Leukotriene A₄ Hydrolyase

INHIBITION BY BESTATIN AND INTRINSIC AMINOPEPTIDASE ACTIVITY ESTABLISHS ITS FUNCTIONAL RESEMBLANCE TO METALLOHYDROLASE ENZYMES*

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Bestatin, an inhibitor of aminopeptidases, was also a potent inhibitor of leukotriene (LT) A₄ hydrolyase. On isolated enzyme its effects were immediate and reversible with a Kᵣ = 201 ± 95 nM. With erythrocytes it inhibited LTB₄ formation >90% within 10 min; with neutrophils it inhibited LTB₄ formation by only 10% during the same period, increasing to 40% in 2 h. Bestatin inhibited LTA₄ hydrolyase selectively; neither 5-lipoxygenase nor 15-lipoxygenase activity in neutrophil lysates was affected. Purified LTA₄ hydrolyase exhibited an intrinsic aminopeptidase activity, hydrolyzing L-lysine-p-nitroanilide and L-leucine-β-naphthylamide with apparent Kᵣ = 156 μM and 70 μM and Vₘₐₓ = 50 and 215 nmol/min/mg, respectively. Both LTA₄ and bestatin suppressed the intrinsic aminopeptidase activity of LTA₄ hydrolyase with apparent Kᵣ values of 5.3 μM and 172 nM, respectively. Other metallohydrolyase inhibitors tested did not reduce LTA₄ hydrolytic/aminopeptidase activity, with one exception; captopril, an inhibitor of angiotensin-converting enzyme, was as effective as bestatin. The results demonstrate a functional resemblance between LTA₄ hydrolase and certain metallohydrolases, consistent with a molecular resemblance at their putative Zn²⁺-binding sites. The availability of a reversible, chemically stable inhibitor of LTA₄ hydrolyase may facilitate investigations on the role of LTB₄ in inflammation, particularly the process termed transcellular biosynthesis.

Leukotriene (LT) B₄ is a lipid mediator which stimulates adhesion of circulating neutrophils to vascular endothelium (1-4), directs their migration toward sites of inflammation (5), and catalyzes the release of their granule constituents (6). Pharmacological restraint of LTB₄ formation may alleviate some inflammatory symptoms, complementing the actions of other agents which inhibit formation of prostaglandins (7). Efforts to modulate leukotriene formation have focused on LTA₄, the product of the 5-lipoxygenase enzyme (8, 9). Numerous inhibitors of 5-lipoxygenase activity have been described (9-13). In contrast, inhibitors of LTA₄ hydrolysis, the terminal, rate-limiting enzyme for LTB₄ biosynthesis, are uncommon (14-17).

Recently, Malfroy et al. (18) and Valee and Auld (19) drew attention to a homologous sequence of amino acids which occur in LTA₄ hydrolyase and the active site of certain peptidases. The disclosure that the enzymes share the unique "signature" sequence common among Zn²⁺-metallohydrolases (20) prompted us to examine their functional resemblance. We report that LTA₄ hydrolyase contains an intrinsic aminopeptidase activity and that bestatin, an aminopeptidase inhibitor, also inhibits LTA₄ hydrolyase. These results provide a basis for improved characterization of the mechanism of formation and the biological role of LTB₄.

EXPERIMENTAL PROCEDURES

Materials—LTA₄, methyl ester, LTB₄, and prostaglandin B₂ were from Cayman Co.; BSA, bestatin ((2S,3R)-3-amino-2-hydroxy-5-phenylbutanoyl-L-leucine), amastatin ((2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl-L-valine-L-valine-L-aspartic acid), epibestatin ((2R,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine), captopril (CYS-1-(3-mercapto-2-methyl-propionyl)-L-proline), L-leucine hydroxamate, dl-methionine hydroxamate, L-lysine p-nitroanilide, L-leucine β-naphthylamide, α-phenanthroline, bacitracin, glycylglycine, and leucine aminopeptidase from porcine kidney microsomes (aminopeptidase M, EC 3.4.11.2) were from Sigma; and phosphoramidon (N³-phenylmalonylamidophenylalanine), and dl-thiorphan (DL-3-mercapto-2-benzylpropanoylglycine) were from Calbiochem; ethyl acetate, acetonitrile, and methanol of HPLC grade were used. The lithium salt of LTA₄ was prepared by saponification of the methyl ester (21). Neutrophils were isolated from human blood (22) and suspended at 2.5 × 10⁷ cells/ml in 0.05 M Hanks' balanced salt solution, 0.02 M HEPES, pH 7.2, with 1.4 mM Ca²⁺ and 0.8 mM Mg²⁺. Recombinant LTA₄ hydrolyse from leukocytes was purified to homogeneity as described (23). Erythrocytes were isolated by filtration through cellulose columns (24).

Incubations—LTA₄ hydrolyse (8 μg/ml) in 0.01 M Tris buffer, pH 8, containing 1 mg/ml BSA and 10⁻¹⁰-10⁻¹⁰ M bestatin were incubated at 25 °C for 10 min prior to incubation with 20 μM LTA₄, for 10 min. Reactions were terminated with 2 volumes of methanol containing prostaglandin B₂ (0.5 μg), a quantitative internal standard. LTB₄ was quantified by chromatography on a C₁₈ column eluted with CH₃OH/H₂O/CH₃COOH (88:24:40:0.1, v/v), LTA₄, LTB₄, and LTB₄ standard solutions were quantified by UV spectrophotometry using extinction coefficients of 40,000 and 60,000 M⁻¹ cm⁻¹, respectively. Other inhibitors of metallohydrolyase enzymes were tested similarly. These included epibestatin, amastatin, L-leucine hydroxamate, DL-methionine hydroxamate (aminopeptidases), captopril (angiotensin-converting enzyme, peptidyl dipeptidase, EC 3.4.15.1), phosphoramidon, thiorphan (neutral endopeptidase, EC 3.4.24.11) glycy-L-tyrosine (carboxypeptidase A, EC 3.4.17.1), and bacitracin (endopeptidases).

The rate of inhibition was determined by incubating 15 μM bestatin with LTA₄ hydrolyase and monitoring its activity in samples withdrawn at intervals from 0 to 30 min. Lineeweaver-Burk plots (10-100 μM LTA₄) were determined in the presence and absence of 1 μM bestatin. Kᵣ values were calculated from Dixon plots (1/min vs. [I]) using Vₘₐₓ from the Lineeweaver-Burk plot. Reversibility was determined by incubating 100 μM bestatin with LTA₄ hydrolyse (14 μg/ml) for 10 min, then monitoring the enzymatic activity before and after gel filtration through a PD-10 column. Selectivity was deter-
mined by incubating lysed neutrophils with 70 μM bestatin and 100 μM arachidonic acid or 10 μM LTA₄ for 10 min. Samples were quenched, extracted, and analyzed for 5-HETE, 15-HETE, and LTB₄. Effects on intact cells were determined by incubating 70 μM bestatin with erythrocytes (2 x 10⁶ cells/ml) or neutrophils (2.5 x 10⁶ cells/ml) at 37 °C. Portions of the incubation mixture were withdrawn and incubated with 10 μM LTA₄ to determine the time dependence of inhibition of LTB₄ formation. Reactions were terminated by the addition of 3 volumes of ethyl acetate, and samples were acidified to pH 3 and extracted three times with ethyl acetate.

Intrinsic aminopeptidase activity of LTA₄ hydrolase, purified to homogeneity, was monitored by incubating enzyme (14 μg/ml) in 0.010 M Tris, pH 8, containing 1 mg/ml BSA, with 0.10-1 mM L-lysine p-nitroanilide. p-Nitroanilide formation was monitored at 405 nm, ε = 10,800 cm⁻¹ M⁻¹. Experiments were performed with 10 μM LTA₄ and 0.5 μM bestatin to determine if they inhibited hydrolysis of L-lysine p-nitroanilide. L-Leucine β-naphthylamide was also used as a substrate; its hydrolysis was quantified by the decrease in absorbance at 248 nm, ε = 28,630 M⁻¹ cm⁻¹. Microsomal rat kidney leucine aminopeptidase M was examined as a catalyst for the enzymatic hydration of LTA₄. L-Lysine p-nitroanilide and L-leucine β-naphthylamide substrates were used to verify peptidase activity.

RESULTS

Bestatin inhibited LTA₄ hydrolase in a rapid, reversible, and dose-dependent manner. The concentration for half-maximal inhibition (IC₅₀) of LTB₄ formation was 4.0 ± 0.8 μM (Fig. 1, lower panel). Lineweaver-Burk plots indicated a mixture of competitive and noncompetitive mechanisms with an apparent Kᵢ = 201 ± 95 nM, mean ± S.D., n = 3 (Fig. 1, upper panel). Inhibition was maximal within 1 min; LTB₄ formation declined from a control value of 1.8 μM to 0.91 μM after 45 s and remained at that level (0.89 ± 0.03 μM, mean ± S.D., n = 5) for 30 min. Preincubation of 15 μM inhibitor and enzyme for 10-90 h at 4 °C did not significantly increase the effect. Of the peptidase inhibitors tested only captopril was as potent as bestatin; all others were ineffective or significantly less potent (Table I). Gel filtration of the inhibitor-enzyme complex through a PD-10 column restored the enzymatic hydration of LTA₄. L-Lysine p-nitroanilide and L-leucine β-naphthylamide substrates were used to verify peptidase activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration for half-maximal inhibition (IC₅₀) μM</th>
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<tbody>
<tr>
<td>LTA₄</td>
<td>L-Lysine p-nitroanilide</td>
</tr>
<tr>
<td>Bestatin</td>
<td>4 x 10⁻⁶</td>
</tr>
<tr>
<td>Amastatin</td>
<td>&gt;10⁻⁶</td>
</tr>
<tr>
<td>Epibestatin</td>
<td>&gt;10⁻⁶</td>
</tr>
<tr>
<td>Captopril</td>
<td>11 x 10⁻⁶</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>1 x 10⁻⁶</td>
</tr>
<tr>
<td>Thiophan</td>
<td>1 x 10⁻⁶</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Inactive</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>Inactive</td>
</tr>
<tr>
<td>L-Leucine hydramidate</td>
<td>Inactive</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>&gt;10⁻⁷</td>
</tr>
<tr>
<td>L-Lysine p-nitroanilide</td>
<td>1 x 10⁻³</td>
</tr>
<tr>
<td>L-Leucine β-naphthylamide</td>
<td>3 x 10⁻³</td>
</tr>
<tr>
<td>LTA₄</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5 x 10⁻⁶</td>
</tr>
</tbody>
</table>

* LTA₄ hydrolase (5 μg/ml) was incubated with inhibitors and 100 μM L-lysine p-nitroanilide at 25 °C, and formation of p-nitroanilide was measured by the increase in absorbance at 405 nm.

** No inhibition was detected at the highest concentration examined, 1 nM.

ND, not determined.

Kᵢ.

formation declined from a control value of 1.8 μM to 0.91 μM after 45 s and remained at that level (0.89 ± 0.03 μM, mean ± S.D., n = 5) for 30 min. Preincubation of 15 μM inhibitor and enzyme for 10-90 h at 4 °C did not significantly increase the effect. Of the peptidase inhibitors tested only captopril was as potent as bestatin; all others were ineffective or significantly less potent (Table I). Gel filtration of the inhibitor-enzyme complex through a PD-10 column restored the enzymatic activity from its inhibited level, 43 ± 4 nmol of LTB₄/mg of enzyme, to 346 ± 40 nmol of LTB₄/mg, a value which was indistinguishable from the control value of 387 ± 4 (mean ± S.D., n = 3).

Bestatin inhibited LTA₄ hydrolase selectively; neither 5-lipoxygenase nor 15-lipoxygenase activity in lysed neutrophils differed significantly from the corresponding control (Table II). It also inhibited LTB₄ formation by intact erythrocytes and neutrophils. The effect was maximal by 10 min in erythrocytes but had reached only 40% after 2 h in neutrophils (Fig. 2). Preincubation of neutrophils with 100 μM bestatin for 15 min at 37 °C prior to stimulation with 5 μM A23187 decreased LTB₄ production from 424 ± 13 to 347 ± 13 nM (p < 0.01). LTB₄ formation declined from 572 ± 33 to 470 ± 19 nM (p < 0.01) in neutrophils treated with 10 μM LTA₄. Inhibition in cells was not reversed by washing, consistent with the cytosolic localization of the enzyme. After two washings with phosphate-buffered saline, incubation for 30 min at 37 °C, and two more washings, LTB₄ formation by erythrocytes remained at 5% of the control value. Results were similar with neutrophils.

LTA₄ hydrolase, purified to homogeneity, contained an intrinsic aminopeptidase activity. The rate of hydrolysis of L-lysine p-nitroanilide was dependent on protein and substrate concentrations with an apparent Kₛ = 156 μM and a Vₘₐₓ = 50 nmol/min/mg enzyme (Fig. 3). The reaction rate was constant for at least 15 min at 25 °C, and, in contrast to LTB₄ formation, mechanism-based inactivation was not evident.

![Fig. 1. Bestatin inhibits purified LTA₄ hydrolase. Lower panel, concentration-dependent inhibition of LTB₄ formation by bestatin, IC₅₀ = 4.0 ± 0.8 μM; upper panel, Lineweaver-Burk plot of 1/substrate (nM) of LTB₄/min/mg of enzyme)⁻¹ versus 1/(sLTA₄)⁻¹ for control enzyme (O) and enzyme plus 1 μM bestatin (●). Apparent Kᵢ = 7.6 ± 0.4 μM LTA₄; Kᵢ = 201 ± 95 nM bestatin.](image-url)
LTA₄ Hydrolase Is Functionally Related to an Aminopeptidase

Lyzed neutrophils (1 ml, 2.5 x 10⁷/ml) with or without 70 μM bestatin were incubated with 100 μM arachidonic acid or 10 μM LTA₄ at 25 °C for 10 min, and products were determined by HPLC. Values are the mean ± S.D., n = 3. Bestatin inhibited LTB₄ formation to a statistically significant degree (*p < 0.02 or **p < 0.01). It did not inhibit formation of lipoxygenase products, 5-HETE and 15-HETE. Bestatin (70 μM) reduced the hydrolysis of 2 mM L-lysine p-nitroanilide to 22% of the control (p < 0.01).

<table>
<thead>
<tr>
<th>Bestatin</th>
<th>Substrate</th>
<th>LTB₄</th>
<th>5,12-diHETES</th>
<th>5-HETE</th>
<th>15-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 μM arachidonate</td>
<td>0.42 ± 0.08</td>
<td>0.21 ± 0.01</td>
<td>2.53 ± 0.35</td>
<td>3.42 ± 0.23</td>
</tr>
<tr>
<td>70 μM</td>
<td>100 μM arachidonate</td>
<td>0.13 ± 0.02*</td>
<td>0.23 ± 0.05</td>
<td>2.57 ± 0.43</td>
<td>4.19 ± 1.43</td>
</tr>
<tr>
<td>None</td>
<td>10 μM LTA₄</td>
<td>1.48 ± 0.09</td>
<td>2.72 ± 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 μM</td>
<td>10 μM LTA₄</td>
<td>0.38 ± 0.02**</td>
<td>2.83 ± 0.20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LTA₄ inhibited the peptidase activity of LTA₄ hydrolase by a mixture of competitive and noncompetitive mechanisms with \( K_i = 5.3 \mu M \), a concentration similar to the \( K_m = 7.6 ± 0.4 \mu M \) for LTA₄ determined under identical conditions. Bestatin inhibited the hydrolysis of L-lysine p-nitroanilide competitively with \( K_i = 172 ± 93 \text{nM} \), a value similar to \( K_i = 201 ± 95 \text{nM} \) for inhibition of LTB₄ formation (Table III). L-Leucine \( \beta \)-naphthylamide was also a substrate for the peptidase activity of LTA₄ hydrolase, with an apparent \( K_m = 70 \mu M \) and a \( V_{max} = 215 \text{nmol/min/mg enzyme} \). A comparison of \( k_{cat}/K_m \) values (estimated from \( V_{max}/K_m \)) for the three substrates shows that the LTA₄ hydrolase catalyzes LTB₄ formation more efficiently than hydrolysis of the two L-leucine derivatives (Table III). The converse was not true: aminopeptidase M did not convert LTA₄ into LTB₄. Aminopeptidase M has 53 and 13 times higher specific activity for L-lysine p-nitroanilide and L-leucine \( \beta \)-naphthylamide, respectively, than does LTA₄ hydrolase.

**DISCUSSION**

LTA₄ hydrolase differs from other cytosolic epoxide hydrolase enzymes in three ways. First, its substrate requirement is rigorous. It hydrolyzes only those compounds with a 5,6-oxide-7,9-trans-11,14-cis configuration (17), but not oxiranes which are substrates for hepatic epoxide hydrolases. Second, its product is not a vicinal diol, typically formed by epoxide hydratase enzymes. Second, its product is not a vicinal diol, typically formed by epoxide hydratase enzymes.
hydrolases, but a dihydroxy compound with a (5S,12R) configuration and a 6-cis olefin geometry. Third, inhibitors of cytosolic hepatic epoxide hydrolase do not inhibit LTA, hydrolase. Recently, amino acid sequence data led to the speculation that LTA, hydrolase may be a member of the Zn\(^{2+}\)-metallohydrolase family (18, 19). Our results demonstrating its inhibition by bestatin and the detection of an intrinsic peptidase activity substantiate that LTA, hydrolase is functionally related to aminopeptidase M, a Zn\(^{2+}\)-containing metallohydrolase. It should be stressed that an intrinsic aminopeptidase activity is detectable in LTA, hydrolase; however, the converse is not true: aminopeptidase M shows no capacity to transform LTA, into LTB\(_4\). Collectively, these data suggest that the family of Zn\(^{2+}\)-containing peptidases, not the epoxide hydrolase family, may offer better targets for inhibition or the catalytic mechanism of LTA, hydrolase.

It is interesting to note that several eicosanoid biosynthetic enzymes, such as peroxidase/cyclooxygenase (25) or 5-lipoxygenase/LTA, synthase (26), have bifunctional catalytic properties associated with a single protein. LTA, hydrolase may also typify this phenomenon.

Bestatin is the first example of a reversible, chemically stable inhibitor of LTA, hydrolase. The only other inhibitors reported are substrate analogs such as LTA, and LTA, derived from eicosatetraenoic and eicosapentaenoic acid, respectively (14–17). Compared with these unstable allicy epoxides, which inactivate the enzyme irreversibly, bestatin may be a more useful tool for clarifying the role of LTB, in inflammation. It is uncertain whether selective inhibition of LTA, hydrolase compares favorably with inhibition of 5-lipoxygenase as a therapeutic tactic. Inhibition of 5-lipoxygenase reduces the formation of both LTB, and the cysteinyl leukotrienes, all of which have some pro-inflammatory properties. However, selective inhibition of LTA, hydrolase may be appropriate in the case of bestatin LTB, formation since LTA, is the investigational leukotriene produced by neutrophils and macrophages (7). It will be informative to determine if any effects of bestatin, in vivo, originate from inhibition of LTB, formation. The availability of a selective LTA, hydrolase inhibitor may also facilitate investigations on transcellular biosynthesis of leukotrienes. Studies of this process have often relied on powerful stimuli such as ionophore A23187 to promote the accumulation and transfer of LTA, from neutrophils to other cells (27–31). Selective inhibition of LTA, hydrolase represents another approach to accumulate LTA, for redistribution among different cell types. It is noteworthy that bestatin inhibits LTB, formation by erythrocytes more effectively than by neutrophils. This trait may be particularly valuable to examine the contribution of erythrocytes to transcellular biosynthesis of LTB, in vitro and in vivo.

The analogs, amastatin and epibestatin, were ineffective under the conditions used, suggesting that structure-activity relationships apply. It is important to stress that amastatin, epibestatin, and hydroxamates inhibited neither LTB, formation nor hydrolysis of L-leucine derivatives. This indicates that the aminopeptidase activity is intrinsically associated with LTA, hydrolase and not a contaminant. Amastatin, and bestatin to a lesser degree, are so-called slow, tight binding inhibitors of aminopeptidase M (32). Although incubation with LTA, hydrolase for 10–90 h enhanced their effects only moderately (<10%) a combination of competitive and slow, tight binding best accounts for the mixed type of inhibition. Mechanism-based inactivation which accompanies turnover of LTA, also complicates the kinetic analysis (33). Among other inhibitors tested only captopril was as potent as bestatin. In view of this it will be interesting to determine if the constitutive aminopeptidase activity of LTA, hydrolase has any physiological relevance, distinct from LTB, formation, such as processing or degradation of biologically active peptides.

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REFERENCES

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