signaling molecule 15-HETE may be produced along two different pathways, suggesting that it has important cellular functions that remain to uncover.

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CRediT authorship contribution statement

AKJ: Conceptualization, Investigation, Methodology, Data curation, Formal analysis Writing- Original draft preparation, **ER**: Conceptualization, Methodology, **DF:** Conceptualization, Methodology Data curation, **JK**: Methodology, Investigation, **JS:** Methodology, **HEC:** Resources, Writing - review & editing, **MH**: Conceptualization, Investigation, Reviewing and Editing, **CW**: Conceptualization, Resources, Writing - review & editing, **GN** Conceptualization, Investigation,

Writing - review & editing, **SED**: Conceptualization, Investigation, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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