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LONG-TERM INTAKE OF MILK PEPTIDES ATTENUATES DEVELOPMENT OF HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS

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Effect of long-term intake of isoleucine-proline-proline (IPP) and valine-proline--proline (VPP), or a sour milk product containing these peptides on development of hypertension was investigated in spontaneously hypertensive rats (SHR). Six-week-old SHR were given: 1) water (control group), 2) IPP and VPP dissolved in water (peptide group) or 3) sour milk containing IPP and VPP (sour milk group) for 12 weeks. Systolic blood pressure (SBP) was measured by tail-cuff method. Development of hypertension was attenuated in the groups receiving tripeptides or sour milk as compared to the control group. At the end of treatment period, SBP was 176 ± 1 mmHg in sour milk group, 181 ± 2 mmHg in peptide group, and 193 ± 1 mmHg in control group ($P < 0.001$). After treatment withdrawal, SBP rose gradually reaching the level of control group within four weeks' follow-up. In functional bioassay of ACE inhibitory activity, effect of the tripeptides on angiotensin I or angiotensin II-induced contraction in rat mesenteric arteries was evaluated. IPP inhibited the angiotensin I -induced contraction, whereas the angiotensin II-induced contraction remained unaltered. In conclusion, long-term intake of IPP and VPP, or a sour milk containing these tripeptides attenuated the development of hypertension in SHR. One possible mechanism underlying this effect is ACE inhibition.

K e y w o r d s: *Milk peptides, hypertension, SHR, ACE inhibition*

INTRODUCTION

Several milk proteins are precursors of peptides which possess various biochemical and physiological properties, including antithrombotic, immunomodulatory and antihypertensive effects (1). These biologically active peptide fragments can be released from milk proteins in enzymatic proteolysis either during gastrointestinal digestion or during fermentation of milk by lactic acid bacteria (2).

Various milk-derived peptides given acutely lower blood pressure in animal experiments (3—6). A dose-dependent reduction in systolic blood pressure (SBP) after single oral administration of tripeptides isoleucine-proline-proline (IPP) or valine-proline-proline (VPP) has been reported in adult spontaneously hypertensive rats (SHR) (3). An antihypertensive effect in SHR has also been observed after single oral administration of a sour milk product containing these peptides (Calpis®) (3).

One possible mechanism for the antihypertensive effect of milk peptides is inhibition of the activity of angiotensin converting enzyme (ACE) (7). IPP and VPP have been reported to possess a weak ACE inhibitory activity in a spectrophotometric assay *in vitro* (8). Other mechanisms for milk peptides have been suggested as well. A dipeptide Tyr-Pro lowers blood pressure in SHR, but the effect does not correlate with its ACE inhibitory activity (5). We have studied blood pressure lowering effect of tetrapeptide α-lactorphin (Tyr-Gly-Leu-Phe), which corresponds to the amino acid sequence 50—53 of milk whey protein α-lactalbumin (9). α-Lactorphin was found to possess a naloxone-sensitive antihypertensive effect in SHR, which suggests that opioid receptors might be involved in the response to α-lactorphin (6).

In the present study, we investigated the effect of long-term oral administration of IPP and VPP, or a sour milk product containing these peptides on blood pressure in young prehypertensive SHR. These milk peptides have previously been reported to possess acute antihypertensive effects after single oral administration (3). Moreover, the ACE inhibitory activity of these peptides was evaluated in a functional bioassay using mesenteric artery preparations of Wistar rats.

MATERIALS AND METHODS

Six-week-old male SHR (Harlan Sprague Dawley, Indianapolis, IN, USA) were used in the long-term experiment investigating the development of hypertension. In the functional bioassay of ACE inhibitory activity, mesenteric artery preparations of female Wistar rats (220—240 g) (Laboratory Animal Centre, University of Helsinki, Finland) were used. The protocol of the study was approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland.

Effect of long-term intake of IPP and VPP and a sour milk containing the tripeptides on blood pressure

At the beginning of the study, the blood pressure- and body weight-matched SHR were divided into three groups (n = 10–11/group) to receive different

treatments *ad libitum* for 12 weeks: 1) a control group drinking tap water, 2) a peptide group receiving IPP and VPP dissolved in drinking water, and 3) a sour milk group receiving a fermented milk product containing IPP and VPP (Valio Ltd, Helsinki, Finland). The sour milk was manufactured from skim milk by inoculation with 10% of *Lactobacillus helveticus* (LBK16H strain).

The rats were weighed weekly. The consumption of drinking fluids and freely accessible feed (R36, Lactamin, Stockholm, Sweden) was monitored throughout the experiment. The contents of energy, nutrients and the tripeptides in different drinking fluids and in the food are shown in *Table 1*. The estimated intake of electrolytes was calculated on the basis of these values. The peptide content in the sour milk and the feed and the electrolyte composition of the sour milk were analysed by Valio Ltd (Helsinki, Finland).

Table 1. Contents of energy, nutrients and tripeptides IPP and VPP in different drinking fluids and in the feed.

Variable	Water	IPP & VPP	Sour milk	Food
Energy, kJ/100 g	0	0	180	1260
Protein, g/100 g	0	0	2.4	18.5
Fat, g/100 g	0	0	0.49	4
Carbohydrate, g/100 g	0	0	7.2	56
Sodium, mg/100 g	0.6	0.6	26	275
Potassium, mg/100 g	0.2	0.2	150	600
Calcium, mg/100 g	2.1	2.1	330	980
Magnesium, mg/100 g	0.2	0.2	33	200
IPP, mg/l	0	16—30	16—18	0
VPP, mg/l	0	16—30	16—18	0

The intakes of IPP and VPP in the sour milk group were calculated on the basis of the daily consumption. In the peptide group, the intakes of the tripeptides were adjusted to correspond the intakes in the sour milk group. The intakes of IPP and VPP were approximately 2.5—3.5 mg/kg/day during the experiment. Previously, these peptides have been shown to reduce blood

pressure acutely after single oral administration at doses ranging from 0.1—5 mg/kg in SHR (3).

After 12 weeks of treatment, all groups received tap water for additional 4 weeks (the follow-up period).

SBP was measured weekly during the 12 weeks' treatment period and every second week during the 4 weeks' follow-up period using a tail cuff blood pressure analyser (IITC Life Science, Model 179, Woodland Hills, CA, USA). Before the measurement, the rats were kept at 30—32°C for 30 min to make the pulsations of the tail artery detectable. When three consecutive blood pressure values were obtained without disturbance of the signal, the arithmetic mean was recorded as the SBP.

During the last week of treatment period, the rats were housed individually in metabolic cages for 24 hours. The consumption of feed and drinking fluids was measured, and the estimated daily intake of electrolytes was calculated. Urine was collected and urinary volume was measured. Urine samples were stored at -80°C until the biochemical determinations were performed. Urinary sodium and potassium concentrations were analysed by flame emission spectrometry (10), and urine calcium and magnesium were determined by flame atomic absorption spectrometry (11) (HUCH, Laboratory Department, Helsinki, Finland). At the end of the experiment, the animals were made unconscious with CO_2/O_2 (70/30%) (AGA, Riihimäki, Finland) and sacrificed by decapitation.

Functional bioassay of ACE inhibitory activity

In a separate set of experiments, 3 mm long sections of the mesenteric artery were cut 5 mm distally from the mesenteric artery-aorta junction. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer buffer (pH 7.4) of the following composition (mmol/l): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2 \times 2H_2O$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \times 7H_2O$ 1.2 and aerated with O_2/CO_2 (96/4%). The rings were equilibrated for 30 min at 37°C with a resting tension of 1.0 g. The presence of intact endothelium in vascular preparations was confirmed by observing a relaxation response to 1 μmol/l acetylcholine (ACh) in 1 μmol/l noradrenaline (NA) precontracted rings. The force of contraction was measured with an isometric force displacement transducer and registered with a polygraph (FTO3 transducer, Model 7P122E Polygraph; Grass Instrument Co, Quincy, MA, USA).

The ACE inhibitory activity of IPP and VPP (0.1—3.3 mmol/l) and captopril (10 μmol/l) was assayed *in vitro* by preincubating mesenteric artery preparations with test substances for 15 min and measuring the response to a single administration of 0.1 μmol/l angiotensin I or angiotensin II. Angiotensins were only administered once in order to avoid tachyphylaxis (12).

Drugs

The following substances were used: IPP and VPP (Peninsula Laboratories Europe Ltd, St. Helens, England), acetylcholine chloride, angiotensin I acetate, angiotensin II acetate, captopril and noradrenaline bitartrate (Sigma Chemical Co, St. Louis, MO, USA). Stock solutions were dissolved in distilled water. All solutions were prepared before use and protected from light.

Statistical analysis

Data for SBP were analysed by two-way analysis of variance (ANOVA) with repeated measures for overall treatment effect. Other data were analysed by one-way ANOVA. The Tukey's test was used for multiple pairwise comparisons of the treatment groups. $P < 0.05$ was considered significant. The results are expressed as means ± SEM.

RESULTS

Effect of long-term intake of IPP and VPP on development of hypertension

SBP was similar in all groups at the beginning of the study (*Fig. 1*). Blood pressure rose gradually during the experiment, and after 10 weeks of treatment, the blood pressure persisted in a stabile hypertensive level in all groups. Intake of milk peptides IPP and VPP or sour milk containing the tripeptides attenuated the development of hypertension in SHR as compared to the control group ($P < 0.01$). After the 12 weeks' treatment period, the SBP level was 12 mmHg lower in the peptide group (181 ± 2 versus 193 ± 1 mmHg, $P < 0.001$) and 17 mmHg lower in the sour milk group (176 ± 1 versus 193 ± 1 mmHg, $P < 0.001$) than in the control group (*Fig. 1*). After the treatment withdrawal, the SBP in the treated groups rose gradually reaching the level of control group within the four weeks' follow-up period (*Fig 1*).

There were no differences in the body weight gain between the groups (*Table 2*). In the group receiving sour milk, the consumption of drinking fluid was higher, whereas the consumption of food was lower than in the control group and in the peptide group. The estimated intake of electrolytes was similar in the peptide group and in the control group, whereas in the group receiving sour milk the estimated intake of sodium was lower and the intakes of potassium and calcium were higher than in the control group (*Table 2*). No differences between the groups were observed in the estimated intake of magnesium. Urinary volume was increased in the sour milk group as compared

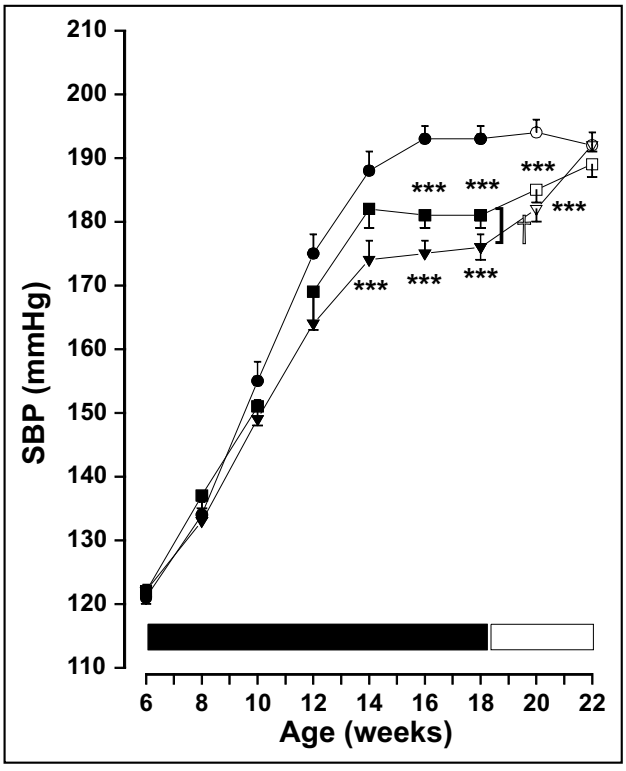

Fig. 1. Effect of long-term intake of IPP and VPP on the development of hypertension in SHR. Circles indicate the control group, squares indicate the peptide group and triangles indicate the sour milk group. Solid bar: the treatment period, open bar: the follow-up period. Data are mean ± SEM (n = 10—11 in each group). ***$P < 0.01$ versus control group, †$P < 0.05$ versus peptide group.

with other groups. Urinary excretion of potassium, magnesium and calcium was higher in the sour milk group as compared to the other groups, but no differences were observed in the sodium excretion (*Table 2*).

Functional bioassay of ACE inhibitory activity in mesenteric arteries

Angiotensin I (0.1 μmol/l) contracted mesenteric arterial preparations similarly to angiotensin II (0.1 μmol/l) (contraction force 0.19 ± 0.03 g after angiotensin I, 0.21 ± 0.03 g after angiotensin II). The angiotensin I-induced contraction was abolished by preincubation with the ACE inhibitor captopril (10 μmol/l) (contraction force 0.01 ± 0.004 g; $P < 0.001$). IPP also dose-dependently inhibited the angiotensin I-induced contraction. The smallest effective concentration which inhibited the contraction was 1 mmol/l (contraction force 0.04 ± 0.01 g; $P < 0.01$). The largest concentration of IPP (3.3 mmol/l) abolished the contraction response to angiotensin I (contraction force 0.02 ± 0.01 g; $P < 0.001$), while the angiotensin II-induced contraction remained unaffected (contraction force 0.29 ± 0.03 g; $P > 0.05$). VPP up to 3.3 mmol/l had no effect on the angiotensin I-contraction.

Table 2. Weight gain, consumption of food and drinking fluid, estimated intake of electrolytes, urine volume and urinary excretion of electrolytes in groups receiving water, IPP and VPP dissolved in water, or sour milk containing IPP and VPP. Variables other than baseline weight were measured at the end of the treatment period.

Variable	Water	IPP & VPP	Sour milk
Weight, g, baseline	138 ± 4	138 ± 5	137 ± 5
Weight g, end of treatment	333 ± 8	334 ± 5	324 ± 10
Consumption of food, g/day	17 ± 1	17 ± 1	6 ± 1***†††
Consumption of drinking fluid, ml/day	33 ± 2	31 ± 3	64 ± 2***†††
Estimated intake of			
Sodium, mmol/d	2.09 ± 0.12	1.98 ± 0.08	1.46 ± 0.07***††
Potassium, mmol/d	2.67 ± 0.15	2.53 ± 0.10	3.39 ± 0.12**†††
Magnesium, mmol/d	1.43 ± 0.08	1.36 ± 0.06	1.37 ± 0.05
Calcium, mmol/d	4.26 ± 0.24	4.05 ± 0.16	6.74 ± 0.23***†††
Urine			
Volume, ml/d	10 ± 1	12 ± 2	35 ± 2***†††
Sodium, mmol/d	1.12 ± 0.06	0.99 ± 0.06	0.97 ± 0.1
Potassium, mmol/d	1.44 ± 0.20	1.36 ± 0.22	2.91 ± 0.1***†††
Magnesium, mmol/d	0.15 ± 0.01	0.15 ± 0.02	0.45 ± 0.01***†††
Calcium, mmol/d	0.02 ± 0.001	0.02 ± 0.001	0.24 ± 0.016***†††

**P < 0.01, ***P < 0.001 versus control; †† P < 0.01, ††† P < 0.001 versus IPP & VPP.

DISCUSSION

We have shown in the present study that long-term intake of milk peptides IPP and VPP attenuated the development of hypertension in young SHR. These tripeptides have previously been shown to reduce blood pressure acutely after single oral administration in SHR (3). Thus, we have now verified that these peptides are effective also during long-term intake. The development of hypertension was also attenuated by a chronic intake of a sour milk containing these peptides. After the 12 weeks of treatment, when hypertension had established and reached a stabile level in all groups, SBP was significantly lower in the peptide group and in the sour milk group than in the control group. After the withdrawal of the treatments, SBP in the peptide and sour milk groups rose gradually reaching the level of the control group, confirming that the antihypertensive effect was due to the treatments.

The antihypertensive mechanism of the tripeptides has been suggested to be ACE inhibition (3, 7, 8). In our functional bioassay in rat vascular preparations, IPP possessed ACE inhibitory activity. However, because this property of IPP was observed only at millimolar concentrations, and VPP did not seem to possess ACE inhibitory activity, other mechanisms underlying the antihypertensive effect of milk peptides may be possible.

In the group receiving sour milk, the development of hypertension was somewhat more extensively attenuated than in the peptide group. Because the intakes of IPP and VPP were similar in these groups, another antihypertensive factor besides the peptides may be present in the sour milk. Milk products contain electrolytes, which can affect blood pressure, eg. calcium, magnesium, potassium, and sodium. Calcium supplementation attenuates the development of hypertension in SHR (13, 14). The estimated intake and the urinary excretion of calcium were higher in the sour milk group than in other groups. Thus, calcium may have played a role in the antihypertensive effect in the sour milk group. Magnesium supplementation has also evoked an antihypertensive effect in some experimental studies (15, 16). In the present study, the urinary excretion of magnesium was highest in the sour milk group. This may be related to the relatively high intake of calcium, which enhances magnesium loss into the urine (17). There is some evidence of a protective effect of potassium on blood pressure (18—20). However, the advantageous effect of supplemental potassium seems to be most potential, when the diet is simultaneously high in sodium (20). In our study, the amount of sodium in the diet was moderate, and no differences were found in the urinary excretion of sodium between the groups. Therefore, magnesium, potassium, and sodium have presumably not had a major influence on our results.

The oral bioavailability of IPP and VPP is not known. Both tripeptides have been detected from abdominal aorta of SHR after single oral administration of a sour milk product containing the peptides (21). Di- and tripeptides can be absorbed intact from the gastrointestinal tract (22, 23). Moreover, peptides possessing a carboxy terminal Pro-Pro -bond seem to be relatively resistant to degradation (24, 25). Hence, some amount of IPP and VPP may have been absorbed as such to elicit an antihypertensive action.

In conclusion, long-term oral administration of IPP and VPP attenuated the development of hypertension in SHR. In addition, a sour milk product containing the tripeptides attenuated the development of hypertension in SHR. One possible mechanism underlying the observed antihypertensive effect is ACE inhibitory activity of the milk peptides. However, the role of other factors, e.g. calcium, cannot be ruled out. Considering the future possibilities of using milk-derived peptides in the non-pharmacological treatment of hypertension, it is essential to confirm the bioavailability of the peptides and perform clinical studies.

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