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Lactotripeptides Inhibiting ACE1 Elevate the Plasma Bradykinin Concentration Acutely in a Placebo-Controlled, Double-Blind, Cross-Over, 4-Week Trial in Healthy Volunteers

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Abstract

This placebo-controlled, double-blind, cross-over intervention with twelve normotensive healthy volunteers tested the effects of milk products containing either 5 or 50 mg of ACE1-inhibitory lactotripeptides (isolecine-proline-proline, Ile-Pro-Pro, and valine-proline-proline, Val-Pro-Pro) and placebo milk drink (with similar taste) on plasma bradykinin levels. The subjects consumed one of the three test products in a random order, double-blinded, and four-week trial. On the first day (day 1) and on the last day (day 29) *i.e.* after four weeks' treatment with one of the products, the acute effect with the same single dose was assayed. Other markers of the renin-angiotensin-aldosterone system (RAAS) were measured from plasma four times on the same days when we also assessed daytime urinary excretion of biomarkers of endothelial function. Neither acute nor prolonged administration of the ACE-1 inhibiting peptide drinks significantly lowered blood pressure of the normotensive subjects. The most important finding was the dose-dependent, and linear increase in plasma bradykinin concentrations after acute dosing on the first day; it was nearly statistically significant also on the day 29 ($p < 0.06$). Other indicators of RAAS or endothelial function did not differ from those of placebo after the acute or prolonged treatments. Our results suggest that even weak inhibitors of ACE-1, such as the lactotripeptides Ile-Pro-Pro and Val-Pro-Pro, are able to diminish the breakdown of bradykinin and therefore increase plasma bradykinin levels. This may partly explain the blood pressure lowering and vasodilatory effects of lactotripeptides, shown by us earlier in mildly hypertensive subjects.

Keywords

Lactotripeptides, Ile-Pro-Pro, Val-Pro-Pro, Bradykinin, Blood Pressure, Human

1. Introduction

Fermented milk products containing casein-derived bioactive tripeptides (lactotripeptides), isoleucine-proline-proline (Ile-Pro-Pro) and valine-proline-proline (Val-Pro-Pro) have been shown to prevent the development of hypertension in different hypertensive animal models by us [1] [2] [3] and others [4] [5]. These and other similar milk products and spreads have been demonstrated to lower blood pressure in long term clinical interventions in mildly hypertensive subjects in Japanese and Finnish populations [6]-[12]. However, trials conducted in The Netherlands detected only minimal or no significant lowering of blood pressure [13] [14] [15] [16], probably, partly because the subjects after the run-in period were no longer hypertensive. Four independent meta-analyses [17] [18] [19] [20] of clinical interventions suggest that the reduction achieved with lactotripeptides in systolic blood pressure is about five mmHg; in diastolic blood pressure it amounts to about three mmHg. In addition to the antihypertensive effect of the peptides, they reduce arterial stiffness [21] and the augmentation index in mildly hypertensive subjects [22]. The clinical findings agree with endothelium dependent vasodilatation seen in experimental studies in hypertensive rat models [23] [24] [25]. Peptide treatment improved vasodilatation induced by bradykinin *ex vivo* in rats possibly by acting on Mas-receptors, while the bradykinin effect was endothelium-dependent and abolished by COX inhibitors [26].

The antihypertensive mechanism of the lactotripeptides seems to be mediated mainly via ACE-1 inhibition and the reduced formation of the strongly vasoconstricting peptide, angiotensin II [4] [5] [24] [25] [26], even though other enzymes acting on the vasculature are also inhibited by these peptides [27].

Recently, an interesting hypothesis on the crucial role of bradykinin in cardioprotection and possibly in the blood pressure lowering effect of ACE-1 inhibitors has been presented. ACE-1 inhibitors have been shown to directly bind to and activate bradykinin receptors [28] [29] [30].

There are some major difficulties in investigating bradykinin's physiological and pathophysiological role *in vivo i.e.* its very short half-life in biological matrices, and lack of simple and reliable assays. However, a combination of liquid-phase extraction, high-performance liquid chromatography and radioimmunoassay was developed [31] and has been used reliably for assaying clinical samples [32]. The present report is the first to confirm that dietary ACE-1 inhibitory peptides may elevate the plasma bradykinin concentration in a clinical intervention in healthy normotensive subjects.

2. Subjects and Methods

Twelve normotensive, non-smoking, healthy male volunteers aged 21 - 26 years participated in this cross-over, double-blind, placebo-controlled, randomized intervention study. Exclusion criteria for participation were BMI > 27 kg/m^2, milk allergy, lactose intolerance, smoking, alcohol or drug abuse, clinical blood abnormalities, use of prescription medicine during 30 days preceding the study and participation in any study involving blood collection during 60 days preceding the study. The protocol was approved by the Ethical Committee of the Lausanne University Hospital (protocol number 158-06).

The subjects came to the Clinical Research Unit of the University Hospital Lausanne at 7 o'clock in the morning of the intervention day. After one hour's rest, systolic and diastolic blood pressure and heart rate were measured in the sitting position. Thereafter, blood samples were drawn from the antecubital vein from the contralateral arm for measurement of bradykinin and other biochemical markers. The urinary bladder was emptied and urine was discarded.

Thereafter the subjects drank within two minutes 100 ml of the test milk product or control (placebo) product. The test liquid product contained either 5 mg or 50 mg (the sum amount) of Ile-Pro-Pro and of Val-Pro-Pro and negligible amounts (less than 0.5 mg) of leucine-proline-proline (Leu-Pro-Pro). The lactotripeptides were added to the test products in the form of peptide powder. The peptides were separated from *Lactobacillus helveticus* Lc1936 fermented milk, concentrated using nanofiltration and dried to produce a powder in Valio Ltd, Finland. The fermented placebo drink was similar (appearance and taste as far as possible), but it did not contain any measurable amounts of the peptides. The study products were provided and analysed by Valio Ltd., Finland.

After the intake of the test/placebo drink, the subjects rested in a sitting position and the blood pressure measurement was repeated after 2.5, 5 and 9 h. After each measurement, blood samples were drawn. Urine was collected during the 10 h period in the ward.

A light lunch was served 4 h after the intake of the test/placebo drink and dinner was served after 9 h.

The procedure was repeated similarly after 28 days' consumption of the peptide/placebo drink (at home once in the morning) containing either none, 5 mg or 50 mg of the peptides. These three different interventions were repeated in a random order for each subject. All procedures were double-blinded.

2.1. Laboratory Measurements

Bradykinin concentrations in plasma were the main outcome and were measured using the method developed originally by Nussberger *et al.* [31] (normal values 0.2 - 7.1 fmol/ml) and used later in clinical studies [32]. Shortly, liquid-phase extraction, high-performance, liquid chromatography and RIA were applied in combination.

Plasma renin activity [33] (normal values 0.2 - 2.0 ng/ml/h), trapping

ACE-activity [34] (normal values 178 - 475 fmol/ml/min), plasma aldosterone [35] (normal values 29 - 76 pg/ml) and urinary aldosterone excretion [36] (normal values 0.5 - 8.0 μg/24 h) during 10 h were measured in the Laboratory of Angiology and Hypertension of University Hospital Lausanne.

Additionally, urinary excretion of 6-keto-prostaglandin $F_{1\alpha}$, 2,3-dinor-prostaglandin $F_{1\alpha}$ (both EIA, Cayman Chemical Co, Ann Arbor, MI, USA), the main urinary metabolites of prostacyclin (PGI_2) as well as nitrate/nitrite (NO_X) (both ELISA) and cyclic GMP (R&D Systems, Abingdon, UK) as indicators of the arginine-nitric-oxide-pathway were analysed in the Medical Faculty, Department of Pharmacology, University of Helsinki from 10 h urine samples collected in the ward during the placebo and the high peptide dose (50 mg) interventions. The measurements were carried out according to the manufacturers' instructions with slight modifications as in our previous study [11].

2.2. Statistical Analyses

The results are presented as mean with standard error of mean (SEM) or with 95 percent confidence intervals. Within-subjects analyses were done using the paired samples t-test. The information from different time points was combined by calculating the area under the curve subtracted by the baseline value ($AUC_{0\text{-}9}$ minus baseline). The statistical significance of linearity in these AUCs was tested using linear mixed models. The 95% confidence intervals for Pearson's correlation coefficient were obtained by bootstrapping.

3. Results

3.1. Blood Pressure and Heart Rate

Because the 5 mg dose of the peptides, administered either as an acute or continuous dosing for four weeks, did not cause any significant changes compared to the placebo drink in any of the variables, the results are presented only for the 50 mg dose. The only exception is when the linearity of the changes is presented.

Neither blood pressure nor heart rate changed significantly vs placebo with the low or high dose of the peptides after acute dosing on either day 1 or day 29. No reduction of blood pressure was seen in the 28 days' intervention in these normotensive subjects (**Table 1**).

3.2. Bradykinin Concentrations in Plasma

Plasma bradykinin, the main outcome, showed quite marked inter-individual and also intra-individual variations during the day. No significant changes in the basal (morning) levels of the peptide groups vs placebo were seen after the 28 days' intervention with either peptide dose. However, the acute study (a single dose in the morning) showed clear, up to three fold higher concentrations vs the basal level in 5 h samples both on day 1 (1.55 ± 0.35 to 4.76 ± 1.03 fmol/ml, $p < 0.05$) and on day 29 (1.49 ± 0.25 to 4.05 ± 1.00 fmol/ml, $p < 0.06$). The increase

Table 1. Blood pressure and heart rate of 12 healthy, normotensive volunteers after the intake of a fermented milk product containing 50 mg of lactotripeptides (Ile-Pro-Pro + Val-Pro-Pro) or placebo on day 1 and on day 29 after 4 weeks' double-blind, cross-over intervention with the same product. Results are expressed as mean +/− SEM.

	Placebo		Test product	
	Day 1	Day 29	Day 1	Day 29
Systolic blood pressure, mmHg				
Baseline	118 ± 1	116 ± 1	116 ± 2	115 ± 1
2.5 h	117 ± 1	118 ± 2	116 ± 2	117 ± 2
5 h	117 ± 2	115 ± 2	116 ± 2	116 ± 2
9 h	120 ± 2	119 ± 2	118 ± 2	120 ± 2
Diastolic blood pressure, mmHg				
Baseline	66 ± 1	66 ± 1	66 ± 1	66 ± 1
2.5 h	66 ± 1	67 ± 1	67 ± 1	67 ± 1
5 h	65 ± 2	64 ± 2	65 ± 1	65 ± 1
9 h	65 ± 1	67 ± 2	68 ± 2	67 ± 1
Heart rate, beats/min				
Baseline	53 ± 2	52 ± 1	55 ± 1	55 ± 2
2.5 h	52 ± 2	52 ± 2	55 ± 2	54 ± 2
5 h	54 ± 2	51 ± 1	53 ± 2	51 ± 1
9 h	52 ± 1	56 ± 2	56 ± 2	57 ± 3

was even more evident when the acute effect of the peptide drink was presented as $AUC_{(0\text{-}9)}$ minus baseline (**Figure 1(a)**) and with calculation of AUC for both doses (5 and 50 mg) which revealed clear linearity between the doses (**Figure 1 (b)**). However, the statistically significant effect of 50 mg was no longer seen on day 29.

3.3. Other Variables of the Renin-Angiotensin-Aldosterone System (RAAS)

Plasma renin activity remained rather stable in all treatment groups both after the acute, single administration of the placebo or peptide drinks on day 1 or 29 or with respect to chronic treatment *i.e.* when comparing baseline concentrations on these days (**Table 2**).

Baseline values for plasma trapping ACE activity were similarly stable during the 28 days' treatment period and no differences between the treatment groups were seen after a single dose (day 1 and 29) (**Table 2**). However, an inverse correlation between the ACE activities and bradykinin levels was evident with the 50 mg dose on day 1 (**Figure 2**).

Plasma aldosterone levels remained similarly stable in all groups throughout the 28 days' treatment and in acute tests.

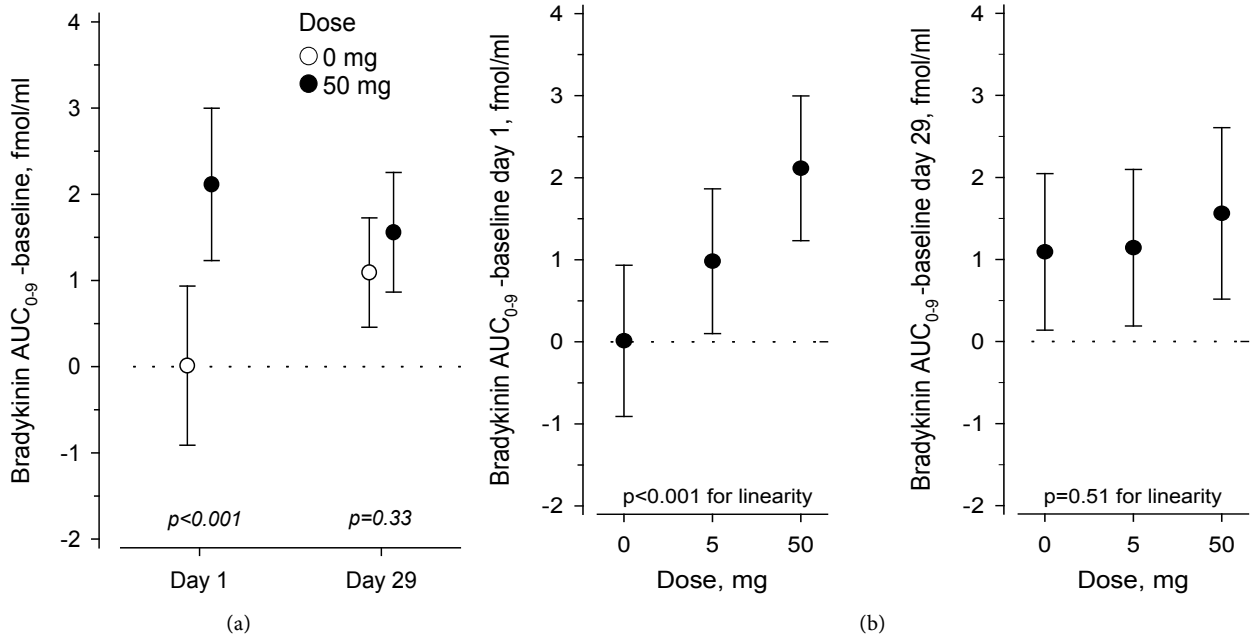

Figure 1. (a) The $AUC_{0\text{-}9}$ minus baseline of plasma bradykinin concentrations (n = 12) after the test drink containing 50 mg of tripeptides (dark dots) or placebo drink without peptides (open circles) on day 1 and day 29 after 4 weeks' double-blind, cross-over intervention. Results are expressed as means with 95% intervals. (b) Linearity of the $AUC_{0\text{-}9}$ minus baseline of plasma bradykinin concentrations (n = 12) after a single dose of the test drinks containing 5 or 50 mg of the tripeptides on day 1 and day 29 after 4 weeks' double-blind, cross-over intervention with the same daily dosages. Results are presented as means with 95% intervals.

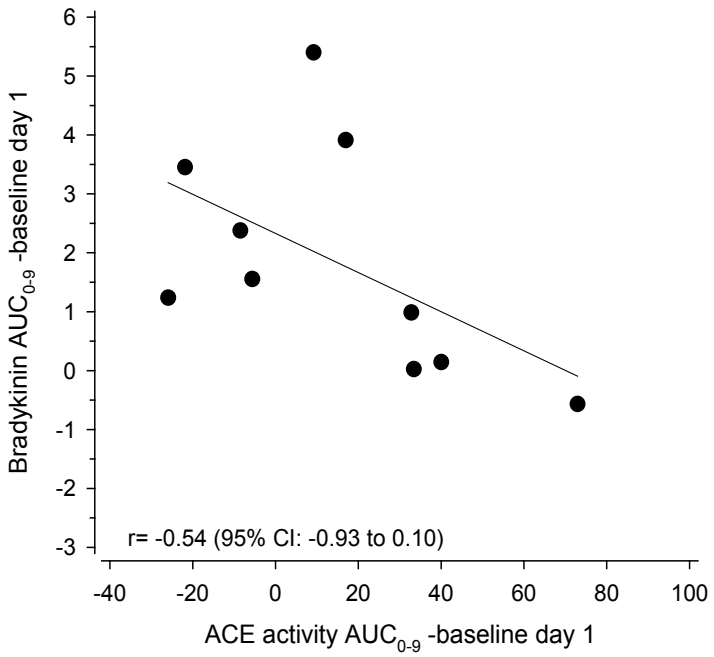

Figure 2. The relationship between individual plasma bradykinin concentrations ($AUC_{0\text{-}9}$ minus baseline) and plasma trapping ACE activity ($AUC_{0\text{-}9}$ minus baseline) on day 1, when bradykinin concentrations increased after a single dose of the test drink containing 50 mg of the tripeptides.

Table 2. Variables of renin-angiotensin-aldosterone system (RAAS) of 12 healthy volunteers after the intake of a fermented milk product containing 50 mg of lactotripeptides (Ile-Pro-Pro + Val-Pro-Pro) or placebo on day 1 and on day 29 after 4 weeks' double-blind, cross-over intervention with the same product. Results are expressed as mean +/− SEM.

	Placebo		Test product	
	Day 1	Day 29	Day 1	Day 29
Plasma renin activity, ng/ml/h				
Baseline (0 h)	0.65 ± 0.08	0.69 ± 0.15	0.63 ± 0.12	0.55 ± 0.09
2.5 h	0.74 ± 0.12	0.85 ± 0.18	0.76 ± 0.10	0.63 ± 0.14
5 h	0.80 ± 0.08	0.69 ± 0.17	0.67 ± 0.11	0.45 ± 0.12
9 h	0.61 ± 0.11	0.61 ± 0.10	0.58 ± 0.09	0.53 ± 0.10
Plasma trapping ACE activity, fmol/ml/min				
Baseline	429 ± 22	421 ± 23	418 ± 25	431 ± 26
2.5 h	439 ± 24	414 ± 35	440 ± 30	446 ± 30
5 h	434 ± 22	436 ± 23	442 ± 22	451 ± 25
9 h	417 ± 23	432 ± 23	421 ± 26	435 ± 26
Plasma aldosterone, pg/ml				
Baseline	50.8 ± 3.4	54.8 ± 4.8	49.5 ± 3.4	52.6 ± 4.2
2.5 h	67.7 ± 8.3	65.4 ± 10.4	68.4 ± 8.7	60.2 ± 5.0
5 h	49.1 ± 3.9	46.4 ± 4.4	50.0 ± 7.3	45.8 ± 4.3
9 h	45.6 ± 4.1	46.6 ± 4.7	53.4 ± 7.2	61.8 ± 8.3

Urine aldosterone excretion during the 10 h collection period varied from subject to subject, but remained at about the same level independent of the treatment (Table 3). A non-significant tendency for linearity (0, 5 and 50 mg peptides; $p = 0.083$) was seen on day 1 (data not shown).

Urinary markers of the vascular arachidonic acid and nitric oxide pathways are presented in Table 3. The basal excretion of prostacyclin metabolites 6-ketoPGF$_{1\alpha}$ and 2,3-dinor 6-ketoPGF$_{1\alpha}$ remained at the same level on day 1 and day 29, and the 50 mg peptide dose did not cause any change in these values (Table 3). This was also the situation for urinary excretion of nitrate/nitrite and cyclic GMP (Table 3).

4. Discussion

The blood pressure lowering effect of milk casein-derived bioactive tripeptides (lactotripeptides) has been demonstrated in animal models [1] [2] [3] [4] [23] [24] [25] as well as in humans (see meta-analyses 18 - 20). The antihypertensive mechanism has been related to ACE1 inhibition [4] [5] [23] [24] [25] and further less formation of angiotensin II, which at least partly explains the favourable effects. However, another mechanism of vasoactive components on the

Table 3. Excretion of biomarkers of endothelial function in urine collected over 10 h from healthy normotensive volunteers (n = 12) after the intake of a fermented milk product containing 50 mg of lactotripeptides (Ile-Pro-Pro + Val-Pro-Pro) or placebo on day 1 and on day 29 after 4 weeks' double-blind, cross-over, intervention with the same product and dose. Results are expressed as mean +/− SEM.

	Placebo		Test product	
	Day 1	Day 29	Day 1	Day 29
Urine aldosterone, µg	1.66 ± 0.21	1.83 ± 0.29	2.34 ± 0.44	1.90 ± 0.29
Urine 6-keto-$PGF_{1\alpha}$, ng	604 ± 40	793 ± 86	660 ± 75	1040 ± 323
Urine 2,3-dinor-6-keto-$PGF_{1\alpha}$, µg	12.1 ± 1.2	13.8 ± 2.9	13.6 ± 1.5	13.4 ± 0.8
Urine nitrate/nitrite, mmol	1130 ± 233	1080 ± 175	1131 ± 211	921 ± 106
Urine cGMP, nmol	263 ± 18	246 ± 17	239 ± 20	238 ± 15

renin-angiotensin system is mediated via bradykinin, whose role until now has been obscure and less extensively studied. Here we approached this issue by measuring plasma bradykinin levels of healthy normotensive subjects after a single dose of fermented milk products containing two concentrations of the lactotripeptides Ile-Pro-Pro and Val-Pro-Pro. The effects of acute dosing were tested on the first and last days of a trial where the volunteers consumed the peptide drinks on a daily basis for four weeks.

Strict and controlled condition was a strength of the study. The subjects spent the whole test day at the ward, drinking and eating were similar each time. Blood pressure measurement and blood sampling were done by the same nurse. The compliance of the consumption of test drinks was excellent. The clinical chemistry measurements were done every time in the same laboratories.

The main finding was that the plasma bradykinin concentration increased dose-dependently after a single dose of the peptide drink on the first, but interestingly, not quite significantly ($p < 0.06$) on the last day after daily use of the test product for four weeks. This can be explained by a kinetic "steady state" of plasma levels of the peptides and their, previously described in an animal study [37]. Thus, on the last day, the acute dosing could no longer exert any significant, measurable effect on this very labile biomarker. Even then, a clear tendency towards an increase in 5 h blood samples was seen with the higher dose (50 mg). Furthermore, with this dose, a negative correlation between bradykinin and ACE1 activity was found on the first day after a single dosing, indicating that there had been clinical inhibition of ACE1. Unfortunately, we were not able to measure active metabolites of bradykinin, such as bradykinin (1 - 5) and (1 - 7) [38] [39]. In fact, the role of the different breakdown products of bradykinin in the antihypertensive mechanism of ACE inhibitors is still a matter of discussion [29] [30].

No changes in blood pressure were seen with either the chronic or acute dosing of the peptide drinks, which can be explained by the fact that the subjects in this trial had normal blood pressure and were not hypertensive as in many trials

where blood pressure lowering effect has been shown [6]-[12]. The lack of blood pressure reduction in the present study is in agreement with some earlier studies where the subjects have been nearly normotensive [13] [14] [15] [16]. No treatment related adverse effects were described by the subjects during the whole trial which lasted three months with three different doses (placebo, 5 or 50 mg of peptides).

We were unable to detect any other treatment effects which could have strengthened these observations of the possible role of bradykinin on its endothelial second messengers such as prostacyclin or on the NO/cyclic GMP pathway. This can be attributable to different factors: firstly, the peptides are too weak ACE-inhibitors to cause measurable changes in variables whose measurement is not only technically difficult but also subject to significant inter-individual and even intra-individual variations; secondly, our healthy, normotensive group was small and bigger changes would have been required for statistically significant differences; thirdly, due to the young age of the volunteers, their cardiovascular system was able to balance possible circulatory effects of the peptides; fourthly, the observed changes in these parameters were too small and clinical effects of the lactotripeptides were not expected in healthy volunteers consuming a regular diet.

However, in our recent experimental study conducted in spontaneously hypertensive rats, in addition to antihypertensive effects, we were able to demonstrate also a marked improvement of endothelium dependent relaxation of mesenteric artery rings by bradykinin in the presence of Ile-Pro-Pro [26]. This may indicate that even though we were not able to observe all of the potential changes in the measured markers of the bradykinin system with weak ACE inhibitory peptides, they may act at the functional level synergistically with bradykinin or the effects may be mediated via the active metabolites of bradykinin [38] [39].

Interestingly, the chemical structure of the first peptide type ACE inhibitor, teprotide, indicates that the carboxy terminal in the tripeptide Ile-Pro-Pro configuration is similar to that present seen in a bradykinin potentiator, B [40]. These observations bring the studied peptides closer to the bradykinin pathway.

Furthermore, treatment with ACE inhibitors has been shown to affect the bradykinin type 2 receptors responsible for vasodilatation [41] [42], and this would then augment the beneficial vascular effects of bradykinin, *i.e.* they would not simply be mediated by preventing the breakdown of bradykinin.

At the cellular level, vascular effects of bradykinin have been suggested to be mediated by stimulation of NO and prostacyclin formation [43]. These mediators were not found to be elevated in the urine collected during the period when bradykinin levels were increased in plasma. This discrepancy can possibly be explained by the quite large variations in their levels and these can be due to either individual or methodological issues *i.e.*, urine without chromatographic separation is not the best matrix for these kinds of measurements. Furthermore, urinary levels probably reflect the long-term balance and changes of these vascular

endothelium-derived variables better than plasma.

Taken together, our present observations with information from the literature and from our earlier experimental findings, suggest that at least part of the favourable vascular effects of ACE-inhibitory lactotripeptides can be associated with bradykinin; these may be mediated in many different ways, and furthermore, it is probable that they act synergistically in the same direction.

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