Eoxins are proinflammatory arachidonic acid metabolites produced via the 15-lipoxygenase-1 pathway in human eosinophils and mast cells

Stina Feltenmark*†, Narinder Gautam‡§, Åsa Brunnström*, William Griffiths¶, Linda Backman*, Charlotte Edenius*, Lennart Lindbom‡, Magnus Björkholm‖, and Hans-Erik Claesson*†**

*Biolipox AB, Berzelius Väg 3, SE-171 65 Solna, Sweden; Departments of †Medical Biochemistry and Biophysics and ‡Physiology and Pharmacology, Karolinska Institutet, SE-171 77 Stockholm, Sweden; §Department of Medicine, Rheumatology Unit, Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden; ¶Institute of Mass Spectrometry, School of Medicine, Swansea University, Singleton Park, Swansea SA2 8PP, United Kingdom; and ‖Department of Medicine, Division of Hematology, Karolinska University Hospital Solna and Karolinska Institutet, SE-171 76 Stockholm, Sweden

Communicated by Bengt Samuelsson, Karolinska Institutet, Stockholm, Sweden, November 2, 2007 (received for review July 27, 2007)

Human eosinophils contain abundant amounts of 15-lipoxygenase (LO)-1. The biological role of 15-LO-1 in humans, however, is unclear. Incubation of eosinophils with arachidonic acid led to formation of a product with a UV absorbance maximum at 282 nm and shorter retention time than leukotriene (LT)C_4 in reverse-phase HPLC. Analysis with positive-ion electrospray tandem MS identified this eosinophil metabolite as 14,15-LTC_4. This metabolite could be metabolized to 14,15-LTD_4 and 14,15-LTE_4 in eosinophils. Because eosinophils are such an abundant source of these metabolites and to avoid confusion with 5-LO-derived LTs, we suggest the names eoxin (EX)C_4, -D_4, and -E_4 instead of 14,15-LTC_4, -D_4, and -E_4, respectively. Cord blood-derived mast cells and surgically removed nasal polyps from allergic subjects also produced EXC_4. Incubation of eosinophils with arachidonic acid favored the production of EXC_4, whereas challenge with calcium ionophore led to exclusive formation of LTC_4. Eosinophils produced EXC_4 after challenge with the proinflammatory agents LTC_4, prostaglandin D_2, and IL-5, demonstrating that EXC_4 can be synthesized from the endogenous pool of arachidonic acid. EXs induced increased permeability of endothelial cell monolayer *in vitro*, indicating that EXs can modulate and enhance vascular permeability, a hallmark of inflammation. In this model system, EXs were 100 times more potent than histamine and almost as potent as LTC_4 and LTD_4. Taken together, this article describes the formation of proinflammatory EXs, in particular in human eosinophils but also in human mast cells and nasal polyps.

15-LO | 14,15-leukotriene

The mammalian lipoxygenases belong to a family of structurally related lipid peroxidizing enzymes that are implicated in the pathogenesis of asthma and other inflammatory disorders. 5-Lipoxygenase (LO) is the key enzyme in leukotriene (LT) synthesis, and this enzyme catalyzes the conversion of arachidonic acid to LTA_4. This unstable metabolite can be hydrolyzed enzymatically to LTB_4 or conjugated with glutathione to form LTC_4, which in turn can be metabolized to LTD_4 and LTE_4. LTC_4 and its metabolites LTD_4 and LTE_4 are collectively referred to as cysteinyl LT, and these mediators are proinflammatory agents and potent bronchoconstrictors (1). In contrast to 5-LO, little is known about the biological function of human 15-LO. Human airway epithelial cells, eosinophils and subsets of mast cells and dendritic cells contain high amounts of 15-LO-1 (2–6). A second form of human 15-LO also exists, which is named 15-LO-2 (7). The amino acid sequence similarity between these enzymes is only 40% (3). IL-4 and/or IL-13 induce the expression of 15-LO-1 in cultured mast cells, monocytes, and epithelial cells (4, 8, 9). Thus, these cytokines may act as important physiological/pathophysiological regulators of 15-LO-1 expression *in vivo*.

15-LO-1 has been suggested to be involved in the pathogenesis of bronchial asthma. The amount of 15-LO-1 is significantly increased in the bronchial submucosa of patients with asthma or chronic bronchitis compared with control subjects (10, 11). The major arachidonic acid metabolite formed via 15-LO-1 is 15-(*S*)-hydroxy-eicosatetraenoic acid [15-(*S*)-HETE], which has been used as a marker for 15-LO activity *in vivo* as well as in various cells and tissues *in vitro*. Human tracheal epithelial cells, chopped human bronchi, and human lung homogenates convert arachidonic acid mainly to 15-HETE (5, 12). Lung specimens from asthmatic subjects produce more 15-HETE than specimens from nonasthmatic subjects (13–15). Increased amounts of 15-HETE have been found in bronchoalveolar lavage fluid (BALF) of antigen-challenged atopic asthmatic patients (16), in sputum samples from asthmatic subjects (17), and in nasal secretions from healthy control subjects (18). Severely asthmatic patients with persistent airway eosinophilia have higher levels of 15-HETE in BALF compared with healthy subjects or with patients with asthma but without eosinophilia (14). The levels of 15-HETE correlate with subbasement membrane thickness, indicating that 15-LO might be associated with airway remodeling (14).

15(*S*)-HETE is formed from 15(*S*)-hydroperoxy-eicosatetraenoic acid [15(*S*)-HPETE], and, in addition to undergoing reduction to 15-HETE, 15-HPETE may also undergo dehydration to form 14,15-epoxy-eicosatetraenoic acid (14,15-LTA_4) (19, 20). Another 15-LO-1-derived metabolite, 5-oxo-15-hydroxy-6,8,11,13-eicosatetraenoic acid, produced by eosinophils, has been reported to be a potent chemotactic agent for human eosinophils (21). However, there are also indications that 15-LO-derived metabolites could play an antiinflammatory role because 15-LO-1 can be involved in the formation of lipoxins (22). In essence, the physiological and pathophysiological role of the 15-LO pathway in humans is far from clarified.

In this report, we describe the formation and biological effects of cysteinyl 14,15-LTs [eoxins (EXs)], which are formed via the 15-LO-1 pathway in human eosinophils, mast cells, and nasal polyps.

Results

Formation of 15LO Products in Isolated Human Eosinophils. Incubation of isolated human eosinophils with arachidonic acid for 5 min

Author contributions: N.G. and Å.B. contributed equally to this work; S.F., W.G., C.E., L.L., M.B., and H.-E.C. designed research; S.F., N.G., Å.B., and L.B. performed research; S.F., N.G., Å.B., W.G., L.L., and H.-E.C. analyzed data; and S.F., W.G., C.E., L.L., and H.-E.C. wrote the paper.

Conflict of interest statement: S.F., Å.B., L.B., C.E., and H.-E.C. are employed by Biolipox AB, a pharmaceutical company.

Freely available online through the PNAS open access option.

**To whom correspondence should be addressed. E-mail: hans-erik.claesson@biolipox.com.

This article contains supporting information online at www.pnas.org/cgi/content/full/0710127105/DC1.

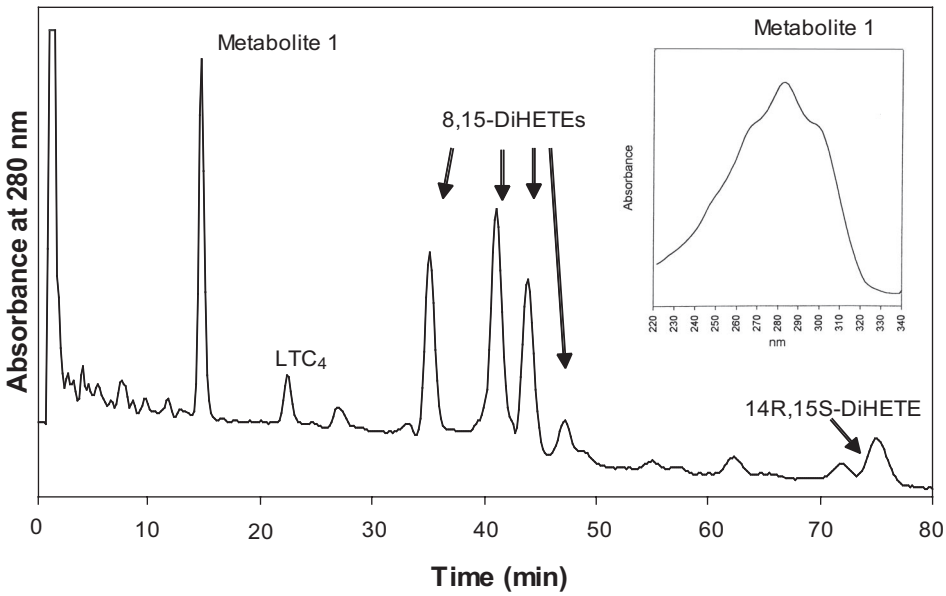

Fig. 1. Formation of arachidonic acid-derived products in human eosinophils. Isolated eosinophils (10×10^6 cells) were resuspended in 1 ml of PBS and preincubated for 2 min at 37°C before stimulation with 10 μM arachidonic acid for 5 min at 37°C. The reaction was terminated by the addition of 3 vol of methanol. After evaporation, the sample was resuspended in a mobile phase and subjected to analysis in a RP-HPLC/UV system by using AcN:MeOH:H_2O:HAc (29:19:72:0.8 by volume), pH 5.6, as a mobile phase. Arrows indicate the retention times of synthetic standards. (*Inset*) UV spectrum of metabolite 1.

led to the formation of 15(*S*)-HETE and 12(*S*)-HETE in a ratio of ≈9:1 (data not shown), which is in agreement with the activity of 15-LO-1 (2). A number of peaks with a UV absorbance maximum of ≈270 nm and retention times between 5 and 7 min were observed in the same chromatogram. To obtain better separation of these more polar metabolites, an acetonitrile-based mobile HPLC phase was used. Five peaks containing a conjugated triene spectrum and UV absorbance maximum at 272 nm were identified (Fig. 1). The retention times of the material in these five peaks corresponded to the retention times for the two double oxygenation products 8(*S*),15(*S*)-5,9,11,13(*Z*,*E*,*Z*,*E*)-DiHETE and 8(*R*),15(*S*)-5,9,11,13(*Z*,*E*,*Z*,*E*)-DiHETE, the two products 8(*S*),15(*S*)-5,9,11,13(*Z*,*E*,*E*,*E*)-DiHETE and 8(*R*),15(*S*)-5,9,11,13(*Z*,*E*,*E*,*E*)-DiHETE derived from 14,15-oxido-LTA_4 (19, 20) and 14(*R*),15(*S*)-DiHETE, respectively. The pattern of products formed was similar to that reported for human airway epithelial cells incubated with arachidonic acid (5). Small amounts of LTC_4, with the characteristic UV spectrum of a conjugated triene but with maximum at 282 nm, were also observed.

In addition to these well known arachidonic acid derived metabolites, a more polar major metabolite (metabolite 1) was observed in the chromatogram, possessing a conjugated triene spectrum, with UV absorbance maximum at 282 nm (Fig. 1 *Inset*). The formation of metabolite 1 was not inhibited by the LT biosynthesis inhibitors MK-886 (10^{-7} M) or BWA4C (10^{-7} M) or the cyclooxygenase inhibitor indomethacin (10^{-6} M) (data not shown). A metabolite with identical UV spectrum and retention time on the RP-HPLC was formed also after incubation of eosinophils with 14,15-LTA_4 (data not shown).

Identification of Metabolite 1 by Liquid Chromatography–Tandem MS (LC-MS/MS). To further analyze the structure of metabolite 1, samples of human eosinophils incubated with exogenous arachidonic acid were subjected to analyzes by LC-MS/MS by using positive-ion electrospray ionization. The resultant product ion spectrum of metabolite 1 ($[M+H]^+$ *m*/*z* 626.2) contained several fragment ions also present in the corresponding spectrum of LTC_4 namely *m*/*z* 479, 308, and 301 (Fig. 2). This finding, in addition to the well known high 15-LO activity in eosinophils and the suggested formation of this metabolite from 14,15-LTA_4, suggested that metabolite 1 could be 14(*R*)-glutathionyl-15(*S*)-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid (14,15-LTC_4). To confirm this hypothesis, synthetic 14,15-LTC_4 was prepared (Biomol Inc) and the product ion spectrum obtained for metabolite 1 was compared with the spectrum obtained for the synthetic 14,15-LTC_4 (Fig. 2). The product ion spectrum of metabolite 1 ($[M+H]^+$ 626.2 *m*/*z*) and its chromatographic retention time were equivalent to that of synthetic 14,15-LTC_4.

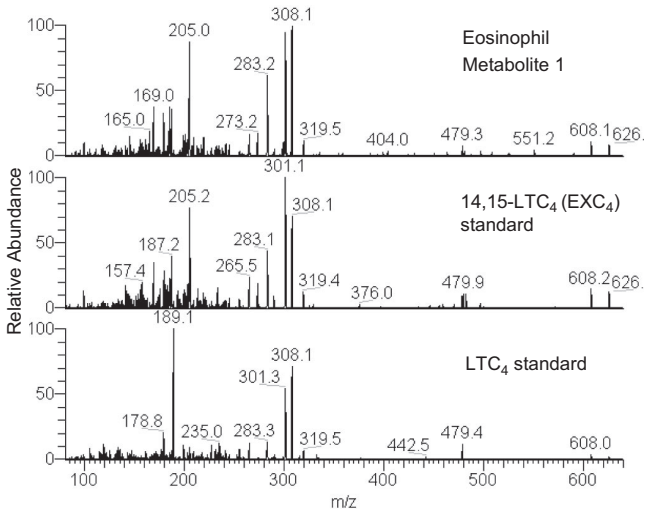

Fig. 2. LC-MS/MS analysis of cysteinyl-containing 15-LO-derived products. Shown is LC-MS/MS spectrum of metabolite 1 formed by eosinophils after incubation with arachidonic acid compared with spectra of synthetic 14,15-LTC_4 (EXC_4) and LTC_4 standards. (*Top*) The spectrum of the product ion scan of 626.2 $[M+H]^+$ derived from a metabolite 1 formed in eosinophils after challenge with arachidonic acid (10 μM) for 5 min at 37°C. (*Middle*) The spectrum of synthetic 14,15-LTC_4 (EXC_4) standard. (*Bottom*) The spectrum of synthetic LTC_4 standard.

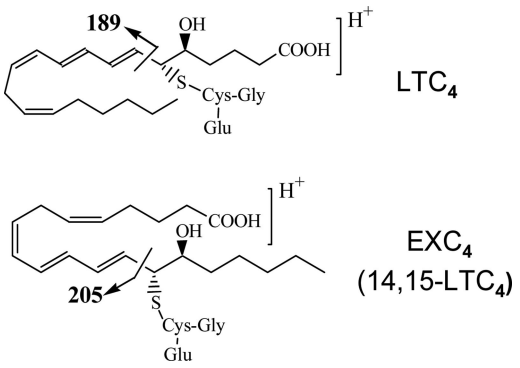

Fig. 3. Fragments distinguishing of cysteinyl 14,15-LTs from cysteinyl LTs in product ion spectra. A prominent difference in the positive-ion mode MS/MS product ion spectra of 5-LO-derived cysteinyl LTs and cysteinyl 14,15-LTs (EXs) are *m/z* 189 and 205. The probable origins of these fragment ions are indicated.

Metabolism of 14,15-LTC$_4$ in Human Eosinophils. To analyze whether eosinophils can metabolize 14,15-LTC$_4$ to 14,15-LTD$_4$ and 14,15-LTE$_4$, eosinophils were incubated with synthetic 14,15-LTC$_4$ or synthetic 14(*R*)-cysteinyl-glycyl-15(*S*)-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid (14,15-LTD$_4$) for 60 min at 37°C. The metabolites formed were analyzed by product ion scans at 497.2 *m/z* for 14,15-LTD$_4$ and 440.2 *m/z* for 14,15-LTE$_4$. The retention time and product ion spectrum of one metabolite formed (metabolite 2) from 14,15-LTC$_4$ were identical to synthetic 14,15-LTD$_4$ [for LC-MS/MS spectrum, see supporting information (SI) Fig. 6*A*], and one metabolite formed (metabolite 3) after incubation with 14,15-LTD$_4$ had identical retention time and product ion spectrum to synthetic 14(*R*)-cysteinyl-15(*S*)-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic (14,15-LTE$_4$) (for the LC-MS/MS spectrum, see SI Fig. 6*B*). There was no significant nonenzymatic metabolism of 14,15-LTC$_4$ and 14,15-LTD$_4$ during 60 min incubation at 37°C (data not shown).

The product ion spectra of 15-LO-derived cysteinyl 14,15-LTs and the corresponding 5-LO-derived cysteinyl LTs were very similar. Interpretation of these spectra indicate that 301 results from the loss of glutathione + H_2O, 308 is protonated glutathione, and 479 results from the loss of the glutamic acid residue and H_2O. However, cysteinyl 14,15-LTs formed a fragment at 205 *m/z*, whereas the corresponding fragment in cysteinyl LTs was at 189 *m/z* (Fig. 3). These fragment ions are probably formed by cleavage of the eicosanoid backbone adjacent to the carbon where the cysteinyl group is positioned, which is at carbon 14 for 14,15-LTs and carbon 6 for LTs (23).

Eoxins: A Suggested Change of Name for Cysteinyl 14,15-LTs. The name LT is traditionally linked to 5-LO products. The previously used name for the 15-LO-derived metabolites synthesized via the 14,15-epoxide in arachidonic acid has been 14,15-LT, although it is not formed via the 5-LO pathway. Because eosinophils are a rich source of these 15-LO-1-derived cysteinyl products, and to avoid confusion with 5-LO-derived products, we suggest the name EXC$_4$, EXD$_4$, and EXE$_4$ instead of 14,15-LTC$_4$, 14,15-LTD$_4$, and 14,15-LTE$_4$, respectively.

Comparison Between the Formation of EXC$_4$ and LTC$_4$ in Eosinophils. Human eosinophils contain abundant amounts of both 15-LO-1 and 5-LO. To examine under which conditions formation of EXC$_4$ and LTC$_4$ were favored, eosinophils were incubated with either arachidonic acid or calcium ionophore A23187 or both. The cells were incubated for 5 min at 37°C, and the amounts of EXC$_4$ and LTC$_4$ were analyzed with RP-HPLC. There was almost no further metabolism of EXC$_4$ to EXD$_4$ or LTC$_4$ to LTD$_4$ in eosinophils after 5 min of incubation (data not shown). Therefore, measurement of these metabolites is considered to reflect the total formation of 15-LO-1-derived EXs and 5-LO-derived cysteinyl LTs, respectively. Incubation of eosinophils with exogenous arachidonic acid led to the formation preferentially of EXC$_4$ but LTC$_4$ was also formed in some samples (Table 1). Activation of the cells with calcium ionophore A23187 exclusively induced formation of LTC$_4$, whereas challenge of eosinophils with both ionophore A23187 and arachidonic acid led to the formation of both EXC$_4$ and LTC$_4$. Although similar amounts of these metabolites were produced in some incubations, the levels of LTC$_4$ were much higher in certain samples. Thus, incubation of the cells with arachidonic acid favor the formation of EXC$_4$, and calcium activation of the cells triggers the 5-LO and strongly favors the formation of 5-LO-derived LTs. Preliminary results indicate that LTC synthase catalyzes both the conversion of LTA$_4$ to LTC$_4$ and EXA$_4$ to EXC$_4$ in eosinophils because the metabolism of EXA$_4$ to EXC$_4$ occurred almost exclusively in the membrane fraction of eosinophils and because Sf9 cells overexpressing human LTC synthase readily converted EXA$_4$ to EXC$_4$ (S.F., R. Morgenstern, T. Bergman, B. Mannervik, and H.-E.C., unpublished work).

Table 1. Biosynthesis of EXC$_4$ and LTC$_4$ in human eosinophils

	Arachidonic acid		A23187		Arachidonic acid plus A23187	
Experiment no.	EXC$_4$	LTC$_4$	EXC$_4$	LTC$_4$	EXC$_4$	LTC$_4$
1	40	0	0	50	30	14
2	32	0	0	40	16	20
3	58	34	0	198	20	322
4	74	12	0	494	34	420
5	34	20	0	82	26	34
6	12	0	0	108	4	18
7	26	6	0	64	14	16
8	46	10	0	326	18	444
9	16	0	0	170	14	24
10	22	38	0	30	18	36
11	48	22	0	264	12	385

Eosinophils were isolated from 11 healthy donors or patients with hypereosinophilia. The cells were suspended in 1 ml of PBS and incubated with 10 μM arachidonic acid, 1 μM ionophore A23187, or both, for 5 min at 37°C, and the supernatants were thereafter subjected to RP-HPLC analysis. The relative amounts of EXC$_4$ and LTC$_4$ were calculated as picomoles per 10^7 eosinophils.

Receptor-Mediated Activation of EXC$_4$ Biosynthesis in Eosinophils. A possible receptor-mediated activation of EXC$_4$ formation was investigated by using the allergen induced mast cell mediators LTC$_4$ and prostaglandin (PG)D$_2$, as well as IL-5, which is a potent eosinophil activator. Isolated eosinophils (10×10^6 per sample) incubated for 5 min at 37°C with LTC$_4$ (1 μM), PGD$_2$ (1 μM), or IL-5 (10 ng/ml) produced 0.09 ± 0.06 ($n = 4$ ± SD), 0.07 ± 0.03 ($n = 4$ ± SD), and 0.21 ($n = 2$, mean value (0.20–0.22) pmol of EXC$_4$ per 10^7 cells, respectively, in comparison with nonstimulated eosinophils (0.02 ± 0.01 pmol of EXC$_4$ per 10^7 cells) ($n = 4$; mean ± SD). These results demonstrate formation of EXC$_4$ from the endogenous pool of arachidonic acid in eosinophils via receptor-mediated mechanisms. In comparison, IL-5 or GM-CSF did not themselves stimulate LTC$_4$ synthesis in eosinophils, but these cytokines enhanced calcium–ionophore-stimulated LTC$_4$ formation in human eosinophils (24).

Biosynthesis of EXC$_4$ and EXD$_4$ in Cord Blood-Derived Mast Cells. Mast cells play a key role in the pathophysiology of asthma. Recently, we have reported that IL-4 induces the expression of 15-LO-1 in

human cord blood-derived mast cells (CBMC) *in vitro* and that mast cells express 15-LO-1 *in vivo* (4). Therefore, it was of interest to investigate whether mast cells could produce EXs. IL-4-primed mast cells were incubated with arachidonic acid (10 μM) for 5 min. Supernatants were collected and analyzed by using an enzyme immunoassay (EIA) against EXC_4. Under these conditions, human mast cells released 0.8 ± 0.6 pmol per 10^6 cells (n = 3; mean ± SD), indicating the capacity to synthesize and release immunoreactive EXC_4 after arachidonic acid stimulation. The identity of EXC_4 was further verified with LC-MS/MS single-reaction monitoring (SRM). Thus, mast cells incubated with arachidonic acid gave rise to a metabolite with the same SRM retention time as synthetic EXC_4 (data not shown). In addition, mast cell samples were analyzed with a specific EXD_4 SRM method to investigate whether the EXC_4 was further metabolized by the cells. Indeed, a metabolite with SRM retention time identical to synthetic EXD_4 was demonstrated (data not shown). Taken together, mast cells possess the capacity to produce both EXC_4 and EXD_4.

Spontaneous Release of EXC_4 by Human Nasal Polyps. Nasal polyposis is a multifactorial disease consisting of tissue infiltration by inflammatory cells such as eosinophils (25). To investigate whether EXC_4 might be synthesized by human nasal polyps, intact surgically removed nasal polyps were washed and incubated in PBS containing protease inhibitor (Complete MINI) and indomethacin at 37°C for 5 or 30 min. Subsequently, the incubation medium was analyzed by using EIA against EXC_4. The results demonstrated that nasal polyps spontaneously released 10.1 and 7.18 pmol of EXC_4 per gram of polyp tissue (each value represents the mean value of two independent experiments) after 5 and 30 min of incubation, respectively. The decreasing levels of EXC_4 over time might be attributable to further metabolism to EXD_4 and EXE_4, which was not detected by the antibodies raised against EXC_4 [cross-reactivity, <1% against EXD_4 (14,15-LTD_4) and EXE_4 (14,15-LTE_4); see *SI Methods*].

Biological Effects of EXs. Increase in vascular permeability leading to plasma leakage is a hallmark of inflammation, and it is well known that the cysteinyl LTs may contribute to this effect in a similar way to histamine and bradykinin (26). To explore the ability of EXC_4 and its metabolites EXD_4 and EXE_4 to induce alterations in vascular permeability, an established *in vitro* permeability assay based on assessment of changes in transendothelial electric resistance (TEER) across human endothelial cell (EC) monolayers was used (27). In this model, maximum stimulus-induced decrease in EC resistance extends down to approximately −70%. The dose–response curve shows that the maximum effect of histamine was obtained at 10^{-5} M in this model system (data not shown). All EXs were capable of inducing an increase in EC monolayer permeability (Fig. 4*A*). Although somewhat less potent than LTC_4 and LTD_4, the EXs were ≈100 times more potent than histamine in this respect (Fig. 4*A*). EXD_4 induced a maximal increase in EC permeability at 10^{-7} M, but a significant increase in EC permeability was also observed at 10^{-8} M (Fig. 4*B*). The kinetics of changes in EC permeability in response to EXs and LTD_4 resemble the pattern observed for directly acting agonists such as histamine and LTC_4 (Fig. 4*B*) (27). LTB_4, on the other hand, requires the presence of granulocytes to affect EC barrier function, and no effect of LTB_4 was observed in this model system.

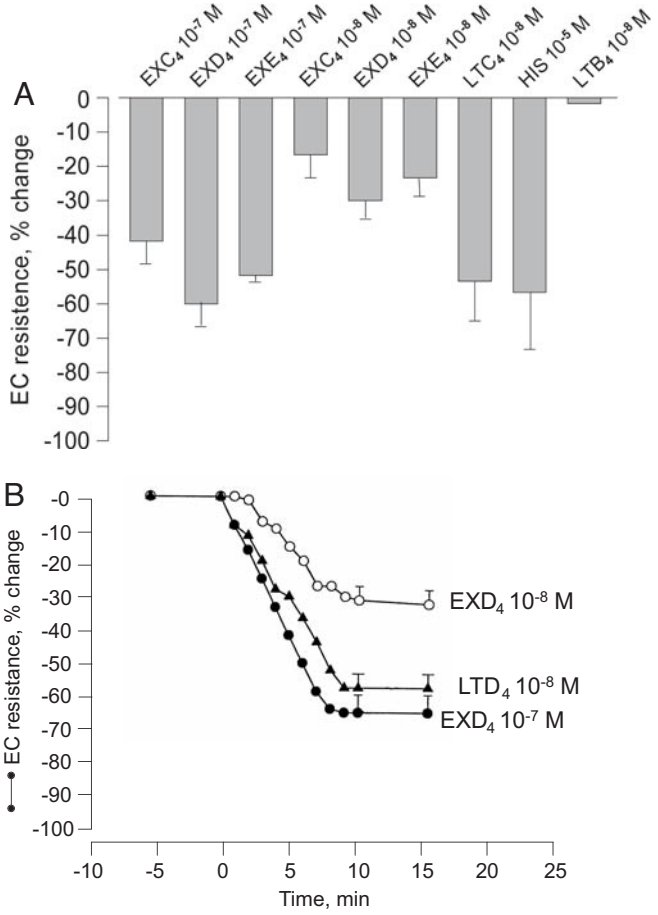

Fig. 4. Alterations in vascular permeability induced by EXs. (*A*) Analysis of rapid changes in EC barrier function in response to EXC_4, EXD_4, EXE_4, LTC_4, LTB_4, and histamine. Analyses were accomplished by continuous registration of TEER with electrodes on each side of the EC monolayer. The maximal decrease in resistance in this model varies typically at approximately −70%. (*B*) Time course for changes in EC barrier function induced by EXD_4 (10^{-8} and 10^{-7} M, respectively) in comparison with LTD_4 (10^{-8} M). Each value represents the mean value of triplicate determinations ± SD.

Discussion

Human airway epithelial cells and eosinophils contain high amounts of 15-LO-1 protein (3, 5, 6). Subsets of human mast cells, macrophages, and dendritic cells also express 15-LO-1 (3, 4, 28). Despite much work in this field of research, the biological role of 15-LO-1 is unclear, except for the function of the enzyme during erythropoiesis (2, 3). In the present report, we show that human eosinophils in particular, but also CBMC and nasal polyps, have the ability to convert arachidonic acid via the 15-LO-1 pathway to metabolites with a conjugated triene spectrum and UV absorbance maximum at 282 nm (Fig. 1). The structures of the eosinophil and mast cell derived metabolites were determined by positive-ion MS/MS and reverse-phase–HPLC/UV (RP-HPLC/UV) spectroscopy and were found to be identical to synthetic 14,15-LTC_4 (EXC_4), 14,15-LTD_4 (EXD_4), and 14,15-LTE_4 (EXE_4) (Figs. 2 and 3). Eosinophils mainly produced 14,15-LTC_4, (EXC_4), and this metabolite could be metabolized further to 14,15-LTD_4 (EXD_4) and 14,15-LTE_4 (EXE_4), as demonstrated by incubation of these cells with synthetic 14,15-LTC_4 (EXC_4) and 14,15-LTD_4 (EXD_4), respectively. Although synthetic 14,15-LTC_4 (EXC_4) has been studied in various biological systems (29, 30) and 14,15-LTA_4 (EXA_4) has been found to be formed in epithelial cells and eosinophils (5, 6), the biosynthesis of cysteinyl 14,15-LTs (EXs) has never been demonstrated to occur from arachidonic acid in human cells and tissues. Rat basophils, however, have been found previously to conjugate synthetic 14,15-LTA_4 to 14,15-LTC_4 (31).

Because eosinophils are a rich source of these now identified metabolites, and to avoid confusion with metabolites produced via the 5-LO pathway, we suggest the name EX instead of 14,15-LT.

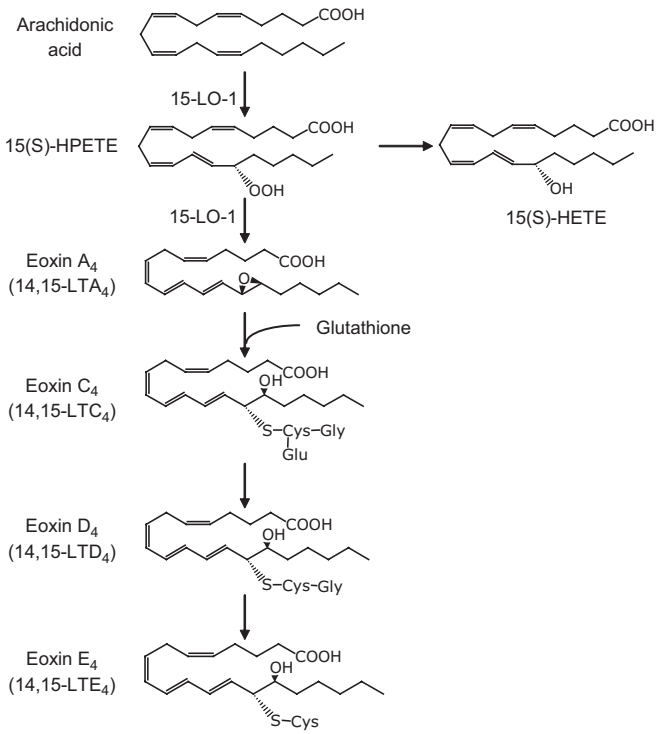

Fig. 5. Proposed metabolic pathway for the formation of EXs.

Thus, it is suggested that the names 14,15-LTA_4, C_4, D_4 and E_4 are replaced by EXA_4, EXC_4, EXD_4 and EXE_4, respectively (Fig. 5).

The mechanism of formation of EXC_4 from arachidonic acid proceeds via 15-HPETE and the intermediate EXA_4 (14,15-LTA_4) (Fig. 5). Incubation of the latter metabolite with eosinophils led to the formation of EXC_4. The formation of EXA_4 in the eosinophils incubations is also based on the detection of the two products 8(*S*),15(*S*)-5,9,11,13(*Z*,*E*,*E*,*E*)-DiHETE and 8(*R*),15(*S*)-5,9,11,13(*Z*,*E*,*E*,*E*)-DiHETE, which are derived from 14,15-oxido-LT A_4 (20). Human 15-LO-1 primarily possesses 15-LO activity but also has ≈10% 12-lipoxygenating activity. It is possible that the intrinsic 12-LO activity of the enzyme catalyses the conversion of 15-HPETE to EXA_4 (20).

Incubation of eosinophils with exogenous arachidonic acid favored the formation of EXC_4 over LTC_4 (table 1), whereas increasing intracellular calcium levels with a calcium ionophore instead triggered 5-LO and led to the exclusive production of LTC_4. Thus, the regulation of 5-LO and 15-LO differs, and the metabolic pathway to be favored will depend on the stimulus. In this respect, the EX (14,15-LT) pathway resembles the PG pathway, occurring readily when free arachidonic acid is available. Receptor-mediated activation of human eosinophils with LTC_4, PGD_2, and IL-5 also triggered the production and release of EXs. Thus, EXs can be produced from the endogenous pool of arachidonic acid in eosinophils.

In a model system with confluent human EC, we demonstrate that all three EXs increase the permeability of the EC monolayer, thus indicating a capacity to induce vascular leakage *in vivo*. The EXs induced increased EC permeability at 10^{-7} and 10^{-8} M and thus were ≈100 times more potent than histamine and almost as potent as cysteinyl LTs in this respect (Fig. 4). In this study, we used an *in vitro* model system for analysis of mediator-induced perturbation of EC monolayer barrier function. Continuous measurement of TEER permits detection of rapid changes in EC permeability and has been shown previously to be a sensitive measure of the barrier capacity of cultured EC monolayers and to mimic changes in vascular permeability *in vivo* (27, 32). The permeability-increasing activity of inflammatory mediators such as histamine and the cysteinyl LTs has long been known. These mediators exert their effect on EC barrier function by triggering intracellular Ca^{2+} mobilization and cytoskeleton rearrangement (33), leading to paracellular gap formation and enhanced macromolecular permeability. It is likely that the EXs stimulate increase in vascular permeability in a similar way. In contrast to the 5-LO-derived cysteinyl LTs, EXC_4 (14,15-LTC_4) and its metabolites appear not to have contractile effects on guinea pig pulmonary parenchymal strip or ileum (29, 30).

Many investigators have reported increased amounts and activity of 15-LO-1 in the airway tissues of patients with asthma compared with control subjects. Healthy smokers, but in particular smokers with chronic bronchitis, also display increased expression of 15-LO-1 in bronchial biopsies. In this report, we show that the 15-LO-1 pathway can generate the proinflammatory EXs (cysteinyl 14,15-LTs) in eosinophils, mast cells, and nasal polyps from allergic subjects, indicating that inhibition of 15-LO-1 might be an attractive target for treatment of inflammatory respiratory disorders such as asthma, rhinitis, and chronic obstructive pulmonary disease (COPD) in humans.

Materials and Methods

Synthetic 14,15-epoxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid (14,15-LTA_4–EXA_4), 14(*R*)-glutathionyl-15(*S*)-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid (14,15-LTC_4–EXC_4), 14(*R*)-cysteinyl-glycyl-15(*S*)-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid (14,15-LTD_4–EXD_4), 14(*R*)-cysteinyl-15(*S*)-hydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid (14,15-LTE_4–EXE_4), 8(*R*),15(*S*)-dihydroxy-5,9,11,13(*Z*,*E*,*E*,*E*)-eicosatetraenoic acid, 8(*S*),15(*S*)-dihydroxy-5,9,11,13(*Z*,*E*,*Z*,*E*)-eicosatetraenoic acid, 8(*S*),15(*S*)-dihydroxy-5,9,11,13(*Z*,*E*,*E*,*E*)-eicosatetraenoic acid, 8(*R*),15(*S*)-dihydroxy-5,9,11,13(*Z*,*E*,*Z*,*E*)-eicosatetraenoic acid, 14(*R*),15(*S*)-dihydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosate-traenoic acid [14(*R*),15(*S*)-DiHETE], 14(*S*),15(*S*)-dihydroxy-5,8,10,12(*Z*,*Z*,*E*,*E*)-eicosatetraenoic acid [14(*S*),15(*S*)-DiHETE], 15(*S*)-hydroxy-5,8,11,13(*Z*,*Z*,*Z*,*E*)-eicosatetraenoic acid [15(*S*)-HETE], LTC_4, LTD_4, and LTE_4 were from Biomol. BWA4C was a gift from Wellcome Research Laboratories, and MK-866 was a gift from Merck Research Laboratories. The isolation of human eosinophils, human umbilical cord blood, and the collection of surgically removed nasal polyps were approved by the ethics committee of Karolinska University Hospital.

Isolation of Human Eosinophils. Venous blood (100 ml) was drawn from healthy volunteers or from patients with hypereosinophilia (no hematological malignancies). Eosinophils were isolated by negative selection by using CD16 antibodies conjugated to magnetic MicroBeads. Details are given in *SI Methods*.

Preparation of $CBMC^{MNC}$ and Isolation of Polyps. $CBMC^{MNC}$ were established as described in ref. 4.

Polyps were surgically removed and kept in PBS for a maximum of 1 h at 4°C. Polyps were separated from surrounding tissue and washed in PBS without Ca^{2+} and Mg^{2+}. The weight of washed wet polyps was 0.7–1 g.

Cell Incubations. *Eosinophils*. Isolated eosinophils (1–20 × 10^6 per sample) were resuspended in PBS (1 ml per sample) and incubated with the indicated substance, as described in *SI Methods*.

Mast Cells. Before incubation, CBMC were primed for 4 days with IL-4 (10 ng/ml) and incubated as described in *SI Methods*.

Human Nasal Polyps. Human washed intact polyps were transferred into clean test tubes containing PBS, protease inhibitor (Complete MINI), and indomethacin (1 μM) in a total volume of 1 ml. Samples were incubated at 37°C for 5 min and 30 min, respectively. Reactions were terminated by rapid cooling to 0°C and subsequent centrifugation at 1,200 × *g*, 5 min. Supernatants were transferred to cryo tubes and stored in −70°C until they were analyzed by EIA.

Analysis of 15-LO-1-Derived Metabolites. RP-HPLC/UV. Samples were evaporated to dryness under a stream of nitrogen and finally resuspended in 200-μl mobile phase [acetonitril:methanol:H_2O:acetic acid, 29:19:72:0.8, pH adjusted to 5.6 with NH_3 for analysis of LTs and cysteinyl 14,15-LTs (EXs), and methanol:water:trifluoroacetic acid 70:30:0.007 for analysis of monohydroxy acids]. The extracted samples were subjected to analysis in a RP-HPLC system as described in *SI Methods*.

LC-MS/MS. LC-MS/MS was performed on a Surveyor MS coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corporation). The cysteinyl 14,15-LTs (EXs) were chromatographically separated from cysteinyl LTs by ≈0.5 min. The mass spectrometer was operated by using an electrospray atmospheric pressure ionization source in positive mode (details are given in *SI Methods*). Two different LC-MS/MS methods (A and B) were used for identification of cysteinyl 14,15-LTs (EXs) produced by eosinophils and mast cells.

LC-MS/MS for Eosinophil Metabolites. LC-MS/MS was used to perform product ion scans of protonated 14,15-LTC_4 (EXC_4), LTC_4, 14,15-LTD_4 (EXD_4), LTD_4, 14,15-LTE_4 (EXE_4), and LTE_4. 14,15-LT C_4 (EXC_4) and LTC_4 at 626.2 *m/z* were fragmented at a collision energy of 22 eV, and 14,15-LTD_4 (EXD_4) and LTD_4 at 497.2 and 14,15-LTE_4 (EXE_4) and LTE_4 at 440.2 *m/z* were all fragmented at 18 eV. Also, mast cell metabolites were detected by LC-MS/MS (details are given in *SI Methods*).

Characterization of an EIA Against 14,15-LTC_4 (EXC_4). An EIA for 14,15-LTC_4 (EXC_4) was developed by Cayman Chemical to quantify small amounts of 14,15-LTC_4 (EXC_4) (see *SI Methods*).

Assessment of EC Barrier Function. Human umbilical vein EC (HUVEC) were isolated and cultured as described previously (27). The cells were seeded onto 3-μm pore size Biomatrix-coated polycarbonate filters (Tissue Culture inserts, 10 mm; NUNC) at a density of 2×10^5 cells and grown to confluent monolayers. The capacity of arachidonic acid metabolites to induce changes in barrier function of EC monolayers was analyzed through continuous registration of TEER (27). For measurement of TEER, the filter insert with EC was transferred to a resistance measurement chamber (Endohm-12; World Precision Instruments) modified to ensure exact positioning of the electrodes in relation to each other and to the filter insert during repeated measurements. The chamber (lower compartment) and the filter insert (upper compartment) were filled with 2 ml and 400 μl of culture medium, respectively. All measurements were made at 37°C with the equipment placed in a cell culture incubator. The electrical resistance of individual EC monolayers was obtained by subtracting the resistance of the corresponding naked filter coated with Biomatrix as measured before seeding of the EC. Thus, TEER values solely represent the resistance of the EC monolayer.

ACKNOWLEDGMENTS. We thank Ms. Hélène Ax:son-Johnson for excellent technical assistance and the staff at the Department of Otorhinolaryngology, Karolinska Hospital, for providing the nasal polyps .This work was supported by Biolipox AB, the Karolinska Institutet, the Swedish Cancer Society, the Swedish Research Council, the Swedish Heart–Lung Foundation, the Foundation Stockholm County Council, and European Commission Sixth Framework Programme Grant LSHM-CT-2004-005033.

1. Claesson HE, Dahlen SE (1999) *J Intern Med* 245:205–227.
2. Kuhn H, Walther M, Kuban RJ (2002) *Prostaglandins Other Lipid Mediat* 68–69:263–290.
3. Kuhn H, O'Donnell VB (2006) *Prog Lipid Res* 45:334–356.
4. Gullikkson M, Brunnström Å, Johannesson M, Backman L, Nilsson G, Harvima IT, Dahlén B, Kumlin M, Claesson H-E (2007) *Biochem Biophys Acta* 1771:1156–1165.
5. Hunter JA, Finkbeiner WE, Nadel JA, Goetzl EJ, Holtzman MJ (1985) *Proc Natl Acad Sci USA* 82:4633–4637.
6. Turk J, Maas RL, Brash AR, Roberts LJ, 2nd, Oates JA (1982) *J Biol Chem* 257:7068–7076.
7. Brash AR, Boeglin WE, Chang MS (1997) *Proc Natl Acad Sci USA* 94:6148–6152.
8. Conrad DJ, Kuhn H, Mulkins M, Highland E, Sigal E (1992) *Proc Natl Acad Sci USA* 89:217–221.
9. Brinckmann R, Topp MS, Zalan I, Heydeck D, Ludwig P, Kuhn H, Berdel WE, Habenicht JR (1996) *Biochem J* 318:305–312.
10. Shannon VR, Chanez P, Bousquet J, Holtzman MJ (1993) *Am Rev Respir Dis* 147:1024–1028.
11. Bradding P, Redington AE, Djukanovic R, Conrad DJ, Holgate ST (1995) *Am J Respir Crit Care Med* 151:1201–1204.
12. Kumlin M, Hamberg M, Granstrom E, Bjorck T, Dahlen B, Matsuda H, Zetterstrom O, Dahlen SE (1990) *Arch Biochem Biophys* 282:254–262.
13. Hamberg M, Hedqvist P, Radegran K (1980) *Acta Physiol Scand* 110:219–221.
14. Chu HW, Balzar S, Westcott JY, Trudeau JB, Sun Y, Conrad DJ, Wenzel SE (2002) *Clin Exp Allergy* 32:1558–1565.
15. Dahlen SE, Hansson G, Hedqvist P, Bjorck T, Granstrom E, Dahlen B (1983) *Proc Natl Acad Sci USA* 80:1712–1716.
16. Murray JJ, Tonnel AB, Brash AR, Roberts LJ, 2nd, Gosset P, Workman R, Capron A, Oates JA (1985) *Trans Assoc Am Physicians* 98:275–280.
17. Profita M, Sala A, Riccobono L, Paterno A, Mirabella A, Bonanno A, Guerrera D, Pace E, Bonsignore G, Bousquet J, *et al.* (2000) *J Allergy Clin Immunol* 105:711–716.
18. Ramis I, Rosello-Catafau J, Bulbena O, Picado C, Gelpi E (1989) *J Chromatogr* 496:416–422.
19. Jubiz W, Radmark O, Lindgren JA, Malmsten C, Samuelsson B (1981) *Biochem Biophys Res Commun* 99:976–986.
20. Maas RL, Brash AR (1983) *Proc Natl Acad Sci USA* 80:2884–2888.
21. Schwenk U, Morita E, Engel R, Schroder JM (1992) *J Biol Chem* 267:12482–12488.
22. Serhan CN, Savill J (2005) *Nat Immunol* 6:1191–1197.
23. Hevko JM, Murphy RC (2001) *J Am Soc Mass Spectrom* 12:763–771.
24. Laviolette M, Ferland C, Comtois JF, Champagne K, Bosse M, Boulet LP (1995) *Eur Respir J* 8:1465–1472.
25. Bachert C, Gevaert P, Holtappels G, van Cauwenberge P (2002) *Curr Allergy Asthma Rep* 2:481–487.
26. Raud J, Lindbom L, Hedqvist P (1992) *Acta Physiol Scand* 146:545–546.
27. Gautam N, Hedqvist P, Lindbom L (1998) *Br J Pharmacol* 125:1109–1114.
28. Spanbroek R, Hildner M, Kohler A, Muller A, Zintl F, Kuhn H, Radmark O, Samuelsson B, Habenicht AJ (2001) *Proc Natl Acad Sci USA* 98:5152–5157.
29. Lewis RA, Drazen JM, Austen KF, Toda M, Brion F, Marfat A, Corey EJ (1981) *Proc Natl Acad Sci USA* 78:4579–4583.
30. Drazen JM, Lewis RA, Austen KF, Toda M, Brion F, Marfat A, Corey EJ (1981) *Proc Natl Acad Sci USA* 78:3195–3198.
31. Sok DE, Han CO, Shieh WR, Zhou BN, Sih CJ (1982) *Biochem Biophys Res Commun* 104:1363–1370.
32. Gautam N, Herwald H, Hedqvist P, Lindbom L (2000) *J Exp Med* 191:1829–1839.
33. Huang AJ, Manning JE, Bandak TM, Ratau MC, Hanser KR, Silverstein SC (1993) *J Cell Biol* 120:1371–1380.