Effect of Protease Inhibitors on Angiotensin-Converting Enzyme Activity in Human T-Lymphocytes

Victor Petrov, Robert Fagard, and Paul Lijnen

The purpose of these investigations was to determine whether the aminopeptidase B and leucine aminopeptidase inhibitor bestatin, the chymase inhibitor chymostatin, the calpain inhibitor E-64, and the neutral serine protease inhibitor leupeptin affect the angiotensin converting enzyme (ACE) activity in T-lymphocytes. ACE activity in homogenates of T-lymphocytes or in intact T-lymphocytes in suspension was measured by determining fluorimetrically histidyl-leucine, formed from the conversion of hippuryl-histidyl-leucine, coupled with o-phtaldialdehyde. The effect of various concentrations (10^{-9} to 10^{-3} mol/L) of the angiotensin-converting enzyme inhibitors lisinopril and captopril and of the various protease inhibitors on ACE activity was studied. Lisinopril and captopril reduced the ACE activity in homogenates of T-lymphocytes in a concentration-dependent manner. Lisinopril exhibited a more pronounced inhibition of ACE in T-lymphocytes than did captopril. Chymostatin and E-64 had no effect on the ACE activity in T-lymphocytes, whereas leupeptin inhibited its activity in a dose-dependent fashion. Bestatin, on the contrary, increased the ACE activity in homogenates of T-lymphocytes as well as in intact T-lymphocytes in proportion to the concentration. Our data showed that the ACE activity in T-lymphocytes was stimulated by bestatin and inhibited by leupeptin, whereas chymostatin and E-64 did not affect the ACE activity in T-lymphocytes. Am J Hypertens 2000;13:535–539 © 2000 American Journal of Hypertension, Ltd.

KEY WORDS: Angiotensin-converting enzyme, aminopeptidases, T-lymphocytes.

Angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase I, has been identified as a membrane-bound enzyme in several types of cells, such as endothelial cells, epithelial cells, fibroblasts, mononuclear cells, T-lymphocytes, macrophages, neurons, and male germinal cells, and is also present in a circulating form in biologic fluids such as plasma, and amniotic and seminal fluids. ACE is thus a widely distributed zinc metallopeptidase. It exists primarily as a membrane-bound glycoprotein, which is anchored to the membrane by its hydrophobic C-terminal part, with the catalytic site facing the extracellular milieu. There are two different forms of membrane-bound ACE, the so-called endothelial form with a molecular mass of 150 to 180 kDa, and the testicular form of about 90 kDa. In addition, a soluble form of ACE exists that is presumably derived from the vascular endothelium. Only one gene per human haploid genome encodes for these different forms of ACE. Reverse transcriptase-polymerase chain reaction experiments with oligonucleotide primers for either the
endothelial or the testicular ACE mRNAs indicate that only the endothelial form of the ACE transcript is present in T-lymphocytes.7

Angiotensin-converting enzyme, initially believed to hydrolyze only angiotensin and bradykinin, is now known to hydrolyze a much broader spectrum of substrates, including substance P, neurotensin, and enkephalins,3,14 the physiologic relevance of which remains to be determined.

It remains also to be elucidated which other aminopeptidases, present on the surface of mammalian cells, affect the ACE activity in T-lymphocytes. Therefore, the aim of the present study was to investigate whether the aminopeptidase B inhibitor bestatin, the chymase inhibitor chymostatin, the calpain inhibitor E-64, or the neutral serine protease inhibitor leupeptin affect the ACE activity in T-lymphocytes.

MATERIALS AND METHODS

Materials The thiol-containing angiotensin-converting enzyme inhibitor (ACEI) captopril [(2S)-1-(3-Mercapto-2-methyl-propionyl)-L-proline], the non-thiol-containing ACEI lisinopril [(S)-Nα-(1-Carboxy-3-phenylpropyl)-Lys-Pro], the chymase inhibitor chymostatin [Nα-Carbonyl-capreomycidine-X-Phe-al-Phe] from a microbial source, the amino-peptidase B inhibitor bestatin or ubinemex [(2S,3R)-3-Amino-2-hydroxycyclohexyl-[S]-alanine], the neutral serine protease inhibitor leupeptin affect the ACE activity in T-lymphocytes.

Assay of ACE in Homogenates of T-Lymphocytes

Homogenates of these T-lymphocytes are prepared by alternately freezing them in liquid nitrogen, then thawing and dissolving in Triton X-100. Therefore, the medium was aspirated and then placed upright in ice for 15 min. Approximately 7.5 mL was aspirated, transferred to a 15-mL Falcon tube, and centrifuged at 300 g at +20°C for 10 min. The supernatant was aspirated and discarded, and the precipitate was detached. Then 0.8 mL of Lympho-Kwik LK-50T was added, the precipitate transferred to an Eppendorf tube and rinsed again with 0.8 mL LK-50T. After mixing with a thermomixer (60 min at 37°C) and centrifugation (2800 g for 2 min), the supernatant was aspirated, the precipitate detached, and 1 mL of RPMI added. After centrifugation (1400 g for 1 min) the supernatant was aspirated and the precipitate detached.

Then 100 μL monoclonal antibody to CD16 and 20 mL was added together with 0.8 mL Lympho-Kwik. After mixing with a thermomixer (60 min at 37°C) and centrifugation (2800 g for 2 min) the supernatant was aspirated and the precipitate detached from the wall; 1 mL RPMI was added and the tubes were centrifuged at 1400 g for 1 min. The supernatant was aspirated, and the precipitate was detached and dissolved into the appropriate medium. The obtained suspension contained only T-suppressor/cytotoxic cells. These T-cells were assayed by a fluorescence-activated cell sorter (FACScan cytometer from Becton Dickinson, San Jose, CA), using SimulTest software package V1.1, and counted routinely with a SysMex Microcell-counter F800 (Toa Medical Electronics, Kobe, Japan).

Homogenates of these T-lymphocytes are prepared by alternately freezing them in liquid nitrogen, then thawing and dissolving in Triton X-100. Therefore, the medium was aspirated and then 2.5 × 10⁶ cells dissolved in 100 μL phosphate-buffered saline (PBS), pH 7.2, containing 9.3 mmol/L Na₂HPO₄, 2.9 mmol/L KH₂PO₄, 3 mmol/L KCl, and 136 mmol/L NaCl. Ten microliters of Triton X-100 (20%) was added. After alternate (3 times) freezing, thawing, and centrifuging (10,000 g for 15 min) of this suspension, 10 μL of the supernatant was used freshly for the assay of ACE.

Assay of ACE in Homogenates of T-Lymphocytes

The fluorimetric assay for ACE in T-lymphocytes is based on the conversion of the substrate analog
hippuryl-L-histidyl-L-leucine to hippurate and L-histidyl-L-leucine, which is quantified spectrofluorometrically (λ<sub>excitation</sub> = 360 nm, λ<sub>fluorescence</sub> = 500 nm) by formation of a fluorescent adduct with o-phtaldialdehyde. Ten microliters of T-cells (2.5 × 10<sup>6</sup> cells/110 μL PBS) or 10 μL of homogenate were incubated with 5 μL of 250 mmol/L hippuryl-L-histidyl-L-leucine in 50 μL buffer, pH 8.3, containing 0.5 mol/L K<sub>2</sub>HPO<sub>4</sub> and 1.5 mol/L NaCl plus 140 μL distilled water for 18 h at 37°C. This reaction was stopped by the addition of 1.45 mL of 0.28 N NaOH. Then 100 μL of 2% o-phtaldialdehyde in methanol was added to each tube and mixed with a vortex. Exactly 10 min later this reaction was terminated by the addition of 200 μL of 3 N HCl and the tubes centrifuged at 15,500 g for 10 min. The supernatant was transferred to a quartz fluorescence cuvette and the cuvette placed in a thermostatted cuvette holder. The fluorescence was read 30 min after the addition of HCl, but within 90 min after its addition, with a LS-50B fluorimeter (Perkin Elmer, Norwalk, CT). The excitation wavelength at a slit of 8 nm was 360 nm and the emission wavelength was 500 nm at a slit of 6 nm, with the use of a filter of 430 nm.

The ACE activity in the T-lymphocytes (n = 6) averaged 8.36 ± 0.50 pmol/10<sup>6</sup> cells/min and was negligible (0.28 ± 0.09 pmol/10<sup>6</sup> cells/min) in the supernatant of these cells. Bestatin, leupeptin, chymostatin, and E-64 did not affect the degradation of histidyl-leucine or the conversion of hippuryl-histidyl-leucine to hippurate and histidyl-leucine in the concentration range studied.

**RESULTS**

**Effect of Lisinopril and Captopril on ACE Activity**

As shown in Figure 1, lisinopril and captopril concentration-dependently inhibited ACE activity in the homogenates of the T-lymphocytes. A complete inhibition of ACE was obtained with 0.1 μmol/L lisinopril, whereas 0.1 μmol/L captopril only inhibited the ACE activity by 49.8% ± 6.5%. Higher concentrations of captopril further inhibited the ACE activity in the T-cell homogenates although to a lesser extent than lisinopril.

**Effect of Chymostatin on ACE Activity**

When homogenates of the T-lymphocytes were incubated with chymostatin (10 μmol/L) for 18 h, no change in ACE activity occurred (8.71 ± 0.03 nmol/10<sup>6</sup> cells/min in control cells, n = 8.71 ± 0.15 nmol/10<sup>6</sup> cells/min in chymostatin-treated cells, n = 4).

**Effect of Bestatin on ACE Activity**

Bestatin dose-dependently increased the ACE activity in homogenates of the T-lymphocytes as well as in intact T-lymphocytes (Figure 2). The percent activation of ACE with 10<sup>-7</sup> to 10<sup>-5</sup> mol/L bestatin was less pronounced (P < .001) in the T-cell homogenates than in the intact T-cells (15.4% ± 2.8% vs 54.1% ± 4.4%, n = 8). Bestatin (10 μmol/L) had, however, no effect on the

**FIGURE 1.** Concentration/response curve for the effect of lisinopril or captopril on the ACE activity in homogenates of T-lymphocytes. Each point represents the mean ± SE from six separate experiments. Basal ACE activity in homogenates of T-lymphocytes averaged 8.36 ± 0.50 pmol/10<sup>6</sup> cells/min.

**FIGURE 2.** Concentration/response curve for the effect of bestatin on the ACE activity in homogenates of T-lymphocytes and intact T-lymphocytes. Values are given as mean ± SE from eight separate experiments.
ACE activity in the microsomal fraction of human T-lymphocytes and in human serum.

Effect of Leupeptin and E-64 on ACE Activity  Leupeptin concentration-dependently inhibited ACE activity in homogenates of T-lymphocytes (Figure 3), whereas E-64 caused only a minor inhibition (on average, 8.6% ± 1.3%), which was independent of dose.

DISCUSSION

Angiotensin-converting enzyme is expressed in peripheral human T(CD2+)-lymphocytes but not in B(CD19+)-lymphocytes.7 The ACE concentration in the two major T-lymphocyte subtypes, CD4 and CD8, is about the same.7 However, the ACE level in peripheral T-lymphocytes is about 2000 molecules/cell, approximately 10 to 20 times less than the three major cell surface proteins, CD3, CD4, and CD8.16

In homogenates of human T-lymphocytes, ACE activity is inhibited by lisinopril and captopril in a concentration-dependent manner (Figure 1), similarly to that in purified human kidney or recombinant endothelial ACE.17 The inhibition of ACE activity by the non–thiol-containing ACEI lisinopril was more pronounced than the one found for the thiol-containing ACEI captopril. However, one should take into account that captopril is known to rapidly form disulfide bands. Hence its true inhibitory potency is difficult to measure in vitro unless the assays are carried out under nitrogen, which was not done in the present study. The ACE activity in human T-lymphocytes is, however, not affected by chymostatin, indicating that chymase is probably not present in human T-cells.

A dual pathway for angiotensin II formation in human hearts in vitro was reported by Urata et al18, approximately 80% of the total angiotensin II formation is due to a previously unknown serine proteinase, whereas ACE-dependent angiotensin II forming activity accounted for only 11% of the total angiotensin II formation. This unknown cardiac serine proteinase is identified as a novel member of the chymase family and is referred to as human heart chymase.19 Although several enzymes such as trypsin, chymotrypsin, tonin, cathepsin G, kallikrein, and rat chymase I can produce angiotensin II from angiotensin I in vitro,20 their physiologic roles in vivo have not been clarified, eg, in the cardiovascular or circulatory system. In addition, some of these enzymes, such as trypsin, chymotrypsin, and rat chymase I also degrade angiotensin II, which makes these enzymes’ physiologic roles in local angiotensin II formation doubtful. As in human T-lymphocytes, no chymase is probably present; the local angiotensin II formation can probably only be ascribed to the action of ACE.

The present study also shows that ACE activity in human T-lymphocytes is not affected by the calpain inhibitor E-64, but is inhibited by the neutral serine proteinase inhibitor leupeptin in a dose-dependent fashion (Figure 3). These findings indicate that some of the neutral proteinases can be involved in the local action of ACE in human T-lymphocytes.

The most unexpected finding of the present study was the stimulation of ACE activity in the homogenates of T-lymphocytes as well as in intact T-lymphocytes by bestatin (Figure 2). The exact mechanism by which bestatin affects the ACE activity in T-lymphocytes remains unknown. However, bestatin does not interfere with the fluorimetric assay of ACE in T-lymphocytes. It does not affect the degradation of histidyl-leucine or the conversion of hippuryl-histidyl-leucine to hippuric acid and histidyl-leucine.

Bestatin is a strong inhibitor of aminopeptidase B and leucine aminopeptidase; the latter two enzymes are present on the surface of mammalian cells,21 including normal fibroblasts, malignantly transformed cells, and lymphocytes.22 There also exist specific binding sites for bestatin on the cell surface of mammalian cells, which leads to the inhibition of leucine aminopeptidase and aminopeptidase B.23

In lymphocytes, these surface membrane-bound proteases may be important in altering the cell surface membrane. This could result in a discharge of a specific signal, initiating a sequence of metabolic alterations inducing changes from the resting to the proliferative stage. Inhibition of these enzymes by bestatin can reverse such a signal and induce an increase in the ACE activity in these T-cells.

However, the effect of bestatin may not be due to specific inhibition of aminopeptidases, but to stimulation of some other biologic activities in the T-lym-
phocytes. Indeed, bestatin is known to exert various biochemical effects on the T-lymphocytes, such as an increase in the proliferation of T-lymphocytes, a stimulation of polysome assembly in T-cell lymphoma, an increase of the activity of DNA polymerase α and terminal deoxynucleotidyl transferase, and a stimulation of uridine uptake and thymidine incorporation into DNA, as well as a bestatin-induced increase in ACE activity, as shown in the present study. However, the mechanism behind this stimulation of ACE by bestatin in T-lymphocytes remains to be elucidated.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the technical and secretarial assistance of Miss T. Coenen, Miss L. Lommelen, Miss Y. Piccart, and Mrs Y. Toremans.

**REFERENCES**