

Nuclear localization of leukotriene A₄ hydrolase in type II alveolar epithelial cells in normal and fibrotic lung

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Brock, Thomas G., Young-Jik Lee, Elana Maydanski, Tessa L. Marburger, Ming Luo, Robert Paine III, and Marc Peters-Golden. Nuclear localization of leukotriene A₄ hydrolase in type II alveolar epithelial cells in normal and fibrotic lung. *Am J Physiol Lung Cell Mol Physiol* 289: L224–L232, 2005. First published April 1, 2005; doi:10.1152/ajplung.00423.2004.—Leukotriene A₄ (LTA₄) hydrolase catalyzes the final step in leukotriene B₄ (LTB₄) synthesis. In addition to its role in LTB₄ synthesis, the enzyme possesses aminopeptidase activity. In this study, we sought to define the subcellular distribution of LTA₄ hydrolase in alveolar epithelial cells, which lack 5-lipoxygenase and do not synthesize LTA₄. Immunohistochemical staining localized LTA₄ hydrolase in the nucleus of type II but not type I alveolar epithelial cells of normal mouse, human, and rat lungs. Nuclear localization of LTA₄ hydrolase was also demonstrated in proliferating type II-like A549 cells. The apparent redistribution of LTA₄ hydrolase from the nucleus to the cytoplasm during type II-to-type I cell differentiation in vivo was recapitulated in vitro. Surprisingly, this change in localization of LTA₄ hydrolase did not affect the capacity of isolated cells to convert LTA₄ to LTB₄. However, proliferation of A549 cells was inhibited by the aminopeptidase inhibitor bestatin. Nuclear accumulation of LTA₄ hydrolase was also conspicuous in epithelial cells during alveolar repair following bleomycin-induced acute lung injury in mice, as well as in hyperplastic type II cells associated with fibrotic lung tissues from patients with idiopathic pulmonary fibrosis. These results show for the first time that LTA₄ hydrolase can be accumulated in the nucleus of type II alveolar epithelial cells and that redistribution of the enzyme to the cytoplasm occurs with differentiation to the type I phenotype. Furthermore, the aminopeptidase activity of LTA₄ hydrolase within the nucleus may play a role in promoting epithelial cell growth.

aminopeptidase; bleomycin; fibrosis; leukotriene B₄

LEUKOTRIENE B₄ (LTB₄) is an intercellular messenger with important roles in both immune defense and inflammatory disease. For example, LTB₄ promotes leukocyte chemotaxis (20), adhesion to endothelium (21, 53), degranulation (13, 19), superoxide anion generation (11, 12), and phagocytosis (38). The overproduction of LTB₄ is associated with a variety of inflammatory lung diseases, including idiopathic pulmonary fibrosis (IPF), acute lung injury, and chronic obstructive pulmonary disease (3, 22, 23, 31, 35).

The synthesis of LTB₄ from arachidonic acid (AA) requires the sequential action of two enzymes: 5-lipoxygenase (5-LO) and leukotriene A₄ (LTA₄) hydrolase. 5-LO catalyzes two sequential reactions to produce the unstable intermediate

LTA₄, which is then modified by the epoxide hydrolase activity of LTA₄ hydrolase to generate LTB₄ (27). Independent of its epoxide hydrolase activity, LTA₄ hydrolase also has an aminopeptidase activity (28, 39) and can be inhibited by aminopeptidase inhibitors, including bestatin (44). LTA₄ hydrolase has a high affinity for short peptides (43), including the enkephalins and dynorphins (24, 41). These aspects of LTA₄ hydrolase have been demonstrated in vitro but have not been found in vivo. In fact, comparison of wild-type mice and those deficient in LTA₄ hydrolase revealed no evidence of a role for the aminopeptidase activity of LTA₄ hydrolase in vivo (9).

LTB₄ is produced primarily by leukocytes because the enzyme 5-LO is largely limited in distribution to these cell types. LTA₄ hydrolase, on the other hand, is found in many cell types, including alveolar epithelial cells (4, 5). In the absence of 5-LO to initiate leukotriene synthesis, these cells cannot, by themselves, generate appreciable LTB₄. However, they can produce LTB₄ through a “transcellular” mechanism, accepting LTA₄ donated by adjacent neutrophils as substrate for LTA₄ hydrolase (25). In this way, the unstable LTA₄ that is secreted by neutrophils is metabolized to LTB₄ before it can break down. Thus LTA₄ hydrolase in cells lacking 5-LO may be important in determining the total amount of LTB₄ produced in tissues containing recruited leukocytes.

The enzyme LTA₄ hydrolase has been shown to be a soluble protein (18, 49). Previously, we reported (7) that, in 5-LO-containing leukocytes, the subcellular distribution of LTA₄ hydrolase was cell type specific, colocalizing with 5-LO in the nucleoplasm of alveolar macrophages and rat basophilic leukemia cells but remaining in the cytoplasm of neutrophils. During that study, we noticed that certain alveolar epithelial cells also appeared to have LTA₄ hydrolase accumulated in the nucleus. Here, we demonstrate that the localization of LTA₄ hydrolase in alveolar epithelial cells depends on the state of differentiation of the cell, being nuclear in type II cells but cytoplasmic in type I cells. The change in localization of LTA₄ hydrolase occurs with differentiation both in vivo and in vitro. Interestingly, the subcellular localization of LTA₄ hydrolase does not appear to affect the capacity of epithelial cells to convert exogenously supplied LTA₄ to secretable LTB₄. However, the proliferation of A549 cells, which have nuclear LTA₄ hydrolase, is inhibited by the aminopeptidase inhibitor bestatin, whereas that of NIH/3T3 cells, which have exclusively cytoplasmic LTA₄ hydrolase, is not.

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MATERIALS AND METHODS

Animals, tissues, and cells. Human lung samples were from biopsies of uninvolved regions from patients with lung cancer (control) or involved regions from patients with IPF, as described (57). The experimental protocol was approved by the University of Michigan Medical Center Institutional Review Board for Approval of Research Involving Human Subjects. Mice were specific pathogen-free 129/Sv mice obtained from the Jackson Laboratory (Bar Harbor, ME). F1 male Fischer 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 American Physiological Society and the University of Michigan Committee on Use and Care of Animals. A549, NIH/3T3, and rat basophilic leukemia (RBL-1) cells were obtained from American Type Culture Collection (Manassas, VA) and grown under 5% CO₂ in DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin.

Bleomycin treatment and immunohistochemical staining. As described (47), mice were anesthetized and subjected to intratracheal injection with 30 μ l of 0.025 U bleomycin (Sigma-Aldrich, St. Louis, MO) diluted in saline. As previously described (7), rat and mouse lungs were removed, inflated, and fixed en bloc with 4% paraformaldehyde overnight at 4°C and processed for paraffin sections. 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 (Inno-Genex, San Ramon, CA). Rabbit polyclonal antibody against LTA₄ hydrolase (a generous gift from Dr. J. Evans, Merck Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada) was prepared in PBS containing 0.1% BSA (titer 1:750) and applied overnight at 4°C. After slides were washed 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, and then treated with avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. 3,3'-Diaminobenzidine was used as peroxidase substrate; preparations were counterstained with Harris' hematoxylin.

Indirect immunofluorescence and confocal microscopy. Cells were cultured on chamber slides, fixed with methanol at -20°C for 30 min, permeabilized with -20°C acetone, air-dried, and then blocked with 1% BSA in PBS containing nonimmune goat serum. Primary antibody (1:500) was prepared in 1% BSA-PBS and applied for 1 h at 37°C. Mounts were washed three times with 1% BSA-PBS, and rhodamine-conjugated goat anti-rabbit secondary (1:250) was added for 1 h at 37°C. After three washes, preparations were mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Fluorescence was visualized with a Nikon Labophot 2 microscope equipped for epifluorescence or with a Bio-Rad MRC-600 laser confocal microscope.

Subcellular fractionation and immunoblot analysis. A549 cells were washed with Dulbecco's balanced salt solution, trypsinized, and resuspended in ice-cold STKM buffer (250 mM sucrose, 50 mM Tris·HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM EGTA) with protease inhibitors (1 mM each phenylmethylsulfonyl fluoride, dithiothreitol, soybean trypsin inhibitor, and leupeptin) at 2 \times 10⁷ cells/ml and subjected to nitrogen cavitation at 400 psi, for 5 min, at 4°C. Maximal breakage of cells was confirmed by lack of trypan blue exclusion. The resulting cavitate was centrifuged at 1,000 g, 10 min at 4°C, to produce crude isolated nuclei and a postnuclear supernatant. The postnuclear supernatant was centrifuged at 100,000 g, 30 min at 4°C, with the resulting supernatant and pellet (resuspended in STKM buffer) designated "cytosol" and "membrane" fractions, respectively. Isolated nuclei were washed and sonicated (30 bursts at 20% duty

cycle) and ultracentrifuged, as described above, to produce nuclear-soluble and -pelletable fractions. Aliquots of fractions were sonicated (10 bursts at 20% duty cycle), and protein concentrations were determined by a modified Coomassie dye-binding assay (Pierce), using BSA as standard. Samples were then subjected to immunoblot analyses as described previously (8). Briefly, samples containing 10 μ g of protein were separated by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane, which was then blocked, washed, and probed with antibody against LTA₄ hydrolase (1:5,000). Detection involved horseradish peroxidase-conjugated goat anti-rabbit secondary (1:5,000) followed by enhanced chemiluminescence (Amersham Biosciences). Multiple exposure times were taken for all blots to ensure that band densities were within the linear range of exposure. The membrane was then stripped and reprobed with anti- β -tubulin (1:200) as a cytoplasmic cytoskeletal marker (32).

Isolation of rat type II pneumocytes. Rat type II cells were isolated by elastase digestion and IgG panning as previously described (17). Briefly, rats were anesthetized, the trachea was cannulated, and the pulmonary circulation was perfused free of blood with a balanced salt solution at 4°C. After multiple whole lung lavages with EGTA (1 mM) in a balanced salt solution, porcine pancreatic elastase (4.3 U/ml; Worthington) was instilled via the trachea to release type II cells. Contaminating cells bearing Fc receptors were removed from the cell suspension by panning on plates coated with rat IgG (Sigma-Aldrich). Cells were plated on chamber slides at 2 \times 10⁵ cells/cm² in DMEM supplemented with penicillin-streptomycin and 10% newborn calf serum and cultured at 37°C in 5% CO₂. The medium was replaced every 2–3 days.

Metabolism of LTA₄ to LTB₄. On days 1 and 7 (after isolation), cells were washed and incubated with various concentrations of LTA₄, freshly hydrolyzed from LTA₄ methyl ester as recommended by supplier (Cayman Chemical, Ann Arbor, MI), in medium. After 30 min at 37°C, the medium was recovered for LTB₄ determination by enzyme immunoassay according to the supplier's instructions (Cayman Chemical). The detection limit for LTB₄ was 4 pg/ml; cross-reactivity for AA, 5-HETE, LTA₄, LTC₄, LTD₄, and LTE₄ was <0.01%. Cells were then washed, scraped, and assayed for LTA₄ hydrolase protein by immunoblot analysis.

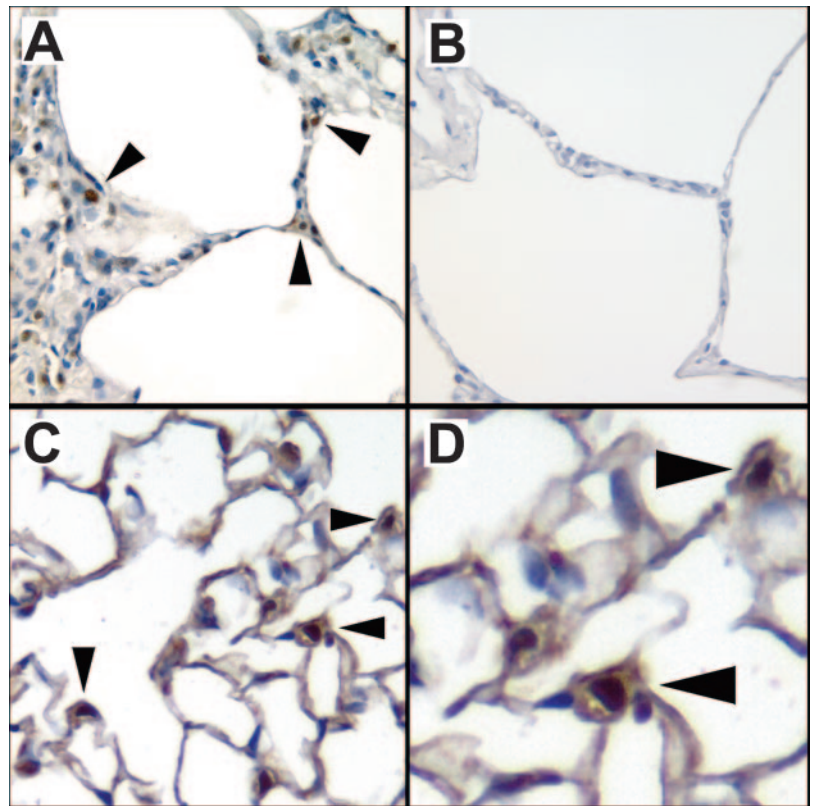
Cell proliferation assays. A549 and NIH/3T3 cells were plated at 4,000 cells/well in 96-well flat-bottomed tissue culture plates in complete media (DMEM, 10% fetal calf serum, and 1% penicillin-streptomycin). After 6 h, cells were quiesced in serum-free media (DMEM and 1% penicillin-streptomycin) for 18 h and then allowed to grow for 24 h in complete medium in the presence of different concentrations (10, 30, 100, 300 μ M, adjusted to 0.5% methanol for each well) of bestatin (Sigma) plus 10 μ Ci of [³H]thymidine (Amersham). As controls, cells were treated with either methanol (0.5%) or 2 μ M of the leukotriene biosynthesis inhibitor MK-866 (Biomol, Plymouth Meeting, PA). We harvested cells onto glass fiber filters using a cell harvester and determined incorporated radioactivity by beta-scintillation counting.

Statistical analysis. Statistical significance was evaluated by one-way ANOVA, using $P < 0.05$ as indicative of statistical significance. Pairs of group means were analyzed using the Tukey-Kramer post hoc test.

RESULTS

Subcellular distribution of LTA₄ hydrolase in human and mouse alveolar epithelial cells. To evaluate the localization of LTA₄ hydrolase in normal alveolar epithelial cells, sections of tissues from untreated human and mouse lungs were evaluated by immunohistochemistry. Human lung tissue showed strong positive (brown) staining for LTA₄ hydrolase in the nucleus of numerous cells, including cells at junctions of the alveolar walls (Fig. 1A). Human lung tissue stained in parallel with

Fig. 1. Subcellular localization of leukotriene A₄ (LTA₄) hydrolase in situ in normal human and mouse alveolar epithelial cells. Normal human (A and B) and mouse (C and D) lung tissues were stained with antibody to LTA₄ hydrolase (A, C, D) or nonimmune serum (B). D provides detail from right side of C. Brown staining indicates positive staining for LTA₄ hydrolase; blue indicates nuclei counterstained with hematoxylin. Arrowheads highlight some cells with nuclear staining for LTA₄ hydrolase. Results are representative of 5 independent experiments.



nonimmune serum was negative (Fig. 1B). In mouse lung sections, positive staining for LTA₄ hydrolase was found in essentially all cells (Fig. 1C). Blue staining of nuclei by the counterstain hematoxylin was particularly evident in flattened cells, whereas brown staining of nuclei was found in cuboidal cells (Fig. 1D). Positive nuclear staining of cuboidal epithelial cells at alveolar wall junctions suggested the presence of LTA₄ hydrolase in the nuclei of type II pneumocytes. Also, it was observed that the majority of alveolar epithelial cells, particularly those in alveolar walls, had blue nuclei, indicating that LTA₄ hydrolase was not in the nucleus of type I pneumocytes.

LTA₄ hydrolase redistribution and activity in rat type II cells in vivo and in vitro. Immunohistochemical staining of normal rat lung tissue indicated that LTA₄ hydrolase was prominent in the nucleus of type II but not type I pneumocytes (Fig. 2A). Both type I and type II cells had positive LTA₄ hydrolase staining in the cytoplasm. Thus, in human, mouse, and rat lungs, nuclear localization of LTA₄ hydrolase was restricted to type II cells, suggesting that LTA₄ hydrolase redistributed from the nucleoplasm to the cytoplasm as type II cells differentiated into type I cells (45). Isolated type II cells are known to differentiate in culture, developing characteristics of type I pneumocytes over several days (6, 51), so the localization of LTA₄ hydrolase was examined in cultured rat type II cells. Freshly isolated type II cells, stained by immunofluorescence, showed strong nuclear accumulation of LTA₄ hydrolase with some cytosolic enzyme (Fig. 2B). After 1 day in culture, the difference between nuclear and cytoplasmic compartments was reduced, although nuclear staining was still clearly greater (Fig. 2C). After 7 days in culture, nuclei were negative for LTA₄ hydrolase, with fluorescence restricted to the cytoplasm (Fig. 2D). The finding that freshly isolated type II pneumocytes

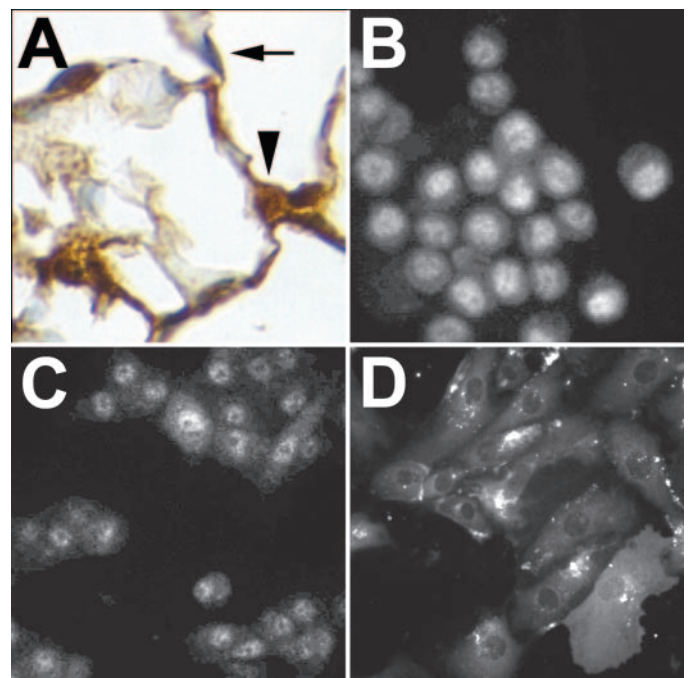


Fig. 2. LTA₄ hydrolase localization in rat alveolar epithelial cells in vivo and in vitro. A: immunohistochemical staining of normal lung from rat. Positive staining for LTA₄ hydrolase is brown, with nuclei counterstained blue. Arrow and arrowhead indicate cells with nuclei that are negative and positive, respectively, for LTA₄ hydrolase. Results are representative of 10 independent experiments. B–D: isolated type II alveolar epithelial cells with LTA₄ hydrolase detected by immunofluorescence. Cells were isolated from lungs of normal rats and fixed and stained at 1 h (B), 1 day (C), or 7 days (D) after they were mounted on slides. Results are representative of 5 independent experiments.

uniformly have nuclear accumulation of LTA₄ hydrolase supports the immunohistochemical localization in type II cells stained in situ. The redistribution of LTA₄ hydrolase in vitro thus appears to be another way in which type II pneumocytes become more type I-like with time in culture.

As noted above, epithelial cells can participate in transcellular metabolism of LTA₄, donated by neighboring leukocytes, to produce LTB₄. It seemed plausible that LTA₄ hydrolase might convert exogenously added LTA₄ to LTB₄ more efficiently when located in the cytoplasm than when deep within the nucleus. Furthermore, this effect might be more pronounced at lower levels of LTA₄. To test this, varying doses of LTA₄ were added to primary rat alveolar epithelial cells that had LTA₄ hydrolase predominantly in the cytoplasm (*day 7* cells) or in the nucleus (*day 1* cells). LTB₄ synthesis from exogenous LTA₄ in *day 7* cells did not differ significantly from that of *day 1* cells, regardless of the concentration of LTA₄ provided (Fig. 3). Immunoblot analysis of cell lysates indicated comparable amounts of total LTA₄ hydrolase protein in *day 1* and *day 7* cells (data not shown). This suggests that, although LTA₄ hydrolase appears to be redistributed from the nucleus to the cytoplasm as type II pneumocytes differentiate and become more type I like, their capacity to produce LTB₄ from exogenous LTA₄ does not greatly change, at least as judged by extracellular measurements of LTB₄.

Subcellular localization of LTA₄ hydrolase in an alveolar epithelial cell line. In addition to nuclear accumulation of LTA₄ hydrolase, type II cells differ from type I cells by having a higher capacity to proliferate. If nuclear localization of LTA₄ hydrolase correlated with proliferative capacity of epithelial cells, then LTA₄ hydrolase should be found in the nucleus of transformed type II cells. The A549 is a continuous cell line derived from a human pulmonary adenocarcinoma that resembles type II alveolar epithelial cells (36). Immunofluorescent staining of proliferating A549 cells for LTA₄ hydrolase resulted in bright speckled fluorescence at the nucleus with less bright diffuse fluorescence in the cytoplasm (Fig. 4A). Bright staining for LTA₄ hydrolase corresponded to staining for DNA using DAPI (Fig. 4B). For comparison, staining for LTA₄ hydrolase in proliferating fibroblast-like NIH/3T3 cells was restricted to the cytoplasm (Fig. 4C), although regions indicated as nuclear by DAPI staining (Fig. 4D) were negative for

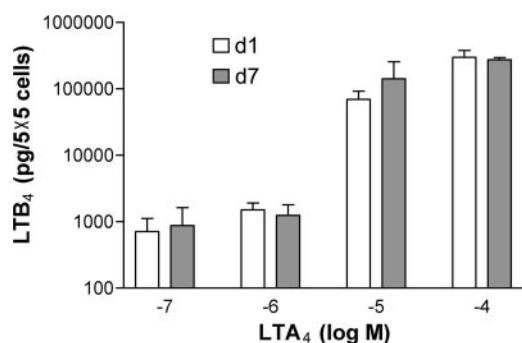


Fig. 3. Metabolism of LTA₄ to LTB₄ by alveolar epithelial cells in culture for 1 (d1) or 7 (d7) days. Isolated type II alveolar epithelial cells were cultured on chamber slides for 1 or 7 days and then washed and given LTA₄ at the indicated concentrations. After 30 min, supernatants were collected and LTB₄ was measured by enzyme immunoassay. Results are representative of 5 independent experiments.

LTA₄ hydrolase. Confocal imaging confirmed that LTA₄ hydrolase was abundant within the nucleus (Fig. 4E) as well as the cytoplasm (Fig. 4F) of A549 cells but restricted to the cytoplasm of NIH/3T3 cells (Fig. 4G). Thus nuclear LTA₄ hydrolase is present in some proliferating epithelial cells but not in fibroblast-like NIH/3T3 cells.

The discovery of nuclear LTA₄ hydrolase in the A549 monoculture allowed further evaluation of localization without contaminating leukocytes, as may be found in isolated type II preparations. To evaluate LTA₄ hydrolase subcellular distribution, A549 cells were fractionated into nuclear and nonnuclear fractions, and these were further separated into soluble and pelletable fractions. Immunoblot analyses indicated the presence of LTA₄ hydrolase predominantly in the soluble nuclear fraction and the cytoplasmic fraction (Fig. 4H). β -Tubulin, a microtubule protein that does not occur in the nucleus, was absent from the soluble nuclear fraction, demonstrating that this fraction was not contaminated with cytoplasmic proteins. Together, these results indicate that LTA₄ hydrolase is soluble and accumulates in the nucleus of proliferating type II-like cells but not fibroblast-like cells.

Because A549 cells, unlike isolated type II pneumocytes, readily proliferate in culture, the importance of LTA₄ hydrolase's aminopeptidase activity on growth could be tested with these cells. Significantly, A549 cells lack detectable 5-LO protein yet express an amount of LTA₄ hydrolase that is similar to that found in NIH/3T3 cells (Fig. 5A, rat basophilic leukemia cell lysate as positive control). As expected, MK-886, an inhibitor of LTA₄ synthesis (16), had no effect on serum-induced growth of either A549 (Fig. 5B) or NIH/3T3 cells (Fig. 5C). In contrast, the aminopeptidase inhibitor bestatin significantly (~22%) inhibited the growth of A549 cells, which have nuclear LTA₄ hydrolase, but not NIH/3T3 cells, which have cytoplasmic LTA₄ hydrolase. These results indicate that aminopeptidase activity is responsible for a portion of the growth potential of A549 cells. Because bestatin is known to inhibit the aminopeptidase activity of LTA₄ hydrolase (44), it is possible that nuclear LTA₄ hydrolase in A549 cells contributes to the growth of those cells through its aminopeptidase activity.

Subcellular localization of LTA₄ hydrolase in bleomycin-treated mouse lung tissue. Proliferation of epithelial and fibroblast cells occurs during the repair process of lungs injured by bleomycin. We asked whether nuclear localization of LTA₄ hydrolase would be linked to proliferation of epithelial, but not fibroblast, cells in bleomycin-damaged lungs using immunohistochemical staining. Bleomycin injury is typically patchy in distribution; at 14 days postbleomycin, tissue sections stained heavily for LTA₄ hydrolase in both uninvolved (Fig. 6A) and fibrotic (Fig. 6B) sites. In regions with relatively normal alveolar architecture, nuclear localization of LTA₄ hydrolase was evident in flattened epithelial cells (Fig. 6A, arrowheads) as well as in cuboidal cells, with few cells showing the blue negative nuclear staining. At fibrotic sites, interstitial cells with predominantly cytoplasmic LTA₄ hydrolase, reminiscent of the distribution in NIH/3T3 fibroblasts (Fig. 4C), were readily observable (Fig. 6B, arrows). These results suggest that nuclear localization of LTA₄ hydrolase is a feature of epithelial cells but not of fibroblast-like cells, associated

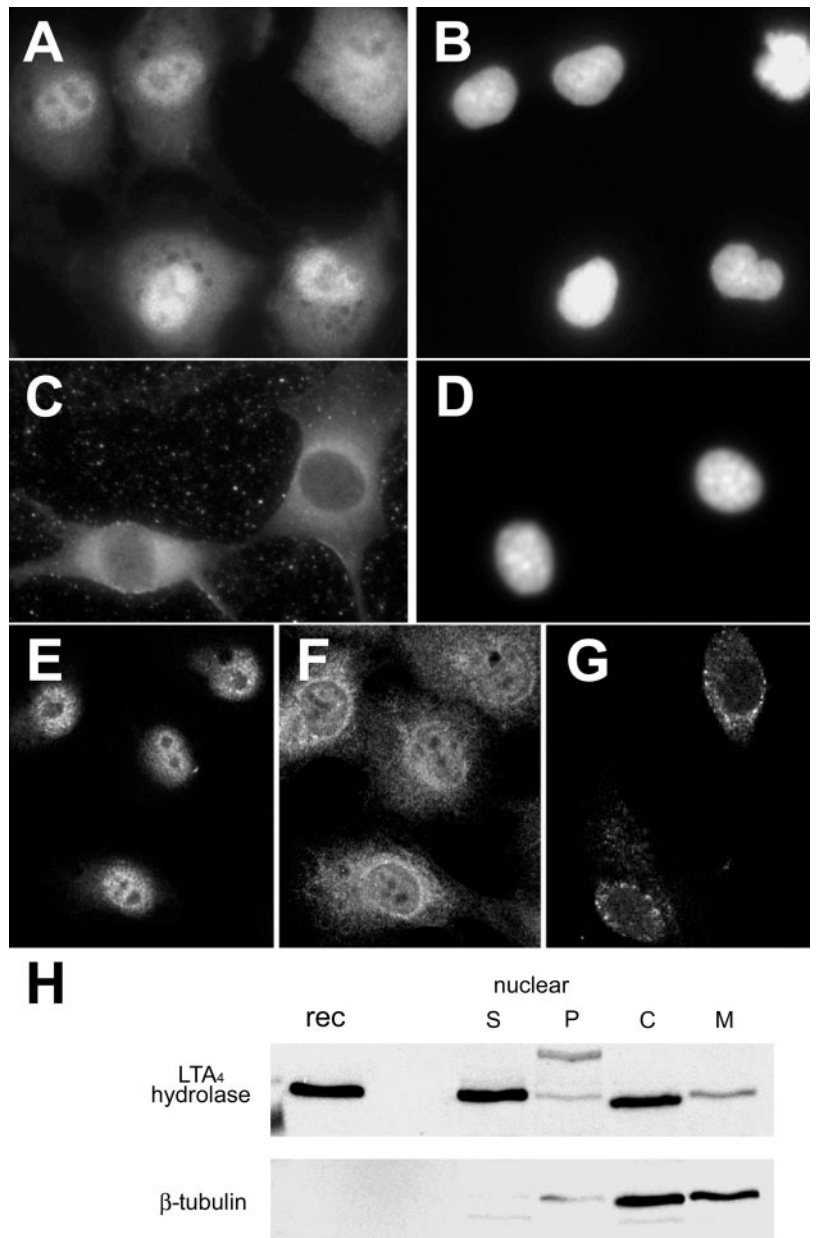


Fig. 4. LTA₄ hydrolase occurs in the nucleus of type II-like A549 epithelial cells but not in fibroblast-like NIH/3T3 (3T3) cells. *A*: localization of LTA₄ hydrolase in A549 cells by indirect immunofluorescent labeling. *B*: localization of DNA in the same field of cells as *A*, using 4',6-diamidino-2-phenylindole (DAPI). *C*: immunofluorescent staining for LTA₄ hydrolase in 3T3 cells. *D*: DNA staining, with DAPI, of the same field of cells as *C*. *E* and *F*: confocal imaging of LTA₄ hydrolase in A549, with optical sections taken at ~7 μm (*E*) and 2 μm (*F*) above slide surface. *G*: confocal imaging of LTA₄ hydrolase in 3T3 cells. *H*: localization of LTA₄ hydrolase in A549 cells by fractionation and immunoblotting. β-Tubulin is used as a cytoplasmic marker. Subcellular fractions (S, nuclear soluble; P, nuclear pelletable; C, cytosolic; M, nonnuclear membrane) were isolated as described in MATERIALS AND METHODS. Rec, recombinant LTA₄ hydrolase standard.

with rapid cell proliferation or tissue repair following bleomycin injury.

At 21 days after bleomycin treatment, staining for LTA₄ hydrolase remained high in fibrotic sites, with the majority of cells having positive-staining nuclei (Fig. 7A). In contrast, staining was diminished and largely restricted to type II cells in uninvolved areas from the same section (Fig. 7B), similar to staining in untreated mouse lung. By 30 days postbleomycin treatment, fibrotic regions remained strongly positive for LTA₄ hydrolase (Fig. 7C), and hyperplastic type II cells with nuclear LTA₄ hydrolase appeared to be delimiting these regions (Fig. 7D). Lung tissue from the same section having normal architecture had normal staining (i.e., like Fig. 1C) for LTA₄ hydrolase (not shown). These findings suggest that nuclear localization of LTA₄ hydrolase remains a feature of hyperplastic epithelial cells at sites of fibrosis or lung repair.

Nuclear localization of LTA₄ hydrolase in human IPF. IPF is characterized by epithelial cell injury and dysfunction (50) as well as LTB₄ overproduction (56). Bleomycin-injured mouse lung is used to model human IPF; therefore, we asked whether the nuclear LTA₄ hydrolase observed in hyperplastic epithelial cells in the mouse model were also found in human disease. Immunohistochemical analysis of lung sections from patients with IPF showed strong nuclear staining of the hyperplastic type II epithelial cells lining the surface of fibrotic areas (Fig. 8). Staining within the fibrotic region itself was faint and diffuse. Strong nuclear staining for LTA₄ hydrolase in type II cells was observed in sections from all eight patients examined. Thus nuclear localization of LTA₄ hydrolase is found in normal type II epithelial cells in humans, mice, and rats, and this subcellular distribution is associated with proliferation in A549 cells, bleomycin-injured mouse lung, and human fibrotic disease.

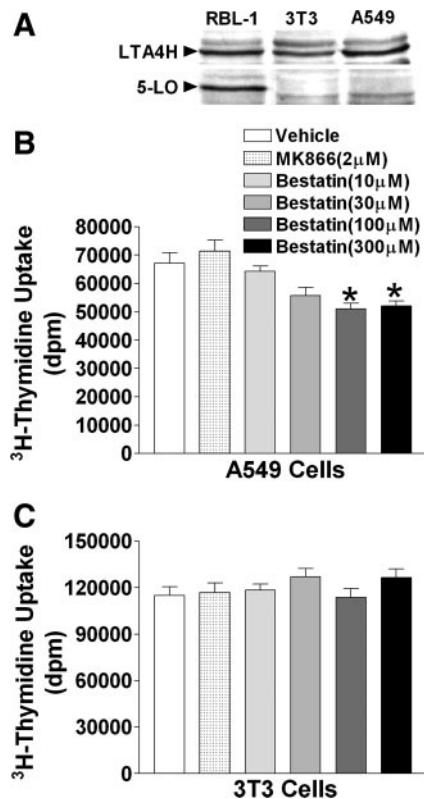


Fig. 5. Cell proliferation of A549, but not 3T3 cells, was inhibited by bestatin. A: immunoblot of cell lysates from RBL-1, 3T3, and A549 cells, probed for LTA₄ hydrolase or 5-lipoxygenase (5-LO). Each lane was loaded with 10 μg of total protein. LTA₄H, LTA₄ hydrolase. B: A549 cells were plated on 96-well plates and treated with vehicle, MK-866, or bestatin at the indicated concentrations. The incorporation of [³H]thymidine was determined by beta-scintillation counting. Results are means (+SE) of 8 wells from a representative experiment. **P* < 0.05. C: incorporation of [³H]thymidine in 3T3 cells, evaluated as described in B.

DISCUSSION

In the present study, we have, for the first time, examined the distribution of LTA₄ hydrolase in alveolar epithelial cells. We found that LTA₄ hydrolase was accumulated in the nucleus of normal type II alveolar epithelial cells in human, mouse, and rat lung, in the type II-like A549 cell line, in lung epithelial cells following bleomycin injury, and in type II alveolar epithelial cells associated with fibrotic regions of lung in patients with IPF. In contrast, LTA₄ hydrolase was predominantly cytosolic in normal type I alveolar epithelial cells *in situ*, in isolated type II cells cultured to become type I-like (45), as well as in fibroblasts. As we found for leukocytes (7), the nuclear import-export of LTA₄ hydrolase appears to be a regulated process. In alveolar epithelial cells, the aminopeptidase activity of nuclear LTA₄ hydrolase may promote growth, and nuclear export of this activity may be linked to transition from a type II to type I cell phenotype.

The functional significance of positioning LTA₄ hydrolase within the nucleus is unclear. The demonstrated biological function for this enzyme in epithelial cells is the synthesis of LTB₄ from donated LTA₄. Freshly isolated type II epithelial cells will readily produce LTB₄ from LTA₄ secreted by either macrophages (48) or neutrophils (25, 61). LTB₄ levels are increased in bronchoalveolar lavage (55) and lung tissue ho-

mogenates (56) from patients with IPF, correlating increased LTB₄ production with prominent nuclear LTA₄ hydrolase in type II cells. For this reason, we speculated that nuclear LTA₄ hydrolase might convert exogenous LTA₄ to LTB₄ better than cytoplasmic enzyme, supporting a contribution by alveolar epithelial cells to lung LTB₄ levels in this disease. However, type II cells at *day 7*, with cytoplasmic LTA₄ hydrolase, secreted as much LTB₄ as type II cells at *day 1*, when LTA₄ hydrolase was accumulated in the nucleus. This indicates that the subcellular distribution of LTA₄ hydrolase did not significantly affect the capacity for transcellular metabolism of LTA₄. Consistent with this, the level of LTB₄ in lung lavage does not increase in mice after treatment with bleomycin (47), although the subcellular distribution of LTA₄ hydrolase does change.

The nuclear localization of LTA₄ hydrolase may affect AA metabolism in other ways. For example, the export of LTB₄ is carrier mediated (33), although the carrier has not been identified. Movement of LTA₄ hydrolase might change its position relative to the carrier and affect LTB₄ export. Also, epithelial cells may themselves produce small levels of LTA₄ (reviewed in Ref. 37). The positioning of LTA₄ relative to 5-LO may be important in determining processing of endogenously produced LTA₄, as in leukocytes (7). Finally, the lipid substrate of LTA₄ hydrolase, LTA₄, is recognized to be an unstable inter-

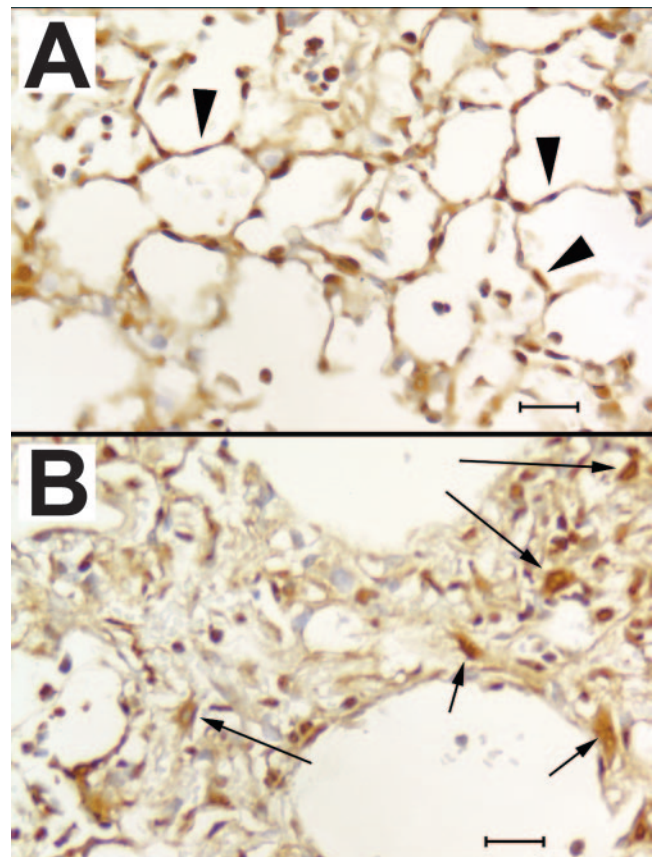


Fig. 6. LTA₄ hydrolase staining of lung tissue from mice 14 days after bleomycin treatment. Representative uninjured (A) and fibrotic (B) regions are from a single lung section of a mouse treated with bleomycin for 14 days (*n* = 2). Arrowheads in A point to flattened cells with nuclear staining. Arrows in B indicate cells with dense cytoplasmic staining for LTA₄ hydrolase. Bars indicate 20 μm.

Fig. 7. Localization of LTA₄ hydrolase in lung tissue from mice treated with bleomycin for 21 days (A and B) or 30 days (C and D). Representative fibrotic (A) and uninvolved (B) regions are from a single lung section of a mouse treated with bleomycin for 21 days ($n = 2$). Detail (D) from indicated (asterisk) area of representative fibrotic region (C) of 30-day treatment highlights cells lining air spaces having nuclear staining for LTA₄ hydrolase (arrowheads).

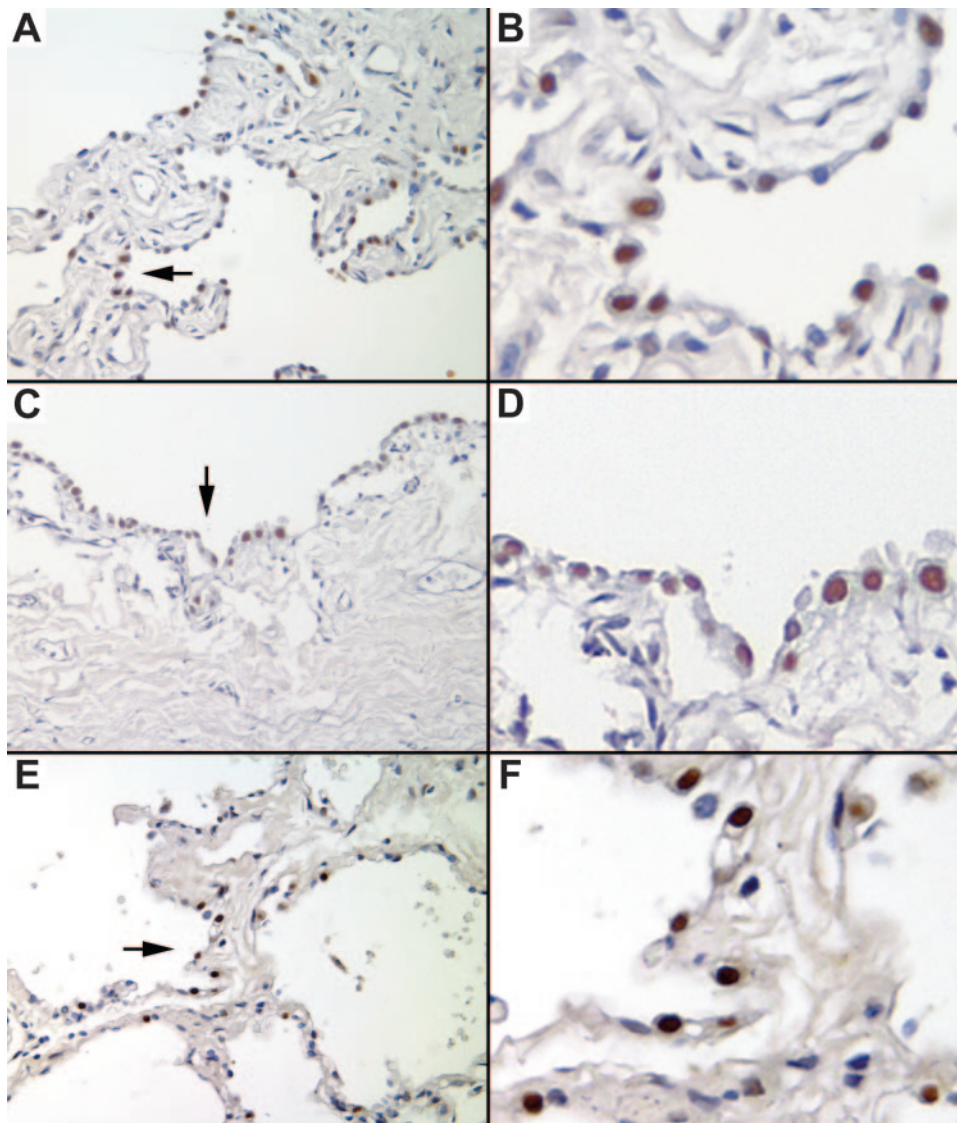
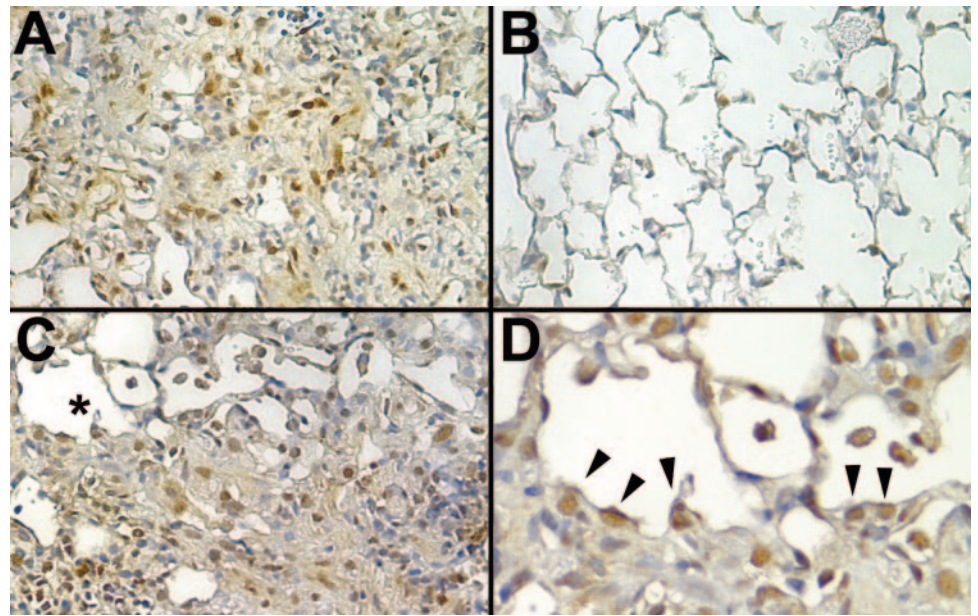


Fig. 8. Nuclear localization of LTA₄ hydrolase in alveolar epithelial cells in lung tissue from patients with idiopathic pulmonary fibrosis. Brown staining indicates positive immunohistochemical staining for LTA₄ hydrolase; blue indicates nuclei counterstained with hematoxylin. Images on right (B, D, and F) are magnifications of field indicated by arrows in images on left (A, C, and E). Results are from 3 different patients and are representative of 8 different patients.

mediate in the leukotriene biosynthetic pathway. However, LTA₄ is stabilized by fatty acid binding proteins, including the epithelial fatty acid binding protein (15), which can move lipids from the cytoplasm into the nucleus (34). Fatty acid binding proteins can interact with transcription factors (58), as can leukotrienes (14). Furthermore, LTA₄ can covalently bind to DNA and RNA, both in vitro and in cells (29). Thus LTA₄ can persist within cells, move into the nucleus, and interact with transcription factors and nucleic acids. The localization of LTA₄ hydrolase within the nucleus may serve to remove LTA₄ by metabolizing it to the rapidly secreted LTB₄, in this way changing its effects on cell function.

One cell function apparently associated with the nuclear localization of LTA₄ hydrolase in epithelial cells is proliferation: the enzyme is strongly nuclear in hyperplastic type II cells in IPF and also in growing A549 cells and leaves the nucleus as type II cells differentiate to become more type I-like, and nonproliferative, in culture. The overexpression of LTA₄ hydrolase in epithelial cells has been linked with tumorigenesis in esophageal adenocarcinoma, and bestatin, an aminopeptidase inhibitor, significantly reduced tumor incidence (10). Interestingly, aminopeptidase inhibitors like bestatin have been shown to suppress DNA synthesis in keratinocytes (52), inhibit proliferation of myeloma cells by intracellular actions (26), and promote the differentiation of U937 histiocytes (40) and lymphocytes (54). Furthermore, enkephalins, which have been shown to be substrates for LTA₄ hydrolase in vitro (24), can be found, with their receptors, in the nuclei and perinuclear regions of epithelial cells (59). Enkephalins inhibit epithelial cell proliferation (60), suggesting that their degradation within the nucleus by a peptidase would allow proliferation. Through either its hydrolase or its aminopeptidase activity, LTA₄ hydrolase appears to promote epithelial cell proliferation when localized within the nucleus, whereas differentiation correlates with its redistribution into the cytoplasm.

In summary, the enzyme LTA₄ hydrolase, a soluble enzyme known to have multiple activities, can be in either the cytoplasm or the nucleoplasm of leukocytes and alveolar epithelial cells. It is likely that redistribution of LTA₄ hydrolase between these two compartments is a regulated process and that it occurs in other cell types as well. Contrary to our expectations, our results indicate that nuclear import of LTA₄ hydrolase does not affect the ability of the cell to convert exogenous LTA₄ to LTB₄. However, bestatin, known as both an aminopeptidase inhibitor and direct LTA₄ hydrolase inhibitor, impairs growth in A549 cells, which have nuclear LTA₄ hydrolase. Together, these results suggest that the aminopeptidase activity of LTA₄ hydrolase plays a role in regulating the growth of type II epithelial cells. Moreover, it may be that the nuclear localization of this activity promotes growth in these cells.

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REFERENCES

- Baker JR, Kylstra TA, and Bigby TD. Effects of metalloproteinase inhibitors on leukotriene A₄ hydrolase in human airway epithelial cells. *Biochem Pharmacol* 50: 905–912, 1995.
- Beset S, Vincourt JB, Amalric F, and Girard JP. Nuclear localization of PAPS synthetase I: a sulfate activation pathway in the nucleus of eukaryotic cells. *FASEB J* 14: 345–354, 2000.
- Biernacki WA, Kharitonov SA, and Barnes PJ. Increased leukotriene B₄ and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax* 58: 294–298, 2003.
- Bigby TD, Lee DM, Meslier N, and Gruenert DC. Leukotriene A₄ hydrolase activity of human airway epithelial cells. *Biochem Biophys Res Commun* 164: 1–7, 1989.
- Bigby TD, Lee DM, Minami M, Ohishi N, Shimizu T, and Baker JR. Characterization of human airway epithelial cell leukotriene A₄ hydrolase. *Am J Respir Cell Mol Biol* 11: 615–624, 1994.
- Borok Z, Danto SI, Lubman RL, Cao Y, Williams MC, and Crandall ED. Modulation of t1 α expression with alveolar epithelial cell phenotype in vitro. *Am J Physiol Lung Cell Mol Physiol* 275: L155–L164, 1998.
- Brock TG, Maydanski E, McNish RW, and Peters-Golden M. Colocalization of leukotriene A₄ hydrolase with 5-lipoxygenase in nuclei of alveolar macrophages and rat basophilic leukemia cells but not neutrophils. *J Biol Chem* 276: 35071–35077, 2001.
- Brock TG, Paine RI, and Peters-Golden M. Localization of 5-lipoxygenase to the nucleus of unstimulated rat basophilic leukemia cells. *J Biol Chem* 269: 22059–22066, 1994.
- Byrum RS, Goulet JL, Snouwaert JN, Griffiths RJ, and Koller BH. Determination of the contribution of cysteinyl leukotrienes and leukotriene B₄ in acute inflammatory responses using 5-lipoxygenase- and leukotriene A₄ hydrolase-deficient mice. *J Immunol* 163: 6810–6819, 1999.
- Chen X, Li N, Wang S, Wu N, Hong J, Jiao X, Krasna MJ, Beer DG, and Yang CS. Leukotriene A₄ hydrolase in rat and human esophageal adenocarcinomas and inhibitory effects of bestatin. *J Natl Cancer Inst* 95: 1053–1061, 2003.
- Claesson HE and Feinmark SJ. Relationship of cyclic-AMP levels in leukotriene B₄-stimulated leukocytes to lysosomal enzyme release and the generation of superoxide anions. *Biochim Biophys Acta* 804: 52–57, 1984.
- Cunningham FM, Shipley ME, and Smith MJ. Aggregation of rat polymorphonuclear leukocytes in vitro. *J Pharm Pharmacol* 32: 377–380, 1980.
- Dahlen SE, Bjork J, Hedqvist P, Arfors KE, Hammarstrom S, Lindgren JA, and Samuelsson B. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proc Natl Acad Sci USA* 78: 3887–3891, 1981.
- Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, and Wahli W. The PPAR α -leukotriene B₄ pathway to inflammation control. *Nature* 384: 39–43, 1996.
- Dickinson Zimmer JS, Voelker DR, Bernlohr DA, and Murphy RC. Stabilization of leukotriene A₄ by epithelial fatty acid-binding protein in the rat basophilic leukemia cell. *J Biol Chem* 279: 7420–7426, 2004.
- Dixon RAF, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, Gillard JW, and Miller DK. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* 343: 282–284, 1990.
- Dobbs LG, Gonzalez R, and Williams MC. An improved method for isolating type II cells in high yield and purity. *Am Rev Respir Dis* 134: 141–145, 1986.
- Evans JF, Dupuis P, and Ford-Hutchinson AW. Purification and characterisation of leukotriene A₄ hydrolase from rat neutrophils. *Biochim Biophys Acta* 840: 43–50, 1985.
- Feinmark SJ, Lindgren JA, Claesson HE, Malmsten C, and Samuelsson B. Stimulation of human leukocyte degranulation by leukotriene B₄ and its omega-oxidized metabolites. *FEBS Lett* 136: 141–144, 1981.
- Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, and Smith MJH. Leukotriene B₄, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 286: 264–265, 1980.
- Gimbrone MAJ, Brock AF, and Schafer AI. Leukotriene B₄ stimulates polymorphonuclear leukocyte adhesion to cultured vascular endothelial cells. *J Clin Invest* 74: 1552–1555, 1984.
- Goetzl EJ, An S, and Smith WL. Specificity of expression and effects of eicosanoid mediators in normal physiology and human diseases. *FASEB J* 9: 1051–1058, 1995.
- Gompertz S, O'Brien C, Bayley DL, Hill SL, and Stockley RA. Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. *Eur Respir J* 17: 1112–1119, 2001.
- Griffin KJ, Gierse J, Krivi G, and Fitzpatrick FA. Opioid peptides are substrates for the bifunctional enzyme LTA₄ hydrolase/aminopeptidase. *Prostaglandins* 44: 251–257, 1992.
- Grimminger F, von Kurten I, Walmrath D, and Seeger W. Type II alveolar epithelial eicosanoid metabolism: predominance of cyclooxygen-

- ase pathways and transcellular lipoxygenase metabolism in co-culture with neutrophils. *Am J Respir Cell Mol Biol* 6: 9–16, 1992.
26. **Grujic M and Renko M.** Aminopeptidase inhibitors bestatin and actinonin inhibit cell proliferation of myeloma cells predominantly by intracellular interactions. *Cancer Lett* 182: 113–119, 2002.
 27. **Haeggstrom JZ, Wetterholm A, Medina JF, and Samuelsson B.** Leukotriene A₄ hydrolase: structural and functional properties of the active center. *J Lipid Mediators* 6: 1–13, 1993.
 28. **Haeggstrom JZ, Wetterholm A, Vallee BL, and Samuelsson B.** Leukotriene A₄ hydrolase: an epoxide hydrolase with peptidase activity. *Biochem Biophys Res Commun* 173: 431–437, 1990.
 29. **Hankin JA, Jones DN, and Murphy RC.** Covalent binding of leukotriene A₄ to DNA and RNA. *Chem Res Toxicol* 16: 551–561, 2003.
 30. **Heilmann I, Pidkowich MS, Girke T, and Shanklin J.** Switching desaturase enzyme specificity by alternate subcellular targeting. *Proc Natl Acad Sci USA* 101: 10266–10271, 2004.
 31. **Henderson WJ.** The role of leukotrienes in inflammation. *Ann Intern Med* 121: 684–697, 1994.
 32. **Hofer D, Shin DW, and Drenckhahn D.** Identification of cytoskeletal markers for the different microvilli and cell types of the rat vomeronasal sensory epithelium. *J Neurocytol* 29: 147–156, 2000.
 33. **Lam BK, Gagnon L, Austen KF, and Soberman RJ.** The mechanism of leukotriene B₄ export from human polymorphonuclear leukocytes. *J Biol Chem* 265: 13438–13441, 1990.
 34. **Lawrence JW, Kroll DJ, and Eacho PI.** Ligand-dependent interaction of hepatic fatty acid-binding protein with the nucleus. *J Lipid Res* 41: 1390–1401, 2000.
 35. **Lewis RA, Austen KF, and Soberman RJ.** Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human disease. *N Engl J Med* 323: 645–655, 1990.
 36. **Lieber M, Smith B, Szakal A, Nelson-Rees W, and Todaro G.** A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 17: 62–70, 1976.
 37. **Luo M, Lee S, and Brock TG.** Leukotriene synthesis by epithelial cells. *Histol Histopathol* 18: 587–595, 2003.
 38. **Mancuso P, Nana-Sinkam P, and Peters-Golden M.** Leukotriene B₄ augments neutrophil phagocytosis of *Klebsiella pneumoniae*. *Infect Immun* 69: 2011–2016, 2001.
 39. **Minami M, Ohishi N, Mutoh H, Izumi T, Bito H, Wada H, Seyama Y, Toh H, and Shimizu T.** Leukotriene A₄ hydrolase is a zinc-containing aminopeptidase. *Biochem Biophys Res Commun* 173: 620–626, 1990.
 40. **Murata M, Kubota Y, Tanaka T, Iida-Tanaka K, Takahara J, and Irino S.** Effect of ubenimex on the proliferation and differentiation of U937 human histiocytic lymphoma cells. *Leukemia* 8: 2188–2193, 1994.
 41. **Nissen JB, Iversen L, and Kragballe K.** Characterization of the aminopeptidase activity of epidermal leukotriene A₄ hydrolase against the opioid dynorphin fragment 1–7. *Br J Dermatol* 133: 742–749, 1995.
 42. **Orning L and Fitzpatrick FA.** Albumins activate peptide hydrolysis by the bifunctional enzyme LTA₄ hydrolase/aminopeptidase. *Biochemistry* 31: 4218–4223, 1992.
 43. **Orning L, Gierse JK, and Fitzpatrick FA.** The bifunctional enzyme leukotriene-A₄ hydrolase is an arginine aminopeptidase of high efficiency and specificity. *J Biol Chem* 269: 11269–11273, 1994.
 44. **Orning L, Krivi G, and Fitzpatrick FA.** Leukotriene A₄ hydrolase. Inhibition by bestatin and intrinsic aminopeptidase activity establish its functional resemblance to metallohydrolase enzymes. *J Biol Chem* 266: 1375–1378, 1991.
 45. **Paine R III and Simon RH.** Expanding the frontiers of lung biology through the creative use of alveolar epithelial cells in culture. *Am J Physiol Lung Cell Mol Physiol* 270: L484–L486, 1996.
 46. **Park H, Park ES, Lee HS, Yun HY, Kwon NS, and Baek KJ.** Distinct characteristic of Gα(h) (transglutaminase II) by compartment: GTPase and transglutaminase activities. *Biochem Biophys Res Commun* 284: 496–500, 2001.
 47. **Peters-Golden M, Bailie M, Marshall T, Wilke C, Phan SH, Toews GB, and Moore BB.** Protection from pulmonary fibrosis in leukotriene-deficient mice. *Am J Respir Crit Care Med* 165: 229–235, 2002.
 48. **Peters-Golden M and Feysa A.** Transcellular eicosanoid synthesis in cocultures of alveolar epithelial cells and macrophages. *Am J Physiol Lung Cell Mol Physiol* 264: L438–L447, 1993.
 49. **Radmark O, Shimizu T, Jornvall H, and Samuelsson B.** Leukotriene A₄ hydrolase in human leukocytes. Purification and properties. *J Biol Chem* 259: 12339–12345, 1984.
 50. **Selman M, King TE, and Pardo A.** Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 134: 136–151, 2001.
 51. **Shannon J, Mason R, and Jennings S.** Functional differentiation of alveolar type II epithelial cells in vitro: Effects of cell shape, cell-matrix and cell-cell interactions. *Biochim Biophys Acta* 931: 143–156, 1987.
 52. **Thielitz A, Bukowska A, Wolke C, Vetter R, Lendeckel U, Wrenger S, Hashimoto Y, Ansoorge S, Gollnick H, and Reinhold D.** Identification of extra- and intracellular alanyl aminopeptidases as new targets to modulate keratinocyte growth and differentiation. *Biochem Biophys Res Commun* 321: 795–801, 2004.
 53. **Tonnesen M, Anderson D, Springer T, Knedler A, Avdi N, and Henson P.** Adherence of neutrophils to cultured human microvascular endothelial cells. Stimulation by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150,95 glycoprotein family. *J Clin Invest* 83: 637–46, 1989.
 54. **Wakabayashi Y, Hashimoto M, Saitoh K, Osawa H, Koike M, and Hirose S.** Effects of bestatin (Ubenimex) on human T-cell colony formation. *Anticancer Drugs* 2: 39–43, 1991.
 55. **Wardlaw A, Hay H, Cromwell O, Collins J, and Kay A.** Leukotrienes, LTC₄ and LTB₄, in bronchoalveolar lavage in bronchial asthma and other respiratory diseases. *J Allergy Clin Immunol* 84: 19–26, 1989.
 56. **Wilborn J, Bailie M, Coffey M, Burdick M, Strieter R, and Peters-Golden M.** Constitutive activation of 5-lipoxygenase in the lungs of patients with idiopathic pulmonary fibrosis. *J Clin Invest* 97: 1827–1836, 1996.
 57. **Wilborn J, Crofford LJ, Burdick MD, Kunkel SL, Strieter RM, and Peters-Golden M.** Fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E₂ and to express cyclooxygenase-2. *J Clin Invest* 95: 1861–1868, 1995.
 58. **Wolfrum C, Borrmann CM, Borchers T, and Spener F.** Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors α- and γ-mediated gene expression via liver fatty acid binding protein: a signaling path to the nucleus. *Proc Natl Acad Sci USA* 98: 2323–2328, 2001.
 59. **Zagon IS, Ruth TB, Leure-duPree AE, Sassani JW, and McLaughlin PJ.** Immunoelectron microscopic localization of the opioid growth factor receptor (OGFr) and OGF in the cornea. *Brain Res* 967: 37–47, 2003.
 60. **Zagon IS, Sassani JW, and McLaughlin PJ.** Opioid growth factor modulates corneal epithelial outgrowth in tissue culture. *Am J Physiol Regul Integr Comp Physiol* 268: R942–R950, 1995.
 61. **Zhou S, Stark JM, and Leikauf GD.** Leukotriene B₄ formation: human neutrophil-airway epithelial cell interactions. *J Appl Physiol* 78: 1396–1403, 1995.