Co-localization of leukotriene A₄ hydrolase with 5-lipoxygenase in nuclei of alveolar macrophages and rat basophilic leukemia cells but not neutrophils

Thomas G. Brock, Elana Maydanski, Robert W. McNish, Marc Peters-Golden

Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan Health System, Ann Arbor, MI

Running Title: Nuclear localization of leukotriene A₄ hydrolase

Corresponding Author: Thomas G. Brock, Department of Internal Medicine, University of Michigan Health System, 6301 MSRB III, Ann Arbor, MI 48109-0642, Tel: 734-763-9077, FAX: 734-764-4556, e-mail: brocko@umich.edu
Summary

The synthesis of leukotriene B₄ from arachidonic acid requires the sequential action of two enzymes: 5-lipoxygenase and leukotriene A₄ hydrolase. 5-Lipoxygenase is known to be present in the cytoplasm of some leukocytes and able to accumulate in the nucleoplasm of others. In this study, we asked if leukotriene A₄ hydrolase co-localizes with 5-lipoxygenase in different types of leukocytes. Examination of rat basophilic leukemia cells by both immunocytochemistry and immunofluorescence revealed that leukotriene A₄ hydrolase, like 5-lipoxygenase, was most abundant in the nucleus, with only minor occurrence in the cytoplasm. The finding of abundant leukotriene A₄ hydrolase in the soluble nuclear fraction was substantiated by two different cell fractionation techniques. Leukotriene A₄ hydrolase was also found to accumulate, together with 5-lipoxygenase, in the nucleus of alveolar macrophages. This result was obtained using both in situ and ex vivo techniques. In contrast to these results, peripheral blood neutrophils contained both leukotriene A₄ hydrolase and 5-lipoxygenase exclusively in the cytoplasm. Following adherence of neutrophils, 5-lipoxygenase was rapidly imported into the nucleus while leukotriene A₄ hydrolase remained cytosolic. Similarly, 5-lipoxygenase was localized in the nucleus of neutrophils recruited into inflamed appendix tissue, while leukotriene A₄ hydrolase remained cytosolic. These results demonstrate for the first time that leukotriene A₄ hydrolase can be accumulated in the nucleus, where it co-localizes with 5-lipoxygenase. As with 5-lipoxygenase, the subcellular distribution of leukotriene A₄ hydrolase is cell specific and dynamic, but differences in the mechanisms regulating nuclear import must exist. The degree to which these two enzymes are co-localized may influence their metabolic coupling in the conversion of arachidonic acid to leukotriene B₄.
Introduction

Leukotriene B\(_4\) (LTB\(_4\))\(^2\) is a lipid mediator with important roles in immune defense, inflammation and disease. For example, LTB\(_4\) stimulates chemotaxis (1), adhesion to endothelium (2,3), degranulation (4,5), superoxide anion generation (6,7) and phagocytosis (8) by neutrophils (PMNs). The overproduction of LTB\(_4\) plays a role in the pathogenesis of a variety of inflammatory diseases, including glomerulonephritis, rheumatoid arthritis, psoriasis, inflammatory bowel disease, acute lung injury, and interstitial lung disease (9-11).

The first step in the synthesis of LTB\(_4\) from arachidonic acid (AA) is mediated by the enzyme 5-lipoxygenase (5-LO), which catalyzes the insertion of molecular oxygen into AA to form 5-hydroperoxyeicosatetraenoic acid as well as its subsequent dehydration to LTA\(_4\) (12,13). LTA\(_4\) is then modified by the epoxide hydrolase activity of the enzyme LTA\(_4\) hydrolase to generate LTB\(_4\) (14). Independent of its epoxide hydrolase activity, LTA\(_4\) hydrolase also has an aminopeptidase activity (15,16).

Several studies have demonstrated that 5-LO is present in the cytoplasm of some cell types and in the nucleoplasm of others (17-19). For example, 5-LO is found in the cytoplasm of peripheral blood neutrophils (17) but predominantly in the nucleoplasm of alveolar macrophages and rat basophilic leukemia (RBL) cells and mast cells (17-19). Furthermore, 5-LO can be induced to move into the nucleus following various stimuli. For example, 5-LO moves into the nucleus of PMNs following adherence to surfaces or recruitment from the blood into sites of inflammation (20). Similarly, 5-LO moves into the nucleus of eosinophils following adherence (21) or in response to treatment with cytokines (22,23). None of the above phenomena are associated with enzyme activation. However, following cell stimulation, 5-LO moves from its site in the cytoplasm or nucleoplasm to become reversibly associated with the nuclear envelope.
and endoplasmic reticulum (24,25). The process of membrane association is calcium dependent (26) and is thought to be essential for the catalytic action of 5-LO.

Our current understanding of the enzyme LTA₄ hydrolase holds that it is a soluble protein, presumably located within the cytoplasm. Since 5-LO can accumulate in the nucleus of leukocytes, we hypothesized that LTA₄ hydrolase might likewise be found within the nucleoplasm. In this study, we demonstrate that the subcellular distribution of LTA₄ hydrolase is cell specific, co-localizing with 5-LO in the nucleoplasm of resting AMs and RBL cells but in the cytoplasm of resting blood PMNs. However, the regulation of nuclear import of LTA₄ hydrolase is distinct from that of 5-LO, since only the latter moves into the nucleus following adherence or recruitment of PMNs.
Experimental Procedures

Animals - F₁ male F-344xBN rats at 6 mo of age were obtained from the National Institute on Aging. The rats were housed individually in specific pathogen free conditions for 2 wk before experimentation. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the University of Michigan Committee on Use and Care of Animals.

Cells – Rat basophilic leukemia cells (RBL-1, American Type Culture Collection) were seeded at 1 x 10⁵ cell ml⁻¹ in Minimal Essential Medium alpha (Gibco, BRL) containing 10% fetal calf serum supplemented with penicillin, streptomycin and amphotericin B (Gibco, BRL). Cells were fed 2 days after seeding and harvested on the third day for experimentation. In some experiments, RBL cells were pelleted and resuspended in medium without serum at 2 x 10⁵ cells ml⁻¹. An equal volume of medium containing 2 µM A23187 was added to stimulate the cells and the cells were maintained for 5 min at 37 °C. Primary AMs were obtained by lung lavage of 6 mo F-344xBN rats, by techniques described previously (27). Human PMNs were isolated from venous blood obtained from healthy volunteers. Purification involved the sequential steps of centrifugation through Ficoll-Paque (Pharmacia Biotech, Inc.), dextran sedimentation, and hypotonic lysis of erythrocytes (20). Viability was assessed by trypan blue exclusion. Cells were >95% neutrophils. For adherence, PMNs were placed on fibronectin-coated glass coverslips in Hank’s balanced salts with calcium and magnesium supplemented with 10 mM HEPES for 30 min at 37 °C. Suspension cultured PMNs were maintained in identical conditions in Teflon tubes. The experimental protocol was approved by the University of Michigan Medical School Institutional Review Board for Approval of Research Involving Human Subjects.
**Human tissues** – Appendix tissues were obtained from anonymous human subjects undergoing surgery for purposes unrelated to this study. Tissues were supplied by the Tissue Procurement Core of the University of Michigan Comprehensive Cancer Center.

**Immunochemical staining** – For immunocytochemistry, RBL cells were diluted to $1 \times 10^5$ cells ml$^{-1}$ with serum-free media, mounted on slides by cyto spun and fixed immediately in -20 °C methanol, 30 min. Mounts were then permeabilized in -20 °C acetone for 3 min and air dried. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue sections were dewaxed in Americlear and rehydrated through decreasing concentrations of ethanol. All materials were then quenched of endogenous peroxidase activity by treatment with 0.3% hydrogen peroxide for 30 min, washed and blocked with Powerblock. Primary antibodies, rabbit polyclonal antibodies raised against human 5-LO and LTA$_4$ hydrolase, were a generous gift from Dr. J. Evans, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada. Antibodies were prepared in PBS containing 0.1% BSA (5-LO 1:750, LTA$_4$ hydrolase 1:1000) and applied overnight at 4 °C. After washing with 0.1% BSA in PBS, slides were probed with secondary antibody (biotinylated goat anti-rabbit, 1:250) for 30 min at 37 °C, washed again, then treated with avidin-biotinylated peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories) for 30 min at room temperature. 3,3’-Diaminobenzidine (DAB) was used as peroxidase substrate; some preparations were counterstained with Harris’ hematoxylin.

Indirect immunofluorescent staining was performed as described previously (28), after fixation and permeabilization, cells were blocked for 30 min at 37°C with 0.1% BSA in PBS and probed with primary antibodies (5-LO, 1:250; LTA$_4$ hydrolase, 1:500; tubulin, 1:1,000) at 37°C for 1 hour. Cells were then washed and reprobed with rhodamine conjugated goat anti-rabbit antibody (titer 1:200), washed, and mounted. In some preparations, nucleic acids were
fluorescently labeled using diamidino-2-phenylindole (DAPI). Samples were viewed with a Nikon Eclipse E600 microscope and imaged with a SPOT Slider digital camera using SPOT Advanced software. Confocal microscopy was performed using a Zeiss LSM 510 microscope equipped with LSM software.

Fractionation and immunoblotting – Enucleation was performed essentially as described (17). Briefly, PBS-washed RBL cells (1 x 10^7 cells) were resuspended in 15% Ficoll in α-MEM containing 20 µg ml⁻¹ cytochalasin B and incubated at 37°C for 30 min. This was layered atop a discontinuous gradient of 15, 16, 17, 20, and 25% Ficoll in α-MEM containing 20 µg ml⁻¹ cytochalasin B and 1 µM EGTA and centrifuged at 100,000 x g, 30 min, 24 °C. Microscopic examination revealed cytoplasts at the 20-25% interface and nucleoplasts in the pellet at the bottom of the Ficoll gradient. Cytoplasts and nucleoplasts were harvested from the gradient, diluted in an equal volume of α-MEM, centrifuged at 1,000 x g, 10 min, 4 °C, and resuspended in TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂) with protease inhibitors (1 mM each phenylmethylsulfonyl fluoride, dithiothreitol, soybean trypsin inhibitor, and leupeptin). An aliquot of the nucleoplasts was sonicated and centrifuged at 100,000 x g, 60 min, 4°C to obtain nuclear soluble and nuclear pelletable fractions. Aliquots of cytoplasts and nucleoplasts were also examined for trypan blue exclusion, counted and stained for tubulin by indirect immunofluorescence and for DNA with DAPI.

Nitrogen cavitation was as described (17), with PBS-washed cells suspended in ice-cold TKM buffer with protease inhibitors at 10^7 cells ml⁻¹ and subjected to nitrogen at 350 psi, 5 min, 4 °C. Total cell breakage was confirmed by the absence of trypan blue exclusion throughout a broad field of cavitate sample. Cavitate was then centrifuged at 1,000 x g, 10 min, 4 °C to pellet nuclei. The low speed supernatant was then centrifuged at 100,000 x g, 60 min, 4 °C to produce
cytosolic (supernatant) and non-nuclear membrane (pellet) fractions. The low speed pellet (nuclei) was resuspended in TKM buffer with 0.25 M sucrose, sonicated with 10 brief bursts at 20% cycle with a Branson sonicator, and centrifuged at 100,000 x g, 60 min, 4 °C to produce nuclear soluble and pelletable fractions. In some experiments, 1 mM calcium chloride was included in the TKM buffer throughout the fractionation procedure.

Protein assay and immunoblot analysis were performed as described previously (29). Briefly, protein concentration was determined by a modified Coomassie dye-binding assay (Pierce Chemical Co.), and 10 µg of each sample were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 5% non-fat dry milk, probed with primary antibody (5-LO, 1:3000; LTA4H, 1:5000), washed and reprobed with secondary antibody (horse radish peroxidase-linked goat anti-rabbit antibody, 1:5,000). Detection was by ECL chemiluminescent reagent (Amersham Pharmacia) and Hyperfilm chemiluminescent film (Amersham Pharmacia).
RESULTS

Subcellular Localization of LTA₄ Hydrolase in RBL Cells – The subcellular distribution of LTA₄ hydrolase, as well as 5-LO, was determined first in the RBL-1 cell line, which can generate significant amounts of LTB₄ (30). By immunocytochemistry, positive staining for LTA₄ hydrolase was evident in both the cytoplasm and nucleus, with the nucleus staining more intensely than the cytoplasm (Fig. 1). Essentially identical results were obtained for 5-LO: weak positive staining was present in the cytoplasm, whereas the nucleus showed much stronger staining. Cells probed in parallel with non-immune serum did not demonstrate any staining.

Indirect immunofluorescent microscopy was used as a second approach to localize LTA₄ hydrolase and 5-LO in individual cells: it avoids potential artifacts due to endogenous peroxidase activity, as may occur with immunocytochemistry, and it allows dual staining for DNA. By this method, the cytoplasm stained poorly for LTA₄ hydrolase and 5-LO, while the nucleus stained strongly for both (Fig. 1). Nuclear staining with DAPI supported localization of both LTA₄ hydrolase and 5-LO to the nucleus. Curiously, the overlay of rhodamine and DAPI signals showed both overlapping and distinct signals for both enzymes, suggesting that the distribution of either enzyme was identical neither to the DNA distribution nor to that of the other enzyme. (Figure 1 here)

While 5-LO has been shown to be soluble within the nucleoplasm of resting cells (17,20), it was not clear from the above analysis whether LTA₄ hydrolase was also within the nucleoplasm of RBL cells or simply associated with the nuclear envelope. To address this question, RBL cells dual stained for LTA₄ hydrolase and DNA were imaged by confocal microscopy. When serial optical sections for both stains were collected simultaneously, LTA₄ hydrolase was found to co-localize with DNA within the nucleus (Fig. 2). However, as
described above, the two signals were not absolutely identical, indicating that the distribution of LTA₄ hydrolase did not completely match that of the DNA. In separate experiments involving confocal imaging of 5-LO and DNA distribution in RBL cells, 5-LO was also found to be intranuclear but not identical in distribution to that of DNA (data not shown).

(Figure 2 here)

While the above techniques indicate immunodetectable protein within nuclei, they cannot determine if the nuclear protein is soluble and the same molecular weight as the cytosolic protein. As one approach to assess these issues, RBL cells were treated with cytochalasin b and centrifuged through a discontinuous Ficoll gradient. By this technique, >90% nucleus-free cytoplasts were obtained at the 20-25% Ficoll interface, as indicated by positive staining for the cytosolic marker α-tubulin (Fig 3B), negative staining for DAPI (not shown) and positive trypan blue exclusion (not shown). Nucleoplasts, characterized as α-tubulin negative (Fig. 3B), DAPI positive (not shown) and trypan blue staining (not shown) were obtained in the pellet. By immunoblot analysis, both cytoplasts and nucleoplasts contained the 78 kD 5-LO protein and the 69 kD LTA₄ hydrolase protein (Fig. 3A). As shown previously (17), nucleoplasts from RBL cells contain 5-LO in both soluble and pelletable fractions. In contrast, LTA₄ hydrolase is restricted to the soluble phase of RBL nucleoplasts (Fig. 3A).

(Figure 3 here)

We have previously demonstrated that, under proper conditions, nitrogen cavitation can effectively disrupt cells while maintaining nuclear integrity (17). When this is done in the absence of calcium, 5-LO protein is most abundant in the cytosolic and nuclear soluble fractions, although it is also detectable in membrane and nuclear pelletable fractions (Fig. 4A and (17,28)). By this technique (and in the same fractions), LTA₄ hydrolase is also predominantly in the
cytosolic and nuclear soluble fractions, although minor amounts can also be detected in the membrane fraction (Fig. 4A).

Because 5-LO can undergo calcium-dependent membrane association following cell stimulation (24,31) and because 5-LO and LTA$_4$ hydrolase function sequentially in LTB$_4$ synthesis, it was of interest to determine if LTA$_4$ hydrolase could demonstrate either calcium-dependent membrane association or membrane association following cell stimulation. As expected, when cells were fractionated in the presence of calcium, the majority of 5-LO protein was found to be associated with the membrane and nuclear pelletable fractions (Fig. 4A). However, in the same samples, LTA4 hydrolase remained predominantly soluble, with a distribution among fractions that matched that found in cells not given calcium. When intact RBL cells were stimulated with 1 $\mu$M A23187 for 5 min, immunofluorescent detection showed that 5-LO appeared to translocate to nuclear membranes, whereas no change in intranuclear distribution of LTA$_4$ hydrolase was found (Fig. 4B).

(Figure 4 here)

Subcellular localization of LTA$_4$ hydrolase in AMs – To determine if nuclear co-localization of LTA$_4$ hydrolase with 5-LO extended to primary leukocytes, we examined rat AMs, which, like RBL cells, can synthesize abundant LTB$_4$ (27). Lung tissue was harvested from normal, untreated rats, inflated and fixed overnight with formalin. Tissue slices were then stained to compare the subcellular localization of LTA$_4$ hydrolase with that of 5-LO in AMs in situ. Positive staining for LTA$_4$ hydrolase, indicated by the brown color developed from the peroxidase substrate DAB, was apparent in both the cytoplasm and the nucleus of AMs, with the nucleus staining being consistently darker (Fig. 5, large arrowhead). This contrasts with the staining of alveolar epithelial cells (Fig. 5, small arrowheads), in which brown staining for
hydrolase was present in the cytoplasm but absent from the nuclei, which were light blue due to counterstaining with hematoxylin. Staining for 5-LO in AMs within a serial section was, like that for hydrolase, heaviest within the nucleus but also evident within the cytoplasm. In serial sections probed with non-immune serum, brown staining was negligible.  

(Figure 5 here)

The subcellular distribution of LTA₄ hydrolase was also assessed in AMs isolated from the lung by lavage. By indirect immunofluorescent microscopy, LTA₄ hydrolase, like 5-LO, was accumulated within the nucleus, although light staining for both enzymes was also detectable within the cytoplasm (Fig. 6). Nuclear localization within given fields was confirmed by positive staining with DAPI. Previously, we have shown that 5-LO is found predominantly in the cytosolic and nuclear soluble fractions of AMs broken by nitrogen cavitation (28). Similarly, LTA₄ hydrolase was most abundant in the same fractions of rat AMs broken and fractionated in the same way (Fig. 7).

(Figure 6 here)

(Figure 7 here)

Subcellular localization of hydrolase in PMNs – In addition to RBL cells and AMs, PMNs synthesize abundant LTB₄. However, unlike RBL cells and AMs, PMNs from peripheral blood have 5-LO solely in the cytoplasm and the import of 5-LO into the nucleus occurs following adherence of PMNs to surfaces or recruitment of PMNs into sites of inflammation (20). When peripheral blood PMNs were maintained in suspension and probed for LTA₄ hydrolase as well as 5-LO protein by indirect immunofluorescent microscopy, both proteins were found to be exclusively cytosolic (Fig. 8). When PMNs were allowed to adhere to a fibronectin-coated
surface for 30 min, 5-LO accumulated strongly within the multi-lobed nuclei, whereas LTA₄ hydrolase remained outside the nucleus (Fig. 8).

(Figure 8 here)

The subcellular distributions of LTA₄ hydrolase and 5-LO in PMNs were also determined in situ, using immunohistochemistry applied to tissue sections. In PMNs situated in vessels in healthy rat lung, positive staining for 5-LO (Fig. 9A) and LTA₄ hydrolase (Fig. 9B) was restricted to the cytoplasm. When sections of tissue from inflamed appendix were probed for 5-LO, strong nuclear staining of recruited polymorphonuclear cells was evident (Fig. 9C), as expected. Serial sections from the same inflamed tissue, probed for LTA₄ hydrolase, showed the darkest staining associated with polymorphonuclear cells, with lighter positive staining associated with mesenchymal tissue and epithelial cells (Fig. 9D,E). In recruited polymorphonuclear cells as well as other tissue cells, hydrolase staining was predominantly cytosolic. Thus, LTA₄ hydrolase can co-localize with 5-LO in the cytoplasm, as in peripheral blood PMNs, or it can segregate from 5-LO, as when it is cytosolic and 5-LO is intranuclear in recruited PMNs.

(Figure 9 here)
Discussion

In the present study, we have used multiple techniques to compare the subcellular distribution of two enzymes, 5-LO and LTA₄ hydrolase, which work sequentially to synthesize LTB₄ from arachidonic acid. We have shown for the first time that LTA₄ hydrolase can be found in the nucleus, where it co-localizes with 5-LO, in resting AMs and RBL cells. In both cell types, lesser amounts of LTA₄ hydrolase and 5-LO were also present in the cytoplasm. We also have found that LTA₄ hydrolase co-localizes with 5-LO in the cytoplasm of peripheral blood PMNs. However, in this cell type, LTA₄ hydrolase does not move into the nucleus with 5-LO following adherence or recruitment of these cells. Finally, we have demonstrated that, unlike 5-LO, LTA₄ hydrolase does not show calcium-dependent membrane association or translocation following cell stimulation.

The finding that LTA₄ hydrolase can accumulate in the nucleus of AMs and RBL cells is particularly surprising, since the current thought is that this enzyme is exclusively cytoplasmic. Indeed, the current characterization for this protein in the Swiss-Prot databank gives its subcellular distribution as “cytoplasmic”. Consistent with this characterization, epithelial and mesenchymal cells in Figs. 5 and 9 lack nuclear staining for LTA₄ hydrolase. This suggests that only certain cell types may show nuclear import of LTA₄ hydrolase.

Nuclear import of small molecules may proceed by diffusion, whereas nuclear import of larger molecules, like the 69 kD LTA₄ hydrolase, requires the presence of a nuclear import sequence, which typically consists of a cluster of basic amino acids (32). Not surprisingly, LTA₄ hydrolase has several such clusters, most notably at (human) 186RKIYK. Interestingly, one of the structural domains of LTA₄ hydrolase, noted by Haeggstrom and colleagues (33), features armadillo repeats. These structural elements are also found on the nuclear import-mediating
importin proteins, suggesting the possibility that LTA₄ hydrolase could instead directly interact with importin-β or the nuclear pore complex. Activation of a nuclear import sequence typically involves a phosphorylation step (34), as a second requirement for nuclear import. Although there is evidence that LTA₄ hydrolase can be phosphorylated at serine 415 (35), it is unclear whether this specific phosphorylation event has any effect on subcellular distribution. Clearly, much work is necessary to better understand the mechanism of nuclear import of LTA₄ hydrolase.

The regulation of the subcellular distribution of LTA₄ hydrolase can be important in many ways. Most obviously, it may affect LTB₄ synthesis. Both AMs and RBL cells, which co-localize 5-LO with LTA₄ hydrolase, efficiently metabolize LTA₄ to LTB₄, as indicated by their ability to secrete large amounts of LTB₄ with negligible amounts of LTA₄. In contrast, PMNs, which can readily segregate 5-LO from LTA₄ hydrolase, secrete significant amounts of LTA₄ as well as LTB₄ (36). It is possible that part of the explanation for the inefficient conversion of LTA₄ to LTB₄ in PMNs lies in the spatial separation of 5-LO and LTA₄ hydrolase.

Another intriguing role for nuclear import of LTA₄ hydrolase may relate to regulating the levels of LTA₄ within the nucleus. Since 5-LO can accumulate in the nucleus, significant amounts of its end product, LTA₄, will be generated within that compartment. LTA₄ apparently has a striking capacity to bind to constituents of DNA (37). This, in turn, may have the potential to affect transcriptional events. By facilitating the conversion of LTA₄ to LTB₄, the co-localization of LTA₄ hydrolase with 5-LO in the nucleus might reduce the amount of intranuclear LTA₄ available to bind to DNA.

Finally, nuclear import of LTA₄ hydrolase might be relevant to the aminopeptidase function of this enzyme. Although mice that were deficient for LTA₄ hydrolase revealed no
evidence for an aminopeptidase role for the enzyme (38), it is possible that such a function only becomes apparent when the enzyme is in a particular subcellular locale. This possibility will require additional investigation.

In summary, we have shown that LTA$_4$ hydrolase can reside, with 5-LO, within the nucleoplasm of AMs and RBL cells. Moreover, the import of LTA$_4$ hydrolase into the nucleus does not necessarily coincide with that of 5-LO, as can be seen in adherent and recruited PMNs. These results indicate that the nuclear import of LTA$_4$ hydrolase is a regulated event that only occurs in some cell types under specific conditions. LTA$_4$ hydrolase is ubiquitously expressed in non-leukocytic cell types as well, and its import in such cell types remains to be evaluated. Furthermore, failure to correctly regulate the subcellular distribution of LTA$_4$ hydrolase may play a role in disease. These possibilities will be the subject of future investigations.
Footnotes

1 This work was supported by Grants R29 AI43574, R21 AI48141 and RO1 HL50496. These investigations were also supported in part by the Tissue Procurement Core of the University of Michigan Comprehensive Cancer Center, Grant #CA46952.

2 Abbreviations: 5-LO, 5-lipoxygenase; AA, arachidonic acid; AM, alveolar macrophage; DAB, diaminobenzidine; DAPI, diamidino-2-phenylindole; LT, leukotriene; NIS, non-immune serum; PMN, polymorphonuclear leukocyte; RBL, rat basophilic leukemia.
References


Figure Legends

**Fig. 1.** Subcellular localization of LTA$_4$ hydrolase and 5-LO by immunostaining. LTA$_4$H: LTA$_4$ hydrolase; NIS: non-immune serum. *(Upper)* Immunocytochemistry. Positive staining is indicated by brown coloration from the peroxidase substrate DAB. *(Lower)* Indirect immunofluorescence. Positive staining for LTA$_4$ hydrolase and 5-LO is indicated by rhodamine (red) fluorescence. Cells were also stained for DNA with DAPI (blue fluorescence). The overlay image represents the combined fluorescence signals of both rhodamine and DAPI fluorescence. Results are representative of five experiments.

**Fig. 2.** Intranuclear co-localization of LTA$_4$ hydrolase and DNA in RBL cells evaluated by confocal microscopy. Cells were dual stained for LTA$_4$ hydrolase and DNA and separate fluorescence signals were collected simultaneously in 1 µm optical sections. Three serial z-sections are presented. Results are representative of 3 experiments.

**Fig. 3.** Localization of 5-LO and LTA$_4$ hydrolase to the nucleus of RBL cells evaluated by enucleation. RBL cells were treated with cytochalasin b and subjected to centrifugation through a discontinuous Ficoll gradient. Fractions containing >90% nucleus-free cytoplasts or >90% cytoplasm-free nucleoplasts were identified by differential staining; an aliquot of nucleoplasts were sonicated and separated into soluble (Ns) and pelletable (Np) fractions by ultracentrifugation. *(A)* Immunoblot analysis of 5-LO and LTA$_4$ hydrolase in enucleation fractions. *(B)* Immunofluorescent characterization of cytoplast and nucleoplast fractions using α-tubulin as a cytosolic marker. Both fields contain similar numbers of plasts. Results are representative of three independent experiments.

**Fig. 4.** Subcellular distribution of 5-LO and LTA$_4$ hydrolase in RBL cells, with or without calcium activation. *(A)* Immunoblot analysis of 5-LO and LTA$_4$ hydrolase in RBL cells
fractionated by nitrogen cavitation and differential centrifugation, with or without calcium. Cells were disrupted as described in Experimental Procedures, with or without 1 mM CaCl₂, to produce cytosolic (C), non-nuclear membrane (M), nuclear soluble (Ns) and nuclear pelletable (Np) fractions. (B) Immunofluorescent localization of 5-LO, LTA₄ hydrolase and DNA in RBL cells after stimulation with 1 µM A23187 (5 min, 37 °C). Results are representative of three independent experiments.

**Fig. 5.** Localization of both LTA₄ hydrolase and 5-LO in lung macrophages in situ. Serial sections of formalin-fixed untreated rat lung tissue were probed with antibodies to LTA₄ hydrolase or 5-LO or with non-immune serum (NIS), with subsequent detection using the peroxidase substrate DAB. Sections were counterstained lightly with hematoxylin. AMs are indicated by large arrowheads; other cells are alveolar epithelial cells (small arrowheads). Results are representative of five independent experiments.

**Fig. 6.** Localization of 5-LO and LTA₄ hydrolase in isolated rat AMs. Cells were obtained by lavage of the alveolar space and immediately fixed and immunostained for either 5-LO or LTA₄ hydrolase. DAPI was used to stain nuclei.

**Fig. 7.** Distribution of LTA₄ hydrolase in rat AM subcellular fractions. Freshly isolated AMs were subjected to nitrogen cavitation, with subsequent fractionation by differential centrifugation as described in Experimental Procedures. Fractions were then probed for LTA₄ hydrolase by immunoblot analysis. Fractions are: cytosolic (C), non-nuclear membrane (M), nuclear soluble (Ns) and nuclear pelletable (Np).

**Fig. 8.** Localization of 5-LO and LTA₄ hydrolase in purified peripheral blood PMNs maintained in suspension or adhered to fibronectin. Human PMNs were purified from peripheral blood by
standard techniques and maintained in Teflon tubes or adhered to fibronectin-coated coverslips for 30 min, 37 °C, then fixed, permeabilized and immunostained for 5-LO or LTA₄ hydrolase. 

**Fig. 9.** Subcellular localization of 5-LO and LTA₄ hydrolase in PMNs in situ: from blood to tissue. (A) 5-LO and (B) LTA₄ hydrolase in PMNs within a pulmonary blood vessel; non-staining cells surrounding PMNs are reticulocytes. (C-E) Recruited PMNs (e.g., arrowheads) in inflamed appendix, stained for 5-LO (C) or LTA₄ hydrolase (D,E). (E) is a serial section stained in parallel with (D) but without counterstaining. Positive staining in all images is brown, from DAB. Counterstaining is with Harris’ hematoxylin (blue staining of nuclei).
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Cavitation: AMs

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Co-localization of leukotriene A4 hydrolase with 5-lipoxygenase in nuclei of alveolar macrophages and rat basophilic leukemia cells but not neutrophils
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