The Hydra head activator in human blood circulation

Degradation of the synthetic peptide by plasma angiotensin-converting enzyme

Michel Roberge, Emanuel Escher*, H. Chica Schaller and Heinz Bodenmüller

Abteilung Biophysik, Max-Planck-Institut für Medizinische Forschung, Jahnstr. 29, 6900 Heidelberg, FRG and **Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada*

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Using methanol extraction combined with HPLC and a new radioimmunoassay, the peptide head activator was detected in human plasma at a concentration of 20–100 fmol/ml. Synthetic head activator incubated with plasma was degraded with a half-life of 7 min. Analysis of sites of enzymatic cleavage and inhibition by captopril showed a major involvement of angiotensin-converting enzyme in this process. Endogenous head activator, on the other hand, was not appreciably degraded upon incubation of plasma in vitro. These findings raise the possibility that the endogenous peptide could bind to a protective carrier molecule and reach potential target tissues via the blood circulation.

Hydra head activator *Radioimmunoassay* *Human plasma* *HPLC* *Neuropeptide* *Angiotensin-converting enzyme*

1. INTRODUCTION

The hydra head activator (HA) is an undecapeptide originally discovered in hydra where it exerts stimulatory effects on growth and differentiation processes involved in head formation [1,2]. Its sequence, Glp-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe, was determined from hydra and sea anemones [3] and subsequently from rich mammalian sources such as human and bovine hypothalamus as well as rat intestine [4]. Investigations on the physiological role of this peptide in mammals indicate that, in addition to other functions, it could be involved in digestion control processes, since it was found to stimulate amylase secretion from rat pancreas in vitro [5]. From its prominent location in the upper part of the small intestine it could be released and reach the pancreas via the blood circulation. Here, we took advantage of a new sensitive radioimmunoassay (RIA) for the HA [6] to demonstrate that this peptide circulates in human plasma at a concentration of 20–100 fmol/ml. Synthetic peptide added to plasma was rapidly degraded. We describe here the predominant role of angiotensin-converting enzyme (ACE, EC 3.14.15.1) in this process. The concentration of endogenous HA, however, remained constant upon incubation of plasma in vitro, thereby suggesting a mechanism for protection of the peptide from proteolytic activity. These findings raise the possibility that the HA could exert actions of physiological significance via the circulation.

2. MATERIALS AND METHODS

2.1. *Peptides and inhibitors*

HA was obtained from Bachem, Switzerland. [^{3}H]Pro^{2,3}-HA (40–60 Ci/mmol) was prepared by catalytic tritiation of (3,4-dehydro-Pro^{2,3})-HA as in [7]. [^{3}H]Phe^{11}-HA (30 Ci/mmol) was obtained upon catalytic tritiation of (4′-I)Phe^{11}-HA in the

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laboratory of Dr J.-L. Morgat, Centre d'Etudes Nucléaires, Saclay, France. Affinity chromatography-purified rabbit lung ACE was a generous gift from Dr Peter Bünning, Freiburg. Captopril (SQ 14,225) was given by Dr Thomas Unger, Heidelberg, teprotide (SQ 20,881) was purchased from Bachem, soybean trypsin inhibitor from Sigma, München, and phenylmethylsulfonyl fluoride from Serva, Heidelberg.

2.2. *Extraction of HA from human plasma and radioimmunoassay*

For determination of the concentration of HA in plasma (see section 3.1), blood from fasting human volunteers was collected in EDTA (1–2 mg/ml). Blood collected in heparin (50 IE/ml) was used as a source of plasma for all other studies. The extraction procedure in [7] was applied without modification. Briefly, endogenous HA was extracted twice in 9-fold excess (v/v) of methanol and further purified by adsorption to C_{18} Sep-pak cartridges (Waters) equilibrated with water. After washing with water and 20% methanol, HA was desorbed with 80% methanol. The HA content of the extracts was determined in a RIA employing ^{125}I-labelled Tyr^{11}-HA as a tracer [6] and the antiserum 12/4 showing N-terminal specificity [7].

2.3. *Assay of HA-degrading activity*

All incubations were performed at 37°C. The reaction was initiated by addition of [^{3}H]Phe^{11}-HA at the indicated concentration and terminated by acidification to pH 2 with 0.5% trifluoroacetic acid (TFA). The C-terminal degradation fragments could be rapidly and conveniently separated from intact HA by applying 1 ml of the acidified mixture on a C_{18} Sep-pak cartridge equilibrated with water. The cartridge was then sequentially washed with 1 ml water and 6 ml of 30% methanol which eluted all the labeled C-terminal fragments of HA. These eluates were collected together in radioactivity vials, frozen to −80°C, lyophilised and counted for radioactivity in a Kontron MR beta counter. Intact HA was eluted with 80% methanol. The cartridge could then be washed with 10 ml methanol, regenerated with 10 ml water and reutilized up to 10 times.

2.4. *HPLC separation and amino acid analysis of HA fragments*

Samples containing high concentration of protein were applied on Sep-pak cartridges equilibrated with water and washed with 2 ml water. The peptides were eluted with 10 ml methanol and concentrated by rotatory evaporation. HPLC separation was performed using a C_8 reverse phase column (LiChrosorb, 5 μm particle size) of 250 × 4 mm column size for work involving synthetic peptide or 250 × 10 mm for analysis of endogenous HA. Elution was carried out with a 40 min linear gradient from 0 to 40% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min for the first column and 4 ml/min for the second one. For determination of radiolabeled fragments, 1-ml fractions were collected and counted for radioactivity. Emerging peaks destined for amino acid determination were collected, dried and hydrolysed in 6 N HCl at 110°C for 24 h. Amino acid analysis was performed after dansylation by thin-layer chromatography [3] and quantitative confirmation was obtained using a Biotronik analyser.

3. RESULTS

3.1. *Concentration of HA in plasma*

The amount of immunoreactive HA was determined after methanol extraction and purification on Sep-pak cartridges in a RIA using ^{125}I-labelled Tyr^{11}-HA as a tracer and antiserum 12/4 which binds preferentially to the N-terminal portion of HA [7]. The concentration found for different individuals was in the range 20–100 fmol/ml. Chromatography on reverse-phase HPLC yielded one major peak of immunoreactivity of retention time identical to that of tritiated synthetic HA (fig.1). The competition line of this material was parallel to that of HA (not shown) and this immunoreactivity was therefore most probably due to HA itself.

3.2. *Degradation of HA in plasma*

The degradation of tritiated HA added to plasma was monophasic, showing a half-life of 7 min (fig.2). Plasma was subjected to gel filtration on a Sephacryl S-300 column (Pharmacia) equilibrated with 25 mM hydroxyethylpiperazine-sulfonic acid, 150 mM NaCl, pH 7.5 at 4°C. The HA-degrading activity was measured by hydrolysis

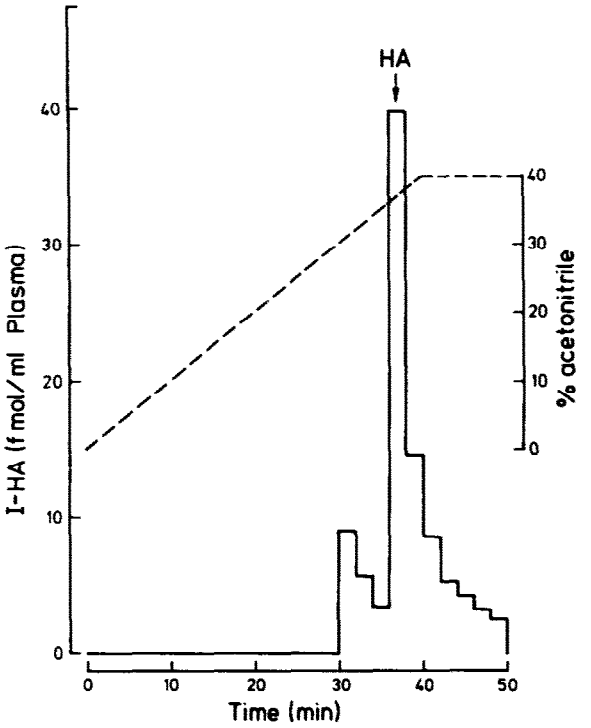

Fig.1. Reverse phase HPLC of human plasma immunoreactive HA (I-HA). Two-min fractions were collected, lyophilised and the content of I-HA was measured as described in section 2.2. The column was calibrated with [^{3}H]Phe^{11}-HA in a separate run.

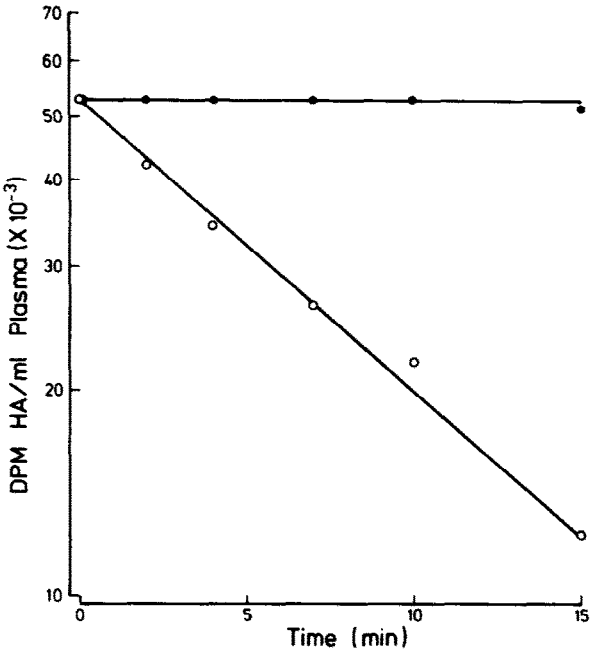

Fig.2. Degradation of HA in plasma. Plasma was preincubated 20 min at 37°C without (○) or with 10^{-6} M captopril (●) and aliquots were removed at various times after addition of 0.9 nM [^{3}H]Phe^{11}-HA. Intact HA was separated from degradation fragments as described in section 2.3.

of [^{3}H]Phe^{11}-HA and eluted as one major peak of K_{av} = 0.25 (fig.3). The active fractions were pooled and referred to as the 'K_{av} = 0.25 peak'. A second, minor peak of activity of M_r 30000 was sometimes detected and then represented less than 15% of the degrading activity recovered from the column. This component was not further characterized in this study. The effect of a number of inhibitors on the degradation of HA by the K_{av} = 0.25 peak is presented in table 1. The inhibitors of serine-dependent proteases, phenylmethylsulfonyl fluoride and soybean trypsin inhibitor showed little or no effect. The ACE inhibitors captopril and teprotide exerted powerful inhibition at low concentrations. The chelating agent EDTA and the sulfhydryl compound dithiothreitol were also potent inhibitors. Captopril at a concentration of 10^{-6} M could completely inhibit the degradation of tritiated HA by plasma over a period of more than 15 min (fig.2). Purified rabbit lung ACE applied on the Sephacryl S-300 column eluted at the same position as the HA-degrading peak (fig.3). These data strongly suggested identity of the K_{av} = 0.25 peak with ACE and to assess this possibility further, we compared the sites of cleavage of HA by these two enzyme activities.

3.3. *Identification of degradation products*

The use of HA tracers tritiated at either end of the peptide proved ideal to identify the peptide fragments produced by the enzyme activity of the plasma. [^{3}H]Pro^{2,3}-HA was used to localize peptide fragments containing the N-terminal sequence of HA and [^{3}H]Phe^{11}-HA to trace the C-terminal peptides. The degradation fragments produced by plasma and by the K_{av} = 0.25 peak were compared to those produced with purified ACE after separation on reverse phase HPLC with a gradient of acetonitrile in 0.1% TFA. These results are shown in fig.4. The degradation pattern of [^{3}H]Pro^{2,3}-HA was similar in all three cases. It showed two peaks

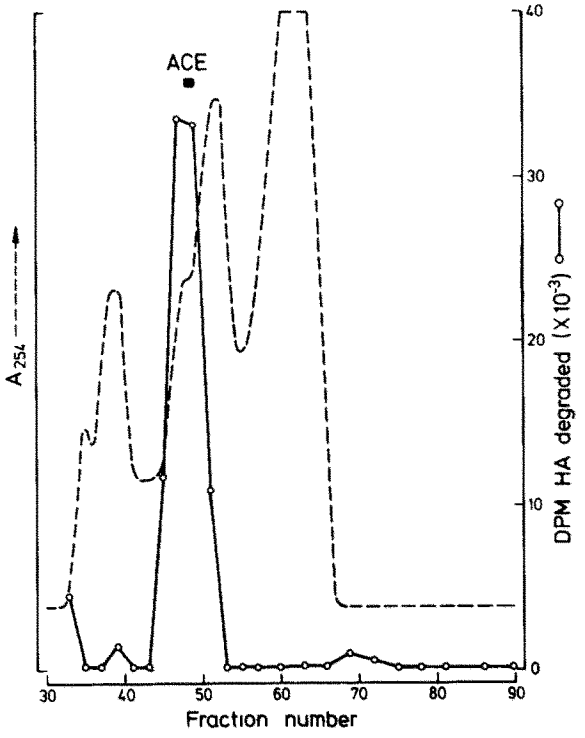

Fig.3. Sephacryl S-300 separation of HA-degrading activity of human plasma. Plasma (4.5 ml) was applied on the column and the elution of proteins was monitored by absorbance at 254 nm (---). The column was equilibrated with 25 mM hydroxyethylpiperazinesulfonic acid, 150 mM NaCl (pH 7.5) at 4°C: 5-ml fractions were collected and 1-ml samples were assayed for HA-degrading activity with 0.6 nM [^{3}H]Phe^{11}-HA as described in section 2.3. In a separate run, purified rabbit lung ACE was applied on the column and its elution was monitored by change in absorbance at 328 nm during incubation with the substrate furanacryloyl-Phe-Gly-Gly [9], and by hydrolysis of [^{3}H]Phe^{11}-HA.

of radioactivity with retention times of 13 and 25 min in addition to intact HA which eluted at 36 min. Following incubation with plasma, the K_{av} = 0.25 peak and purified ACE, [^{3}H]Phe^{11}-HA degradation yielded one common peak of retention time 28 min apart from intact HA. In addition, a peak of radioactivity eluting at 13 min was produced upon incubation with plasma and the K_{av} = 0.25 peak, but not after incubation with purified ACE. To determine the amino acid composition of these fragments, unlabeled HA (50 nmol/ml) was incubated with purified ACE and the degradation

Table 1

Effect of inhibitors on the degradation of HA by the K_{av} = 0.25 peak[a]

Inhibitor	Concentration	% Inhibition
Phenylmethylsulfonyl-fluoride	10^{-3} M	5.2
Soybean trypsin inhibitor	0.1 mg/ml	1.2
Captopril	10^{-7} M	92.5
	10^{-8} M	62.1
Teprotide[b]	10^{-5} M	99.2
	10^{-6} M	97.8
EDTA	10^{-3} M	99.6
Dithiothreitol	10^{-3} M	91.1

[a] Fractions 44–51 from Sephacryl S-300 separation were pooled and 1 ml preincubated 20 min at 37°C with the various inhibitors at the indicated concentrations. The reaction was initiated by adding 0.6 nM [^{3}H]Phe^{11}-HA and terminated after 3 h by acidification with 0.5% TFA. The extent of degradation was measured as described in section 2.3

[b] Teprotide: Glp-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

peptides were separated by HPLC and monitored by absorbance at 210 nm. Fig.5 shows the chromatographic profile generated after 2 h incubation. The retention times and amino acid analysis of these peaks are summarized in table 2. The results show cleavage of HA at the Gly^5-Ser^6, Lys^7-Val^8 and Ile^9-Leu^{10} peptide bonds. The reaction yielded the dipeptides Leu-Phe and Val-Ile. This is consistent with the known dipeptidyl carboxypeptidase activity of ACE. The dipeptide Ser-Lys was not detected but was probably also produced since the fragments Glp-Pro-Pro-Gly-Gly-Ser-Lys and Glp-Pro-Pro-Gly-Gly were produced. We suspect that this dipeptide eluted in the flow-through of the column because of its hydrophilic properties. The time course study of generation of these degradation products (fig.6) shows a disappearance of HA proportional to the appearance of Leu-Phe in the medium. The Glp-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile fragment first accumulated but its concentration began to drop after 2 h as Val-Ile was formed. This demonstrates the sequential removal of Leu-Phe and Val-Ile from HA. The fragment Glp-Pro-Pro-Gly-Gly-Ser-Lys which should be produced upon cleavage of Val-Ile was not detected in our

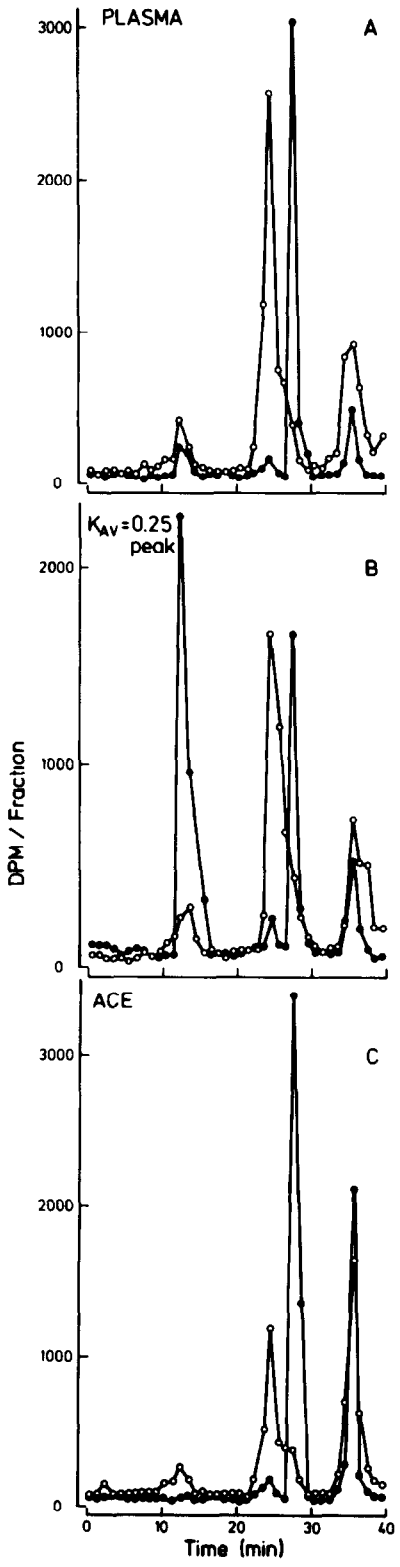

Fig.4. Reverse phase HPLC of degradation fragments produced by incubation of [^{3}H]Pro^{2,3}-HA (○) and [^{3}H]Phe^{11}-HA (●) with plasma (A), the K_{av} = 0.25 peak (B) and purified ACE (C). The tritiated peptides were incubated 30 min with 100 μl plasma, 3 h with 1 ml K_{av} = 0.25 peak and 90 min with 0.7 mg/ml purified ACE. HPLC separation was performed as in section 2.4.

chromatograms. It was possibly very rapidly degraded to Glp-Pro-Pro-Gly-Gly and Ser-Lys which accumulated in the medium and not lost on HPLC separation because quantitation of the peptide fragments did not show any loss of N-terminal peptide. Comparison of these results with those obtained by incubating radioactive HA with plasma and with the K_{av} = 0.25 peak (fig.4) shows

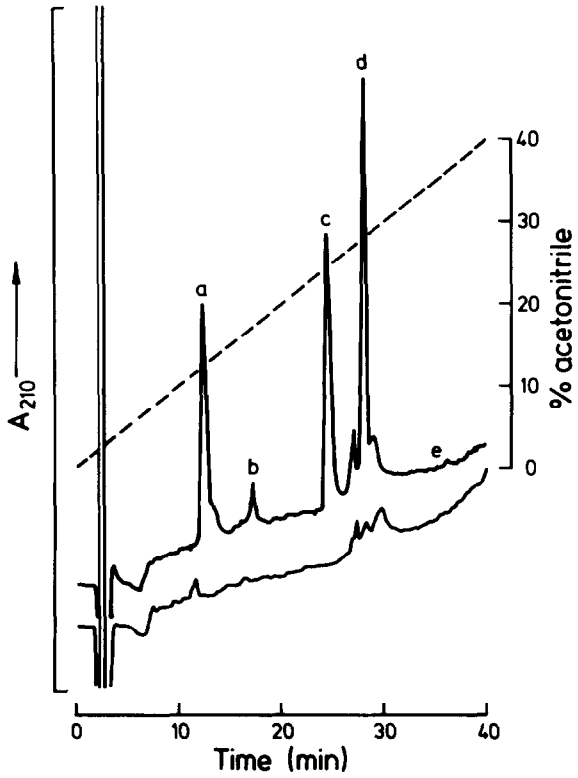

Fig.5. Reverse phase HPLC of HA degradation by purified ACE. HA (50 nmol/ml) was incubated 2 h at 37°C with 0.7 mg/ml ACE in 50 mM hydroxyethylpiperazinesulfonic acid, 300 mM NaCl (pH 7.5) and 5 nmol were submitted to HPLC as in section 2.4 (upper curve). Control incubations were performed without substrate (lower curve).

Table 2

HPLC separation of peptides derived from degradation of HA by angiotensin-converting enzyme

Peak	Retention time (min)	Peptide[a]
a	13	Glp-Pro-Pro-Gly-Gly
b	18	Val-Ile
c	25	Glp-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile
d	28	Leu-Phe
e	36	Glp-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe

[a] Determined by amino acid analysis as described in section 2.4

that the proline-labeled peaks of retention times 13 and 25 min correspond to peaks a and c and that the phenylalanine-labeled peak of retention time 28 min is Leu-Phe (peak d). With the exception of fragment Val-Ile which is not labeled and therefore could not be detected by the tracer method, the fragments produced by the enzyme activity of the plasma and the K_{av} = 0.25 peak correspond to those produced by ACE and are the result of a dipeptidyl carboxypeptidase activity. The only difference, namely the phenylalanine-labeled peak which elutes at 13 min, was identified as tritiated Phe on the following basis: HPLC of progressively

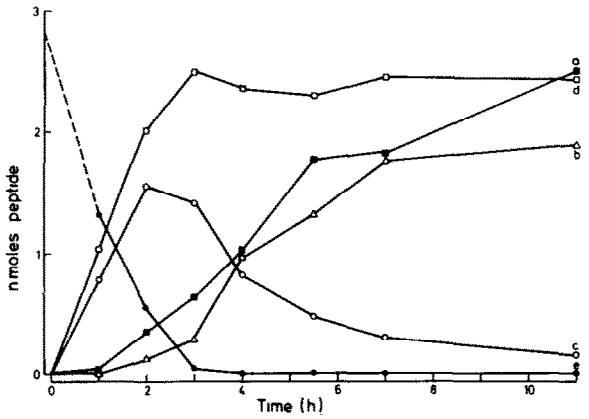

Fig.6. Time course of degradation of HA by ACE. HA (50 nmol/ml) was incubated with purified ACE (0.7 mg/ml) in 50 mM hydroxyethylpiperazinesulfonic acid, 300 mM NaCl (pH 7.5) and aliquots were submitted to HPLC at different times. The amount of peptide in each peak was determined by quantitative amino acid analysis as described in section 2.4. The peptides are labeled a–e and their amino acid composition is given in table 2.

shorter C-sequences of HA starting from Ser-Lys-Val-Ile-Leu-Phe to Phe showed that only free Phe eluted at this position. It was most probably produced by cleavage of the fragment Leu-Phe rather than by a carboxypeptidase activity on HA itself. This interpretation is strengthened by the observation that the appearance of radioactivity in the Phe peak was proportional to the disappearance of radioactivity in the Leu-Phe peak at time points where no more labeled HA was present in the medium (not shown). Moreover, the Phe peak was not produced in the presence of concentrations of captopril which inhibit ACE but not most of the other peptidases. It has been indeed demonstrated that dipeptide-degrading activity often copurifies with ACE isolated from plasma even after several isolation steps of the converting enzyme [8]. The affinity chromatography-purified rabbit lung ACE used in this study was devoid of such a contaminating dipeptidase activity.

3.4. *Degradation of endogenous HA*

To determine whether plasma endogenous HA is degraded with the same kinetics as exogenous synthetic peptide, fresh plasma was incubated at 37°C and aliquots were removed at different times for determination of HA content by RIA after methanol extraction and HPLC purification. The concentration of endogenous HA did not decrease with time, suggesting that the peptide occurs in plasma in a form which is protected from enzyme degradation.

4. DISCUSSION

Here, we have found that the peptide HA occurs

in human plasma at a concentration of 20–100 fmol/ml. This value is comparable to most of the other peptide hormones whose unstimulated plasma concentrations are usually within the range 5–150 fmol/ml. Synthetic tritiated HA was degraded by a plasma enzyme which was identified as ACE on the following basis: (i) purified ACE and the HA-degrading activity eluted at the same position on Sephacryl S-300 gel filtration; (ii) the HA-degrading activity was inhibited by the ACE inhibitors captopril and teprotide at concentrations allowing for extensive inhibition of ACE while leaving most of other protease activities unaffected [10], and by EDTA which is also a potent inhibitor of the Zn^{2+}-dependent ACE [11]; (iii) the degradation patterns of both ACE and the plasma HA-degrading enzyme were similar, liberating dipeptidyl residues from the C-terminus of HA.

ACE is known to occur in human plasma [11,12]. However, plasma is not the main source of ACE activity of the organism. In fact, most of the enzyme is bound to the luminal surface of endothelial cells with particularly high concentrations in the lungs. The half-life of angiotensin I in plasma is of the order of minutes while in vivo, it is transformed to angiotensin II in the 3–5 s required for the blood to pass through the pulmonary circulation [13]. At physiological concentrations, bradykinin is also almost completely inactivated on passage through the lung circulation [14]. It is therefore to be expected that the half-life of 7 min estimated for HA in human plasma would be considerably shorter upon injection of the peptide in the blood circulation. Indeed, we have observed that the half-life of HA injected in the venous circulation of anesthetized rats, which includes a clearance part in addition to enzymatic degradation, is less than 30 s while the half-life measured in plasma in vitro was similar to that in human plasma (unpublished). However, the endogenous circulating HA was found to be very stable against proteolytic degradation. This could be due to adsorption of HA to a larger molecule. This possibility is substantiated by preliminary experiments which indicate that an important proportion of the plasma peptide is adsorbed to a protein fraction which elutes on molecular sieve columns in the high molecular mass range. This association could protect the HA from degradation. The peptide can be desorbed from this fraction by methanol extraction, a procedure normally used for HA isolation. The behavior of this extracted molecule both on HPLC and in the RIA indicates identity with synthetic HA.

More investigations will be required to characterize this carrier molecule and the type of binding which renders the peptide resistant to proteolytic degradation.

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