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Hemorphins derived from hemoglobin have an inhibitory action on angiotensin converting enzyme activity

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The hemorphins are opioid active peptides, which are enzymatically released from the beta-chain of hemoglobin. In this paper we report an inhibitory effect of these peptides on angiotensin converting enzyme (ACE) activity, known to be involved in blood pressure regulation. The hemorphins were found to be quite stable in tissue extracts containing ACE, and their importance as naturally occurring ACE inhibitors is discussed.

Hemorphin; ACE-activity; Inhibition; Blood pressure regulation; HPLC; Mass spectrometry

1. INTRODUCTION

The hemorphins are hemoglobin-derived peptides with affinity for opioid receptors [1,2]. They were originally isolated from enzymatically treated bovine blood [2] and the smallest active fragment Tyr-Pro-Trp-Thr (hemorphin-4) represents fragment 34–37 of the beta-chain of bovine hemoglobin. The same fragment occurs in the human protein, at the position 35–38 of the beta-, gamma-, delta-, or epsilon- chain [2]. The N-terminal, Tyr-Pro, is also found in the beta-casomorphins, which have previously been shown to inhibit ACE activity [3]. Recently, an opioid active fragment of hemoglobin was isolated from the human pituitary gland [4]. This peptide (LVV-hemorphin-6) corresponds to the sequence at position 32–40 of the beta-chain of the human entity. Its physiological significance is unknown, so far, but since it is found in large amounts in the pituitary, it is likely to also occur in the circulation.

Angiotensin converting enzyme (ACE) is a peptidyl dipeptide hydrolase (EC 3.4.15.1), which liberates the octapeptide angiotensin II from the decapeptide angiotensin I, by cleaving off the C-terminal dipeptide His-Leu [5]. Moreover, it inactivates the vasodilator bradykinin, by sequentially removing its 2 C-terminal dipeptides [6]. Angiotensin II is a strong vasoconstrictor agent, which also stimulates the release of the salt-retaining hormone aldosterone from the adrenal cortex [7]. These actions elevate the blood pressure. The development of ACE inhibitors like captopril [8], enalapril and lisinopril [9] has made it possible to treat hypertension in a new effective way. A characteristic of these inhibitors is that they have a proline residue in the C-terminal of their amino acid sequence. The beta-casomorphins, which also contain a proline residue, have already been shown to inhibit ACE activity [3]. Since the hemorphins have the same N-terminal, Tyr-Pro, it is of interest to investigate their action on ACE activity, as well.

The degradation of circulating angiotensin I to angiotensin II mainly occurs by the ACE bound to the vascular walls of the blood vessels in the lungs [10,11]. Therefore, we investigated the stability of the synthesized peptides, LVV-hemorphin-6 and hemorphin-6 towards membrane bound lung enzymes, including ACE. Incubations were performed with and without different protease inhibitors. The peptide fragments were separated by HPLC and analysed by mass spectrometry. The influence of the hemorphins on ACE activity was examined by measuring their inhibitory effect on the hydrolysis of hippuryl-histidyl-leucine, a specific substrate for ACE activity.

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2. MATERIALS AND METHODS

2.1. *Materials*

The synthetic peptides used in this study were synthetized by Dr G. Lindeberg, Department of Immunology, University of Uppsala, Uppsala, Sweden. The ACE substrate hippuryl-histidyl-leucine (hip-His-Leu) was from Cambridge Research Biochemicals and the ^{14}C-labelled hip-His-Leu was purchased from NEN Products (Stockholm, Sweden). The protease inhibitors amastatin, phosphoramidon, EDTA, phenylmethylsulphonylfluoride (PMSF), parahydroxymercuribenzoic acid (pHMB) and diisopropylfluorophosphate (DFP) were from Sigma, and captopril was from Squibb. All other chemicals and solvents were of analytical grade from commercial sources. A Pharmacia/LKB system was used for the RP-HPLC separation. The instrument was equipped with a Silica gel C-18 column (Ultrapac Column TSK ODS 120 T, 4.6×250 mm, particle size 5.0 μm).

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2.2. *Enzyme preparation*

The enzyme extract was prepared from human lung tissue, which was obtained at autopsy from patients with no known lung disease. The time between death and autopsy did not exceed 48 h. Within less than 1 h after autopsy the tissue was stored at −70°C. When preparing the extract, 80 g of the tissue was thawed and homogenized in 5 vols (400 ml) of 50 mM Tris-HCl buffer at pH 7.8. The solution was centrifuged in a Beckman J-2-21 centrifuge at $15000 \times g_{max}$ for 40 min. The pellet was washed in 20 mM Tris-HCl buffer, pH 7.8, with subsequent centrifugation, 3 times. The membrane-bound enzymes were solubilized by stirring the pellet material with buffer containing 0.5% Triton X-100 at 4°C for 16 h. After a final centrifugation, the enzyme containing supernatant was stored in aliquots at −70°C.

2.3. *Inhibition studies*

The ACE activity was monitored by measuring the hydrolysis of hip-His-Leu. The enzyme extract was incubated, in polyethylene tubes with 100 μM unlabelled and 15000 cpm of ^{14}C-labelled hip-His-Leu (spec. act. 3.0 mCi/mmol) for 0, 20, 40, 80 or 120 min at 37°C, and buffered at pH 7.8 with 20 mM Tris-HCl, all in a final volume of 100 μl. Amastatin (20 μM) and phosphoramidon (10 μM) were added to avoid degradation of the inhibiting peptides. For the inhibition assay, bradykinin, Tyr-Pro, hemorphin-4, hemorphin-6 and LVV-hemorphin-6, in a final concentration of 100 μM, were used. The reaction was terminated by adding 100 μl of 1 M hydrochloric acid. To extract the released product, hippuric acid, 2 ml ethylacetate/heptane (1:1) was added, vortexed for 15 s and centrifuged for 10 min at $500 \times g$ in a 2161 Midispin R centrifuge (LKB, Bromma, Sweden). A 1 ml aliquot of the upper layer was removed for counting in a Packard liquid scintillation counter.

2.4. *Conversion studies*

Nanomolar concentration of the peptides, hemorphin-6 and LVV-hemorphin-6, were incubated at 37°C in Eppendorf tubes with the enzyme extract in the presence of various protease inhibitors, such as PMSF (0.2 mM), pHMB (0.2 mM), EDTA (1.0 mM), DFP (0.2 mM), amastatin (20 μM), captopril (10 μM) and phosphoramidon (10 μM) in a final volume of 100 μl. The reaction mixture was buffered with 20 mM Tris-HCl buffer at pH 7.8. The incubation time varied from 1 h up to 7 h. The reaction was terminated by adding 200 μl ice-cold ethanol, followed by centrifugation in a Beckman Microfuge B for 5 min. The supernatant was evaporated in a Savant Vac concentrator (Hicksville, NY, USA) prior to RP-HPLC separation of the generated products.

2.5. *Reversed phase high performance liquid chromatography (RP-HPLC)*

The degradation products were resolved on a Silica gel C-18 column with a nonlinear gradient of acetonitrile (ACN), containing 0.04% trifluoroacetic acid (TFA). The gradient was 15–60% of ACN solution in 40 min and 60–80% in 10 min. The flow rate was 0.5 ml/min and fractions of 0.5 ml were collected and evaporated prior to mass spectrometry analysis.

2.6. *Mass spectrometry analysis*

The generated peptide fragments were analysed by plasma desorption mass spectrometry (PDMS) [12] on a BioIon 20 instrument (Applied Biosystems AB, Sweden).

3. RESULTS AND DISCUSSION

Since the hemorphins might be formed in plasma during physiological or pathological degradation of hemoglobin, it is interesting to determine their action on ACE activity. Through this mechanism they may have a part in the regulation of blood pressure. Before studying the inhibitory effect on ACE activity of the hemorphins, the stability of the peptides in the enzyme extract was examined. When hemorphin-6 was incubated with the lung enzyme extract, the Thr-Gln bond was cleaved and the hemorphin-4 fragment was formed (Table I, Fig. 1). The hydrolysis was inhibited by EDTA and phosphoramidon, but was not affected by any other of the enzyme inhibitors used (see Section 2.4). This indicates that a thermolysin-like metalloendopeptidase (enkephalinase or EC 3.4.24.11) [13], is responsible for the cleavage. The tetrapeptide,

Table I

Hemorphins hydrolyzed in vitro by human lung extract

Peptide	Structure and cleavage point
Hemorphin-6	Tyr-Pro-Trp-Thr↓-Gln-Arg
LVV-Hemorphin-6	Leu-Val-Val-Tyr-Pro-Trp-Thr↓-Gln↓-Arg

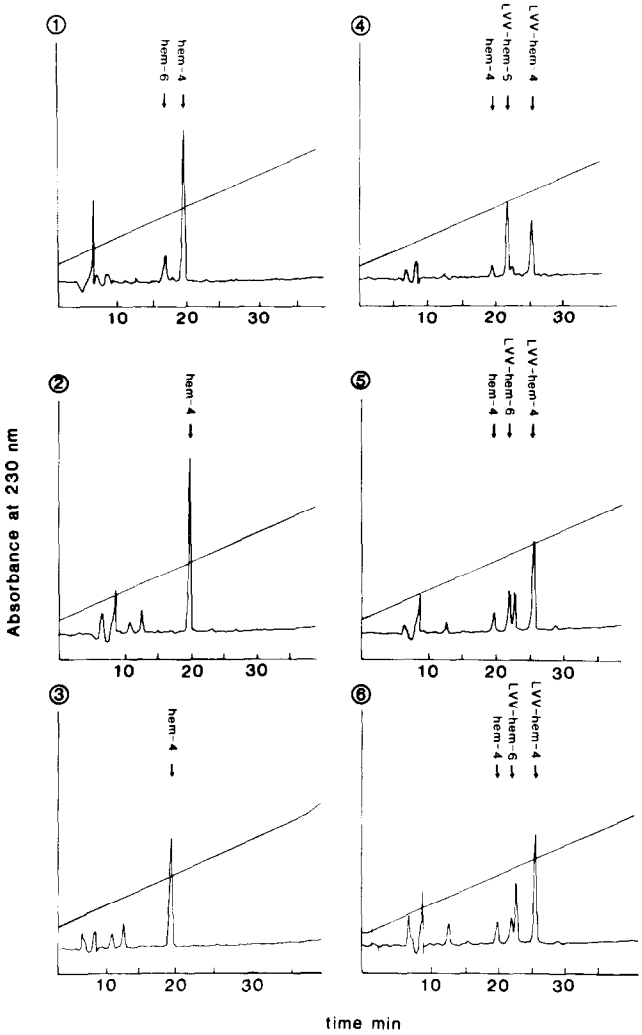

Fig. 1. HPLC separations of hemorphin-6 (1–3) and LVV-hemorphin-6 (4–6) and their degradation products after 1, 3, and 7 h of incubation. The identity of the peptide fragments was confirmed by mass spectrometry.

Table II

ACE activity in the presence of bradykinin and hemorphin related peptides, all in 100 μM concentration

Peptide	Hippuric acid released pmol/40 min	% inhibition
Control	437.9	0
Bradykinin	9.4	98
Tyr-Pro	343.1	22
Hemorphin-6	161.7	63
LVV-Hemorphin-6	62.3	86

hemorphin-4, was very stable and remained even after 7 h of incubation (Fig. 1).

The longer peptide, LVV-hemorphin-6, was incubated with the same enzyme extract and for the same incubation times. After 1 h, with no inhibitor present, Gln-Arg was released (Table I, Fig. 1). Continued incubation up to 7 h gave no further cleavage of the LVV-hemorphin-4 fragment. In the presence of phosphoramidon, there was no change in the cleavage pattern, suggesting another enzyme responsible for the hydrolysis of the Thr-Gln bond, than that cleaving hemorphin-6. None of the peptidase inhitors (captopril, amastatin, pHMB or PMSF) had any action on the cleavage pattern, while EDTA showed an unexpected effect. The hemorphin-4 fragment was released, thus indicating that the Val-Tyr bond is cleaved by an enzyme sensitive to metal ions. Since hemorphin-6 was not seen when LVV-hemorphin-6 was incubated, the cleavage of the Thr-Gln bond is likely to precede the hydrolysis of the Val-Tyr bond. Accordingly, in the presence of phosphoramidon hemorphin-6 remained unconverted following one hour of incubation with the lung extract, whereas about 40% of LVV-hemorphin-6 was converted to LVV-hemorphin-4. Both hemorphin-6 and LVV-hemorphin-4 remained stable up to 7 h.

All inhibitory experiments were performed in the presence of amastatin and phosphoramidon within a time course of 2 h. The most potent of the hemorphin related peptides assayed, appeared to be LVV-hemorphin-6, which inhibited the ACE activity by 86% (at 100 μM) compared to 98% for bradykinin (100 μM) at 40 min of incubation (Table II and Fig. 2). Compared to hemorphin-6, the LVV-hemorphin-6 was 1.4 times more potent and 4.0 times more potent than Tyr-Pro at the same concentration and incubation time. A definite inhibitory action was also seen by the hemorphin-4, which is the most stable metabolite. The fragment was prepared by enzyme incubation of hemorphin-6 and purified by HPLC and thereby the concentration could not be precise.

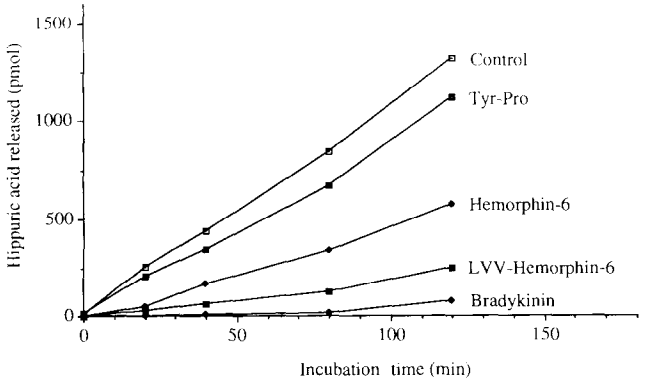

Fig. 2. Time courses of the hydrolysis of hip-His-Leu by human lung ACE activity in the presence of bradykinin and hemorphin related peptides.

When attempts were made to isolate LVV-hemorphin-6 from plasma [3], it was not successful, probably because of its EDTA content. In this study we observed that EDTA enhanced the degradation of that very peptide, so it is quite possible that it occurs in plasma as well as in the pituitary. In conclusion, the most important hemorphin was LVV-hemorphin-6. Its stability was high towards the enzyme activities in the lung extract and its inhibitory action on ACE activity was the strongest of the hemorphins assayed. Hemorphin-6 was less stable and appeared to be less potent in inhibiting the ACE activity. Further investigation needs to be done with the metabolites, LVV-hemorphin-4 and hemorphin-4. The natural concentrations of these peptides in plasma also need to be determined and thereby their possible importance in blood pressure regulation.

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