Volume 297, number 1,2, 139–142 FEBS 10644 February 1992

Bestatin inhibits covalent coupling of [^{3}H]LTA$_4$ to human leukocyte LTA$_4$ hydrolase

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Received 5 September 1991; revised version received 2 December 1991

The covalent coupling of [^{3}H]LTA$_4$ to human leukocyte LTA$_4$ hydrolase is inhibited in a concentration-dependent fashion by pre-incubation with bestatin. This inhibition correlated with the concentration-dependent inhibition by bestatin of LTB$_4$ and LTB$_5$ synthesis by LTA$_4$ hydrolase. Epibestatin, a diastereomer of bestatin, neither inhibited LTB$_4$ or LTB$_5$ production by LTA$_4$ hydrolase nor prevented the covalent coupling of [^{3}H]LTA$_4$ to the enzyme. In contrast, captopril inhibited both LTB$_4$ synthesis by LTA$_4$ hydrolase and covalent coupling of [^{3}H]LTA$_4$ to the enzyme.

LTA$_4$ hydrolase; Bestatin; [^{3}H]LTA$_4$ covalent coupling; Human leukocyte

1. INTRODUCTION

Leukotriene (LT)B$_4$ is a product of activated leukocytes which has potent autocrine chemotactic and chemokinetic properties [1–3]. The synthesis of LTB$_4$ is catalysed by the enzyme LTA$_4$ hydrolase, which is a unique 70,000 Da cytosolic protein with no structural or functional similarities to other epoxide hydrolases [5–9]. LTA$_4$ and LTA$_5$ are the only epoxide substrates identified for LTA$_4$ hydrolase [10]. LTA$_3$ has been shown to be a potent inhibitor which irreversibly inactivates the enzyme [11]. Mechanism-based inactivation of LTA$_4$ hydrolase by LTA$_4$ itself occurs with covalent modification of the enzyme, which is stoichiometric with inactivation [12]. In contrast to 5-lipoxygenase, the first committed enzyme in the leukotriene synthetic pathway, LTA$_4$ hydrolase is ubiquitously distributed in most tissues [13,14]. A zinc binding site in LTA$_4$ hydrolase was recently identified which has strong homology to that of aminopeptidases such as *E. coli* aminopeptidase N and human kidney aminopeptidase M [15–17]. LTA$_4$ hydrolase has been shown to contain 1 mol of zinc per mole of enzyme and to have an intrinsic aminopeptidase activity in addition to its LTA$_4$ hydrolytic function [17,18]. The amino acid residues of LTA$_4$ hydrolase involved in aminopeptidase activity and LTA$_4$ hydrolysis have been proposed to be centred around the zinc binding domain since zinc is essential for both activities [16–18]. Pre-incubation of LTA$_4$ hydrolase with LTA$_4$ also reduces both activities in accordance with the model of one active site for both enzymatic activities [19]. Furthermore, bestatin, a zinc aminopeptidase inhibitor, selectively and reversibly inhibits both the aminopeptidase and the LTA$_4$ hydrolytic activities of LTA$_4$ hydrolase [20]. Selective inhibition of LTB$_4$ and LTB$_5$ production and covalent coupling of [^{3}H]LTA$_4$ to human leukocyte LTA$_4$ hydrolase by bestatin and captopril is presented in the following paper. The results visualize competition at a single active site of LTA$_4$ hydrolase capable of both LTA$_4$ hydrolytic and aminopeptidase functions.

Abbreviations: LTA$_3$, 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11-*cis*-eicosatetraenoic acid; LTA$_4$, 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTA$_5$, 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14,17-*cis*-eicosatetraenoic acid; LTB$_4$, 5(*S*),12(*R*)-dihydroxy-8,10-*trans*-6,14-*cis*-eicosatetraenoic acid.

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2. MATERIALS AND METHODS

2.1. *Materials*

LTA$_4$ and LTA$_5$ methyl esters, LTB$_4$ (Merck Frosst), LTA$_5$ methyl ester (BioMol.), [14,15-^{3}H]LTA$_4$ methyl ester (42 Ci/mmol) (Du Pont-New England Nuclear), prostaglandin B$_2$ (PGB$_2$), bovine serum albumin (BSA), bestatin ((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine), epibestatin (2*R*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine), captopril ((2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline) (Sigma) and methanol of HPLC grade were used. Rainbow molecular weight markers were from Amersham. Sodium salts of LTA$_4$, LTA$_5$ and [14,15-^{3}H]LTA$_4$ were prepared by saponification of their methyl esters in methanol:10 N NaOH (9.5:0.5, v/v) for 1 h at 20–25°C. Human leukocyte LTA$_4$ hydrolase was purified 50-fold from freshly drawn blood by ammonium sulphate precipitation (40–80%) and DEAE-5PW high pressure liquid chromatography (HPLC) fractionation as described previously [5]. The human leukocyte cytosol preparation used was the 30–60% ammonium sulphate fraction from the 10,000 × *g* supernatant of buffy coat concentrates (Red Cross Montreal) prepared as described previously [21]. Protein was determined by a modification of the method of Bradford [22].

2.2. *LTA$_4$ hydrolase activity assays*

Human leukocyte cytosol (400 μl of a 3.2 mg/ml 30–60% ammonium

sulphate-precipitated fraction) was pre-incubated in a 500 μl total volume of 100 mM Tris-HCl, pH 8.0, 1 mg/ml BSA with either 5 μl dimethyl sulphoxide (DMSO) (control) or 5 μl of a 100-fold concentrate of compound dissolved in DMSO for 10 min at 20–25°C. LTA_4 or LTA_5 (10 μM final concentration) was added and incubation continued for a further 10 min at 20–25°C. The reaction was terminated by the addition of 0.5 ml of ethanol containing 1 nmol/ml PGB_2, samples held on ice for 10 min then centrifuged at 13,000 × *g* for 5 min at 4°C. A 100 μl aliquot of the supernatant was analysed for LTB_4 formation by reverse phase HPLC on a C_{18} Waters Novapak column (3.9 × 150 mm) eluting isocratically at 0.8 ml/min with a 70:30:0.1 (methanol:water:acetic acid) pH 5.4 solvent and monitoring absorbance of products at 270 nm. PGB_2 internal standard eluted at 5.7 min, *all-trans*-LTB_3, epi-*all-trans*-LTB_4 and LTB_4 eluted at 7.9, 8.8 and 10.3 min, respectively.

2.3. *[^{3}H]LTA_4 labeling of LTA_4 hydrolase*

Partially pure human leukocyte LTA_4 hydrolase (40 μl of a 0.1 mg/ml DEAE-5PW HPLC purified fraction) or human leukocyte cytosol (40 μl of a 3.2 mg/ml 30–60% ammonium sulphate-precipitated fraction) were pre-incubated in a 50 μl total volume of 200 mM Tris-HCl, pH 8.0 with either 0.5 μl DMSO (control) or 0.5 μl of a 100-fold concentrate of compound in DMSO for 10 min at 20–25°C. [^{3}H]LTA_4 (440,000 dpm, 0.2 μCi, 0.1 μM final concentration) was added and incubation continued for a further 10 min at 20–25°C. The reaction was terminated by the addition of 6 μl of 100% TCA, samples held on ice for 10 min then centrifuged at 13,000 × *g* for 5 min at 4°C. Pellets were washed once with 100 μl of 1 M Tris-HCl, pH 8.0, and resuspended in 50 μl of a denaturing polyacrylamide gel sample buffer containing 6.7 mM Tris-HCl, pH 6.8, 0.13% sodium dodecyl sulphate, 1.3% glycerol, 0.08 M β-mercaptoethanol, and Bromophenol blue. Samples were boiled for 2 min and 15 μl aliquots electrophoresed on 8 × 8 cm 8–16% or 10% polyacrylamide Novex mini-gels according to the method of Laemmli [23]. Gels were fixed for 1 h in 10% methanol, 10% acetic acid, enlightened, (Enlighten, Dupont NEN) for 30 min, dried at 50°C for 8 h and exposed to Kodak XAR film in an intensifying cassette for 48–120 h. Fluorographs were scanned using an LKB 2202 laser densitometer for quantitation of radiolabeling of LTA_4 hydrolase. The absorbance of the LTA_4 hydrolase band in a boiled enzyme lane on each gel (enzyme boiled for 2 min prior to pre-incubation with DMSO) was subtracted from the LTA_4 hydrolase band absorbances of other samples. The boiled enzyme control absorbance varied on different fluorographs from 0–25% of control [^{3}H]LTA_4 labeling.

3. RESULTS

Bestatin inhibited covalent coupling of [^{3}H]LTA_4 to a partially purified preparation of human leukocyte LTA_4 hydrolase. Pre-incubation of the enzyme with 100, 10 or 1 μM bestatin resulted in approximately 90, 50 or 20% inhibition of [^{3}H]LTA_4 labeling of LTA_4 hydrolase, respectively. As shown in Fig. 1, pre-incubation of the partially purified human leukocyte LTA_4 hydrolase preparation with 10 μM LTA_3, an irreversible inhibitor of LTA_4 hydrolase [11], resulted in approximately 90% inhibition of [^{3}H]LTA_4 labeling of the enzyme.

Bestatin inhibited LTB_4 formation by LTA_4 hydrolase in a human leukocyte cytosol fraction in a concentration-dependent fashion with 50% inhibition of activity at approximately 10 μM bestatin (Fig. 2). In this human leukocyte cytosolic preparation, [^{3}H]LTA_4 labeling of LTA_4 hydrolase was also inhibited in a concentration-dependent fashion by bestatin with 50% inhibition of labeling at approximately 10 μM (Fig. 3). Similar results for bestatin inhibition of labeling were obtained using [^{3}H]LTA_4 methyl ester instead of

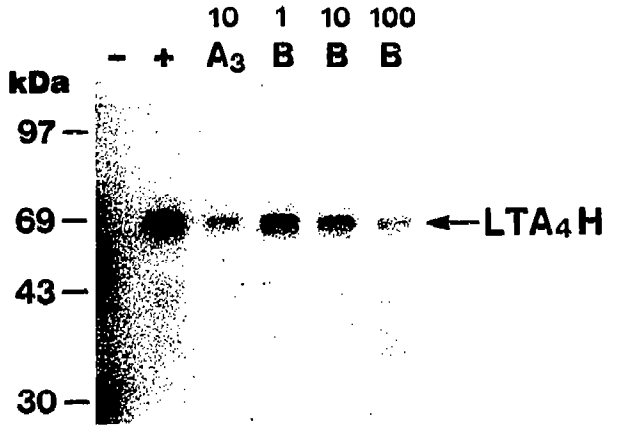

Fig. 1. Inhibition by bestatin of [^{3}H]LTA_4 labeling of partially purified human leukocyte LTA_4 hydrolase. Partially purified human leukocyte LTA_4 hydrolase (40 μl of a 0.1 mg/ml DEAE-5PW HPLC purified fraction) was pre-incubated with either DMSO (control) (+), or 10 μM LTA_3 (A_3), 1, 10 or 100 μM bestatin, then incubated with 0.1 μM [^{3}H]LTA_4 as described in Materials and Methods. The intensity of the [^{3}H]LTA_4 labeling of LTA_4 hydrolase was quantitated by densitometric analysis of the fluorograph and expressed as the % of control labeling. An aliquot of enzyme boiled for 2 min prior to pre-incubation with DMSO and [^{3}H]LTA_4 served as a boiled control (−). Positions of rainbow molecular weight markers (Amersham) are shown on the left.

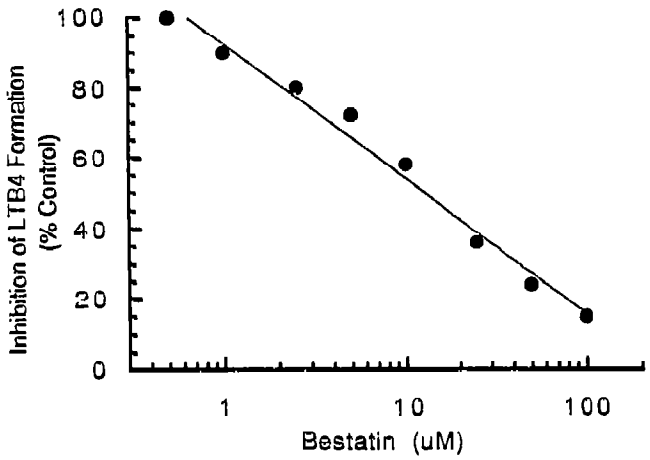

Fig. 2. Inhibition by bestatin of LTB_4 formation by human leukocyte cytosol. A human leukocyte cytosol fraction (30–60% ammonium sulphate-precipitated proteins) was pre-incubated with DMSO (control) or varying concentrations of bestatin in DMSO for 10 min at 20–25°C. LTA_4 was added to a final concentration of 10 μM and following a further 10 min incubation at 20–25°C LTB_4 formation was determined as described in Materials and Methods. Data is expressed as the % of control LTB_4 formation and is the mean of duplicate determinations where the range was ± 5%. The graph is representative of 2 such titrations.

Table I

Selective inhibition of LTB_4 or LTB_5 formation by human leukocyte LTA_4 hydrolase

Inhibitor conc. (μM)	% Inhibition of LTB_4 or LTB_5 formation				
	Bestatin		Epibestatin		Captopril
	LTB_4	LTB_5	LTB_4	LTB_5	LTB_4
100	85	85	−8	3	60
10	47	36	−3		11
1	15	11	5		−4

A human leukocyte cytosol fraction (30–60% ammonium sulphate-precipitated proteins including LTA_4 hydrolase) was pre-incubated with 100, 10 or 1 μM bestatin, captopril, epibestatin or DMSO (control) for 10 min at 20–25°C. LTA_4 or LTA_5 was added to a final concentration of 10 μM and following a further 10 min incubation LTB_4 formation was determined as described in Materials and Methods. Data is expressed as the % of control LTB_4 or LTB_5 formation and is the mean of 2–4 determinations where the range was ±7%.

$[^3H]LTA_4$ (data not shown). Less than 25% inhibition of $[^3H]LTA_4$ labeling of LTA_4 hydrolase was seen when 100 μM bestatin was incubated for 10 min with the cytosol after covalent coupling of the $[^3H]LTA_4$ to the enzyme (data not shown).

In the first report of the inhibition of LTA_4 hydrolase activity by bestatin it was shown that epibestatin did not inhibit enzyme activity in contrast to bestatin and captopril [20]. This result was confirmed for the LTA_4 hydrolase activity in our human leukocyte cytosolic fraction (Table I). As demonstrated in Table I, in complete agreement with the previous study [20], bestatin was a more potent inhibitor than captopril, while epibestatin, up to a concentration of 100 μM, showed no inhibition of LTB_4 formation. The profile of selective inhibition of LTB_4 formation by these compounds correlated well with their inhibition of $[^3H]LTA_4$ labeling of the enzyme (Fig. 4). Quantitation of $[^3H]$ incorporation into LTA_4 hydrolase by laser densitometric scanning of the fluorograph shown in Fig. 4 and also from a number of other fluorographs gave averages of 89% (+/− 5%, S.E.M.), −10% (+/− 28% S.E.M.), and 80% (+/− 15%, S.E.M.) for inhibition of $[^3H]LTA_4$ labeling of LTA_4 hydrolase by 100 μM bestatin, 100 μM epibestatin and 100 μM captopril, respectively. These values are in close agreement with the selectivity of inhibition of LTB_4 formation by these compounds (Table I).

LTA_5 had previously been shown to be both a substrate and an inhibitor of LTA_4 hydrolase [24]. In addition, LTA_5 has been shown to inhibit covalent coupling of $[^3H]LTA_4$ to LTA_4 hydrolase [24]. In our human cytosolic fraction, bestatin inhibited LTB_5 formation from LTA_5 in a concentration-dependent fashion with 50% inhibition at approximately 20 μM bestatin (Table I and data not shown). Epibestatin at 100 μM concentration showed no inhibition of LTB_5 formation from LTA_5 (Table I).

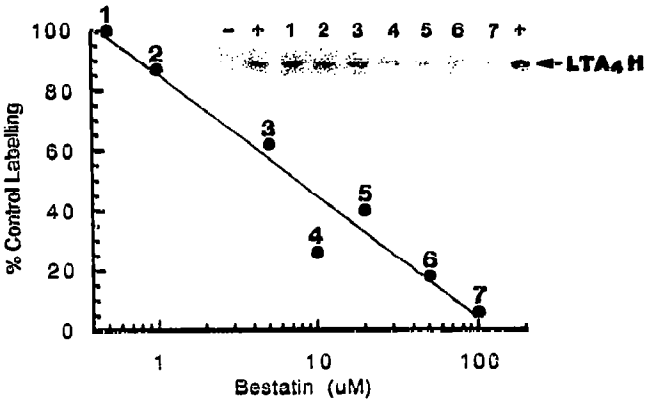

Fig. 3. Inhibition by bestatin of $[^3H]LTA_4$ labeling of human leukocyte LTA_4 hydrolase. A human leukocyte cytosol fraction (30–60% ammonium sulphate-precipitated proteins) was pre-incubated with DMSO control (+) or varying concentrations of bestatin in DMSO followed by incubation with $[^3H]LTA_4$ as described in Materials and Methods. The intensity of the $[^3H]LTA_4$-labeled LTA_4 hydrolase band was quantitated by densitometric analysis of the fluorograph and expressed as % of control labeling. The inset shows the LTA_4 hydrolase region from the fluorograph with boiled enzyme DMSO control (−), the DMSO control (+), and 0.5 μM (1), 1 μM (2), 5 μM (3), 10 μM (4), 20 μM (5), 50 μM (6) and 100 μM (7) bestatin pre-incubations. The graph is representative of 2 such titrations vs. $[^3H]LTA_4$ and 2 similar titrations vs. $[^3H]LTA_4$ methyl ester.

4. DISCUSSION

The intriguing discovery in 1990 that in addition to its epoxide hydrolase activity LTA_4 hydrolase has a zinc-dependent aminopeptidase activity has resulted in considerable interest in the significance of these two activities for the one enzyme [15–18]. Professor Bengt Samuelsson originally disclosed data on October 2, 1990 at the New York Academy of Science and British Medical Society conferences on 'Advances in the Understanding and Treatment of Asthma' confirming that LTA_4 hydrolase contains 1 mol of zinc per mol of enzyme. He presented more studies demonstrating the importance of the zinc binding domain of LTA_4

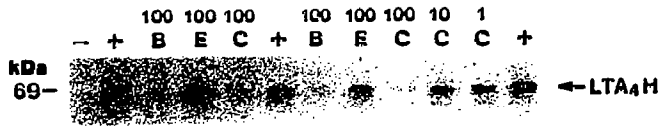

Fig. 4. Selectivity of inhibition of $[^3H]LTA_4$ labeling by bestatin and captopril in contrast to epibestatin. A human leukocyte cytosol fraction (30–60% ammonium sulphate-precipitated proteins) was pre-incubated with DMSO control (+) or 100 μM bestatin (100, B), 100 μM epibestatin (100, E), 100 μM captopril (100, C), 10 μM captopril (10, C) or 1 μM captopril (1, C) followed by incubation with $[^3H]LTA_4$ as described in Materials and Methods. The figure shows the fluorograph of $[^3H]LTA_4$-labeled LTA_4 hydrolase with boiled enzyme control (−). The intensity of the $[^3H]LTA_4$ labeling of LTA_4 hydrolase was quantitated by densitometry and expressed as the % of control labeling.

hydrolase for both epoxide hydrolysis and aminopeptidase activity in a plenary lecture given on May 16, 1991 at the Washington Prostaglandins, Leukotrienes, Lipoxins and PAF meeting. This lecture, in combination with the elegant report documenting the selective reversible inhibition of both LTA_4 hydrolysis and aminopeptide cleavage activities of LTA_4 hydrolase by bestatin [20], stimulated the work described in the present paper.

We show here the selective inhibition by bestatin of both LTB_4 and LTB_5 formation and [^{3}H]LTA_4 labeling of LTA_4 hydrolase in a human leukocyte cytosolic fraction. Previously it had been definitively demonstrated that the incorporation of [^{3}H]LTA_4 methyl ester into LTA_4 hydrolase was stoichiometric with the inactivation of the enzyme at concentrations up to 200 μM [^{3}H]LTA_4 methyl ester [12]. Our method of quantitation of [^{3}H]LTA_4 incorporation into LTA_4 hydrolase by laser densitometric scanning of fluorographs does not permit a stroichiometric analysis of the extent of labeling vs. enzyme inhibition. However, the present study was carried out at 0.1 μM [^{3}H]LTA_4 (or 0.1 μM [^{3}H]LTA_4 methyl ester), a concentration well below the concentration at which stoichiometric labeling and inactivation of LTA_4 hydrolase were previously observed [12]. Therefore, we believe that in our studies there would be little chance for indiscriminate binding of the epoxide to the enzyme.

Our results strongly imply that the site of mechanism-based inactivation of the enzyme by LTA_4 is the site at which bestatin inhibits LTB_4 and LTB_5 formation by LTA_4 hydrolase. As would be expected, pre-incubation of LTA_4 hydrolase with bestatin is able to protect the enzyme from subsequent mechanism-based inactivation by LTA_4 (personal communication, Frank Fitzpatrick). The present data visualizing inhibition of [^{3}H]LTA_4 labeling of human leukocyte LTA_4 hydrolase augments and enhances the hypothesis that there is a single site on the enzyme that catalyses both epoxide hydrolysis and aminopeptide cleavage. If a protein substrate is discovered for the aminopeptidase activity of LTA_4 hydrolase it would be of great interest to determine if preincubation with this protein would also selectively inhibit [^{3}H]LTA_4 covalent coupling to the enzyme.

Acknowledgements: The authors acknowledge many stimulating discussions with Dr. Frank Fitzpatrick and the inspiring plenary lecture given by Prof. Bengt Samuelsson on May 16, 1991.

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