Determination of leukotriene A_4 stabilization by S100A8/A9 proteins using mass spectrometry

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Abstract **Leukotriene A_4 (LTA_4) is the precursor for the formation of bioactive leukotrienes, but is highly susceptible to nonenzymatic hydrolysis. Although it is chemically reactive, LTA_4 participates in the process of transcellular metabolism, which requires the transfer of LTA_4 from one cell to another for the production of additional leukotrienes. Due to the susceptibility of LTA_4 to hydrolysis, various methods have been used to measure the half-life of LTA_4 in the presence of different proteins in efforts to understand how it is transported between cells. In this work, a new liquid chromatography mass spectrometry technique was developed to improve upon these previous assays that analyzed LTA_4 directly. The new technique derivatizes LTA_4 to stable compounds for analysis and removes the potential for sample decomposition between analytical runs. This assay was used in measuring the capabilities of the S100A8/A9 protein complex isolated from human neutrophils to stabilize LTA_4.** **It was determined that the S100A8/A9 protein complex protects LTA_4 from hydrolysis in a Ca^{2+} dependent manner and increases LTA_4 half-life to in excess of 35 and 5 min at 4°C and 37°C, respectively.**—Rector, C. L., and R. C. Murphy. **Determination of leukotriene A_4 stabilization by S100A8/A9 proteins using mass spectrometry.** ***J. Lipid Res.*** **2009.** 50: **2064–2071.**

Supplementary key words transcellular metabolism • leukotriene biosynthesis • S100 protein

Leukotrienes are a class of biologically active metabolites from arachidonic acid that play a role in various physiological processes. These compounds are biosynthesized within cells via a 5-lipoxygenase (5-LO) enzymatic pathway (**Scheme 1**) (1–3). Leukotriene biosynthesis is initiated by the activation of cytosolic phospholipase A_2, which in turn releases arachidonic acid from the *sn*-2 position of membrane phospholipids (4, 5). The free arachidonic acid is then presented to 5-LO by 5-LO activating protein, FLAP (6, 7). Within 5-LO, molecular oxygen is added to carbon-5 of arachidonic acid through a stereospecific free radical process to produce 5*S*-hydroperoxy-eicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5-HPETE) (8). 5-LO can then transform 5-HPETE to leukotriene A_4 (LTA_4), which possesses a reactive conjugated triene epoxide moiety.

Individual cells can also possess two additional enzymes to which LTA_4 is a substrate for the formation of additional leukotrienes. LTA_4 hydrolase converts LTA_4 to leukotriene B_4 (LTB_4) by the addition of water (9). LTC_4 synthase utilizes LTA_4 to produce leukotriene C_4 (LTC_4) by addition of glutathione to C-6 of the LTA_4 epoxide (10). Although LTA_4 is the precursor to other leukotrienes, it still remains a chemically reactive molecule. The conjugated triene epoxide renders LTA_4 extremely susceptible to degradation to Δ^6-*trans*-LTB_4s or 5,6-dihydroxyeicosatetraenoic acids (diHETE) via nonenzymatic hydrolysis (11). At pH 7.4, the half-life of LTA_4 has been measured at less than 3s at 37°C (12).

Whereas some cells contain the entire enzymatic cascade to produce leukotrienes from phospholipids, many cells are only capable of producing leukotrienes if provided the precursors from neighboring cells by a process termed transcellular biosynthesis (13). For example, erythrocytes contain LTA_4 hydrolase, but lack 5-LO to produce LTA_4 (14). When provided with exogenous LTA_4, these cells readily produce LTB_4. Endothelial cells and platelets, which contain LTC_4 synthase but no 5-LO, convert LTA_4 to LTC_4 (15–17). Studies have shown that in human neutrophils, greater than 50% of LTA_4 generated after cellular

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Abbreviations: CID, collision induced dissociation; diHETE, dihydroxyeicosatetraenoic acid; 5,6-(EtO,OH)-ETE, 5,6-ethoxy-hydroxyeicosatetraenoic acid; FPLC, fast-protein liquid chromatography; 5-HPETE, 5*S*-hydroperoxy-eicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid; 5-LO, 5-lipoxygenase; LTA_4, 5*S*-*trans*-5,6-oxido-eicosa-7*E*,9*E*,11*Z*,14*Z*-tetraenoic acid, leukotriene A_4; LTB_4, 5*S*, 12*R*-dihydroxy-eicosa-6*Z*,8*E*,10*E*,14*Z*-tetraenoic acid, leukotriene B_4; LTC_4, 5*S*-hydroxy, 6*R*-(*S*-glutathionyl)-eicosa-7*E*,9*E*,11*Z*,14*Z*-tetraenoic acid, leukotriene C_4; 5-OH, 12-EtO-LTB_4, 5-hydroxy, 12-ethoxy-Δ^6-*trans*-LTB_4; 5-oxoETE, 5-oxo-eicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid; PMN, polymorphonuclear leukocyte; RP-HPLC, reversed phase high performance liquid chromatography; RP/LC/MS, reversed phase liquid chromatography mass spectrometry; SPE, solid phase extraction.

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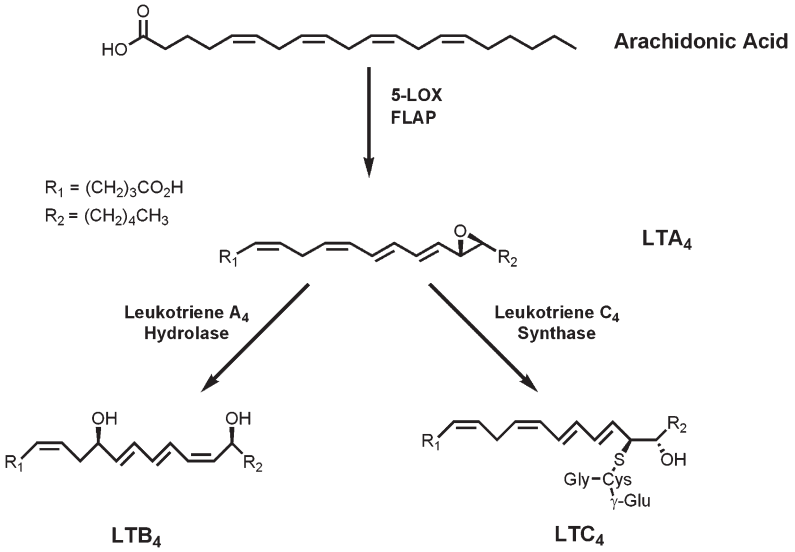


Scheme 1. Leukotriene synthesis pathway.

stimulation is released from the neutrophils to participate in transcellular metabolism (18). The susceptibility of LTA_4 to nonenzymatic hydrolysis indicates that some type of protection must be afforded to LTA_4 during its transport following synthesis for the transcellular processes.

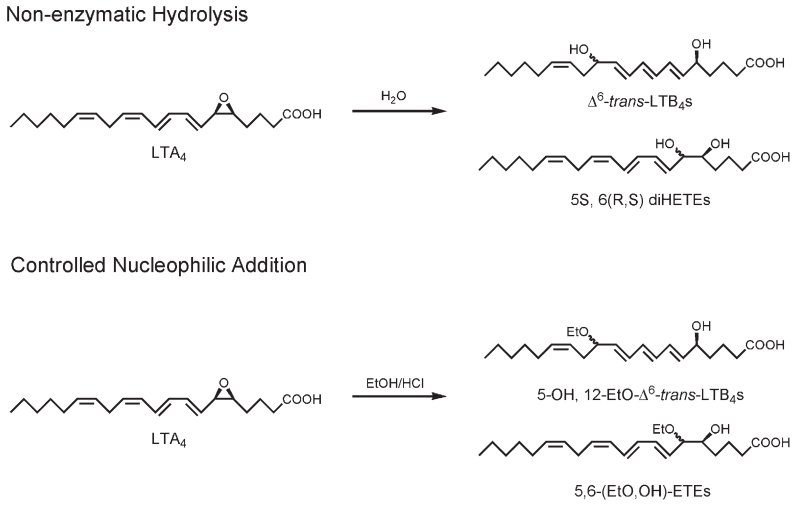
Previous work has focused on assessing the ability of various proteins to stabilize LTA_4 against nonenzymatic hydrolysis. Serum albumin increases the half-life of LTA_4 to more than 20 min at 25°C at protein concentrations found in plasma (12). Fatty acid binding proteins were later studied and found to also increase LTA_4 half-life (19, 20). However, neither of these proteins is found in large concentrations within neutrophils. The LTA_4 stabilizing protein in neutrophils is currently unknown, yet neutrophils are a major source of LTA_4 in transcellular production of leukotrienes. A possible protein of interest in neutrophils is the S100A8/A9 complex, which is a major arachidonate binding protein (21).

The LTA_4 half-life measurements in the previous studies were performed by measuring LTA_4 directly by reversed phase HPLC (RP-HPLC) with UV spectroscopy utilizing the unique chromophore of the triene epoxide. The possibility remained that the LTA_4 could still undergo nonenzymatic hydrolysis despite the use of alkaline solvent conditions. In this report, a new method of measuring LTA_4 half-life is described that measured chemically stable ethoxy derivatives of Δ^6-*trans*-LTB_4 and 5,6-diHETE using reversed phase liquid chromatography mass spectrometry (RP-LC/MS). In addition, the stabilization ability of the S100A8/A9 protein complex, which is found in large quantities within neutrophils, was analyzed.

EXPERIMENTAL PROCEDURES

LTA_4 half-life assay

For determination of LTA_4 stabilization, a solution (200 μl) was made with the free acid of LTA_4 (5 μM final concentration), 1.7 μM protein or stabilizing agent in 0.1 M phosphate buffer, pH 7.4. LTA_4 free acid was produced by hydrolysis of LTA_4 methyl ester using a 4:1 acetone:NaOH mixture as previously described (22). For conditions with Ca^{2+}, a final concentration of 60 μM Ca^{2+} was present from the addition of a stock $CaCl_2$ solution. At various times, 25 μl aliquots were removed from the larger solution and the nonenzymatic hydrolyses were stopped by addition of 25 μl of



Scheme 2. Formation of stable products by nucleophilic addition to leukotriene A_4.

1M HCl in absolute ethanol. The reaction mixture was allowed to stand for 1 min, then adjusted to approximately pH 3 by addition of 1M NaOH, and finally diluted to a total volume of 150 μl with water. An internal standard of 5-oxo-eicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5-oxoETE), (20 ng), was then added. The individual samples were then prepared to remove proteins with solid phase extraction (SPE) cartridges (Strata C18-E, 100 mg/1ml; Phenomenex, Torrance, CA). The SPE cartridges were conditioned by running 1 ml of methanol and then 1 ml of water through each cartridge. After the samples were added to the cartridges, 2 ml of water was run through the columns before 1 ml of methanol was eluted and collected. The methanol was removed in vacu, and then 500 μl of CH_2Cl_2 was added to each sample, which usually contained residual water. The CH_2Cl_2 layer was removed from the extractions and dried down under a N_2 stream. The ethoxy derivatives from LTA_4 were then dissolved in a 100 μl of methanol:water (1:1) for LC/MS/MS analysis.

Mass spectrometric analysis of ethoxy, hydroxy derivatives and determination of LTA_4 half-life

An aliquot of each sample (80 μl) was injected into a RP-HPLC system, and the 5-hydroxy, 12-ethoxy-Δ^6-*trans*-LTB_4s (5-OH, 12-EtO-LTB_4s) and the 5,6 ethoxy, hydroxyeicosatetraenoic acids [5,6-(EtO,OH)-ETEs] from LTA_4 were separated using a C18 column (Gemini, 2 × 150 mm, 5 μm; Phenomenex) eluted at a flow rate of 200 μl/min with a solvent system composition starting as 75% aqueous ammonium acetate (1 mM, pH 5.7, solvent A) and 25% methanol also 1 mM in ammonium acetate (solvent B). The starting solvent composition was held for 2 min after injection and then programmed to 90% solvent B in 6 min where it was held for 5 min prior to recycling to 25% solvent B. The 5-OH, 12-EtO-LTB_4s and 5,6-(EtO,OH)-ETEs were monitored using the collision induced dissociation (CID) spectra *m/z* 363.4→317.2 and 5-oxoETE was followed using *m/z* 317.2→203.2. For determination of the half-life of LTA_4 in the presence of different stabilizing agents, the peak area ratio (5-OH, 12-EtO-LTB_4s:5-oxoETE was determined from LC/MS/MS chromatograms at the various time points. The natural logarithm of the peak area ratios were then plotted versus time. The rate of decay was determined as the slope of the linear regression of a logarithmic plot, which in turn was used to determine the half-life from the standard half-life equation: LTA_4 half-life = ln 2 /-(rate of decay).

Neutrophil cytosol and S100 proteins isolation

Human polymorphonuclear leukocytes (PMNs) were isolated from peripheral blood of healthy volunteers as described previously (23). Isolated PMNs were taken up in homogenization buffer (1.7×10^8 cells/ml). Homogenization buffer was prepared with 1 Complete mini tablet (Roche Applied Sciences, Indianapolis, IN) in 7 ml 0.2 M phosphate buffer with 0.1 M NaCl, pH 7.4. The ice-cooled cells were disrupted using a Sonics Vibracell VCX 600 sonicator (Newtown, CT) at 24% amplitude (4 × 15s). The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was then isolated and further centrifuged at 100,000 g for 1 h at 4°C. This supernatant was collected and used as the neutrophil cytosolic fraction. S100 proteins were isolated by purifying the cytosol fractions using fast-protein liquid chromatography (FPLC) with a Superdex 75 column (GE Healthcare, Piscataway, NJ). The elution solvent was 0.01 M PBS with 0.2 M NaCl, pH 7.4 at a flow rate of 1 ml/min.

Analysis of S100 proteins by Coomassie staining and immunostaining on Western blots

Cytosol fractions or purified S100s were separated by SDS-PAGE gels (4-20% acrylamide gradient). For denatured gels, the samples contained 30 mM DTT and were heated at 95°C for 5 min. Coomassie staining was performed with Gel Code Blue stain (Pierce, Rockford, IL) following the directions from the manufacturer.

For Western blotting, the proteins were electrolytically transferred to a membrane with a mixture of 25 mM Tris buffer, 192 mM glycine and 0.1% SDS and 20% methanol. Nonspecific binding of antibodies was blocked by washing the membrane with 5% powdered milk in water for 30 min. The membrane was then incubated with rabbit anti-S100A8 (calgranulin A) or anti-S100A9 (calgranulin B) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) at 1:1000 dilution overnight in 0.01 M PBS buffer, pH 7.4 containing 5% BSA at 4°C. After several washes in 20 mM Tris buffer with 0.5 M NaCl, 0.05% Tween 20 (3 × 15 min), the bound antibodies were detected by rinsing the membrane with HRP conjugated donkey anti-rabbit IgG antibody (Affinity Bioreagents, Golden, CO) at 1:2500 in Tris buffer for 1 h. The membrane was then again washed 3 × 15 min with Tris buffer, 0.05% Tween 20. HRP labeled proteins were then detected using a Pierce ECL Western blotting kit.

RESULTS

Nucleophillic addition to LTA_4

The susceptibility of LTA_4 to nonenzymatic hydrolysis is due to the epoxide conjugated triene moiety. In aqueous conditions, the epoxide moiety can be protonated, which in turn leads to the opening of the epoxide to form a delocalized carbocation to which water can add to form stable 5,12 diHETEs and 5,6 diHETEs (11). The hydrolysis reaction is a typical nucleophillic addition of water. To form unique stable products at designated time points, a more reactive nucleophile was added to the reaction mixtures. The addition of 1M HCl in absolute ethanol provided another nucleophile to react with LTA_4. The acidic conditions caused the rapid opening of the epoxide, which allowed the addition of ethanol to form unique ethoxy products that are similar to the nonenzymatic hydrolysis products of LTA_4. These ethoxy products are chemically stable like the nonenzymatic products of LTA_4 hydrolysis and therefore remove the chemical liability inherent in LTA_4 during analysis.

The ethoxy derivatives were then analyzed by LC/MS and LC/MS/MS to determine whether the acidic ethanol that added to LTA_4 formed products truly similar to those produced through nonenzymatic hydrolysis. The ethoxy derivatives were separated using RP-HPLC into two distinct chromatogram peaks. LC/MS of both chromatogram peaks generated $[M\text{-}H]^-$ carboxylate anions at *m/z* 363.3. The CID of the $[M\text{-}H]^-$ ion of the less lipophilic peak in the RP-HPLC produced the spectrum shown in **Fig. 1A**. The CID of *m/z* 363.3 resulted in product ions of *m/z* 345 and a prominent *m/z* 317 that correspond to losses of water and ethanol, respectively, from the parent carboxylate ion. Additional product ions were seen at *m/z* 255, *m/z* 273, and *m/z* 299 that result from the additional neutral loss combinations of water, ethanol, and carbon dioxide from the parent carboxylate ion. The major fragment ion produced from the carboxylate ion was *m/z* 203. A similar minor product was seen in spectra analyses of 5,12 diHETE that

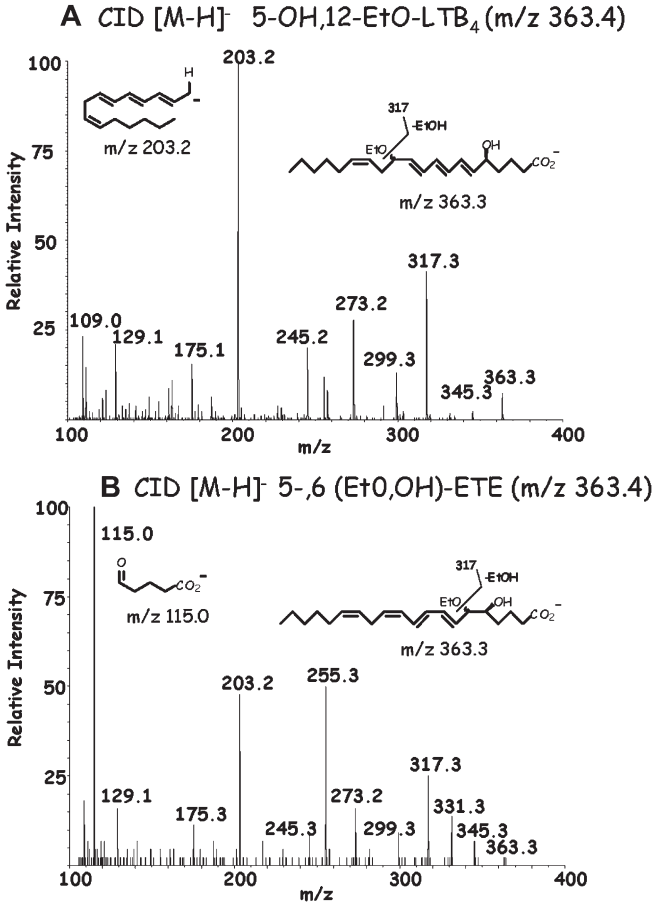


Fig. 1. Collision induced dissociation of the carboxylate anion $[M\text{-}H]^-$ of ethoxy derivatives of leukotriene B_4 from nucleophilic addition of acidic ethanol to leukotriene A_4. A: 5-OH, 12-EtO-LTB_4s. B: 5,6-(EtO,OH)-ETEs.

arose from the dehydration involving the C-12 hydroxyl group to form the 5-oxoETE, then cleavage of the C-5,C-6 bond with loss of a neutral ketene (24).

A second more lipophilic peak was seen in the RP-HPLC chromatogram of the ethoxy derivatives also with a $[M\text{-}H]^-$ ion of *m/z* 363.3. The CID of this peak is shown in Fig. 1B. The CID of the precursor ion *m/z* 363.3 resulted in the formation of many of the same product ions seen in the less lipophilic peak described above. Distinct peaks of *m/z* 345 and *m/z* 317 were formed from the neutral loss of water and ethanol. In addition, product ions of *m/z* 255, *m/z* 273, and *m/z* 299 were again seen that are the result of various neutral loss combinations of water, ethanol, and carbon dioxide from the parent carboxylate ion. The major *m/z* 203 fragment was also seen in this peak; however, it was not the major fragment ion. The predominant product ion was *m/z* 115. In LC/MS/MS scans of 5,6-diHETEs, the major fragmentation ion was also *m/z* 115 that comes from the cleavage of the C-5,C-6 carbon bond with transfer of the hydrogen atom from the 5-hydroxy group to C-8 (24). Based on the fragmentation patterns from the two ethoxy derivative peaks, the two derivatives separated in the same manner as the nonenzymatic hydrolysis peaks of LTA_4. The less lipophilic peak was the 5-OH, 12-EtO-LTB_4s and the more lipophilic peak was the 5,6-(EtO,OH)-ETEs.

Validation of the new assay

In order to validate the new derivatization assay, the ability of previously studied agents to stabilize LTA_4 was measured and then compared with previously reported values. The first set of studies involved a 50% ethanol mixture in 0.1 M phosphate buffer at pH 7.4. Previous work had shown that the high organic composition of this mixture increased the half-life of LTA_4 to approximately 70 min at 4°C when measuring the LTA_4 directly by UV spectroscopy (12).

In this study, LTA_4 was placed in the 50% aqueous ethanol mixture and aliquots were removed at corresponding time points (**Figure 2**). Acidic ethanol was immediately added to the aliquots to form ethoxy derivatives from the opening of the epoxide. After neutralizing the samples with NaOH, an internal standard of 5-oxoETE was added to each sample. The samples were then analyzed by RP-LC/MS/MS while monitoring for the unique transitions *m/z* 363.3 → *m/z* 317.2 for the 5-OH, 12-EtO-LTB_4s and

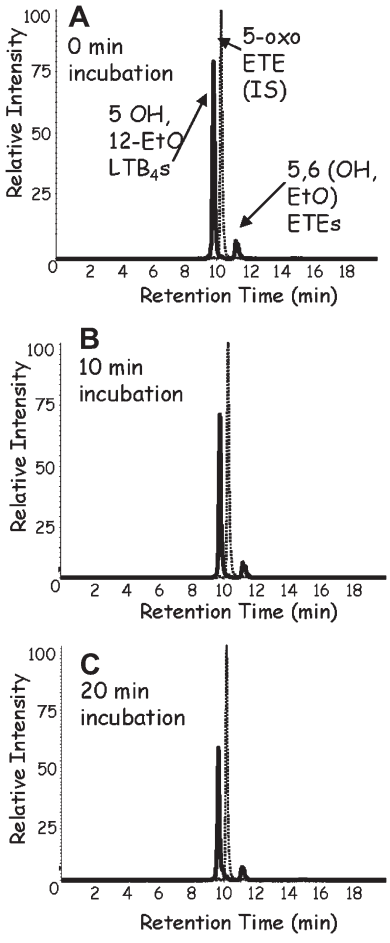


Fig. 2. Tandem LC-MS assay of LTA_4 stabilization by using ethanol:water (1:1) at 4°C. LC/MS/MS chromatograms are shown for time points taken at intervals of (A) 0 min, (B) 10 min, and (C) 20 min. Collision induced dissociations monitored during analysis: (solid line) 5-OH, 12-EtO-LTB_4s and 5,6-(EtO,OH)-ETEs *m/z* 363.4 → 317.2 (broken line) 5-oxoETE *m/z* 317.2 → 203.2.

5,6-(EtO,OH)-ETEs and m/z 317.2 → m/z 203.2 for 5-oxo-ETE. The LC/MS/MS chromatograms from monitoring the half-life of LTA_4 in the presence of 50% aqueous ethanol revealed two peaks for the 5-OH, 12-EtO-LTB_4s and 5,6-(EtO,OH)-ETEs (shown as the solid lines and the internal standard shown as the dotted line in Fig. 2). The peak area from the 5-OH, 12-EtO-LTB_4s ion transition was then ratioed to that from the 5-oxoETE ion transition. The natural logarithm of ion transition for the 5,OH,12-EtO-LTB_4s was then plotted versus time to determine the rate of LTA_4 decay via hydrolysis (**Fig. 3**). Using this technique, the half-life of LTA_4 in the presence of 50% aqueous ethanol was determined to be 68 ± 4 min (SEM, n = 6) whereas the measured half-life in PBS under the assay conditions employed was 5 ± 1 min (Fig. 3). In separate experiments, the signal for the 5-OH, 12-EtO-LTB_4s (m/z 363.3 → 317.2) as well as the signal from the more abundant product ion (m/z 363.3 → 203) were used to define the half-life of LTA_4 and compared. As expected, the signal (and corresponding response factor) for the later transition was higher, but the calculated half-life of LTA_4 was the same within experimental error (± 4 min). Furthermore, using the less abundant hydrolysis product 5,6-(EtO,OH)-ETE observed at the longer retention time yielded the same estimated half-life (± 1 min) for LTA_4 stabilized by ethanol.

The new assay was then used to measure the stabilization of LTA_4 in the presence of a protein, specifically BSA. Previous work had shown the capability of 5 mg/ml human albumin to increase the half-life of LTA_4 at 25°C to approximately 19 ± 2.5 min (12). In this study, LTA_4 was added to a 5 mg/ml mixture of BSA in 0.1 M PBS buffer, pH 7.4. At various intervals, aliquots were removed and the remaining LTA_4 was derivatized with acidic ethanol. LC/MS/MS analysis was performed as described above to determine the half-life in the presence of BSA. With the new assay, 5 mg/ml BSA was determined to increase the half-life of LTA_4 to 18 ± 3 min at 25°C (SEM, n = 6) (Data not shown). The half-lives measured with this new assay were similar to the values measured using UV absorbance to monitor LTA_4 directly.

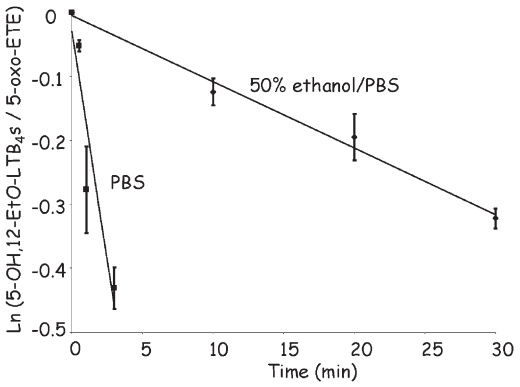


Fig. 3. LTA_4 stabilization by 50% ethanol in 0.1 M phosphate buffer, pH 7.4 (◆) or 0.1 M PBS buffer, pH 7.4 (■) measured by LC/MS/MS. Linear regression of the natural logarithm of the ratio of the measured abundance of 5-OH, 12-EtO-LTB_4s (m/z 363.3 → 317.2) to 5-oxo-ETE abundance (m/z 317.2 → 203.2) during the time course of the LTA_4 stability study (SEM, n = 6 for 50% ethanol and n = 3 for PBS).

LTA_4 stabilization by S100A8/A9 protein complex isolated from PMNs

The new LC/MS/MS assay was then used to measure the ability of the S100A8/A9 protein complex isolated from PMNs to stabilize LTA_4. Previous reports indicated that the S100A8/A9 complex is the major fatty acid binding protein within neutrophils (21). The protein complex has been shown to have a high affinity for arachidonic acid (25). In this work, the ability of the S100 complex to increase the half-life of LTA_4 is measured in the same manner as the work performed with BSA as the stabilizing protein.

First, the S100A8/A9 protein complex had to be isolated from human PMNs. Approximately 200 × 10^6 PMNs were obtained from a blood draw for the protein isolations. After disrupting the cells, a series of centrifugations were used to isolate a solution of the cytosolic proteins. The proteins were then separated by FPLC size exclusion chromatography using an isocratic mobile phase while collecting fractions every 2 min. The fractions were then analyzed by immunoassays to determine which fractions contained the S100A8 and S100A9 proteins. Individual Western blots were performed using antibodies for S100A8 and S100A9 (**Fig. 4A**). From the analysis, it was obvious the S100 proteins were found primarily in two isolated fractions from the protein separation. The purity of the protein in the fractions was assessed by Coomassie gel staining of the FPLC fractions 7 and 8 that were shown to contain the S100 proteins by Western blotting (Fig. 4B). From the gel, the individual monomers of S100A8 and S100A9 were visible as distinct bands. The S100A8 monomer had a

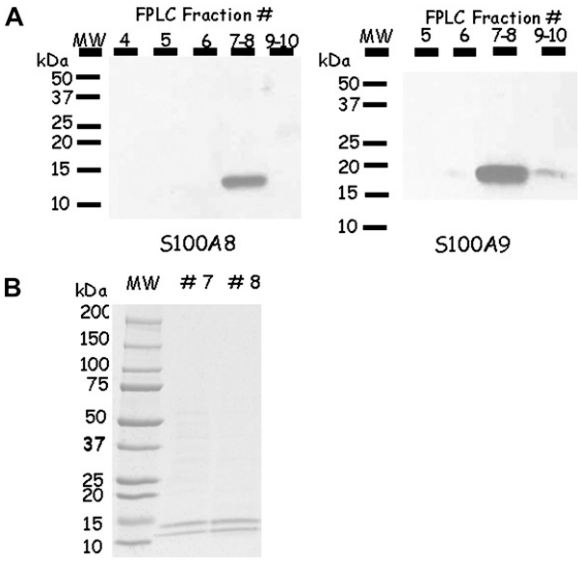


Fig. 4. A: Western blot for S100A8 and S100A9 proteins in the isolated FPLC fractions of human PMN cytosol. FPLC fractions collected using 0.1 M PBS w/ 0.2 M NaCl at 1 ml/min. Proteins separated using 4 → 20% Tris-HCl gels. B: Coomassie gel staining of FPLC fractions 7 and 8. Proteins separated using 4 → 20% Tris-HCl gel.

molecular weight of 10.8 kDa and the S100A9 monomer a mass of 13.2 kDa (26). The reducing conditions of the gel and presence of DTT caused the separation of the heterodimeric S100A8/A9 complex typically found within unstimulated PMNs.

With the protein complex isolated, the half-life of LTA_4 in the presence of various concentrations of the S100A8/A9 complex was measured using the LC/MS/MS assay. The ability of the S100A8/A9 complex to bind arachidonic acid is known to be a Ca^{2+} dependent process (27). The S100A8/A9 heterodimer is the normal state of S100A8/A9 proteins in nonstimulated PMNs and is unable to bind fatty acids (25). However, in the presence of Ca^{2+}, two S100A8/A9 heterodimers associate to form a heterotetrameric complex that possesses fatty acid binding abilities. In this study, the half-life of LTA_4 was measured with both Ca^{2+} present and absent from the protein mixtures. Because the S100A8/A9 proteins used in this study were isolated from the cytosol of nonstimulated human neutrophils, little endogenous arachidonic acid would be present in the partially purified protein to compete with added LTA_4 due to the poor fatty acid binding properties of the S100A8/A9 proteins in the presence of low Ca^{2+} ion concentrations (25) and the low levels of free arachidonate present in the neutrophil cytosol (23). For the experiments with Ca^{2+}, the isolated S100 proteins were incubated with Ca^{2+} for 30 min prior to the addition of LTA_4 to allow for the formation of the heterotetrameric complex. Following the incubation, LTA_4 was added to the protein mixture for half-life studies at 4°C and 37°C. At progressing times, aliquots were removed and reacted with acidic ethanol to form ethoxy derivatives in the same manner as BSA described earlier. The LC/MS/MS analyses of the ethoxy derivatives demonstrated the stabilizing effect of the S100A8/A9 protein complex toward LTA_4, particularly in the presence of Ca^{2+} (**Fig. 5**).

The half-life of LTA_4 was measured at both 4°C and the more biologically relevant 37°C. The S100A8/A9 complex showed a concentration and Ca^{2+} dependent stabilization of LTA_4. At 4°C, LTA_4 (5 μM) had a half-life of 5 min in PBS buffer with no stabilizing proteins. The inclusion of S100 proteins at concentrations of 2.6 μM and 1.7 μM in the absence of Ca^{2+} demonstrated minimum stabilization of LTA_4 (5 μM) in comparison to buffer alone. A 40 μg/ml mixture of the isolated cytosolic proteins, which contained approximately 40–45% S100 proteins, also showed little stabilization of LTA_4 in the absence of Ca^{2+}. However, a significant increase in the ability of the S100A8/A9 protein to stabilize LTA_4 was seen with the addition of an excess of Ca^{2+} to the reaction mixtures. In a concentration-dependent manner, a S100A8/A9 mixture at a concentration of 2.6 μM and 1.7 μM increased the half-life of LTA_4 (5 μM) to 37 min and 22 min, respectively, showing the dependency of the binding protein concentration on LTA_4 stabilization as observed previously for fatty acid binding proteins (19). Even the isolated 40 μg/ml cytosolic protein mixture increased the LTA_4 half-life to 13 min (Fig. 5A). In protein stabilization studies, it was likely that only a fraction of the added LTA_4 was stabilized by binding (saturating concentrations of S100 proteins were not employed) and therefore, unbound LTA_4 would be immediately hydrolyzed (Fig. 3) and only bound LTA_4 would be hydrolyzed based on rate of leaving the protein complex.

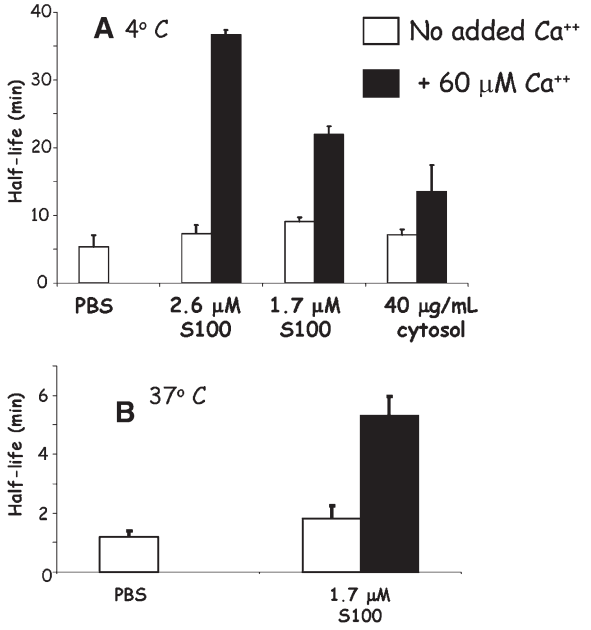


Fig. 5. Measured leukotriene A_4 half-life in the presence of S100A8/A9 proteins isolated from human PMNs in the absence (striped bars) and presence (solid bars) of 60 μM $CaCl_2$ and starting with a 5 μM LTA_4 solution. A: LTA_4 half-lives measured at 4°C (SEM, n = 3). B: LTA_4 half-lives measured at 37°C (SEM, n = 6).

The LTA_4 stabilization was observed not only at lower temperatures. If the reaction mixtures were heated to 37°C, the half-life of LTA_4 was considerably shorter. In the absence of Ca^{2+}, the half-life of LTA_4 in PBS buffer and 1.7 μM S100A8/A9 protein was measured as approximately 1.5 min. The inclusion of Ca^{2+} with 1.7 μM S100A8/A9 protein increased the half-life of LTA_4 to over 5 min (Fig. 5B). This increase in LTA_4 half-life would be a reasonable amount of time for possible transport of LTA_4 during transcellular metabolism pathways.

DISCUSSION

The detailed mechanism by which leukotriene transcellular metabolism occurs is still undetermined. One of the unresolved questions is how LTA_4 is stabilized long enough for transport between donor and acceptor cells for the production of leukotrienes. Previous studies have shown that LTA_4 is susceptible to nonenzymatic hydrolysis. At 37°C, the half-life of LTA_4 has been reported as approximately 3 s in PBS buffer, pH 7.4 (12). Therefore, it is generally assumed that the reactive molecule is bound to a stabilizing protein. Several studies have focused on identifying these potential stabilizing proteins. Albumin was the first protein identified that was capable of increasing the half-life of LTA_4 to the order of minutes (12). Subsequent work found various fatty acid binding proteins that were also capable of stabilizing LTA_4 (19, 20).

These previous studies relied upon the analysis of the LTA_4 molecule itself using RP-HPLC. The aqueous solvent system was kept sufficiently alkaline to reduce the rate of LTA_4 hydrolysis. However, even under these conditions, hydrolysis of LTA_4 could still occur. The studies of albumin and fatty acid binding proteins relied on unique HPLC retention time and UV spectrophotometry to monitor LTA_4 utilizing the distinct chromophore at 280 nm from the epoxide triene moiety (12, 19, 20). In the study with fatty acid binding proteins, LC/MS/MS analysis was also used to both specifically monitor LTA_4 and to increase the sensitivity of the assay in relation to the UV analysis (20). Although mass spectrometry successfully increased the sensitivity in specific analysis of LTA_4, it still suffered from the potential drawback of additional LTA_4 hydrolysis in the RP-HPLC solvents during runs and in the times awaiting analyses.

In this work, a new assay was developed for measuring the ability of different reagents and proteins to stabilize LTA_4. The new method utilized LC/MS/MS; therefore, it had higher sensitivity and specificity associated with mass spectrometry over absorbance spectroscopy. The advantage of this new technique over the previous mass spectrometric assay was rapid conversion of LTA_4 into chemically stable 5-OH, 12-EtO-LTB_4s and 5,6-(EtO,OH)-ETEs by an acidic ethanol mixture that were then analyzed. Precedence for the derivatization of LTA_4 with an acidic alcohol was used in the initial identification of the structure of LTA_4 (11).

The nucleophilic addition of acidic ethanol opened the epoxide ring of LTA_4 to produce derivatives that were similar to nonenzymatic hydrolysis products. Under RP-HPLC conditions, the 5-OH, 12-EtO-LTB_4s eluted before the 5,6-(EtO,OH)-ETEs, which was the same elution order as the Δ^6-*trans*-LTB_4s and 5,6-diHETEs. LC/MS/MS analysis of the products displayed spectra with similar fragmentation patterns as nonenzymatic 5,12- and 5,6-diHETES (24). Both sets of regioisomers from the ethoxy derivatives and nonenzymatic products had fragment ions resulting from various neutral loss combinations of ethanol, water, and carbon dioxide. The most abundant fragment from the 5,6 products of the derivatization and nonenzymatic hydrolysis were *m/z* 115, which likely resulted from a charge-remote fragmentation of the C-5,C-6 bond. The 5,12 derivatives and hydrolysis products also had similar fragmentation patterns, but the most abundant fragments differed. With Δ^6-*trans*-LTB_4s, the major LC/MS/MS fragment was *m/z* 195 that resulted from a charge-driven fragmentation of the C-11, C-12 bond. The 5-OH, 12-EtO-LTB_4s had a major fragment of *m/z* 203 without any formation of an *m/z* 195. In the CID spectra of Δ^6-*trans*-LTB_4s, a minor *m/z* 203 fragment was previously described (24).

Once the products from the LTA_4 derivatization were identified by RP-LC/MS/MS, the next step was to incorporate the derivatization technique into a LC/MS/MS assay for determining LTA_4 half-lives. The mass fragmentation chosen for monitoring both the 5-OH, 12-EtO-LTB_4s and the 5,6-(EtO,OH)-ETEs were *m/z* 363.3 → *m/z* 317.2 because these were unique ions from the loss of the ethoxy substituent from both the 5,12 and 5,6 products. Although this was not the most predominant fragmentation pathway for either product, it did provide a method to ensure the products measured came only from the LTA_4 derivatization. If greater sensitivity were required in future work, the precursor/product ions *m/z* 363.3 → *m/z* 203.2 and *m/z* 363.3 → *m/z* 115.0 could be monitored simultaneously for the 5-OH, 12-EtO-LTB_4s and 5,6-(EtO,OH)-ETEs, respectively.

The first two systems tested with the new LC/MS/MS assay were two that had been previously used in the initial measurements of LTA_4 half-lives. The LTA_4 half-life in 50% aqueous ethanol was measured at 76 ± 5 min, which corresponded well with the previously measured value of approximately 70 min in the same system (12). The half-life of LTA_4 was measured as 18 ± 3 min in 5 mg/ml BSA at 25°C. This compared well to the value of 19 ± 2.5 min measured previously (12).

Because the results of the new LC/MS/MS assay matched well with previously reported LTA_4 half-lives under different conditions, it was used in an effort to determine the stabilizing protein found within stimulated PMNs. The only fatty acid binding protein that has been reported to be expressed by the human PMNs is the S100A8/A9 complex (21). S100A8/A9 proteins constitute approximately 40–45% of the cytosolic proteins within PMNs (28). Upon stimulation of PMNs and monocytes, the protein complex has been shown to move to the plasma membrane and secreted (29–31). This protein complex is found as a heterodimer in nonactivated PMNs; however, in the presence of Ca^{2+} from stimulated neutrophils, two of the S100A8/A9 heterodimers associate to form a heterotetrameric complex. Studies with [^{3}H]labeled arachidonic acid have demonstrated that only the heterotetrameric complex is capable of binding fatty acids (25). The S100 complex shows a high specificity toward polyunsaturated fatty acids and the greatest affinity for arachidonic acid with a K_d of 0.13 μM.

The S100 proteins were isolated using a simple size exclusion chromatography technique that was shown to provide the proteins with good purity as demonstrated by Coomassie gel staining. Next, the ability of the purified proteins to stabilize LTA_4 was measured. These experiments indicated that the S100A8/A9 complex stabilized LTA_4 in a protein concentration and Ca^{2+} dependent manner. At both 4°C and 37°C, the S100 complex (1.7 μM) provided only slight protection of LTA_4 was compared with 0.1 M PBS, pH 7.4 buffer. However, if the S100 proteins were incubated with Ca^{2+} for 30 min prior to their use in stabilizing LTA_4, a significant increase in the half-life of LTA_4 was seen. This stabilization was shown to be protein concentration dependent as demonstrated with the LTA_4 half-life increases with the two purified S100 samples as well as the more dilute PMN cytosolic mixture at 4°C. Even at the more biologically relevant temperature of 37°C, the S100 proteins were able to increase the half-life of LTA_4 to in excess of 5 min. This seems a reasonable amount of time for LTA_4 to potentially be passed from a donor PMN to an acceptor cell for use in transcellular metabolism.

One matter of concern with the values obtained from the experiments with the S100 proteins was the measured half-life of LTA_4 being approximately 1 min in PBS at 37°C in comparison to the previously reported half-life of approximately 3s under these conditions (12). This original value was extrapolated from a series of experiments with different percentages of aqueous ethanol. Although this original value was only from extrapolation, it is believed the difference in this experiment comes from a difference in the reaction set-up. The LTA_4 that was added to the reaction mixtures was in a (4:1) acetone:0.25 M NaOH mixture. The reaction mixtures used in the experiments described here were only several hundred μl of solution. It is believed that the addition of even the small quantities of the LTA_4 mixture into these mixtures was probably overloading the buffering capacity of the PBS, thereby increasing its pH. The more alkaline buffer then stabilized LTA_4 to some extent. The original experiments with UV spectroscopy used larger quantities of solutions for their experiments so the potential of overrunning the buffering capacity of the solutions was reduced.

This new LC/MS/MS technique is advantageous over previously used methods. The primary advantage of this new assay is the ability to perform the half-life experiments with low quantities of proteins and LTA_4 due to the high sensitivity and selectivity associated with LC/MS/MS. Thereby, it should permit the studies of additional proteins and reagents. The discovery that the highly abundant S100A8/A9 in the human neutrophil is capable of stabilizing LTA_4 suggests that these proteins and formation of their complex may play an important role in transcellular biosynthesis of leukotrienes in vivo, mediated by the human neutrophil.

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