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ORIGINAL ARTICLE

Metabolism of Anandamide into Eoxamides by 15-Lipoxygenase-1 and Glutathione Transferases

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Abstract Human 15-lipoxygenase-1 (15-LO-1) can metabolize arachidonic acid (ARA) into pro-inflammatory mediators such as the eoxins, 15-hydroperoxyeicosatetraenoic acid (HPETE), and 15-hydroxyeicosatetraenoyl-phosphatidylethanolamine. We have in this study investigated the formation of various lipid hydroperoxide by either purified 15-LO-1 or in the Hodgkin lymphoma cell line L1236, which contain abundant amount of 15-LO-1. Both purified 15-LO-1 and L1236 cells produced lipid hydroperoxides more efficiently when anandamide (AEA) or 2-arachidonoyl-glycerol ester was used as substrate than with ARA. Furthermore, L1236 cells converted AEA to a novel class of cysteinyl-containing metabolites. Based on RP-HPLC, mass spectrometry and comparison to synthetic products, these metabolites were identified as the ethanolamide of the eoxin (EX) C_4 and EXD_4. By using the epoxide EXA_4-ethanol amide, it was also found that platelets have the capacity to produce the ethanolamide of EXC_4 and EXD_4. We suggest that the ethanolamides of the eoxins should be referred to as eoxamides, in analogy to the ethanolamides of prostaglandins which are named prostamides. The metabolism of AEA into eoxamides might engender molecules with novel biological effects. Alternatively, it might represent a new mechanism for the termination of AEA signalling.

Keywords 15-Lipoxygenase-1 · Anandamide · Endocannabinoid · Inflammation · Pain · Arachidonic acid · Glutathione

Abbreviations

LO	Lipoxygenase
ARA	Arachidonic acid
AEA	Anandamide
EX	Eoxin
LT	Leukotriene
2-AG	2-Arachidonoyl-glyceryl ester
PPAR	Peroxisome proliferator-activated receptor
GSH	Glutathione
GST	Glutathione-S-transferase

Introduction

Lipoxygenases (LOs) are a family of enzymes catalyzing the positional as well as stereo-specific introduction of molecular oxygen into *cis*-1,4-pentadiene structures found in unsaturated fatty acids or derivatives thereof [1–3] leading to the formation of a hydroperoxide. Oxidation of arachidonic acid (ARA) by 5-lipoxygenase (5-LO) (EC 1.13.11.34), the key enzyme in leukotriene synthesis, leads to the formation of the unstable epoxide leukotriene (LT) A_4. This compound can be conjugated with glutathione to form LTC_4 by LTC_4 synthase (EC 4.4.1.20) [4] or hydrolyzed by LTA_4 hydrolase (EC 3.3.2.6) to form LTB_4. Leukotriene C_4, and its metabolites LTD_4 and LTE_4, are proinflammatory agents, potent bronchoconstrictors and

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inducers of plasma leakage [5] whereas LTB_4 is a potent chemoattractant [5]. In analogy to the formation LTC_4, a pathway for the formation of eoxins, a class of structurally related pro-inflammatory lipid mediators, has been described [6, 7]. The oxidation of ARA, catalyzed by human 15-lipoxygenase-1 (15-LO-1) (EC 1.13.11.33), leads in two consecutive steps to the unstable epoxide eoxin (EX) A_4 which can be conjugated to glutathione to yield EXC_4, a reaction very similar to the formation of LTC_4 [6, 8]. The conjugation of glutathione to lipid epoxides is performed by glutathione transferases, including LTC_4-synthase (LTC4S), microsomal glutathione S-transferase 2 (mGST-2) and soluble glutathione transferases [6, 9–11].

In contrast to 5-LO, less is known about the physiological or pathological role of 15-LO-1. Thus, the finding that eoxins are proinflammatory might shed some light onto the role of 15-LO-1 in inflammatory processes. Furthermore, 15-HPETE and 15-HETE-phosphatidylamine have also been found to possess pro-inflammatory effects [12, 13]. On the other hand, 15-LO-1 metabolites have also been demonstrated to possess anti-inflammatory roles [14]. The expression of human 15-LO is selective and cells such as human eosinophils, airway epithelial cells, reticulocytes, mast cells and dendritic cells has been demonstrated to express 15-LO-1 [7, 15, 16]. The enzyme 15-LO-1, or its rodent ortholog 12/15-LO, has been implicated in pro- as well as anti-inflammatory processes [3, 17]. Two reports describe that ovalbumin sensitized 12/15-LO null mice have reduced airway inflammation, reduced cytokine production and less proliferation of airway epithelial cells after exposure of allergen [18, 19]. Other pathological processes where 15-LO-1 has been suggested to play a role includes cytokine release from airway epithelial cells [20], cardiac inflammation [21], propagation of cancer metastasis [22], atherosclerosis [23–25], Alzheimer's disease [26], insulin resistance and diabetic nephropathy [27, 28]. Furthermore, pharmacological evidence suggests that 15-LO-1 is involved in neuronal cell death induced by oxidative stress [29]. Unlike 5-LO, 15-LO-1 and 12/15-LO has a broad substrate specificity [15] and the enzyme can metabolize membrane bound phospholipids [27] and other conjugated lipids like endocannabinoids and vanilloids to their corresponding mono-hydroxy metabolites [30–35].

Endocannabinoids are a family of endogenous lipid-derived mediators of which arachidonyl ethanolamide (AEA) (anandamide) and 2-arachidonoylglycerol (2-AG) are amongst the most characterized substances. Both AEA and 2-AG are agonists of the G-protein coupled cannabinoid receptors (CB) 1 and 2 [36, 37]. AEA and other endocannabinoids also bind to the vanilloid receptor channel TRPV-1 (VR-1), an ion channel that mediates the pungent effect of the red hot chilli pepper component capsaicin [37, 38].

Oxygenation of endocannabinoids or vanilloids by lipoxygenases has been described in several reports and the monohydroxy derivatives of AEA seem to signal through CB1/2, TRPV-1 and PPAR-α [30–35]. The lipid class *N*-acyltaurines (NAT) was recently discovered and arachidonoyltaurine has also been shown to function as a substrate for lipoxygenases but not for cyclooxygenases [39]. On the other hand, cyclooxygenase-2 (COX-2) (EC 1.14.99.1) has been reported to metabolize AEA and 2-AG to their corresponding prostaglandin-ethanolamide and glycerol esters with high efficiency [40–42]. The pharmacology of these COX-2 metabolites is rapidly being defined and they are now collectively called prostamides [43]. The oxygenation of endocannabinoids by either cyclooxygenases or lipoxygenases is an expanding research area and there is a great likelihood that the oxygenation pathways for endocannabinoids will intersect with eicosanoid pathways [44, 45].

We report herein the in-vitro identification of a novel class of cysteinyl-containing eoxin-like metabolites derived from AEA, i.e. eoxin-ethanolamides, which in analogy to the prostamides are named eoxamides. This pathway of AEA metabolism may lead to a new class of biological active endocannabinoid metabolites or alternatively, a new pathway for terminating AEA signalling.

Materials and Methods

Materials

ARA, AEA and LNA were purchased from NuCheck. HPLC solvents were from Rathburn Chemicals (Walkerburn UK). Synthetic 13-hydroxyoctadeca-9*Z*,11*E*-dienoic acid (13-HODE) and 15-hydroxyeicosa-5*Z*,8*Z*,11*Z*,13*E*-tetraenoic acid (15-HETE) were from Biomol. Diphenyl-1-pyrenylphosphine (DPPP) was from Molecular Probes, Invitrogen. Tissue culture medium, antibiotics, fetal calf serum and Dulbeccos phosphate buffered saline (D-PBS) were from Gibco (Paisley, Scotland, UK). 14,15-epoxy-5,8,10,12 (*Z*,*Z*,*E*,*E*)-eicosatetraenoyl-EA (EXA_4-EA) was provided by Mats Hamberg (Karolinska Institutet, Sweden). All other chemicals and reagents were from Sigma-Aldrich.

Chemical Synthesis of Eoxin Amides

14-Glutathionyl-15-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoyl-EA (EXC_4-EA) was synthesized from EXA_4-EA by conjugation to glutathione under alkaline conditions. Briefly, EXA_4-EA was dried under nitrogen and resuspended in 50 mM NaOH and 5 mM GSH to a final

concentration of 1 mM. The reaction was allowed to proceed for at least 3 h at room temperature. The purity and quantization of the produced EXC_4-EA was performed with RP-HPLC and mass spectrometry. 14(*R*)-cysteinyl-glycyl-15(*S*)-hydroxy-5,8,10,12 (*Z*,*Z*,*E*,*E*)-eicosatetraenoyl-EA (EXD_4-EA) was synthesized as described above with the exception that glutathione was exchanged for Cys-Gly (Sigma-Aldrich, Sweden).

15-LO-1 Fluorescent Enzyme Assay

The 15-LO-1 activity was measured in 96-well black plates (Optiplate, Corning) essentially as described [46]. Briefly, purified 15-LO-1 diluted in D-PBS at 3.18 μg/mL (2.8 μg/mL final concentration) or D-PBS alone was added to each well. Thereafter was 5 μL 2 mM LNA added to each well and the plate was incubated at room temperature for 10 min. The reaction was terminated with 1 volume of methanol containing 0.25 mM DPPP for fluorescent detection of lipid hydroperoxides or with one volume of methanol without DPPP for RP-HPLC-analysis of metabolites as described below. Fluorescence was measured 30 min after the addition of DPPP using a Spectramax Gemini fluorescence plate reader (Molecular Devices) with an excitation wavelength of 363 nm and an emission wavelength of 380 nm.

15-LO-1 Cell-based Assay

The human Hodgkin lymphoma cell line L1236 was grown in a humidified atmosphere at 5 % CO_2 in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum and L-glutamine. The cells were washed twice in D-PBS before suspension in D-PBS at a final concentration of 10×10^6 cells/mL. Substrate diluted in ethanol was added to yield a final concentration of 50 μM (or as indicated) and the samples were thereafter incubated for typically 2 min at 37 °C. The reaction was terminated by the addition of two volumes of methanol and the samples were stored at −20 °C for at least 60 min before RP-HPLC analysis.

Analysis of Lipoxygenase Products by RP-HPLC

Plates were centrifuged at 2,500 rpm for 5 min to remove precipitated proteins. The resulting supernatants was used for the analysis of monohydroxy acids, eoxins and cysteinyl-containing metabolites of AEA by injecting an aliquot of the supernatant onto a Onyx Monolithic C18 column (100 × 4.6 mm from Phenomenex) at a flow rate of 1.2 mL/min using a Waters Alliance 2795 system. Products were eluted by a 1-min isocratic period followed by a 20-min linear gradient from 20:27:53:0.8 to 35:35:30:0.8 (acetonitrile:methanol:H_2O:acetic acid (pH adjusted to 5.6 with NH_3) (by volume).The retention times for all metabolites were compared to authentic standards and qualitative measurements were performed using a 2996 photodiode-array (PDA) detector to verify the spectrum of peaks.

Analysis of products from cellular incubations with EXA_4-EA were performed by injecting an aliquot of the supernatant onto a C18 NovaPak (3.9 × 150 mm) column from Waters coupled to a Waters Alliance 2695 system equipped with a 2996 PDA UV-detector using a flow rate of 1.2 mL/min. Products were eluted by a 4-min isocratic period followed by a 10-min linear gradient from 25:15:60:0.8 to 45:45:10:0.8 [acetonitrile:methanol:H_2O:acetic acid (pH adjusted to 5.6 with NH_3] (by volume). In cases with large sample volumes, the supernatants were evaporated under reduced pressure and the residues dissolved in methanol and transferred to a test tube. Samples were thereafter evaporated to dryness under a stream of nitrogen, and finally resuspended in 300 μl of the appropriate mobile phase.

Nanospray Mass Spectrometry Analysis

After termination of incubations, samples were centrifuged (1400×*g*, 6 min) and the supernatants were diluted with water to contain maximum 25 % methanol and transferred to a washed and equilibrated extraction cartridge, Oasis HLB 1 cc 10 mg (Waters AB, Sweden). The columns were washed with water and eluted with 200 μl methanol to retrieve the metabolites. Reverse phase HPLC was performed on a Waters Alliance 2690 with a Nova Pak C_{18} column (2.1 × 150 mm, Waters AB). The initial mobile phase was 100 % A (0.01 % acetic acid adjusted to pH 5.6 with ammonia) at a flow rate at 0.4 mL/min. A linear gradient was started after 5 min, reaching 36 % B (60:40 acetonitrile: methanol) at 20 min. The mobile phase was isocratic at 64:36, A:B for 100 min. Column effluent was monitored using diode array detection (PDA 996, Waters AB, Sweden). UV spectra were acquired between 200 and 340 nm. The material in the peaks I and II was collected with a fraction collector (FC II, Waters AB, Sweden) and further analyzed by mass spectrometry. The collected fractions were dried to some extent under N_2 and subsequently dissolved in methanol:water (1:1). Mass spectrometry was performed on a Quattro Micro or a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) operating in positive ion mode with a capillary voltage at 2.2 kV. MS/MS were obtained using collision energy of 20 eV using argon as the collision gas.

Statistical Analysis

Raw data were imported into Graph Pad PRISM 5.0 and all graphs and chromatograms were generated by the use of this software. Statistical analyses were performed by Students two-tailed unpaired paired *t* test.

Results

Comparison of Different Lipid-derivatives as Substrate for Human 15-LO-1

Human 15-LO-1 is known to use a variety of lipids as substrates, albeit with different efficient substrate utilization, i.e. different K_m and V_{max} [47]. We have previously described a fluorescent method for detecting 15-LO-1 derived lipid hydroperoxides [46]. We used this method to investigate different conjugated neutral derivatives of ARA and LNA, i.e. ethanolamides and glycerol esters, as substrates for 15-LO-1. Purified human enzyme was incubated with different substrates as outlined in Fig. 1. The fluorescence intensity obtained with ethanolamide of ARA and LNA was significantly higher than the fluorescence obtained with the free fatty acid. Incubation of the glycerol ester of ARA, but not that of LNA, led to significant higher fluorescence as compared to the free acid. The difference between ARA and AEA as substrate for 15-LO-1 was further investigated in a cellular assay by dose–response curves followed by analysis of the monohydroxy products using RP-HPLC. We have previously described that the Hodgkin lymphoma cell line L1236 express high levels of active endogenous 15-LO-1 and that the cells can produce eoxins [8]. Thus, L1236 cells were incubated with various concentrations of ARA or AEA and as depicted in Fig. 2, both substrates induced a robust formation of 15-HETE and 15-HETE-EA, respectively. The apparent K_m ($K_{m(app)}$) was calculated to 108 or 39 μM when using AEA or ARA, respectively. The difference observed in the formation of lipid-hydroperoxides as judged by fluorescence (Fig. 1) could not be observed in the cell-based assay. The cause

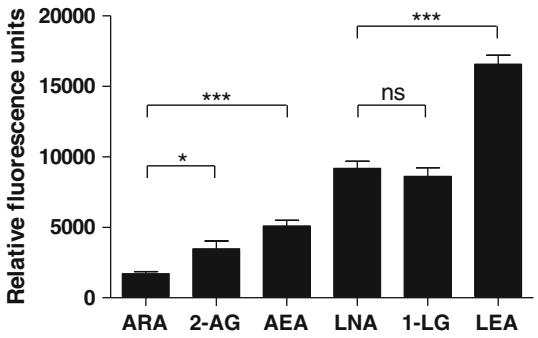

Fig. 1 Formation of lipid hydroperoxides by 15-LO-1. Purified human 15-LO-1 was incubated with 200 μM of the indicated substrate. The reaction was quenched with one volume of methanol containing 125 μM DPPP after 10 min and the lipid hydroperoxide was allowed to react with DPPP for 30 min before the fluorescence was determined. The results are the means ± SEM from three independent experiments each performed in triplicates. *ARA* arachidonic acid, *2-AG* 2-arachidonoylglycerol, *AEA* anandamide, *LNA* linoleic acid, *1-LG* 1-linoleoyl-glycerol, *LEA* linoleoylethanolamide

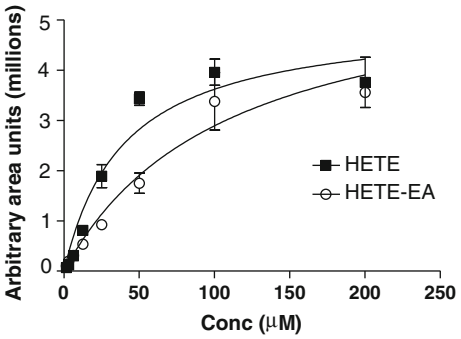

Fig. 2 Formation of monohydroxy metabolites in L1236 cells. L1236 cells (5×10^6 cells/mL) were incubated with the indicated concentrations of ARA or AEA. The amount of 15-HETE or 15-HETE-EA as well as 12-HETE or 12-HETE-EA was quantified by RP-HPLC. The formation of 15- and 12-HETE is presented as the sum of the two metabolites and the results are the means ± SEM ($n = 3$) from two independent experiments. *Open circles* indicate the sum of 12- and 15-HETE-EA and *solid squares* indicate the sum of 12- and 15-HETE

for this might be that the lipid-hydroperoxides were not metabolized in the enzyme assay due to the fact that they were rapidly reduced to the monohydroxy metabolites in the presence of DPPP. The metabolism of the lipid-hydroperoxides in the cell-based assay is most likely very different as compared to the metabolism using purified enzyme. Epoxides could be formed by dehydration or hydroperoxide isomerize activity of 15-LO-1. Furthermore, the epoxides might be non-enzymatically hydrolysed to several dihydroxy products or conjugated to glutathione by a glutathione transferase, as described for the formation of eoxins in L1236 cells [7].

Analysis of Anandamide Metabolites in L1236 Cells

The characterization of ARA metabolites in L1236 cells has been described [8] but no report have so far characterized metabolism of AEA in L1236 cells. In order to characterize the metabolism of AEA in L1236 cells, we investigated the formation of dihydroxy-AEA such as 8(*S*),15(*S*)-, 8(*R*),15(*S*)- and 14(*R*),15(*S*)-dihydroxy-AEA as well as putative cysteinyl-containing AEA metabolites by RP-HPLC after incubation of cells with AEA. As shown in Fig. 3, incubations with AEA led to the formation of metabolites with a characteristic UV-absorbance maximum at 270 nm with two shoulders, thus indicating the presence of a conjugated triene in these samples (Fig. 3, peaks 4–6). These metabolites are thus likely to be dihydroxy-metabolites of AEA. Interesting was the fact that we observed two major and one minor metabolite with an UV-absorbance maximum at 280 nm and two shoulders after incubation of L1236 cells with AEA (Fig. 3, peaks 1–3). These characteristics of the UV-spectrum indicate a triene conjugated with glutathione. The retention time of these mediators were

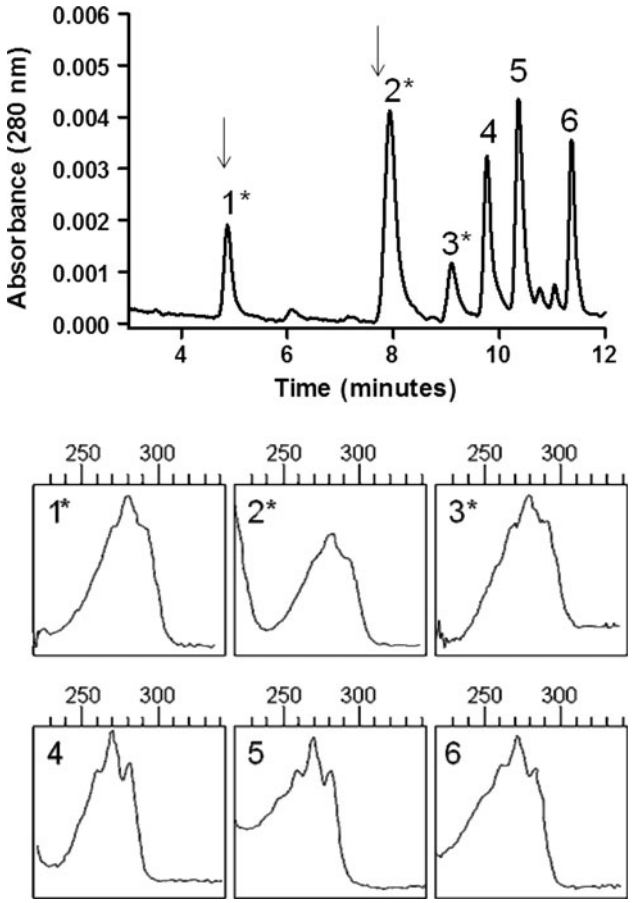

Fig. 3 Chromatographic profile of anandamide metabolites in L1236 cells. L1236 cells (1.0×10^7 cells/mL) were incubated with 50 μM of AEA. An aliquot of the supernatant (200 μL) was injected onto a Onyx monolithic C18 column and the formation of eluting cysteinyl-containing metabolites was analyzed by their absorbance at 280 nm. *Upper panel* shows the elution profile and absorbance at 280 nm. *Asterisks* at peaks 1, 2 and 3 denote products with UV-absorbance maximum at 280 nm. The elution of synthetic EXC_4-EA and EXD_4-EA were analyzed using the same chromatographic system and the elution times are indicated by *arrows*. *Lower panel* shows the UV-spectra for the indicated peaks

different from other cysteinyl-containing ARA-derivates such as the cysteinyl-containing leukotrienes or the eoxins (data not shown), indicating that these metabolites were unique metabolites of AEA. The most polar AEA metabolite formed with a UV-absorbance maximum at 280 nm (peak 1) was analyzed by mass spectrometry. In a positive ion mode MS scan it had the 669 *m*/*z* present, which correspond to AEA conjugated with glutathione. The material in peak 1 gave rise to a MS/MS daughter ion spectrum, containing 308 *m*/*z* which correspond to the glutathione part (Fig. 4a). The fragmentation occurred mainly in the peptide part. Thus, this gives no information about the position of glutathione in the fatty acid, although nothing in the MS/MS spectra contradicts the theory that the glutathione is positioned on the omega-7 carbon.

In order to verify the identity of the products in peak 1, we synthesized 14,15-epoxy-eicosatetraenoyl-EA and chemically conjugated glutathione. RP-HPLC and MS/MS analysis of this synthetic compound demonstrated identical retention time and UV-spectrum after RP-HPLC analysis (data not shown) as well as identical molecular mass and similar MS/MS fragmentation pattern (Fig. 4b) to the product in peak 1, thus demonstrating that the product in peak 1 is indistinguishable to synthetic 14-glutathionyl-15-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoyl-EA (EXC_4-EA). The stereochemistry at carbon 14 and 15 has not yet been determined. The stereochemistry of the double bonds is based upon the UV-spectrum and has not yet been experimentally verified.

A second AEA-metabolite with a UV-spectrum similar to EXC_4-ethanol amide and a retention time of 8 min was also observed after incubation of L1236 cells with AEA (Fig. 3, peak 2). The mechanism of formation and metabolism of leukotrienes and eoxins suggested that this metabolite could be the ethanol amide equivalent of EXD_4. Thus, we synthesized EXD_4-EA from 14,15-epoxy-eicosatetraenoyl-EA by conjugating the dipeptide Cys-Gly (by a similar addition as described for the conjugation of glutathione). The product of this reaction displayed a similar retention time and UV-spectrum as observed for the less polar compound eluting at 8 min (data not shown) thus suggesting that this metabolite is the EXD_4-ethanol amide (EXD_4-EA) metabolite of AEA (Fig. 5).

Furthermore, a third metabolite with similar UV-spectrum as the other AEA-metabolites was also detectable in L1236 cells after incubation with AEA (Fig. 3, peak 3). The identity of this minor metabolite is unclear but our hypothesis is that this metabolite is EXE_4-EA. EXE_4, another cysteinyl-containing eoxin, was identified after incubations of L1236 cells with EXA_4 [8]. The retention time and UV-spectrum of the third product suggests that the identity of this metabolite is EXE_4-EA.

Based upon the structural similarity to the eoxins, we suggest that these novel AEA-metabolites should be called eoxamides, in analogy to the nomenclature for prostaglandins and the corresponding prostaglandin-ethanol amides, i.e. the prostamides.

Comparison Between the Formation of Eoxins and Eoxamides in L1236 Cells

In order to compare the capacity of L1236 cells to produce cysteinyl-containing 15-LO-1 metabolites derived from ARA or AEA, we quantified the formation of eoxins and eoxamides by RP-HPLC analysis. Since incubation of 15-LO-1 with 2-arachidonoyl glycerol (2-AG) led to significantly higher fluorescence (Fig. 1) than ARA, presumably through the formation of 2-AG lipid hydroperoxides,

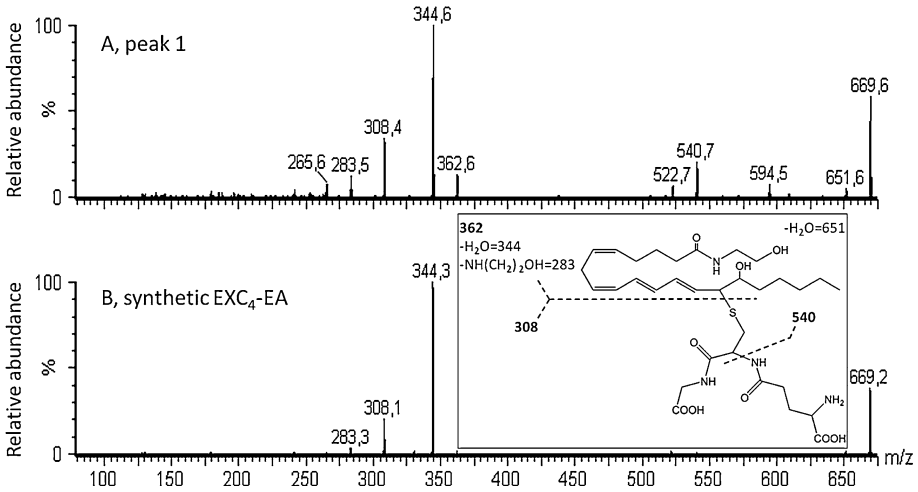

Fig. 4 Identification of anandamide metabolites by MS/MS. L1236 cells were incubated with AEA and the samples were thereafter purified by solid phase extraction and analyzed with RP-HPLC. Peak 1 were collected and further analyzed by mass spectrometry. Peak 1 and synthetic EXC_4-EA had corresponding retention times. The samples were introduced to a triple quadrupole mass spectrometer by nanospray infusion. The MS spectra contained a $[M + H]^+$ at 669 and subsequently MS/MS daughter ion scan at *m/z* 669 was performed. The MS/MS spectra of the material in peak 1 are displayed in *panel* **a**. *Panel* **b** shows spectrum of components in the fraction collected from the HPLC analysis of synthetic EXC_4-EA. The insert in *panel* **b** also shows the structure and suggested fragments of synthetic EXC_4-EA. Spectra A were recorded on a Quattro Ultima (Waters) and spectrum B on a Quattro Micro (Waters)

we also included 2-AG in our studies of cysteinyl-containing 15-LO-1 metabolites. As summarized in Fig. 6, addition of 50 μM of substrate to cells led to a robust formation of cysteinyl-containing metabolites of both ARA and AEA. The cells had higher capacity to convert AEA to EXC_4/D_4-EA than to metabolize ARA to EXC_4/D_4. However, considerably lower amount of cysteinyl-containing 2-AG metabolites were detected.

Metabolism of EXA_4-Ethanol Amide by Human Platelets and L1236 cells

Platelets are well-known producers of eicosanoids and especially TXA_2 and 12-HETE through the actions of COX1/TXAS and 12-LO, respectively [48]. Platelets express LTC_4-synthase and can convert exogenous added LTA_4 to LTC_4 [49]. Thus, to further characterize the extent of eoxamide formation in other cells, we incubated human platelets with EXA_4-EA and analyzed the metabolites formed by RP-HPLC. As demonstrated in Fig. 7, platelets readily produced both EXC_4-EA and EXD_4-EA upon incubation with EXA_4-EA as indicated by the presence of two metabolites with an UV-absorbance maximum at 280 nm (Fig. 7, peak 1 and 4) that co-eluted with synthetic standards of EXC_4-EA and EXD_4-EA (indicated by arrows in Fig. 7). Furthermore, several metabolites with UV-absorbance maximum at 270 nm could be detected after incubation of platelets with EXA_4-EA. These metabolites (peaks 2, 3 and 5) are likely to be dihydroxy metabolites of AEA. Figure 8 shows the profile of cysteinyl-containing EXA_4-EA metabolites formed at different incubation period in both platelets (Fig 8a) and in L1236 cells (Fig. 8b). The major product formed in platelets at all time points was EXD_4-EA (Figs. 7, 8a). The metabolism of EXC_4-EA and EXD_4-EA in L1236 appeared to be more extensive than in platelets since formation of a third less polar metabolite, presumably EXE_4-EA, was observed to a higher extent in L1236 cells than in platelets (Figs. 3, 7, 8b). This metabolite could only be observed in platelets at incubation times of 10 min or longer, and then only to a low extent. The total formation of cysteinyl-containing metabolites of EXA_4-EA was higher in incubations with L1236 cells than in incubations with platelets. However, the amount of protein is different in L1236 cells as compared to platelets and the values are not corrected for the amount of protein in each cell. Thus, the efficacy of the cells to form eoxamides cannot be judged from these data. Studies using purified glutathione transferases in order to identify enzymes capable of synthesizing eoxamides and to investigate enzyme kinetics are currently ongoing.

Discussion

Endocannabinoids have, since their discovery, been the subject of extensive research as well as pharmaceutical intervention [50]. The tonus of AEA is regulated by de novo synthesis as well as with hydrolyses by FAAH or monoacylglycerol lipases and it has been demonstrated that pharmaceutical blockade of AEA hydrolysis have analgesic effects [51–54]. By blocking the degradation of AEA, one would anticipate higher levels of AEA and thus also

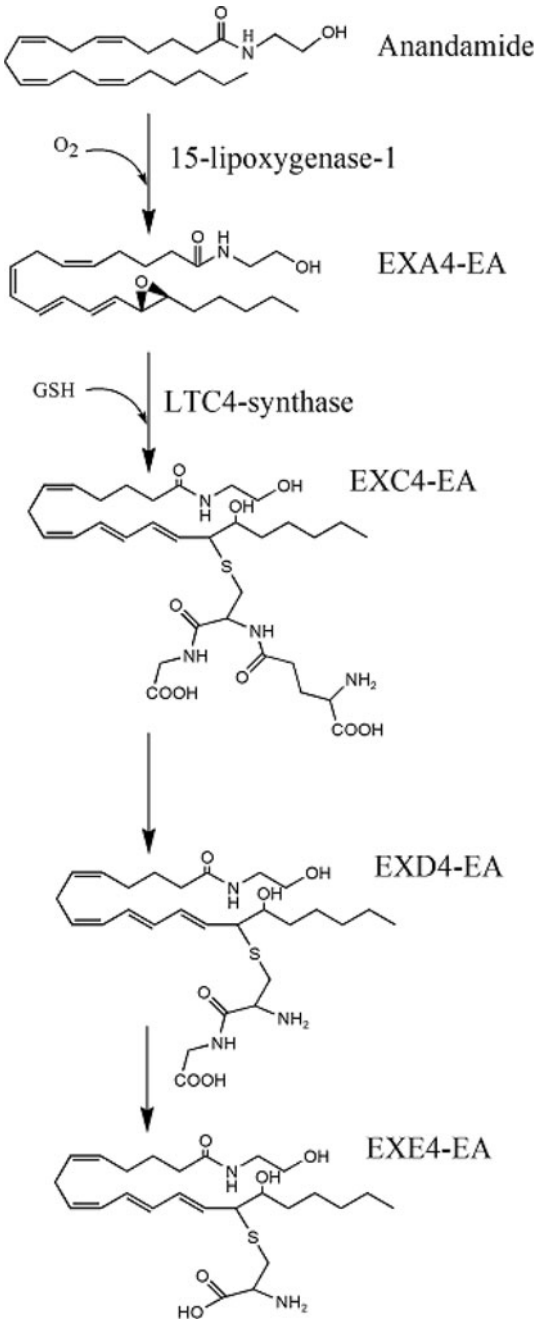

Fig. 5 Pathway for the formation of eoxamides

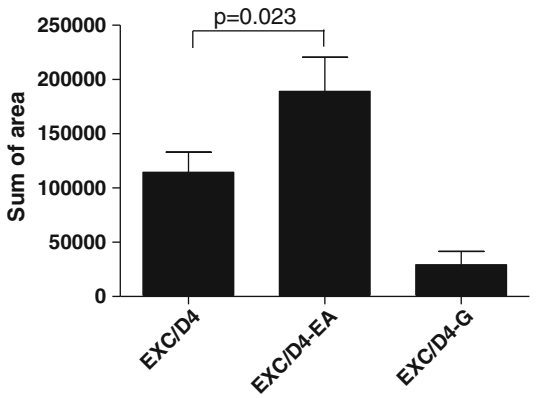

Fig. 6 Relative cellular formation of cysteinyl-containing metabolites formed from ARA, AEA or 2-AG in L1236 cells. L1236 cells (1.0×10^7 cells/mL) were incubated with 50 μM of ARA, AEA or 2-AG. An aliquot of the supernatant (200 μL) was analyzed RP-HPLC. The formation of cysteinyl-containing metabolites were analyzed by their absorbance at 280 nm and by comparison of retention times to synthetic standards. The results are presented as the sum of cysteinyl-containing metabolites ±SEM ($n = 3$) from three individual experiment, each performed in duplicate

several applications for these pharmaceuticals in disorders where AEA signalling is involved in the pathophysiology of the disease. Neuropathic pain is one area where intervention of endocannabinoids might prove to be efficacious [55]. Our finding that 15-LO-1 can utilize neutral lipids such as AEA and 2-AG to the same extent, or even better than the corresponding free fatty acid (Figs. 1, 2), indicates that 15-LO-1 mediated oxygenation of endocannabinoids can be extensive as compared to metabolism of ARA in cells expressing 15-LO-1. Furthermore, we describe herein the formation of previously unknown cysteinyl-containing metabolites of AEA by the concerted action of 15-LO-1 and a glutathione transferase (Figs. 3, 4 and 5). The formation of cysteinyl-containing metabolites of AEA exceeded the formation of cysteinyl-containing metabolites from ARA (Fig. 6), supporting the fact that 15-LO-1 mediated metabolism of AEA can be extensive. Apart from the L1236 cells, platelets were also identified as a cellular source of eoxamides (Figs. 7, 8), but only if provided with the epoxide-precursor 14,15-LTA_4-EA. This is analogous to the formation of LTC_4 by platelets after addition of exogenous LTA_4 [56], so-called transcellular metabolism. Cells that express both 15-LO-1 and an appropriate glutathione transferase can indeed synthesize eoxamides from endogenous sources, as seen in L1236 cells. Platelets which lack 15-LO-1 are dependent on transcellular metabolism of EXA_4 for EXC_4 synthesis. If transcellular metabolism also occurs for eoxamides remains to be determined. The fact that there is metabolism of EXC_4-EA to EXD_4-EA and possibly also to EXE_4-EA (Figs. 3, 8) suggests that the pathway for metabolism of eoxamides are similar to the metabolism of cysteinyl-leukotrienes. The metabolism of eoxamides to a stable end-product such as EXE_4-EA could lead to a way to measure the formation of these novel metabolites in vivo.

Endocannabinoids have been described as having multiple effects within the CNS of which some are due to binding of endocannabinoids to cannabinoid-receptor expressing neurons whereas other effects are due to neuroinflammation [50]. The recent finding that monoacylglycerol lipase (MAGL) can hydrolyze 2-arachidonoylglycerol to generate

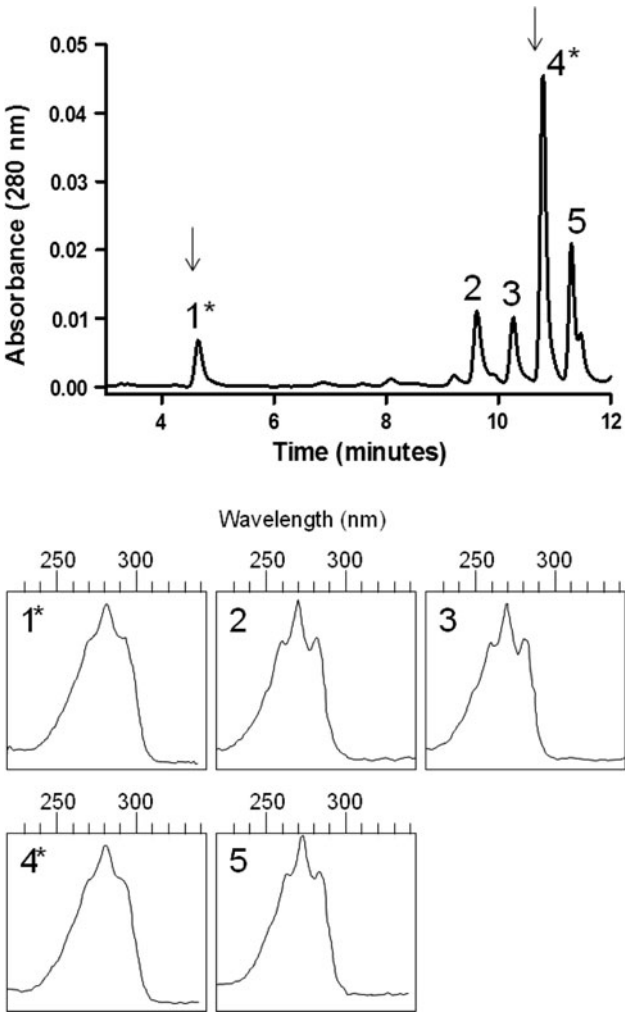

Fig. 7 Chromatographic profile of metabolites formed in platelets after addition of EXA_4-EA. Human washed platelets (approximately 4.0×10^8 cells/mL) were incubated with 50 μM of EXA_4-EA for 5 min before termination with acidic methanol. After centrifugation, an aliquot of the supernatant (200 μL) was injected onto a Nova Pak C18 RP-HPLC column. Metabolites were eluted using a different gradient as compared to Fig. 3 (described in "Materials and Methods"). *Upper panel* shows the elution profile and absorbance at 280 nm. The formation of metabolites was analyzed by comparing their retention times to synthetic standards as well as their absorbance at 280 or 270 nm. *Asterisks* at peak *1* and *4* denote products with UV-absorbance maximum at 280 nm. The elution times of synthetic EXC_4-EA and EXD_4-EA are indicated by *arrows* at 4.7 and 11 min, respectively. The *lower panel* shows the UV-spectra for the indicated peaks

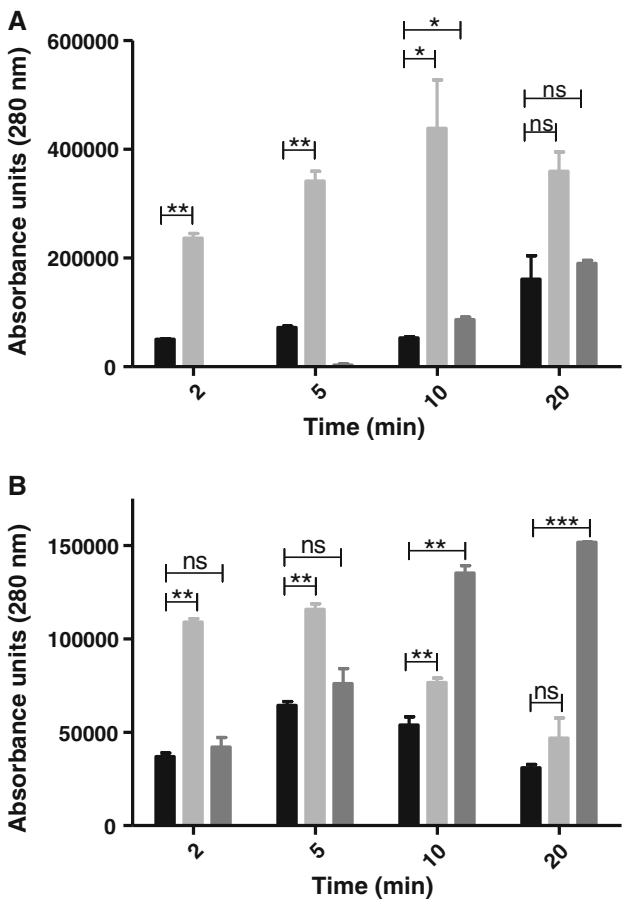

Fig. 8 Comparison between the cellular formation of eoxamides in platelets and L1236 cells after addition of EXA_4-EA. Human washed platelets (approximately 4.0×10^8 cells/mL) (**a**) or L1236 cells (1.0×10^7 cells/mL) (**b**) were incubated with 50 μM of EXA_4-EA for the indicated times before termination with acidic methanol. The formation of cysteinyl-containing metabolites were analyzed by comparing their retention times to synthetic standards as well as their absorbance at 280 nm. The results are presented as the sum of cysteinyl-containing metabolites ±SEM ($n = 3$) from one representative experiment out of two. *Black bars* EXC_4-EA, *light dark grey bars* EXD_4-EA and *dark grey bars* EXE_4-EA

ARA as a precursor for neuroinflammatory prostaglandins has also shed light on the role of endocannabinoids in neuroinflammation [45]. It is noteworthy that 15-LO-1 is expressed within the CNS [26] and that formation of leukotrienes in the CNS has been known for a long time [57, 58]. The recent finding that leukotriene receptor antagonists is effective in an animal model of multiple sclerosis [59] further highlights the link between CNS, neuroinflammation and cysteinyl-containing leukotrienes.

The Hodgkin lymphoma cell line L1236 has high endogenous expression of 15-LO-1. The expression of 15-LO-1 has also been confirmed in several Reed-Sternberg positive cells by immunohistochemistry [8] and one hypothesis could be that 15-LO-1 plays a regulatory role in controlling immunological responses in the inflammatory process. Apart from their role within the CNS, cannabinoids are also known to be able to affect the immune system and CB1 and 2 are expressed in immune cells with B-lymphocytes being one of the cell types that most abundantly express CB1 and 2 [60, 61]. Polymorphonuclear neutrophils, T-cells and monocytes have also been shown to express CB1 and 2. Thus, the possibility for

immunomodulatory functions for enzymes/receptors involved in endocannabinoid signalling, or termination thereof, is evident. Several pharmacological active compounds that interfere with the endocannabinoid system display plethoric effects affecting both systemic as well as peripheral processes including body weight control, neuropathic pain, inflammation and cytokine release [50]. Our finding of the eoxamides identifies a new pathway for the formation of AEA metabolites. If these unique compounds display any biological role themselves remains to be investigated. However, it is interesting to note that several metabolites of AEA such as the lipoxygenase derived monohydroxy acids [62] or the cyclooxygenases derived prostamides bind to existing receptors or exerts their effect through yet unidentified receptors.

The catabolism of endocannabinoids by FAAH can have a profound effect on pain sensation in animals [53]. The formation of eoxamides is a new example of the catabolism of AEA and the concerted action of 15-LO-1 and glutathione S-transferases or LTC_4-synthase to yield EXC_4-EA might be a new mechanism to terminate AEA signalling. Given the fact that 15-LO-1 has an expression profile restricted to higher primates and rabbits [47], the described metabolism of AEA in this report could be unique to man and other species that express 15-LO-1. We suggest that the described peroxidation and subsequent glutathionylation of AEA should be considered as an alternative pathway to FAAH/MAGL-pathways for elimination and secretion of AEA and as a new pathway for the generation of novel and potentially biological active AEA-metabolites.

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