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Characterization of New Milk-derived Inhibitors of Angiotensin Converting Enzyme *In Vitro* and *In Vivo*

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Inhibition of angiotensin converting enzyme (ACE) has been observed with a variety of different peptides, and peptide fragments with inhibitory capabilities have been identified within many different proteins, including milk proteins. The purpose of this study therefore was to identify new short peptides with inhibitory properties from the primary structure of milk proteins and to characterize them *in vitro* and *in vivo*, since no milk derived ACE inhibitors have previously been evaluated for their ability to inhibit ACE *in vivo*. *In vitro*, 8 of 9 dipeptides were found to be competitive inhibitors of ACE. The IC_{50} was significantly lower when an angiotensin I-like substrate was used, than when a bradykinin-like substrate was used. Using three different *in vivo* models for ACE inhibition, a very moderate effect was observed for three of the new peptides, but only for up to 6 or 12 minutes. Nothing was observed with two reference compounds that are reported to be hypotensive ACE-inhibitors derived from milk proteins. This raises the question whether the mechanism of hypotensive action is straightforward inhibition of ACE *in vivo*.

Keywords: Angiotensins; Angiotensin converting enzyme; Bradykinin; Michaelis–Menten enzyme kinetics; Milk derived peptides; Rats

Abbreviations: ACE, angiotensin converting enzyme; Ang., angiotensin; HHL, Hippuryl-histidyl-leucine; HPA, Hippuryl-phenyl-alanyl-arginine

INTRODUCTION

Several inhibitors of angiotensin converting enzyme (ACE, E.C. 3.4.15.1.) have been found in fermented milk.[1] They are formed by the action of bacterial proteinase activity on milk proteins, notably casein, resulting in peptide fragments that are inhibitory towards the enzyme. Some of the identified peptides have also been shown to lower blood pressure in hypertensive rats.[1,2] The milk derived peptides with ACE inhibitory potential have IC_{50}-values in the micromolar range, which is comparable to that of some of the inhibitory peptides that have served as lead structures in the development of inhibitors used nowadays in the clinic against hypertension and cardiac failure. The best characterized peptides found in fermented milk are Ile-pro-pro (IC_{50} = 5 μM), Val-pro-pro (9 μM) and Lys-val-leu-pro-val-pro (5 μM). ACE has a variety of naturally occurring substrates including angiotensin I (resulting in formation of angiotensin II, a potent pressor agent), bradykinin (a depressor agent, which is degraded into inactive fragments by ACE) and other peptides with little structural resemblance. Great structural diversity is also observed among the inhibitors identified up to now. For peptide inhibitors, the inhibition characteristics are determined mainly by the C-terminal sequence, especially the last two or three amino acids, which is partly explained by the fact that ACE is a C-terminal dipeptidase,[3,4] whereas the N-terminal part of the peptide has been reported to play a minor role. The ACE inhibitors used nowadays in the clinic today is mainly short lipophilic peptide analogues (often based on the common theme X-proline, where X for instance can be cysteine; captopril is a structural analogue of cysteinyl-proline,[3,4] even though the naturally occurring peptidic inhibitors have great diversity. The variety of structures that may inhibit ACE thus

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leads to the speculation that in any protein, a high number of peptides with inhibitory activity towards ACE may be embedded. In the present study we therefore tried to identify peptides from the primary structure of milk protein with inhibitory activity towards ACE and characterize their pharmacological potential towards ACE *in vitro* using both an angiotensin I-like substrate and a bradykinin-like substrate. The use of the two different substrates seemed justified, as it has previously been shown that inhibitors of ACE have IC_{50}-values that are reported to be dependent on the substrate used[5,6]; this in turn calls for testing of effects *in vivo* using models based on both angiotensin I and bradykinin metabolism. A direct effect of the milk-derived inhibitors on ACE itself rather than blood pressure has never been evaluated *in vivo*, so this work is also aimed at the study of the milk-derived inhibitors on ACE in living rats, by using three different models indirectly quantifying conversion of angiotensin I to angiotensin II, or deactivation of bradykinin, respectively.

MATERIALS AND METHODS

Chemicals

Peptides that were included in this study are summarized in Table I. Rabbit lung ACE and the dipeptides were obtained from Sigma Chemicals Inc. (Ohio, USA). Lysyl-valyl-leucyl-prolyl-valyl-proline (KVLPVP) was obtained from Schafer-N (Copenhagen, Denmark.). Isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP) were obtained from AnaSpec Inc. (San Jose, USA). Bradykinin, angiotensin I and angiotensin II were from Calbiochem (Darmstadt, Germany). Hexamethonium chloride was from ICN Biomedicals (Costa Mesa, USA). Hippuryl-histidyl-leucine

TABLE I The peptides used in this study

Peptide	Source containing peptide in primary sequence*	Comment
Ala-asp	LF: f73-74, f241-242, f408-409, f514-515.	A peptide, which is not expected to be an inhibitor, because of its hydrophilic nature.
Ala-pro	α_{s1}CN: f41-42. βCN:f118-119. LF: f50-51, f256-257, f511-512.	C-terminal part of FFVAP, which is reported to be an inhibitor of ACE ($IC_{50} = 6\,\mu M$).[15] See comment for Phe-pro.
Ala-tyr	α_{s1}CN: f158-159, 173-174. LF: f184-185.	Analog of VY.
Phe-pro	βCN: f77-78, f126-127, f172-173. αs_1CN: f43-44. αs_2CN: f107-108.	Like captopril and enalaprilat a dipeptide based on he sequence X-pro, where X is a hydrophobic residue. X-pro sequences were proposed as lead structures for ACE inhibitors.[4]
Leu-trp	αs_1CN: f213-214.	C-terminal part of TTMPLW, which is reported to be an inhibitor of ACE ($IC_{50} = 16\,\mu M$).[16]
Leu-tyr	α_s2CN: f114-115. βCN: f207-208. LF: f337-338.	Analog of VY.
Ser-tyr	κCN: f58-59.	Analog of VY.
Tyr-tyr	α_{s1}CN: f180-181.	Analog of VY.
Val-tyr	βCN: f74-75. α_{s2}CN: f98-99.	C-terminal part of IVY, which is reported to be an inhibitor of ACE ($IC_{50} = 12\,\mu M$).[17]
Ile-pro-pro	βCN: f129-131.	Shares C-terminal structure with many of the venom-derived peptides, which inhibit ACE. The peptide has been characterized *in vitro* against ACE ($IC_{50} = 5\,\mu M$) and caused a blood pressure reduction of up to. approx. 30 mmHg in SHR.[9]
Lys-val-leu-pro-val-pro	βCN: f184-189.	Has been characterized *in vitro* against ACE ($IC_{50} = 5\,\mu M$). Caused a reduction of up to approx. 30 mmHg in SHR upon oral administration.[7]

* α_{s1}CN = alpha-S1 casein[18]; α_{s2}CN = alpha-S2 casein[19] βCN = beta casein[20]; κCN = kappa casein[21]; LF = lactoferrin.[22]

(angiotensin I-like substrate for ACE) was from Sigma and hippuryl-phenylalanyl-arginine (bradykinin-like substrate for ACE) was from Bachem AG (Bubendorf, Switzerland).

In Vitro ACE Inhibitory Activity

The inhibitory activity towards ACE was assayed as described elsewhere.[7] In brief, an artificial substrate (hippuryl-histidyl-leucine or hippuryl-phenylalanyl-alanine) is used, which upon enzymatic cleavage liberates hippuric acid, which can easily be assayed spectrophotometrcially after extraction into an organic phase, evacuation and resuspension in water. For reference, 100 percent enzymatic activity was defined as the activity observed using buffer without any inhibitor present, and zero percent inhibition was defined as the activity observed when the enzyme was absent (enzyme solution replaced by water). Data for inhibition as a function of log C (molar concentration of inhibitor) were then fitted to the Michaelis–Menten equation in order to obtain IC_{50}-values. The curve fitting program used was GraphPad Prism (GraphPad Inc., San Diego, USA).

Animals and Arterial Cannulations

Male Sprague Dawley rats, age 12–14 weeks, obtained from Møllegaard & Bomholtgaard A/S (Ejby, Denmark) were used. They were allowed to acclimatize for at least 10 days upon arrival and had free access to standard rat pellet food and tap water. Lights were on from 6 a.m. to 6. p.m. in the animal facility. The experimental procedure was as follows: the rat was anaesthetized by intraperitoneal injection of 1.5 g urethane per kg bodyweight. A heparinized polyethylene tubing coupled to a pressure transducer (model P23xl, SpectraMed BV, Bilthoven, The Netherlands) was inserted into the left carotid artery for recording of arterial blood pressure. An injection tube was inserted in the right jugular vein. If subject to angiotensin I infusion, the rats also had the left jugular vein cannulated and coupled to an infusion pump. Before administration of peptides the rat was treated with 5 mg hexamethonium chloride per kg bodyweight to block ganglion activity. Injection volumes were 1 ml/kg and after each injection a wash-in of approximately 0.5 ml/kg volume was given. The methods for ACE inhibition *in vivo* were based on the ability of ACE to convert angiotensin I to angiotensin II or to inactivate bradykinin.

Method 1: Angiotensin Injections

The ability of ACE to convert angiotensin I to angiotensin II was measured as previously described with some modification.[8] This method was employed since the sensitivity to angiotensin II in itself may vary during the experiment. Rats received 0.3 μg/kg angiotensin I followed by 0.15 μg/kg angiotensin II and the ratio of the two pressor responses were recorded. This dual administration was repeated at least twice to assess a reference ratio. A saline peptide was then administered at 5 mg/kg. At 6, 12, 20, and 30 min after peptide administration the rat received injections of angiotensin I and angiotensin II and the recorded parameter was:

$$activity = \frac{\Delta P_I / \Delta P_{II}}{\Delta P_{I,0} / \Delta P_{II,0}} \quad (1)$$

where ΔP_I and ΔP_{II} are pressor responses to angiotensin I and angiotensin II after administration, respectively, and where $\Delta P_{I,0}$ and $\Delta P_{II,0}$ are the pressor responses before peptide administration. The reason for only having four time-points during the 30 min period was to avoid volume overloading in the animals.

A control group receiving saline without peptide served as a negative control. Thus, to test if the peptide in this model affects *in vivo* ACE activity, the inhibition as described above when peptide was administered was compared to the inhibition in the control group, rather than comparing the post-injection function of ACE with the pre-injection effect. Each rat was administered one peptide; three experiments (N = 3 rats) were carried out for each peptide and if an effect was observed the group size was increased to N = 5 rats.

A group receiving Captopril was included as a positive control, to confirm that the test system is sensitive to *in vivo*-active ACE inhibitors.

Method 2: Angiotensin I Infusion

The rats received a continuous infusion of angiotensin I at 2.5 μg/ml using an infusion speed of 10 ml/kg/h, raising the blood pressure approximately 20 mmHg from baseline immediately (Perfusor EDL2, B. Braun AG, Melsungen, Germany). In this method there is no way of compensating for any changes in sensitivity to angiotensin II. A group receiving saline infusion served as negative control. A group receiving Captopril served as positive control. N = 3 in the groups which, increased to N = 5 if effects were observed.

Method 3: Bradykinin Injections

The rats received bradykinin injections at 0.2 μg/kg. A peptide was administered and at 6, 12, 20 and 30 min after administration the injection of bradykinin was repeated. A group receiving saline served as negative control, and a group receiving Captopril

served as positive control. N = 3 in the groups which increased to N = 5 if effects were observed.

Statistics

Graph Pad Prism 2.01 (GraphPad Software Inc.) was used to fit the *in vitro* data to the Michaelis–Menten dynamic equation, thereby calculating IC_{50} values and Hill slopes for the peptides *in vitro*. For the *in vivo* studies, unpaired student's t-test was used to compare test parameters to the saline parameters.

To test if the IC_{50} values using HHL as substrate for ACE are correlated to the IC_{50} values using HPA as substrate, Spearman's rank test was used to allow testing of an association, which may not be linear. This test was also used to test a correlation of Hill slopes. To compare means of IC_{50}-values and Hill slopes, the Mann–Whitney test was used.

RESULTS

In general, the peptides fitted well to the Michaelis–Mentel dynamic model ($r^2 > 0.95$) *in vitro* showing competitive inhibition and the results are shown in Table II. Concentration-response curves for 8 of 9 of the peptides could be constructed *in vitro*. The potencies of the peptides were not greater than those previously described (such as IPP and KVLPVP, $IC_{50} = 5\,\mu M$ for both peptides.[7,9] Several of the peptides showed Hill coefficients significantly lower than 1. The IC_{50} value is lower when HHL is used as a substrate, than when HPA is used ($p < 0.001$) and the lower the IC_{50} using HHL, the lower the IC_{50} using HPA ($p < 0.05$) for a peptide. Likewise, the Hills slopes were correlated ($p < 0.05$).

TABLE II ACE inhibition *in vitro* using an angiotensin I-like substrate (HHL, hippuryl-histiduyl-leucine) or a bradykinin-like substrate (HPA, hippuryl-phenylalanyl-arginine)

	HHL as substrate		HPA as substrate	
Peptide	$IC_{50}(\mu M)$	Hill slope	IC_{50} (μM)	Hill slope
val-tyr	11	0.77	533	0.52
leu-trp	15	0.56	120	0.66
leu-tyr	44	0.75	534	0.71
ala-pro	21	0.91	222	0.86
ala-tyr	40	1.00	787	0.71
tyr-tyr	9	0.53	244	0.46
phe-pro	205	0.66	1104	0.50
ser-tyr	41	0.70	1650	0.58
ala-asp	>1600	ND	>3000	ND

Sigmoidal dose-response curves were fitted to the dynamic Michaelis–Menten equation to obtain IC_{50} and Hill slopes. ND: not determined.

No effects were observed for any peptide in the angiotensin I infusion experiments, or when bradykinin was used for injection. With the tandem injections of angiotensin I and angiotensin II, a modest effect could be detected for VY, LY, KVLPVP and LW. Table III summarizes the *in vivo* results. Figure 1 shows the inhibition as a function of time for the dipeptide val-tyr.

DISCUSSION

Since ACE is an enzyme with two active sites, our interpretation of a Hill coefficient lower than unity, is that binding of one inhibitor to the enzyme at one active site decreases the affinity for the inhibitor at the other active site, which has been observed with other inhibitors.[10] If this holds for any substance with affinity for the active site of ACE, including also the natural substrates, it may be a part of an allosteric

TABLE III *In vivo* effects of peptides in the three different models

	Ang I / Ang II injections¶		Ang I infusion¶	BK injection¶
Peptide	Inh. max %	t_{max}(min)¶	Reversion†	ΔP(mm Hg)‡
val-tyr	16%**	6	None	None
leu-tyr	8%**	12	None	None
leu-trp	12%*	6	None	None
ser-tyr	None	n/a	None	None
phe-pro	None	n/a	None	None
ala-asp	None	n/a	None	None
tyr-tyr	None	n/a	None	None
ala-tyr	None	n/a	None	None
ala-pro	None	n/a	None	None
lys-val-leu-pro-val-pro	11%*	6	None	None
Val-pro-pro	None	n/a	None	None
Ile-pro-pro	None	n/a	None	None
Captopril	83%***	6	100%***	−22 mm Hg***

The milk-derived peptides show little or no effects, whereas the effects of captopril, a reference ACE inhibitor is pronounced. Doses are 5 mg/kg. N = 3 in all groups and increased to N = 5 when effect was observed. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). † The angiotensin I infusion increases baseline blood pressure by approximately 25 mmHg, and reversion is calculated based on the ability of the inhibitor to restore the pre-infusion baseline blood pressure. ‡ ΔP is the depressor response to an injection of bradykinin, which in itself does not cause any effects unless ACE is inhibited. ¶ AI = angiotensin I, AII = angiotensin II, BK = bradykinin, t_{max} = the time needed to achieve maximal inhibition.

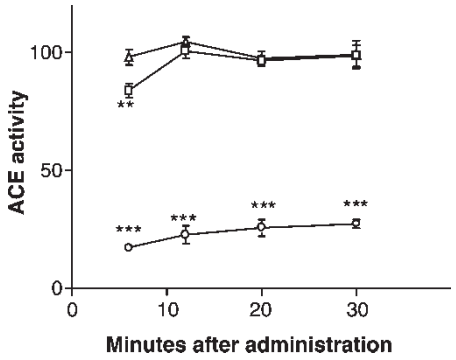

FIGURE 1 The acitivity of ACE *in vivo* after intravenous administration of 5 mg/kg of either captopril (○) or valyl-tyrosine (□), or vehicle (△). The model used is based on injection of angiotensins. Error bars represent the SEM values, N = 5. ($^{**}P < 0.01$, $^{***}P < 0.001$).

regulatory mechanism. If, for example, angiotensin I is present in high concentrations, negative cooperativity in this way may result in lower levels of angiotensin II formed than compared to a situation with no negative cooperativity.

In this study, HHL is used as an angiotensin I-like substrate and HPA is a bradykinin-like substrate. The observation that IC_{50} is lower when HHL is used, suggests that the ACE inhibitors of this study preferentially interfere with angiotensin metabolism, i.e. it takes more inhibitor to interfere with bradykinin catabolism than angiotensin conversion. It is partially reflected in the *in vivo* results obtained here, that the inhibitors generally have preference for interference with angiotensin metabolism rather than bradykinin metabolism *in vitro*, as modest inhibition is observed with angiotensin I conversion but no effect is observed with bradykinin.

The fact that the IC_{50} value is highly dependent on the choice of substrate makes it difficult to compare the *in vitro* characteristics in different studies, where other substrates may be used for the *in vitro* characterization. Hippuryl-glycyl-glycine and dansyl-triglycine, are examples of other substrates used. In this study the IC_{50} values using the two different substrates were correlated, indicating that the IC_{50} hierarchy is conserved in the two groups. The same is true for the Hill slopes. Thus, a peptide that has a low IC_{50} in the group analysed using Hip-his-leu, is also likely to show a low IC_{50} in the group analysed using Hip-phe-arg, even though the IC_{50} values in the latter group generally are higher.

The *in vivo* models in this study are sensitive to captopril (see Table III and Figure 1; similar results were observed with lysinopril but are not shown). The captopril doses reported to be hypotensive doses in telemetric or tail cuff studies (using hypertensive rats), are in magnitude comparable to or higher than the 5 mg/kg used here.[11–13] In these animals, no major differences in the renin-angiotensin system have been detected, when compared to their normotensive counterparts, even though the effects of ACE inhibitors are pronounced. This indicates that the physiological effects of ACE inhibitors in rats may be detected with normotensive rats as ACE-inhibition (disability to metabolise bradykinin or convert angiotensin) in lower doses than when a hypotensive response can be detected using hypertensive rats. Thus, the failure of the new peptides used here to produce an *in vivo* inhibition of ACE makes them unlikely to be hypotensive agents in hypertensive animals. Conversely it is somewhat surprising that only very moderate levels of inhibition of ACE are seen in this experiment even with the known inhibitors IPP, VPP and KVLPVP, which are reported to be hypotensive in relevant cardiovascular models.[7,2] Since *in vivo* inhibition of ACE is thought to be correlated to a hypotensive effect, it seems more likely that the milk-derived inhibitors do not act directly as inhibitors of ACE but through other pathways if they are hypotensive after oral or intravenous treatment. A peptide showing virtually no inhibitory activity against ACE but with pronounced hypotensive effect has been produced from a milk source.[14] The reason why we did not observe strong *in vivo* effects here may simply be that the peptides are degraded rapidly. Many peptides are degraded within seconds or minutes in the blood stream unless they are somehow modified. Angiotensin I itself is a good example of this. The ACE inhibitors used in modern therapy are all synthetic peptides, which have been made resistant to hydrolysis. Investigations on the plasma half-life of ile-pro-pro and the dipeptides are being carried out.

The mechanism of action for the known milk derived inhibitors which are reported to be hypotensive (ile-pro-pro and lys-val-leu-pro-val-pro) remains obscure. One possible explanation is that they might in fact be pro-drugs, which upon ingestion need activation into *in vivo* active compounds much like the newer types of clinically used ACE inhibitors, such as enalapril or trandolapril. To confirm this, active metabolites of the inhibitors must be identified, for example through administration of radioactively labelled peptide accompanied by chromatographic fractionation of plasma in order to obtain the labelled metabolite. Another possibility is that the inhibitors are active against ACE *in vitro* only and that their mode of hypotensive action must be sought elsewhere.

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