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On the biosynthesis of specialized pro-resolving mediators in human neutrophils and the influence of cell integrity

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ABSTRACT

Neutrophils are key players in inflammation initiation and resolution. Little attention has been paid to the detailed biosynthesis of specialized pro-resolving mediators (SPM) in these cells. We investigated SPM formation in human polymorphonuclear leukocytes (PMNL), in broken PMNL preparations and recombinant human 5-lipoxygenase (5-LO) supplemented with the SPM precursor lipids 15-Hydroxyeicosatetraenoic acid (15-HETE), 18-Hydroxyeicosapentaenoic acid (18-HEPE) or 17-Hydroxydocosahexaenoic acid (17-HDHA). In addition, the influence of 5-LO activating protein (FLAP) inhibition on SPM formation in PMNL was assessed.

Intact human PMNL preferred ARA over DHA for lipid mediator formation. In contrast, in incubations supplemented with the SPM precursor lipids DHA-derived 17-HDHA was preferred over 15-HETE and 18-HEPE. SPM formation in the cells was dominated by 5(*S*),15(*S*)-diHETE (800 pmol/20 mio cells) and Resolvin D5 (2300 pmol/20 mio cells). Formation of lipoxins (<10 pmol/20 mio cells), E-series (<70 pmol/20 mio cells) and other D-series resolvins (<20 pmol/20 mio cells) was low and only detected after addition of the precursor lipids. Upon destruction of cell integrity, formation of lipoxins and 5(*S*),15(*S*)-diHETE increased while formation of 17-HDHA- and 18-HEPE-derived SPMs was attenuated. Recombinant 5-LO did not accept the precursors for SPM formation and FLAP inhibition prevented the formation of the 5-LO-dependent SPMs. Together with the data on FLAP inhibition our results point to unknown factors that control SPM formation in human neutrophils and also render lipoxin and 5(*S*),15(*S*)-diHETE formation independent of membrane association and FLAP when cellular integrity is destroyed.

1. Introduction

Specialized pro-resolving mediators (SPM) are a group of polyhydroxylated polyunsaturated fatty acid (PUFA) derivatives which are thought to play an integral part in the resolution of inflammation. Together with pro-inflammatory lipid mediators such as leukotrienes and prostaglandins, SPM are released from immune cells at specific time points during the time-course of zymosan-induced peritonitis in mice - a model system for inflammation resolution. Here, SPMs repress the invasion of neutrophils into the inflamed tissue at the same time promoting the influx of monocytes, stimulate efferocytosis of dying neutrophils by macrophages and coordinate the egress of the phagocytes into the lymphatics thus restoring tissue function and homeostasis resulting in resolution of inflammation [1]. A large body of evidence

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LO, lipoxygenase; HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HDHA, hydroxydocosahexaenoic acid; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; LT, leukotriene; LLOQ, lower limit of quantification; PMNL, polymorphonuclear leukocyte; PUFA, polyunsaturated fatty acid; SPM, specialized pro-resolving mediator; FLAP, 5-LO activating protein.

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from animal models shows that exogenous application of individual SPMs such as D-series resolvins (RvD), protectin D1 and lipoxins can positively influence resolution and ameliorate disease severity [2]. Even though these data are promising, little attention has been paid to the details of SPM biosynthesis in humans in the past. The in vivo half live and distribution of SPMs is rather short which rules out direct therapeutic application [3–5]. Therefore, for the successful development of resolution supportive therapies, the in-depth knowledge of the participating cells and the molecular details of SPM biosynthesis in humans is important.

SPMs have been detected in various fluids and tissues of the human body such as blood, breast milk, adipose and muscle tissues, spleen and lymph nodes and have been inversely correlated with disease severity in some studies, for review see [6]. Furthermore, SPMs were found in human blister exudates during resolution in an experimental model of skin inflammation [7,8]. However, reported SPM concentrations in humans are often very low which renders SPM research an analytically challenging issue.

SPMs are either formed from the ω-6 PUFA arachidonic acid (ARA) or the ω-3 PUFAs eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) by sequential oxidation steps. These steps are carried out by 5-lipoxygenase (LO) together with 12-LO or 15-LO. Alternatively, formation routes of the intermediates can also involve cytochrome P450 enzymes, (acetylated) cyclooxygenase-2 or autoxidation [9,10]. Fig. 1 shows the proposed biosynthetic pathways for SPMs in detail.

5-LO also plays an integral part in the biosynthesis of pro-inflammatory leukotrienes from ARA [11]. What controls the switch from pro-inflammatory to pro-resolving lipid mediator formation by 5-LO remains elusive so far. It has been discussed that 5-LO operates in the cytosol of macrophages during SPM formation instead of translocation to the nucleus which is crucial for leukotriene formation [12]. This would be a way to separate pro-inflammatory from pro-resolving lipid mediator formation in leukocytes. However, we could recently show that the 5-LO activating protein (FLAP) is essential for LXA_4 and RvD1 biosynthesis in intact neutrophils and monocytes [13,14]. Since FLAP is an integral membrane protein, cytosolic SPM formation does not really fit. If FLAP dependency also applies to other 5-LO-dependent SPMs such as D- and E-series resolvins and maresins is not known.

Lipoxygenases are expressed in different leukocyte subtypes in humans: Active 5-LO can be found in myeloid cells such as monocytes, macrophages, eosinophils and neutrophils while platelets express 12-LO [11]. 15-LO-1 is present in alternatively activated (M2) macrophages

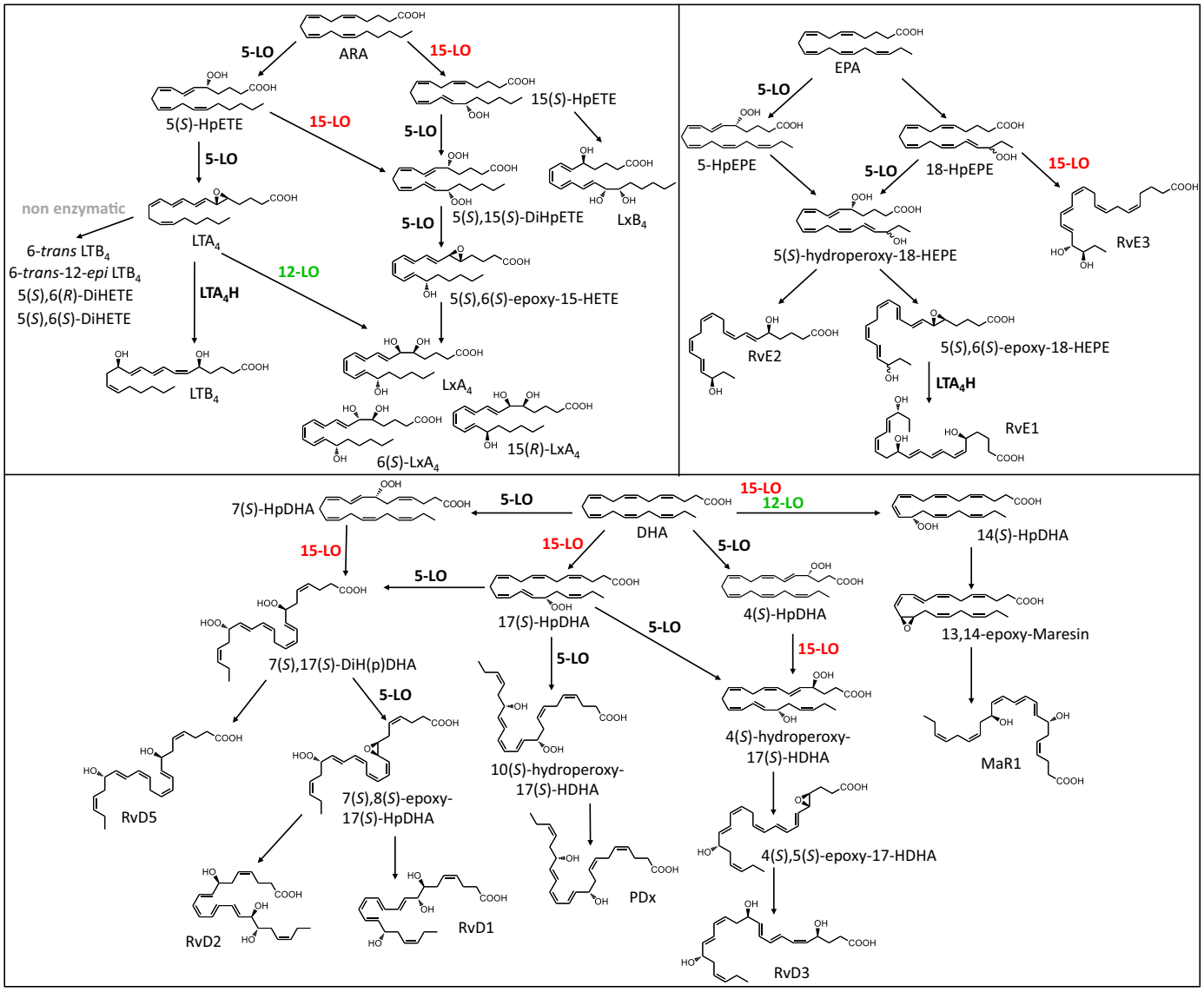

Fig. 1. Suggested formation routes for LO-derived leukotrienes and SPMs. Note: The enzymes are only indicative, because several oxylipins can be formed by different pathways as well as by autoxidation.

following IL-4 or IL-13 stimulation as well as liver X receptor activation and in eosinophils [15,16]. 15-LO-2 is expressed in M2 macrophages as well and its expression can be further elevated by persistent stimulation of TLR-2 and -4 [17]. Although alternatively activated macrophages co-express 5- and 15-LO-1/-2, SPM formation is low in these cells compared to pro-inflammatory leukotrienes, prostaglandins and 15-LO-derived monohydroxylated PUFAs (15-HETE, 17-HDHA) released alongside [17,18]. These data show that SPM release from a single cell is not efficient and clearly argues for transcellular SPM biosynthesis where alternatively activated macrophages are probably the source of 15-LO-dependent SPM precursors while a second 5-LO expressing leukocyte subpopulation takes over the final oxidation steps. Or vice versa, neutrophil-derived 5-H(*p*)ETE might be taken up by 15-LO-expressing macrophages. Indeed, interaction of macrophages with neutrophils plays an important role in inflammation resolution and neutrophils supplemented with 15-HETE or 17-HDHA have been shown to release lipoxins and RvD1, respectively upon stimulation [13,19–21].

Since expression of active 5-LO is limited to leukocytes, leukocytic SPM formation is an important hallmark to estimate human SPM formation capacity. In the present study, we took a closer look on SPM formation in human neutrophils. For this, polymorphonuclear leukocytes (PMNL) supplemented with ARA, DHA or the monohydroxylated SPM precursor PUFAs (15-HETE, 15-HpETE, 18-HEPE, 17-HDHA, 17-HpDHA) were stimulated with Ca^{2+} ionophore and lipid mediator formation was monitored and compared to cells stimulated in absence of these fatty acid precursors. Furthermore, SPM formation in broken cell preparations as well as from recombinant 5-LO was investigated and FLAP dependency was tested. This should answer the question whether 5-LO is able to accept monohydroxylated PUFAs for SPM formation on its own, whether FLAP dependency in PMNL applies to 5-LO-dependent SPM formation in general and whether other factors apart from 5- and 15-LOs play a role in PMNL-dependent SPM biosynthesis in humans.

2. Materials and methods

2.1. Drugs, chemical reagents and other materials

Dextran, $CaCl_2$ and calcium ionophore (A23187) were obtained from Sigma-Aldrich (Steinheim, Germany). PBS was purchased from Gibco Life Technologies (Paisley, UK). EDTA and SDS were purchased from Merck KGaA (Darmstadt, Germany). Peroxide free ARA, 15(*S*)-HETE, 15(*R*)-HETE, 17(*S*)-HDHA, 17(*R*)-HDHA, 18(*R/S*)-HEPE, the oxylipin standards as well as the deuterated standards were bought from Cayman Chemical (Ann Arbor, MI, USA). UPLC grade methanol, D-(+)-glucose, HPLC grade *n*-hexane and ATP (disodium salt) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Ultrapure tris(hydroxymethyl)aminomethane (TRIS), acrylamide 4 K solution 30%, dimethyl sulfoxide (DMSO) were purchased from AppliChem GmbH (Darmstadt, Germany). LC-MS grade methanol, acetonitrile and acetic acid were bought from Fisher Scientific (Schwerte, Germany). HPLC grade ethyl acetate was from VWR (Darmstadt, Germany).

2.2. Purification of polymorphonuclear leukocytes (PMNL) from human leukocyte concentrates

Human PMNL were freshly isolated from leukocyte concentrates (Institut für Transfusionsmedizin und Immunhämatologie, DRK-Bluspendedienst, Frankfurt, Germany). Blood was drawn with the informed consent of the patients. For PMNL purification, total leukocytes were separated from erythrocytes by dextran sedimentation for 30 min. After this, leukocyte subsets were isolated on NycoPrep cushions (PAA Laboratory, Pasching, Austria) by centrifugation (10 min without deceleration) at 800*g*. After this, the supernatant as well as the separation medium were removed resulting in the crude PMNL pellet. To get rid of remaining erythrocyte contaminations in the PMNL fraction, the cell pellet was then subjected to hypotonic lysis with ice-cold water for 45 s. After this, the lysis was stopped by addition of PBS followed by centrifugation. The resulting erythrocyte free PMNL pellet was then resuspended in PGC buffer (PBS + 1 mg/mL glucose), counted and kept on ice until further processing.

2.3. PMNL homogenates and 100,000 g supernatants (S100)

PMNL homogenates were prepared by sonicating (Sonoplus HD 200, sonotrode MS72, BANDELIN electronic GmbH & Co, Berlin, Germany) a suspension of freshly isolated PMNL in ice-cold PBS/EDTA (PBS + 1 mM EDTA) 3 times at 90% intensity for 10 s at 4 °C. Cell concentration was 10^7 human PMNL per 500 μL PBS/EDTA. To prepare PMNL 100,000 *g* supernatants (S100), PMNL homogenates were additionally subjected to an ultracentrifugation step at 100,000 *g* (70 min, 4 °C). The resulting supernatants were then further used for activity assays.

2.4. Recombinant 5-LO

Recombinant human 5-LO was expressed in *Escherichia coli* BL21 (DE3). For this the cells were transformed with the plasmid pT3-5-LOX (kind gift of Prof. Olof Rådmark, Karolinska Institutet, Stockholm, Sweden). The protein was expressed and purified as previously described using an ÄKTA Xpress system (GE Healthcare, Uppsala, Sweden) [22].

2.5. Lipid mediator formation assays

Activity assays with intact cells were carried out with 10^7 freshly isolated human PMNL resuspended in 500 μL PGC buffer (PBS + 1 mg/mL glucose) supplemented with $CaCl_2$ (final concentration 1 mM). Lipid mediator formation was triggered by addition of 10 μM SPM precursor oxylipins (15(*S*)-HETE, 15(*S*)-HpETE, 15(*R*)-HETE, 17(*S*)-HDHA, 17(*S*)-HpDHA, 17(*R*)-HDHA or 18(*R/S*)-HEPE) or ARA plus 5 μM Ca^{2+} ionophore A23187 for 15 min at 37 °C in a water bath. The reaction was terminated by addition of 500 μL ice-cold methanol. After sonication, the cell supernatants were then frozen at −80 °C until further analysis by LC-MS/MS. In incubations where the FLAP inhibitor Mk-886 was employed, PMNL were pre-incubated with the inhibitor or its vehicle control (DMSO) for 15 min (37 °C, water bath) prior to the addition of Ca^{2+} ionophore A23187.

For activity assays with broken PMNL preparations, homogenates or S100 corresponding to 10^7 PMNL were diluted in 500 μL PGC buffer supplemented with $CaCl_2$ (final concentration 2 mM) per sample. For assays employing purified human 5-LO, 1.5 μg of the recombinant protein were diluted in 500 μL PGC buffer supplemented with $CaCl_2$ (1 mM). After addition of ATP (final concentration 1 mM), the samples were pre-heated for 30 s in a water bath. After this, the reaction was started by addition of 10 μM SPM precursor oxylipins (15(*S*)-HETE, 15(*S*)-HpETE, 15(*R*)-HETE, 17(*S*)-HDHA, 17(*S*)-HpDHA, 17(*R*)-HDHA or 18(*R/S*)-HEPE), ARA or DHA for 10 min at 37 °C. Finally, the reaction was terminated by addition of 500 μL ice-cold methanol. For further processing, all samples were sonicated after assay termination and immediately frozen at −80 °C until further analysis by LC-MS/MS. Table 1 shows the impurities and autoxidation products of the different SPM precursor oxylipins and their effective concentrations in the activity assays.

2.6. Extraction and analysis of lipid mediators

Eicosanoids and other oxylipins were analyzed as described [23–27]. In brief, antioxidants and inhibitors as well as and internal standards were added to the sonicated cell suspension. Cell suspensions from incubations with hydroperoxy PUFAs were additionally treated with 25 μL SnCl2 (10 mg/mL in methanol) to reduce the hydroperoxy PUFAs to their corresponding hydroxyl PUFAs. Following protein precipitation the lipid mediators were extracted by solid phase extraction on a mixed

Table 1
HydroxyPUFA impurities, autoxidation products and effective concentrations used in the activity assays. HydroxyPUFAs were incubated in PGC buffer without addition of cells, cell preparations or recombinant 5-LO during the activity assays (background controls). HydroxyPUFA concentrations are shown in nM. Effective concentrations of the monohydroxyPUFA SPM precursors are marked in bold red. Oxylipins in concentrations below 0.01% of the total monohydroxyPUFAs (15-HETE, 18-HEPE, 17-HDHA) added were considered unspecific and are depicted as <0.01% in light grey. nd, not determined.

[nM]	15-HETE		17-HDHA		18-HEPE
	(*S*)	(*R*)	(*S*)	(*R*)	(*R/S*)
5(*R/S*)-HETE	<0.01%	<0.01%	<0.01%	<0.01%	nd
12(*R/S*)-HETE	1	15	15	1	nd
15(*R/S*)-HETE	**12689**	**12292**	4	3	20
5(*S*),15(*S*)-DiHETE	17	10	<0.01%	< 0.01%	<0.01%
5(*S*),12(*S*)-DiHETE	8	13	<0.01%	< 0.01%	nd
8(*S*),15(*S*)-DiHETE	2	2	<0.01%	< 0.01%	<0.01%
5(*S*),6(*R*)-DiHETE	<0.01%	<0.01%	<0.01%	< 0.01%	nd
5(*S*),6(*S*)-DiHETE	<0.01%	<0.01%	<0.01%	< 0.01%	nd
LTB_4	<0.01%	<0.01%	<0.01%	< 0.01%	nd
6-trans-LTB_4	<0.01%	<0.01%	<0.01%	< 0.01%	nd
6-trans-12-epi-LTB_4	<0.01%	<0.01%	<0.01%	1	nd
LxA_4	<0.01%	<0.01%	<0.01%	< 0.01%	<0.01%
15(*R*)-LxA_4	<0.01%	<0.01%	<0.01%	< 0.01%	<0.01%
6(*S*)-LxA_4	<0.01%	<0.01%	<0.01%	< 0.01%	<0.01%
LxB_4	<0.01%	<0.01%	<0.01%	< 0.01%	<0.01%
5(*R/S*)-HEPE	<0.01%	<0.01%	<0.01%	< 0.01%	nd
12(*R/S*)-HEPE	<0.01%	<0.01%	<0.01%	< 0.01%	nd
15(*R/S*)-HEPE	28	<0.01%	10	< 0.01%	nd
18(*R/S*)-HEPE	<0.01%	5	26	5	**11011**
RvE1	<0.01%	<0.01%	1	1	<0.01%
18(*R*)-RvE2	<0.01%	<0.01%	<0.01%	< 0.01%	7
18(*S*)-RvE3	<0.01%	<0.01%	<0.01%	<0.01%	5
18(*R*)-RvE3	<0.01%	<0.01%	<0.01%	<0.01%	1
4(*R/S*)-HDHA	<0.01%	<0.01%	<0.01%	<0.01%	nd
7(*R/S*)-HDHA	<0.01%	<0.01%	<0.01%	<0.01%	nd
14(*R/S*-)HDHA	<0.01%	<0.01%	214	1	nd
17(*R/S*)-HDHA	<0.01%	<0.01%	**7708**	**5664**	<0.01%
RvD1	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
17(*R*)-RvD1	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
RvD2	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
RvD3	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
RvD5	<0.01%	<0.01%	<0.01%	2	<0.01%
MaR1	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
7epi-MaR1	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
PDx	<0.01%	<0.01%	<0.01%	2	<0.01%

mode anion exchange/reversed phase column (Bond Elute Certify II, Agilent). Separation was carried out on a Zorbax Eclipse Plus C18 reversed phase column (2.1 × 150 mm, particle size 1.8 μm, pore size 9.5 nm). Detection was carried out following electrospray ionization in negative mode by a triple quadrupole mass spectrometer (Sciex QTRAP 5500) operated in scheduled selected reaction monitoring mode. Details on the sample preparation procedure, LC-MS/MS parameters and method characterization are described in the Supplemental Information.

2.7. SDS-PAGE/Western blotting

Western Blotting samples were obtained by lysis of freshly isolated PMNL or the pellet resulting after 100,000 *g* centrifugation of the PMNL homogenates (P100) with SDS buffer (2.2% SDS, 11% glycerol, 56 mM TrisHCl). For this, the boiling buffer was added to the cell pellets followed by further incubation of the samples at 96 °C for 5 min to assure complete protein denaturation. S100 protein lysates were obtained by addition of ice-cold methanol to the S100 fraction followed by overnight precipitation of the proteins at −20 °C. The resulting protein pellet was also lysed in hot SDS buffer as described above. Protein concentrations of the lysates were determined using the Pierce bicinchoninic acid method according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL, USA) employing a microplate reader (infinite M200, Tecan Group Ltd., Crailsheim, Germany). Equal quantities of the cell lysates were separated on 10% or 16% polyacrylamide gels via electrophoresis (SDS-PAGE) and proteins were electrophoretically blotted onto nitrocellulose membranes (Odyssey, LI-COR Biosciences, Bad Homburg, Germany). Membranes were then incubated in Odyssey blocking reagent (LI-COR Biosciences, Bad Homburg, Germany) followed by treatment with the respective primary antibodies directed against either 5-LO (sc-515,821, Santa Cruz Biotechnology, Heidelberg, Germany), 15-LO-1 (ab119774, abcam, Cambridge, UK), 15-LO-2 (sc271290, Santa Cruz Biotechnology, Hamburg, Germany), FLAP (ab53536, abcam, Cambridge, UK) or β-actin (sc-47,778 or sc-1616, Santa Cruz Biotechnology, Heidelberg, Germany). After several washing steps and incubation with the corresponding IRDye680- or IRDye800-conjugated secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany), immunoreactive bands were visualized on the Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

3. Results

3.1. Formation of lipoxygenase-derived lipid mediators in human PMNL stimulated with Ca^{2+} ionophore (A23187)

In order to investigate the formation of SPM from human neutrophils, the PMN fraction was isolated from leukocyte concentrates. In addition to neutrophils, PMNL may contain small numbers of eosinophils which express 15-LO-1 in addition to 5-LO. To ascertain proper cell isolation and processing as well as to enable correct data interpretation, the expression patterns of 5-LO as well as 15-LO-1, 15-LO-2 and FLAP were assessed in intact PMNL as well as PMNL 100,000 *g* supernatants (S100) and pellets (P100) (Fig. 3E). As expected, intact human PMNL expressed high amounts of 5-LO and FLAP. When the cell integrity was destroyed and the corresponding homogenates were subjected to 100,000 *g* centrifugation, 5-LO was found in the soluble fraction (S100) whereas the integral membrane protein FLAP was found in the pellet (P100). All donors displayed low 15-LO-1 expression confirming the presence of small numbers of eosinophils in our preparations. Comparable to 5-LO, 15-LO-1 was found in the soluble fraction after 100,000 *g* centrifugation. 15-LO-2 was not detected in any donor.

Before conducting the experiments, different stimuli were tested for their SPM inducing capacity in human PMNL (Fig. 2A–E). From this, we chose Ca^{2+} ionophore as most potent inducer of lipoxin and resolvin biosynthesis (Fig. 2F). First, we investigated the lipid mediator formation from endogenous lipid stores in human PMNL. For this, PMNL were stimulated with 5 μM Ca^{2+} ionophore A23187 for 15 min at 37 °C (Fig. 3A). While PMNL formed substantial amounts of ARA-derived lipid mediators, formation of EPA- and DHA-based hydroxylated PUFAs was considerably lower. The monohydroxylated ARA metabolites 5- and 12-HETE showed the highest formation of all lipid mediators measured (5.3 ± 0.8 pmol/10^6 cells and 6.9 ± 2.7 pmol/10^6 cells) followed by LTB_4 (2 ± 0.5 pmol/10^6 cells). The 5-LO-derived non-enzymatic hydrolysis products of LTA_4 [5(*S*),6(*S*)-diHETE, 5(*S*),6(*R*)-diHETE, 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 - summed up in the graph as LTA_4] and the 5-LO/12-LO double oxygenation product 5(*S*),12(*S*)-diHETE were found in low amounts of 1.25 ± 0.3 pmol/10^6 cells and 1.4 ± 1 pmol/10^6 cells, respectively. Formation of 15-LO-dependent 15-HETE and 5(*S*),15(*S*)-diHETE was even lower (0.4 ± 0.04 pmol/10^6 cells and 0.5 ± 0.2 pmol/10^6 ± cells, respectively). No lipoxins were detected in the incubations. Compared to the ARA metabolites, EPA-derived lipid mediator formation was much lower. Only small amounts of 5- and 12-HEPE (0.2 ± 0.03 pmol/10^6 cells and 0.4 ± 0.2 pmol/10^6 cells, respectively) as well as 18-HEPE (0.2 ± 0.1 pmol/10^6 cells) were detected. In addition, even lower amounts of 15-HEPE, RvE1 and 18(*R*)-RvE3 were found. Comparable to EPA, formation of DHA-derived lipid mediators was very low. Again, the 12-LO product 14-HDHA showed the highest formation of all DHA products (0.2 + 0.06 pmol/10^6 cells) followed by 17-HDHA (0.03 ± 0.01 pmol/10^6 cells), 7-HDHA, 4-HDHA as well as RvD5. RvD1, RvD2,

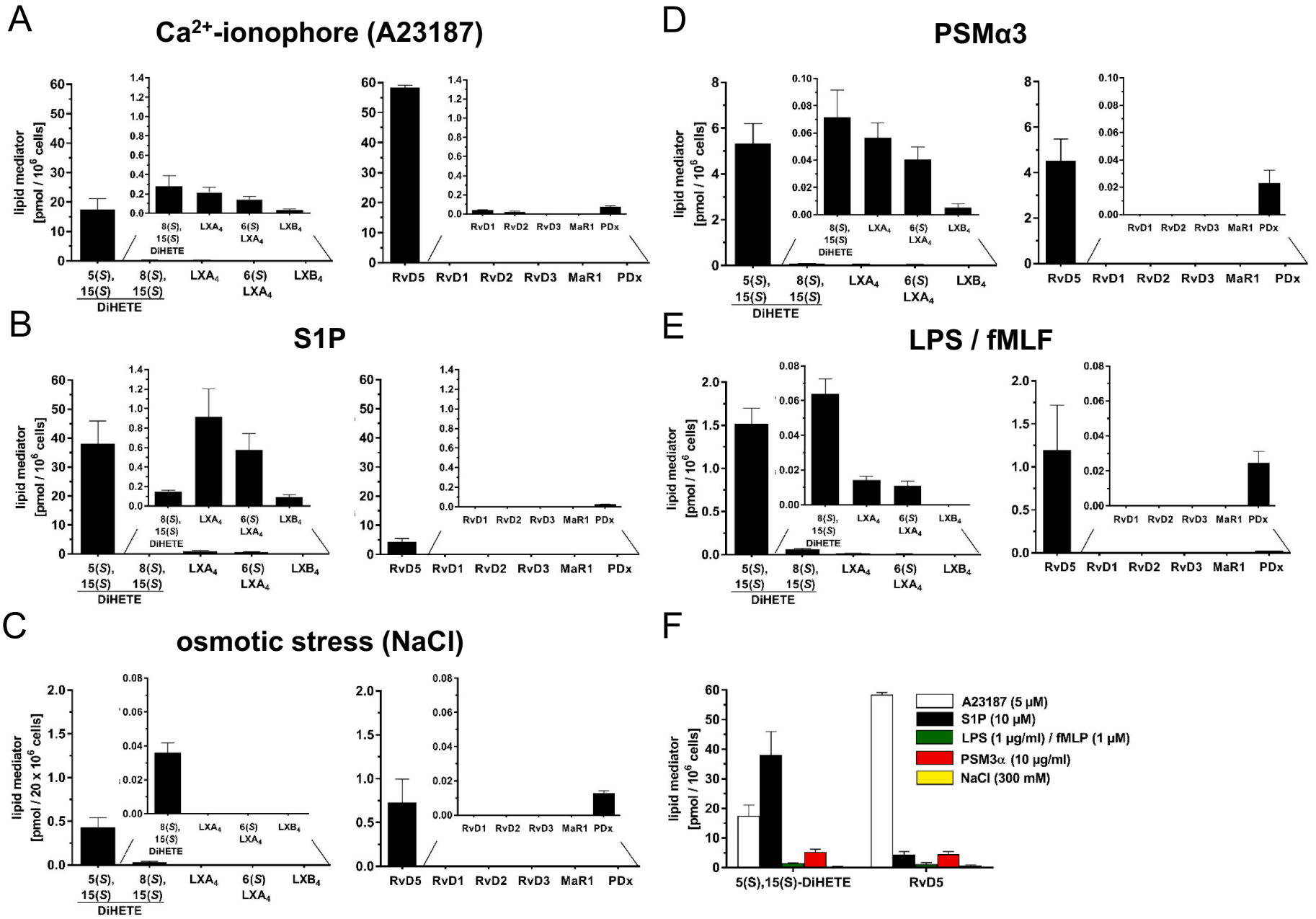

Fig. 2. Comparison of lipid mediator formation from different stimuli. Cells were stimulated with either (A) 5 µM Ca^{2+} ionophore, (B) 10 µM sphingosine-1-phosphate (S1P), (C) osmotic stress (0.3 M NaCl), (D) 10 µg/ml phenol-soluble modulin α3 (PSMα3) or (E) 1 µM N-formyl-leucyl-phenylalanine (fMLF) at 37 °C in presence of 10 µM 15(*S*)-HETE or 17(*S*)-HDHA. fMLF incubations were additionally primed with 1 µg/ml bacterial lipopolysaccharides (LPS) for 20 min at RT prior stimulation. After 15 min of stimulation, the reactions were terminated by addition of ice-cold methanol and lipid mediators in the samples were quantified by LC-MS/MS. (F) Comparison of 5(*S*),15(*S*)-diHETE and RvD5 formation efficiency of the different stimuli. All data are depicted as mean + SEM from 3 independent experiments.

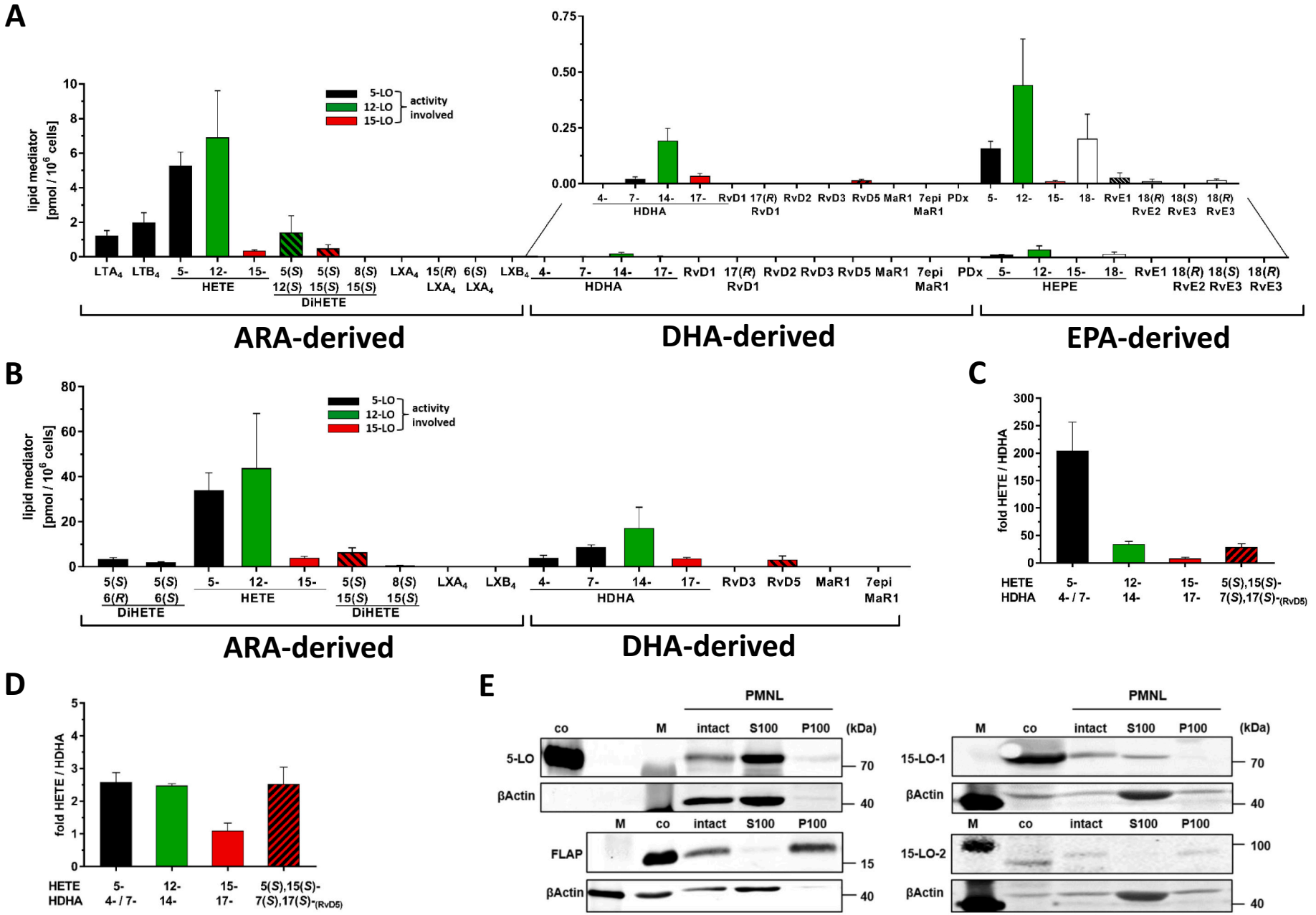

Fig. 3. Enzyme expression and lipoxygenase activities in stimulated human PMNL. Lipid mediator formation in PMNL was stimulated with 5 μM Ca^{2+} ionophore (A) or with 2.5 μM Ca^{2+} ionophore in presence of an ARA/DHA mix (10 μM each) (B) for 15 (A) or 10 (B) minutes at 37 °C. After this, the reaction was terminated by addition of ice-cold methanol and lipid mediators in the samples were quantified by LC-MS/MS. Data are depicted as mean + SEM from 6 (A) and 4 (B) independent experiments. Ratio of corresponding ARA and DHA LO metabolites (HETE:HDHA) from A (C) and B (D). (E) Western Blots from PMNL lysates. After isolation the cells were either directly lysed (intact) or homogenized by sonication followed by 100,000 *g* centrifugation resulting in a soluble fraction (S100) and the pelleted insoluble membrane fraction (P100). Proteins in the S100 were precipitated with methanol. S100 proteins and P100 pellets were then solubilized in boiling SDS buffer and equal quantities of the lysates were electrophoretically separated followed by Western Blotting. Recombinant human 5-LO or FLAP, 15-LO-1 and 15-LO-2 overexpressing 239 T cells were used as positive controls. One representative experiment out of 4 is shown. Co, control; M, molecular weight marker.

RvD3, PDx and maresins were below the detection limit in these incubations. Comparison of the corresponding ARA and DHA mono- and dihydroxylated PUFAs for each lipoxygenase action showed that the ARA metabolites were always more abundant in PMNL (Fig. 3C). 5-LO-derived 5-HETE formation was 205-fold higher compared to the sum of both corresponding DHA metabolites 4- and 7-HDHA. 12-HETE was about 34-fold higher compared to 14-HDHA and 15-HETE was 8-fold higher than 17-HDHA. Also, 5(*S*),15(*S*)-diHETE exceeded RvD5 formation by 29-fold.

This dominance of ARA over DHA products might be due to a net enzymatic preference of ARA in PMNL. To investigate this further, we supplemented PMNL with a mixture of ARA and DHA (10 μM each) during stimulation with Ca^{2+} ionophore (Fig. 3B). This exogenous PUFA supplementation resulted in an overall increase in hydroxylated PUFAs with the DHA metabolites being more heavily affected. Nevertheless, ARA-derived lipid mediators still dominated the 5- and 12-lipoxygenation products although the gap between DHA and ARA metabolites was much smaller (3-fold for 5-HETE and 2-fold for 12-HETE and 5(*S*),15(*S*)-diHETE) compared to PMNL stimulated in absence of exogenous PUFAs (Fig. 3D). Interestingly, 15-LO-derived 15-HETE and 17-HDHA were formed in equal amounts under these conditions. Although simultaneous supplementation with ARA and DHA resulted in an upregulation of 5(*S*),15(*S*)-diHETE and its DHA-derived analogue RvD5, other SPMs were not detected in our incubations.

3.2. Lipid mediator formation in human PMNL supplemented with 15-HETE, 18-HEPE or 17-HDHA

PMNL alone did not form substantial amounts of SPM upon stimulation with Ca^{2+} ionophore even though 5-LO and 15-LO-1 expression was found in the incubations. Apparently, endogenous formation of the hydroxylated PUFAs that serve as 5-LO substrates for the formation of SPMs (15-H(*p*)ETE, 17-H(*p*)DHA, 18-H(*p*)EPE) was too low. In order to mimic possible transcellular biosynthetic pathways where SPM precursors are released from cells or tissues expressing 15-LO and taken up by PMNL, we treated PMNL with the precursors for lipoxins (15(*S*)- and 15(*R*)-HETE), E-series resolvins (18(*R/S*)-HEPE), D-series resolvins and protectins (17(*S*)- and 17(*R*)-HDHA). For this, PMNL were stimulated with Ca^{2+} ionophore in the presence of each precursor (10 μM).

15(*S*)-HETE supplementation led to a substantial increase in the 5-LO-dependent 15-HETE metabolite 5(*S*),15(*S*)-diHETE from 0.5 ± 0.2 pmol/10^6 cells treated with ionophore only to 40.1 ± 10.2 pmol/10^6 cells in precursor supplemented cells (Fig. 4A). In contrast to the ionophore only treated cells, LXA_4, 6(*S*)-LXA_4 and LXB_4 formation was detectable in the precursor-supplemented preparations but lipoxin yields were about 75, 110 and 570 times lower (LXA_4 0.5 ± 0.1 pmol/10^6 cells; 6(*S*)-LXA_4 0.4 ± 0.1 pmol/10^6 cells; LXB_4 0.07 ± 0.02 pmol/10^6 cells) compared to 5(*S*),15(*S*)-diHETE. When 17(*S*)-HDHA was used instead of 15(*S*)-HETE, RvD5 levels were substantially elevated from 0.02 ± 0.005 pmol/10^6 cells to 114.2 ± 25.1 pmol/10^6 cells. Interestingly, utilization of 17(*S*)-HDHA was about 4 times more efficient compared to 15(*S*)-HETE and even about 30 times higher than 18(*R/S*)-HEPE (Fig. 6C). In addition to RvD5, only small amounts of RvD1 (0.7 ± 0.2 pmol/10^6 cells) and PDx (0.3 ± 0.1 pmol/10^6 cells) were detected. 18(*R/S*)-HEPE supplementation resulted in small amounts of RvE1 (3.4 ± 0.3 pmol/10^6 cells), 18(*R*)-RvE2 (1.1 ± 0.4 pmol/10^6cells), 18(*S*)-RvE3 (3.2 ± 1.1 pmol/10^6cells) and 18(*R*)-RvE3 (0.2 ± 0.03 pmol/10^6cells). Furthermore, we compared both 15-HETE and 17-HDHA enantiomers and found that formation of 15(*R*)-LXA_4 from 15(*R*)-HETE was twofold higher compared to LXA_4 while formation of 17(*R*)-RvD1 from 17(*R*)-HDHA was about half compared to RvD1 derived from 17(*S*)-HDHA (Fig. 4B).

In order to investigate if the capacity for SPM biosynthesis substantially differs between the hydroxy PUFAs (15-HETE, 17-HDHA, 18-

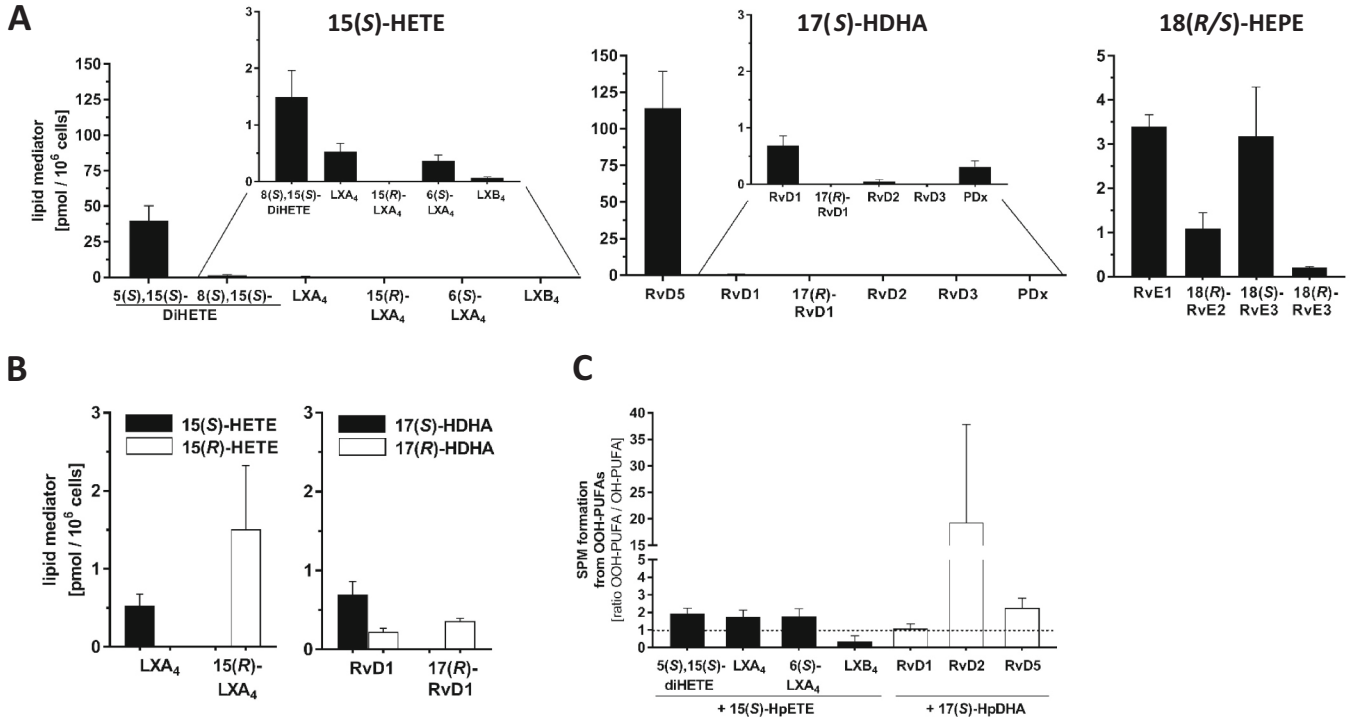

Fig. 4. Lipid mediator formation in intact human PMNL supplemented with 15-HETE, 18-HEPE or 17-HDHA. (A) Freshly isolated human PMNL were resuspended in PGC buffer (2×10^7 cells/mL) followed by stimulation with 5 μM Ca^{2+} ionophore at 37 °C. In addition, 10 μM of the respective monohydroxylated PUFA were added. The reaction was terminated after 15 min by addition of ice-cold methanol. The lipid mediators formed in these incubations were then quantified by LC-MS/MS. Data are depicted as mean + SEM from 4 to 5 independent experiments. (B) Comparison of LXA_4 and RvD1 formation upon stimulation with the 15-HETE or 17-HDHA enantiomers. (C) Comparison of SPM formation from 10 μM hydroxyl PUFA and hydroperoxy PUFA precursors in intact human PMNL. Data are depicted as mean + SEM from 3 independent experiments.

HEPE) and their corresponding hydroperoxy precursors (15-HpETE, 17-HpDHA, 18-HpEPE), we compared SPM formation from 10 μM 15(*S*)-HETE and 15(*S*)-HpETE as well as from 17(*S*)-HDHA and 17(*S*)-HpHDA in intact PMNL (Fig. 4C). Since 18-HpEPE is not commercially available, *E*-series resolvins could not be tested in this assay. Formation of 5(*S*),15(*S*)-diHETE, LXA_4 and 6(*S*)-LXA_4 was increased by a factor of 2 in 15(*S*)-HpETE compared to 15(*S*)-HETE treated cells, while LXB_4 formation was lower from the hydroperoxy precursor. D-resolvin formation from 17(*S*)-HpDHA was more efficient for RvD5 (2-fold) and RvD2 (20-fold) but had no influence on RvD1 formation.

3.3. Influence of cell integrity on SPM formation in human PMNL

In the following experiments, PMNL cell integrity was destroyed by sonication to investigate if 5-LO-dependent SPM formation is based on an intact cellular environment or if 5-LO can form SPM independently of other cellular factors in neutrophils. Cell homogenates corresponding to 2×10^7 cells/mL were supplemented with 10 μM of the SPM precursor lipids (15-HETE, 18-HEPE or 17-HDHA) and the SPM synthesis capacity was determined in presence of 1 mM ATP. In addition, the homogenates were further subjected to 100,000 *g* centrifugation in order to remove the membrane fraction. Then, the resulting soluble fraction (S100) containing 5-LO and 15-LO-1 as well as other soluble PMNL proteins was tested for SPM formation in presence of 10 μM SPM precursors and 1 mM ATP. Intact PMNL stimulated with Ca^{2+} ionophore and supplemented with the SPM precursors served as controls.

First, we had a look on the total precursor recovery (proportion of the sum of unchanged monohydroxy PUFAs plus corresponding metabolites to the total monohydroxy PUFAs added) in the samples to assure that our method captured all relevant metabolites formed from the SPM precursors in the PMNL incubations (Fig. 5). Total precursor recovery of 15(*S*)- and 15(*R*)-HETE, 18(*R/S*)-HEPE and 17(*R*)-HDHA added up to almost 100% or more in intact PMNL. Interestingly, 17(*S*)-HDHA total precursor recovery was only 74% in intact cells suggesting that not all metabolites were captured in our LC-MS/MS method. Total precursor recovery in PMNL homogenates was about 100% for all hydroxylated PUFAs. A loss of lipids due to adherence to plastic ware or cellular debris that might hamper lipid analysis could therefore be ruled out. With the exception of 17(*S*)-HDHA (87%), recovery in S100 preparations was again 100%.

Next, we investigated the formation of lipid mediators from PMNL homogenates and S100 preparations compared to intact cells (Fig. 6A). Unexpectedly, destruction of PMNL cell integrity led to a 5-fold increase in 5(*S*),15(*S*)-diHETE (241.9 ± 12.5 pmol/10^6cells) and a 9-fold increase in LXA_4 formation (4.8 ± 0.4 pmol/10^6cells) in 15(*S*)-HETE treated homogenates compared to intact cells. 8(*S*),15(*S*)-diHETE, 6(*S*)-LXA_4 and LXB_4 levels also increased. Removal of insoluble cell components such as membrane fragments and organelles by 100,000 *g* centrifugation (S100) resulted in even higher 5(*S*),15(*S*)- and 8(*S*),15(*S*)-diHETE levels and had only a low inhibitory effect on the elevated LXA_4 and 6(*S*)-LXA_4 formation compared to the homogenates. Interestingly, PMNL homogenates supplemented with 17(*S*)-HDHA or 18(*R/S*)-HEPE showed the opposite effect. Here, biosynthesis of RvD1, RvD5, RvE1 and 18(*R*)-RvE2 that was detected in the intact cells was inhibited in the homogenates about 2- to 3-fold upon destruction of cell integrity. In contrast, formation of PDx was not impaired. When we used PMNL S100 preparations instead of the homogenates in the activity assay, resolvin and PDx formation was completely abrogated (Fig. 6A).

For comparison, leukotriene formation from ARA in intact PMNL, homogenates and S100 was investigated (Fig. 6B). For this, intact cells and cell preparations were supplemented with 10 μM ARA instead of the monohydroxy PUFAs. Interestingly, destruction of cell integrity impaired 5-HETE formation while 15-HETE and LTB_4 levels were 2-fold elevated. 12-HETE formation was not influenced.

In contrast to intact PMNL where 17(*S*)-HDHA is preferentially metabolized, destruction of cell integrity and 100,000 *g* centrifugation reversed this effect: In homogenates utilization of 15(*S*)-HETE exceeded that of 17(*S*)-HDHA by 24-fold. Even more, in S100 preparations 15(*S*)-HETE was preferred 126-fold over 17(*S*)-HDHA (Fig. 6C, D).

3.4. Influence of FLAP inhibition on SPM formation in human PMNL

FLAP dependency has been shown for lipoxin, 5(*S*),15(*S*)-diHETE and RvD1 formation [13,28]. To investigate this further, intact PMNL were pre-treated with 3 μM of the FLAP inhibitor Mk-886 followed by stimulation with 5 μM Ca^{2+} ionophore in presence or absence of 15(*S*)-HETE, 17(*S*)-HDHA or 18(*R/S*)-HEPE (10 μM). Cells treated with 10 μM ARA instead of the monohydroxy PUFAs were used as control. Inhibition of FLAP resulted in an overall shift from 5-LO to 12−/15-LO products in PMNL treated with ARA (Fig. 7B): 5-LO-derived 5-HETE and LTB_4 were strongly inhibited (residual activity below 10%) while 15-HETE levels were elevated about 3.2-fold. 12-HETE formation was also augmented by Mk-886 treatment although to a lower extent (~1.6-fold). When cell integrity was disrupted in ARA supplemented PMNL, the inhibitory effect of MK-886 on 5-LO activity was completely abolished and no shunting into other LO pathways was detectable. Intact PMNL stimulated with Ca^{2+}-ionophore in the absence of exogenously added fatty acids displayed a lower formation of 5-HETE and LTB_4 as well as an increase in 15-HETE but no effect on 12-HETE was detectable (Fig. 7A). This effect was also found for the DHA- and EPA-derived monohydroxylated PUFAs.

In intact PMNL supplemented with 15(*S*)-HETE, 17(*S*)-HDHA or 18(*R/S*)-HEPE instead of ARA, formation of 5(*S*),15(*S*)-diHETE, LXA_4, 6(*S*)-LXA_4, RvD1, RvD5 and RvE1 was potently inhibited by Mk-886 supporting the FLAP dependency of their biosynthesis (Fig. 7B).

Destruction of cell integrity led to a loss of inhibition of 5(*S*),15(*S*)-

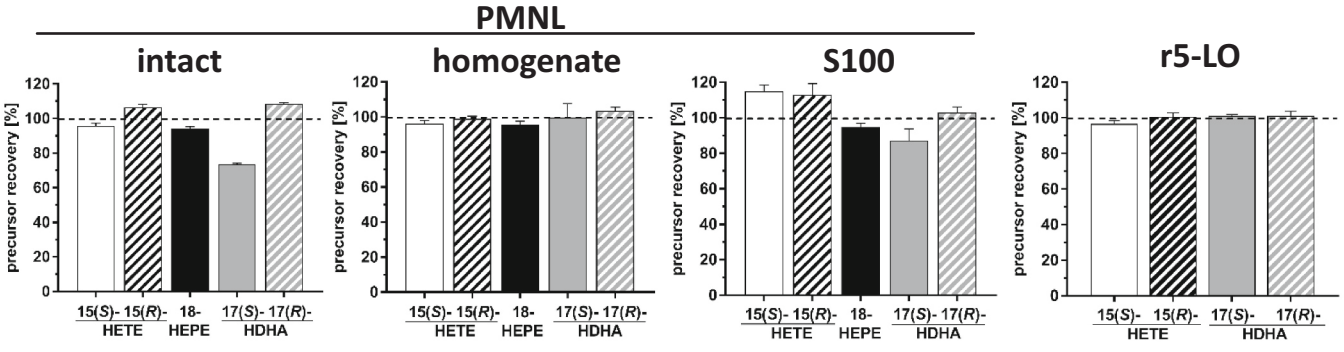

Fig. 5. Total precursor recovery. Percent recovery for each monohydroxylated PUFA in intact PMNL, PMNL homogenates, PMNL 100,000 *g* preparations (S100) as well as with recombinant 5-LO. Values were calculated as the sum of the non-metabolized monohydroxylated PUFA plus the metabolites in relation to the total monohydroxylated PUFA added to each assay.

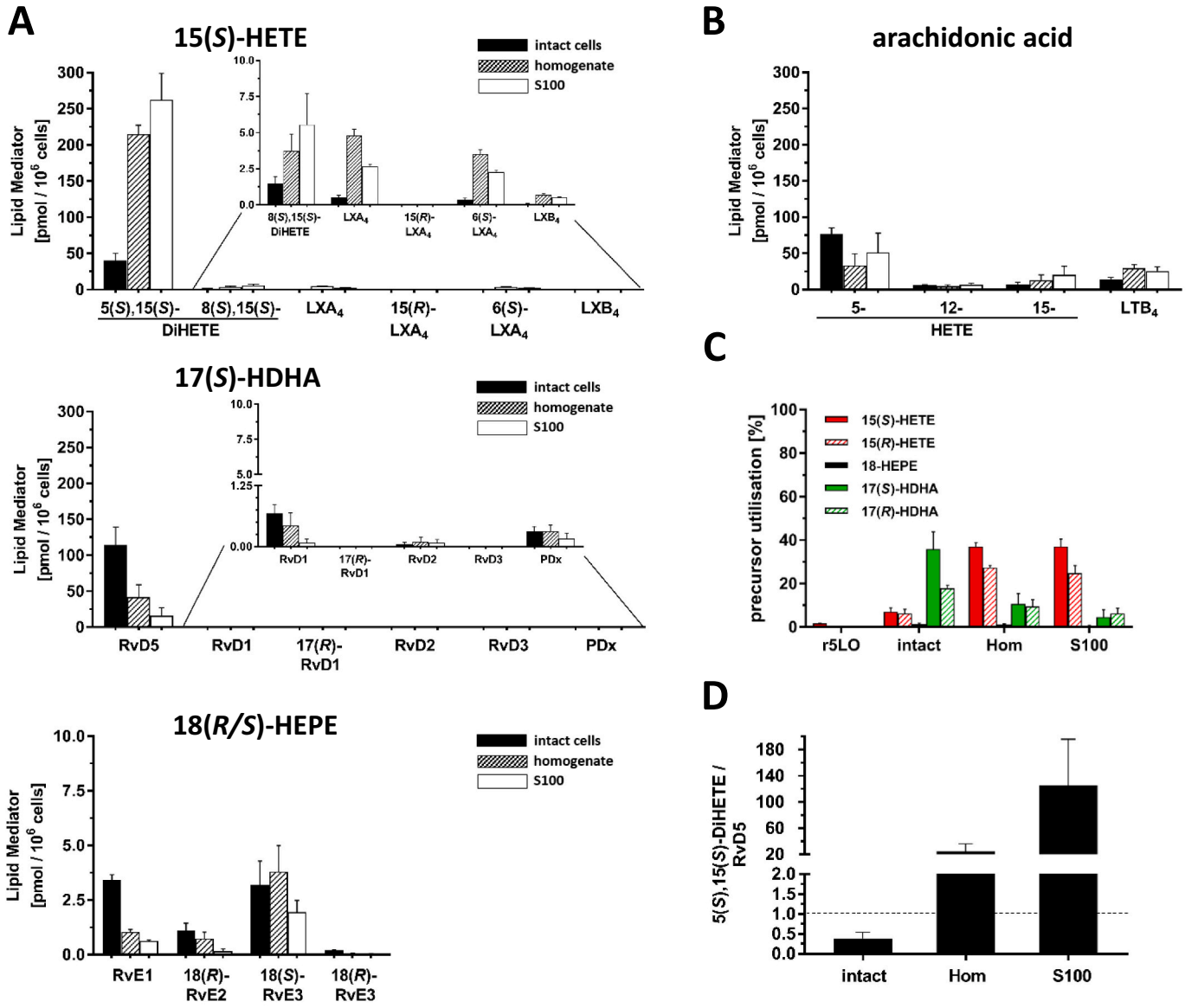

Fig. 6. Influence of cell integrity on SPM formation in human PMNL supplemented with 15(*S*)-HETE, 18(*R/S*)-HEPE or 17(*S*)-HDHA. (A) Freshly isolated PMNL were homogenized by sonication followed by 100,000 *g* centrifugation. Resulting homogenates and 100,000 g supernatants (S100) corresponding to (2×10^7 cells/mL) were supplemented with 10 μM 15(*S*)-HETE, 18(*R/S*)-HEPE or 17(*S*)-HDHA in presence of 1 mM ATP and Ca^{2+}. Intact PMNL (2×10^7 cells/mL) stimulated with 5 μM Ca^{2+} ionophore and supplemented with 10 μM of the respective monohydroxylated PUFA served as controls. The reaction was terminated after 15 min by addition of ice-cold methanol. Lipid mediators formed in these incubations were then quantified by LC-MS/MS. (B) Cells were treated with 10 μM ARA instead of the monohydroxylated PUFAs and lipid mediator formation was quantified via LC-MS/MS [45]. Data are depicted as mean + SEM from 4 to 8 independent experiments. (C) Precursor utilization calculated as sum of all metabolites detected from a respective monohydroxylated PUFA in relation to the total monohydroxylated PUFA added. (D) Ratio of 5(*S*),15(*S*)-diHETE to the corresponding DHA metabolite RvD5.

diHETE, RvD1 and RvD5 formation in homogenates (residual activities being 91%, 110% and 89%, respectively). Interestingly, inhibition of LXA_4 and 6(*S*)-LXA_4 formation was not completely rescued (residual activity 66%) in the cell homogenates. Further 100,000 *g* centrifugation completely abolished the inhibitory effect of Mk-886 seen in the intact cells and also elevated the formation of RvD5 and PDx. As expected, formation of 8(*S*),15(*S*)-diHETE and PDx was barely affected by MK-886 treatment since 5-LO is not involved in their biosynthesis.

3.5. Recombinant human 5-LO alone is not able to form SPM

Due to the unexpected upregulation of lipoxin and 5(*S*),15(*S*)-diHETE formation in PMNL homogenates and S100 preparations, recombinant purified human 5-LO (r5-LO) was assayed in presence of 15 (*S*)-HETE or 17(*S*)-HDHA and compared to ARA.

Upon ARA supplementation, r5-LO released a number of hydroxylated products (Fig. 8A). The non-enzymatic hydrolysis products of LTA_4, 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 (855 ± 51 pmol/3 μg protein and 1406 ± 88 pmol/3 μg protein) were the most abundant products found in the incubations followed by 5-HETE (370 ± 38 pmol/3 μg protein). In addition, small amounts of LTA_4-derived 5(*S*),6(*R*)- and 5(*S*),6(*S*)-diHETE were formed. When r5-LO was supplemented with 15(*S*)-HETE the enzyme released minute amounts of 5(*S*),15(*S*)-diHETE (86 ± 32 pmol/3 μg protein) and even lower amounts of lipoxins. After addition of 17(*S*)-HDHA no oxidation products were detected. Total precursor recovery for 15(*S*)-HETE and 17(*S*)-HDHA in these incubations was almost 100% with negligible precursor utilization (Fig. 5).

In addition to the monohydroxy precursors, SPM formation from

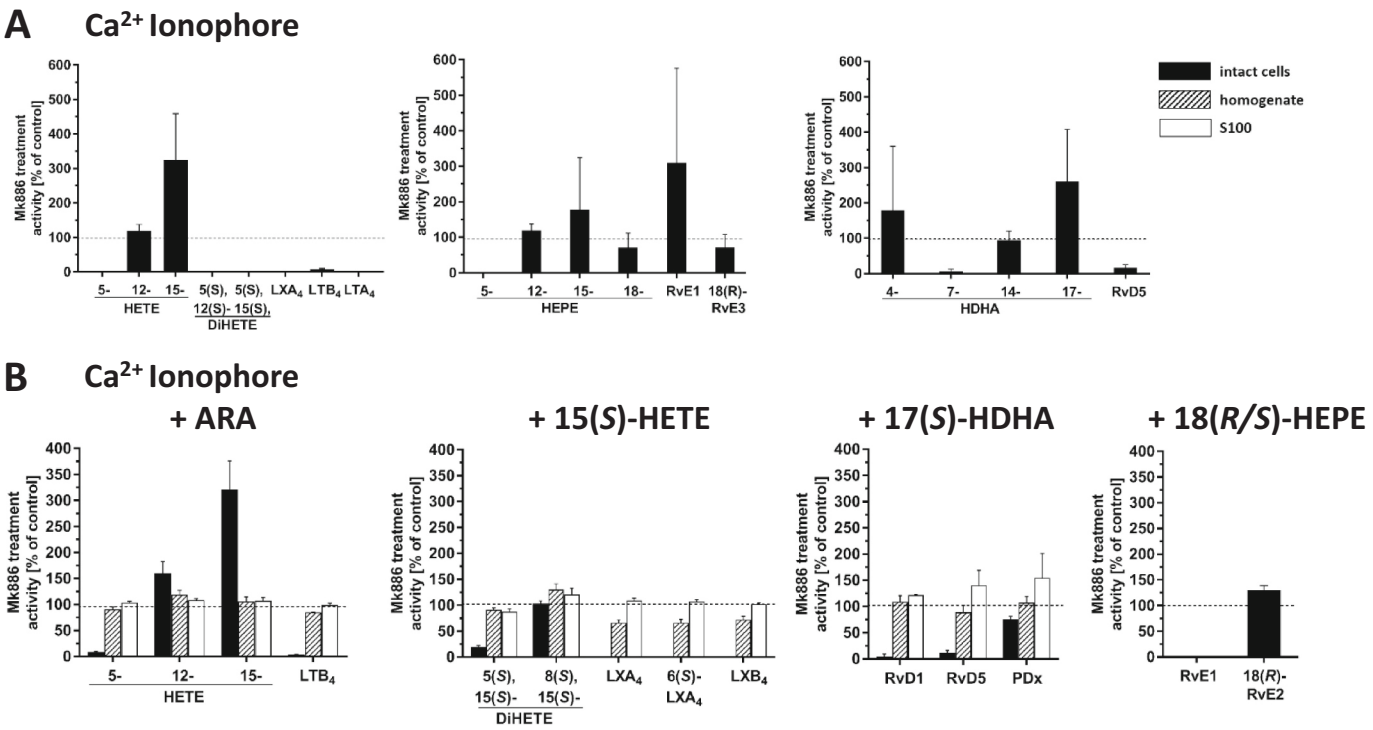

Fig. 7. Influence of FLAP inhibition on SPM formation in human PMNL supplemented with 15(*S*)-HETE, 18(*R/S*)-HEPE or 17(*S*)-HDHA. (A) Intact PMNL were resuspended in PGC-buffer and preincubated with Mk-886 (3 μM) for 15 min followed by stimulation with 5 μM Ca^{2+} ionophore for 15 min at 37 °C. (B) Either 2 × 10^7/mL intact or homogenized PMNL or PMNL homogenates centrifuged at 100,000 *g* (S100) were preincubated with the FLAP inhibitor Mk-886 (3 μM) for 15 min. The reaction was started by addition of 10 μM of 15(*S*)-HETE, 18(*R/S*)-HEPE, 17(*S*)-HDHA or ARA together with 5 μM Ca^{2+} ionophore (intact cells) or 1 mM ATP (homogenates, S100). Reactions were terminated after 15 min with ice-cold methanol and the lipid mediators formed were quantified using LC-MS/MS. Data on lipid mediator formation in ARA treated PMNL were measured according to [45]. Data are depicted as mean + SEM from 3 to 4 independent experiments.

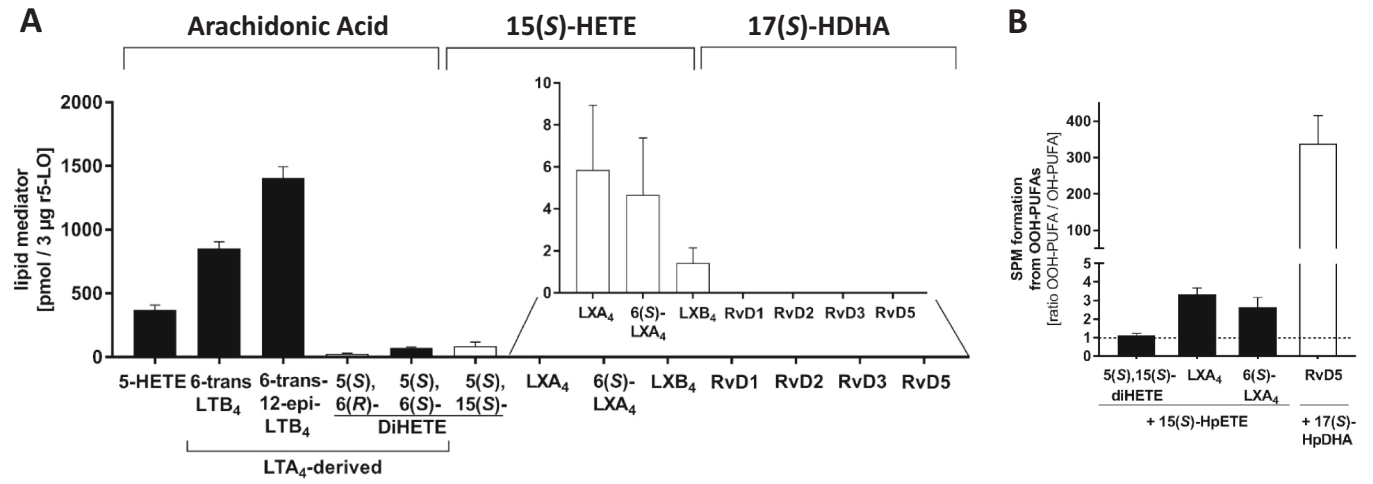

Fig. 8. Lipid mediator formation by human recombinant 5-LO supplemented with 15(*S*)-HETE, 17(*S*)-HDHA or ARA. Human recombinant 5-LO was isolated from *E. coli* by gel filtration. For each sample 1.5 μg of the purified enzyme were diluted in 500 μl PGC buffer. (A) The reaction was started by addition of 10 μM of 15(*S*)-HETE, 17(*S*)-HDHA or ARA together with 1 mM ATP in presence of Ca^{2+}. Reactions were terminated after 15 min with ice-cold methanol and the lipid mediators formed were quantified by LC-MS/MS. Data are depicted as mean + SEM from 4 independent experiments. (B) Comparison of SPM formation from 10 μM hydroxyl PUFA and hydroperoxy PUFA precursors by recombinant human 5-LO. Data are depicted as mean + SEM from 3 independent experiments.

their corresponding hydroperoxy precursors by isolated human 5-LO was studied (Fig. 8B). We found that formation of 5(*S*),15(*S*)-diHETE did not differ between 15(*S*)-HETE and 15(*S*)-HpETE while LXA_4 and 6(*S*)-LXA_4 formation was 2–3 times more efficient from the hydroperoxy PUFA. LXB_4 was below the LLOQ in these incubations. Furthermore, neither 17(*S*)-HDHA nor 17(*S*)-HpDHA were converted into the trihydroxylated D-series resolvins RvD1, RvD2 and RvD3. In contrast, isolated 5-LO readily accepted 17(*S*)-HpHDA for the formation of RvD5 while 17(*S*)-HDHA was hardly converted by the enzyme.

4. Discussion and conclusions

4.1. Discussion

In the present study we investigated the formation of SPM in human neutrophil preparations (PMNL). As expected, the cells displayed abundant 5-LO and FLAP expression (Fig. 3E). Expression of 15-LO-1 was detected as well, probably due to low numbers of eosinophils present in the PMNL fraction, while 15-LO-2 was absent in the preparations. In contrast to this, it was recently found that neutrophils from human peripheral blood isolated by CD16 positive selection express 15-LO-2 while eosinophils exclusively express 15-LO-1 [29]. In our hands, PMNL showed a close band running above 15-LO-2 in the Western Blots. We can therefore not rule out that a post-transcriptionally modified 15-LO-2 was expressed in our neutrophil preparations.

We found that PMNL primarily form ARA-derived lipid mediators such as leukotrienes and 5-HETE from endogenous sources whereas 15-HETE, 5(*S*),15(*S*)-diHETE and the formation of monohydroxylated EPA and DHA derivatives as well as RvD5 was very low (Fig. 3A). Lipoxins, E-series resolvins, maresins, protectins as well as other D-series resolvins were not detected in these incubations. The high levels of 12-lipoxygenation products such as 12-HETE and 14-HDHA were probably due to inevitable platelet contaminations in our preparations.

The formation of low amounts of DHA- and EPA-derived metabolites in Ca^{2+} ionophore stimulated PMNL (Fig. 3A) seems to be due to a low abundance of EPA and DHA in PMNL membranes since supplementation of the cells with these fatty acids strongly increased the formation of EPA- and DHA-derived oxylipins (Fig. 3B). This co-incubation levelled the difference between 15-HETE and 17-HDHA formation suggesting that 15-LO has no substrate preference in PMNL. Nevertheless, PMNL still preferred ARA over DHA for 5- and 12-lipoxygenation. This is in line with our previous finding of a preference for ARA over DHA in the formation of LO-derived lipid mediators in human monocyte-derived M1 and M2 macrophages [17].

With the exception of RvD5, SPMs were not formed by PMNL even if the cells were supplemented with ARA and DHA. RvD5 is a 5-LO/15-LO dioxygenation product that can be considered as the DHA-derived analogue to 5(*S*),15(*S*)-diHETE. This lack of SPM formation is surprising since PMNL release substantial amounts of 5-HETE, 15-HETE and 5,15-diHETE under these conditions. Apparently, neither the abundant amounts of 5-H(*p*)ETE/7-H(*p*)DHA formed in PMNL were converted into SPMs by 15-LO-1 present in the preparations nor was 15-H(*p*)ETE/17-H(*p*)DHA efficiently metabolized by 5-LO. Therefore, our data suggest that PMNL alone are not able to synthesize significant amounts of trihydroxylated SPMs such as lipoxins, RvD1, RvD2 and RvD3 from endogenous sources although 5-LO-derived precursors are available.

In order to mimic the transcellular 15-LO/5-LO route of SPM formation, we added the SPM precursors 15-HETE, 17-HDHA or 18-HEPE to PMNL and stimulated the cells with Ca^{2+} ionophore (Fig. 4A). Exogenous 15-HETE has been shown to switch the substrate utilization of 5-LO from ARA to 15-HETE products in neutrophils and eosinophils when both lipids are present at the same time thereby reducing the formation of leukotrienes and 5-HETE [30–35].This would argue for a lipid mediator switch in PMNL upon accumulation of extracellular 15-LO products. In addition to this preference for 15-HETE, we show here that PMNL also change their lipid 5-lipoxygenation preference. Upon addition of 15-HETE, 17-HDHA or 18-HEPE, SPM formation from DHA-derived 17(*S*)-HDHA was 4-fold more efficient compared to 15(*S*)-HETE. Again, 5(*S*),15(*S*)-diHETE and its DHA analogue RvD5 clearly dominated the SPMs released in PMNL supplemented with 15(*S*)-HETE or 17(*S*)-HDHA, respectively. Although formation of lipoxins, RvD1, RvE1, 18(*R*)-RvE2 and PDx was detectable upon supplementation with the monohydroxy precursors, the levels formed were very low. Our data show that even upon exogenous addition of substantial quantities, the SPM-precursors 15-HETE, 17-HDHA and 18-HEPE are scarcely used for the formation of SPM. Thus, the capacity to form pro-inflammatory lipid mediators such as leukotrienes or 5-HETE exceeds that for SPM formation in PMNL by far. Exceptions from this are the dihydroxylated 5-/15-lipoxygenation products 5(*S*),15(*S*)-diHETE and RvD5. In line with these data, we recently published that TLR-4-stimulated human M2 macrophages which co-express 15-LO-1 and -2 release substantial amounts of 5(*S*),15(*S*)-diHETE and RvD5 but only low concentrations of other SPMs such as lipoxins, *E*-series resolvins, RvD1 and RvD2 upon supplementation with a mix of ARA, EPA and DHA [17]. Apparently, oxylipins formed in human neutrophils, eosinophils and macrophages are dominated by the dihydroxylated ARA and DHA derivatives 5(*S*),15(*S*)-diHETE and RvD5 (7(*S*),17(*S*)-diHDHA) derived from the sequential dioxygenation by 5-LO and 15-LO. 5-LO's downstream epoxidase activity on 5(*S*)-hydroperoxy,15(*S*)-HETE and 7(*S*)-hydroperoxy-17(*S*)-HDHA seems to be poor. Judged by the amounts of lipid mediators formed in intact PMNL, precursor turnover can therefore be classified as follows: 17-HDHA > ARA = 15-HETE > DHA > 18-HEPE for 5-LO products formed by dioxygenation and 18-HEPE > 15-HETE = 17-HDHA > ARA = DHA for the trihydroxylated products mainly formed by 5-LO's epoxidation reaction.

Regarding ARA, 5-LO catalyses LTA_4 formation via two reactions: First 5(*S*)-HpETE is formed by insertion of molecular oxygen (deoxygenation reaction). If 5(*S*)-HpETE stays in the active center, epoxidation of the hydroperoxide follows resulting in the formation of LTA_4 [11]. Due to a superior substrate capture rate (k_{cat}/k_M) 5-LO prefers ARA over 5(*S*)-HpETE. Therefore, LTA_4 accumulation can be observed when ARA availability is limited [35–37]. It seems as if 5-LO's preference for substrates carrying an unoxidized C atom in position 5 also applies to 15-HETE/17-HDHA in comparison to 5(*S*)-hydroperoxy-15(*S*)-HETE/7(*S*)-hydroperoxy-15(*S*)-HDHA in our incubations favoring the formation of dihydroxylated products instead of trihydroxylated ones. In accordance with this, it was recently shown that recombinant 5-LO does not accept 5(*S*),15(*S*)-diHpETE for the formation of LXA_4 due to a much slower substrate capture rate of 5(*S*),15(*S*)-diHpETE compared to 15(*S*)-HETE [38].

There is a lack of comprehensive studies comparing SPM formation capacity from monohydroxy PUFAs (15-HETE, 17-HDHA, 18-HEPE) with their hydroperoxy precursors (15-HpETE, 17-HpDHA, 18-HpEPE) in cellular systems. It was recently shown, that the turnover of 15(*S*)-HETE and 15(*S*)-HpETE by isolated 5-LO is comparable and differs only by a factor of 1.5 [39]. In line with this, earlier studies compared lipoxin formation from 15-HETE or 15-HpETE in PMNL and found little difference in the product quantities [20,21,40]. Since this issue has never been addressed systematically for a larger number of SPM, we compared formation of 5(*S*),15(*S*)-diHETE, lipoxins and D-series resolvins in intact human PMNL und with isolated recombinant human 5-LO. In line with recent studies on isolated 5-LO [39], 5(*S*),15(*S*)-diHETE, lipoxins and D-resolvin formation was only moderately elevated upon treatment with the hydroperoxy precursors in intact PMNL. The same was true for lipoxin and 5(*S*),15(*S*)-diHETE formation from isolated recombinant 5-LO. Of note, the use of 17(*S*)-HpDHA instead of 17(*S*)-HDHA strongly elevated the formation of RvD5 by the recombinant enzyme. This shows again that 5-LO only accepted the monohydroxy/monohydroperoxy PUFAs while the epoxidation reaction at position 5/6 or 7/8 is not efficiently executed.

Two alternative conclusions can be drawn from these data: A) Formation of SPMs other than 5(*S*),15(*S*)-diHETE and RvD5 is highly

inefficient in humans due to a decidedly slow substrate capture rate of the dihydroperoxylated PUFAs by 5-LO epoxidase activity. B) Unknown factors might exist which are triggered by stimuli other than Ca^{2+} ionophore that allosterically control the substrate acceptance of 5-LO to facilitate a higher turn-over of bulkier, more hydrophilic substrates.

Since cellular 5-LO activity is regulated in a complex manner by factors such as the intracellular calcium level [41], phosphorylation [42] and the cellular redox tone [43], we also investigated the activity of the PMNL LOs in broken cell preparations. This better reflects the biosynthetic capacity and usually correlates with LO protein expression [11]. Furthermore, disruption of PMNL cell integrity can mimic cell death and lysis during inflammatory processes which is known to contribute to the switch of inflammation to resolution. Interestingly, destruction of PMNL integrity followed by 100,000 *g* centrifugation led to the gradual inhibition of 17(*S*)-HDHA- and 18(*R/S*)-HEPE-derived SPMs while 15(*S*)-HETE-derived 5(*S*),15(*S*)-diHETE and lipoxin formation strongly increased (Fig. 6). On the other hand, when recombinant human 5-LO was treated with 17(*S*)-HDHA or 15(*S*)-HETE the enzyme did not accept these substrates for further oxidation.

These data clearly show that the utilization of the SPM precursors 18-HEPE and 17-HDHA needs an intact cellular environment. In contrast to resolvins, formation of lipoxins and 5(*S*),15(*S*)-diHETE was dramatically enhanced in homogenates and S100 preparations treated with 15(*S*)-HETE (Fig. 6A). However, recombinant human 5-LO alone only poorly accepted 15(*S*)-HETE as substrate whereas ARA was readily converted by the recombinant enzyme. We have recently shown that isolated human 5-LO accepts 18-HEPE for further conversion into RvE2, RvE3 and RvE1 isomers in the presence of phosphatidylcholine (PC) [27]. Since PC was not added to incubations of recombinant 5-LO in this study, it can be speculated that presence of PC might trigger acceptance of 15(*S*)-HETE as well suggesting that binding to phospholipid bilayers in the presence of Ca^{2+} and ATP might be sufficient to induce changes in 5-LO that allow the acceptance of bulkier, more hydrophilic lipids such as 15-HETE, 17-HDHA and 18-HEPE. Indeed, it is known that the activity of purified recombinant 5-LO is upregulated in presence of PC vesicles [36]. Furthermore, membrane association of 5-LO has been shown to change the substrate specificity of the enzyme allowing the utilization of 12(*S*)-HETE and 15(*S*)-HETE as substrates [44]. This also fits our recent findings that a 5-LO triple tryptophan mutant deficient in membrane binding and translocation shows attenuated lipoxin formation in intact HEK293 cells [13].

Taken together, these data strongly argue for a dependency of SPM biosynthesis on 5-LO translocation in intact cells but does neither explain the elevated lipoxin nor the attenuated resolvin levels in broken PMNL preparations. Since elevated lipoxin levels were also observed in PMNL S100 supernatants which do not contain membrane fragments, it seems that an additional cytosolic factor in PMNL is involved in lipoxin formation.

Total precursor recovery encompassing the LO-derived metabolites plus the unchanged remains of the respective monohydroxylated PUFA averaged almost 100% for both 15-HETE enantiomers, 17(*R*)-HDHA and 18(*R/S*)-HEPE in intact PMNL as well as in the broken cell preparations (Fig. 5). This suggests that the detection method we used captured the vast majority of oxidation products formed from the respective precursor in PMNL and also ruled out a substantial loss of precursor lipids due to adherence to plastic ware or cell debris. In contrast, recovery of 17(*S*)-HDHA was only around 70% in intact cells and 80% in S100 preparations suggesting that one or more unknown oxidation products exist which were not covered by our method. Since S100 preparations which are devoid of cell membrane debris also showed reduced recovery of 17(*S*)-HDHA, elevated membrane incorporation can be ruled out as main cause for the reduced recovery in our assays. The nature of the unknown 17(*S*)-HDHA metabolite(s) has to be elucidated in the future.

In addition to 5-LO translocation and membrane association, we have recently shown that FLAP is essential for LXA_4 and RvD1 formation in neutrophils which are exogenously supplied with 15-HETE and 17-HDHA [13]. Formation of 5(*S*),15(*S*)-diHETE has been shown to be FLAP-dependent as well [28]. FLAP is an integral membrane protein of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) family. It is thought to transfer substrate to 5-LO thereby being essential for the enzyme's activity in intact cells. In addition, FLAP serves as a membrane anchor for 5-LO [11]. In this study we could support the FLAP dependency of RvD1, LXA_4 and 5(*S*),15(*S*)-diHETE biosynthesis. Furthermore, we show here that formation of LXB_4, RvE1 and RvD5 are also sensitive to inhibition by Mk-886.

4.2. Conclusions

Biosynthesis of pro-resolving lipid mediators in humans is far from understood. Here, we could show that SPMs formed in human neutrophils are dominated by the dihydroxylated ARA and DHA derivatives 5(*S*),15(*S*)-diHETE and RvD5 derived from the dioxygenation by 5-LO and 15-LO. In contrast, 5-LO's downstream epoxidase activity, a prerequisite for lipoxin and resolvin biosynthesis, is poor. Furthermore, we show that FLAP is important for biosynthesis of all 5-LO-dependent SPMs measured in intact PMNL and that membrane association is a prerequisite for the acceptance of monohydroxy PUFAs by 5-LO. In addition, we show that neutrophils prefer ARA over DHA for SPM biosynthesis while 17-HDHA is preferred over 15-HETE and 18-HEPE for the generation of the dioxygenation products. Of note, formation of E- and D-series resolvins was restricted to intact cells, whereas biosynthesis of lipoxins and 5(*S*),15(*S*)-diHETE was strongly upregulated upon destruction of cell integrity. Together with the data on FLAP inhibition our results point to unknown factors that control SPM formation and also render lipoxin and 5(*S*),15(*S*)-diHETE formation independent of membrane association and FLAP when cellular integrity is destroyed.

CRediT authorship contribution statement

Malwina Mainka: Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Sven George:** Investigation, Writing – review & editing. **Carlo Angioni:** Investigation, Formal analysis, Writing – review & editing. **Roland Ebert:** Investigation, Writing – review & editing. **Tamara Goebel:** Investigation, Writing – review & editing. **Nadja Kampschulte:** Investigation, Formal analysis, Writing – review & editing. **Andre Krommes:** Investigation, Writing – review & editing. **Andreas Weigert:** Writing – review & editing. **Dominique Thomas:** Investigation, Formal analysis, Writing – review & editing. **Nils Helge Schebb:** Supervision, Funding acquisition, Resources, Writing – review & editing. **Dieter Steinhilber:** Supervision, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **Astrid Stefanie Kahnt:** Supervision, Conceptualization, Project administration, Investigation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors state no conflict of interest. This article has not been published elsewhere and is approved by all authors. If accepted it will not be published elsewhere without the written consent of the copyright-holder.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2021.159093.

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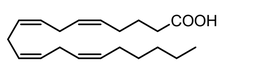

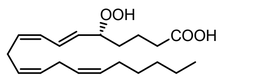

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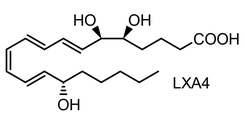

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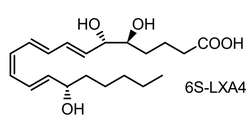

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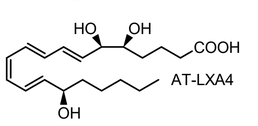

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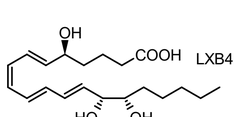
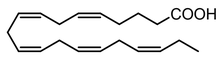
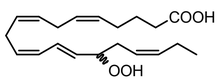
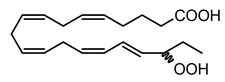
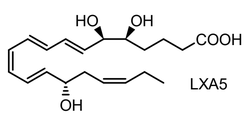


LXB4









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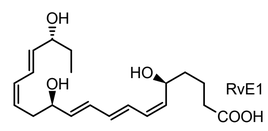
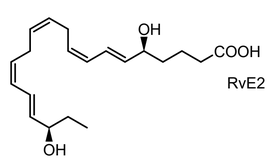
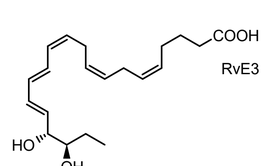


RvE1



RvE2

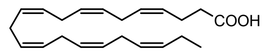


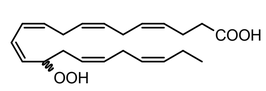
RvE3

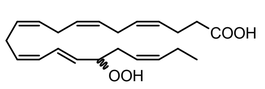
LTB4

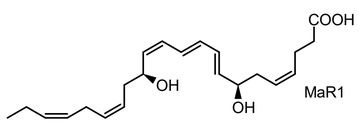
LTA4

RvE3

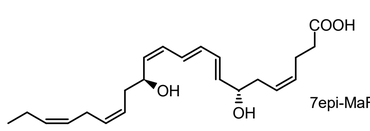




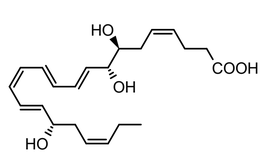




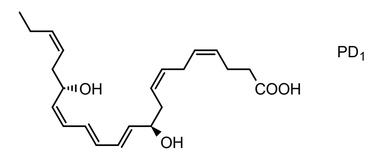
MaR1



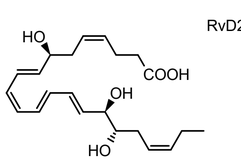
7epi-MaR1



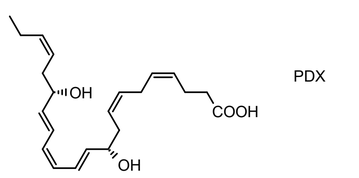
RvD1



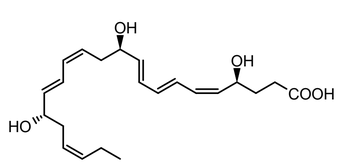
PD1



RvD2



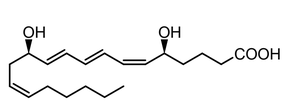
PDX



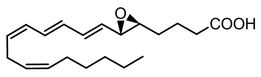
RvD3
(cayman)

RvD5

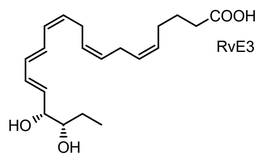
17RRvD1



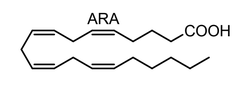
LTB4



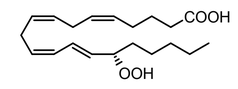
LTA4



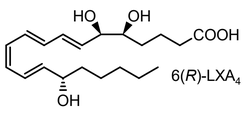
RvE3



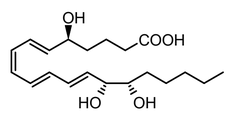
ARA



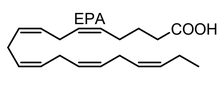
15-HpETE



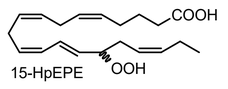
6(*R*)-LXA$_4$



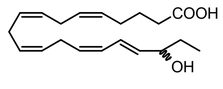
LXB$_4$



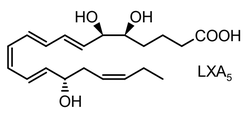
EPA



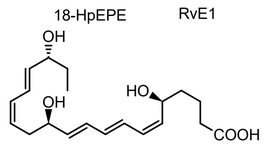
15-HpEPE



18-HpEPE



LXA$_5$



RvE1

RvE3

RvE2

DHA

14-HpDHA

MaR1

RvD1

17-HpDHA

(N)PD$_1$

RvD2

RvD5

RvD3

PDX

ARA

EPA

15-HpETE

15-HpEPE

6(*R*)-LXA$_4$

LXA$_5$

RvE1

LXB$_4$

RvE3

RvE2

5,15-HpETE

5(6)ep,15-HpETE

LXA$_4$

15(*R*)-LXA$_4$

6(S)-LXA$_4$

5,18-DiHEPE

5,6-Ep-18-HEPE