Original Article

Casein Hydrolysate Containing the Antihypertensive Tripeptides Val-Pro-Pro and Ile-Pro-Pro Improves Vascular Endothelial Function Independent of Blood Pressure-Lowering Effects: Contribution of the Inhibitory Action of Angiotensin-Converting Enzyme

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Accumulating evidence shows that deterioration of vascular endothelial function underlies the pathophysiology of cardiovascular diseases following lifestyle-related diseases. Both Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), which are tripeptides derived from proteolytic hydrolysate of milk casein, inhibit angiotensin-converting enzyme (ACE), suggesting that both VPP and IPP may improve vascular endothelial function, because many ACE inhibitors are known to improve endothelial function. We investigated the effects of ACE-inhibitory food component in humans with mild hypertension, since there has been no report on such effects. The study was conducted by the placebo-controlled, double-blind crossover method in 25 male subjects with mild hypertension. After casein hydrolysate containing both VPP and IPP were administered for 1 week, reactive hyperemia of the left upper forearm was measured using plethysmography as an index of vascular endothelial function. Since one subject dropped out, we analyzed the data of 24 subjects. The reactive hyperemia of the left upper forearm was produced by a 5 min occlusion using inflation of a cuff. The maximum blood flow during reactive hyperemia was 20.8 ± 6.7 mL/min/100 mL tissue in the placebo group, whereas it increased remarkably to 30.0 ± 10.4 mL/min/100 mL tissue in the group administered casein hydrolysate containing both VPP and IPP ($p < 0.001$). There was no change in systemic blood pressure, indicating that the improvement of the vascular endothelial function attributable to VPP and IPP is independent of hemodynamic changes. We conclude that casein hydrolysate containing VPP and IPP improves the vascular endothelial dysfunction in subjects with mild hypertension. The continuous intake of VPP and IPP could help to prevent cardiovascular diseases in hypertensive subjects. (*Hypertens Res* 2007; 30: 489–496)

Key Words: reactive hyperemia, endothelial function, angiotensin-converting enzyme

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Introduction

It has recently been recognized that lifestyle-related diseases such as hyperlipidemia, hypertension and diabetes mellitus precede cardiovascular diseases such as angina pectoris, myocardial infarction and cerebral vascular diseases. Lifestyle-related diseases, which are tightly linked to dietary habits and lifestyles, are on the rise worldwide and have contributed to an increase in cardiovascular events. The lifestyle-related diseases often overlap, and when the lifestyle-related diseases complicated, culminate in the progression of arteriosclerosis. The progression of arteriosclerosis consequently increases in the risk for cardiovascular diseases. There are many cellular explanations for the linkage between the lifestyle-related diseases and cardiovascular diseases. It is well known that the lifestyle-related diseases commonly deteriorate vascular endothelial function (1), one of the key factors for the progression of cardiovascular diseases. Indeed, vascular endothelial cells protect blood vessels and prevent arteriosclerosis by releasing various vasoactive substances that, in addition to their vasoactive properties, have the ability to inhibit monocyte adhesion and/or thrombus formation (2). Therefore, it is very important to protect or restore the endothelial function in patients with lifestyle-related diseases. Angiotensin-converting enzyme (ACE) inhibitors (ACEIs) have been known to decrease the incidence of cardiovascular diseases (3), while many ACEIs have been reported to improve vascular endothelial functions (4, 5). If common human foods could safely provide the ACE-inhibitory actions, their consumption could be a powerful strategy to prevent the incidence of cardiovascular diseases in patients with lifestyle-related diseases. Indeed, sardine peptide (6) derived from sardines, casein dodecapeptide, and dried bonito oligopeptide (7) have been demonstrated to be a source of natural food-derived ACEIs. Intriguingly, two tripeptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), contained in Calpis fermented milk and proteolytic hydrolysate of milk casein also have the effects of ACEIs (8, 9), and we have reported that long-term intake of tablets, fermented milk, or other beverages containing both VPP and IPP resulted in blood pressure-lowering effects in subjects with mild hypertension (10), suggesting that VPP and IPP, with their ACE-inhibiting activities, can improve vascular endothelial function independent of their blood pressure-lowering effects, although to date there has been no report on the improvement of endothelial dysfunction by ACEIs derived from food components.

Therefore, in the present study, we conducted a placebo-controlled crossover study to determine the effect of short-term intake of casein hydrolysate containing both VPP and IPP on the endothelial function in subjects with mild hypertension.

Fig. 1. The trial profiles. Twelve patients received casein hydrolysate containing both Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) and 12 received the placebo in the first treatment period after enrollment. No significant difference was detected in the value of age, body height, body weight, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse rate, blood glucose, HbA1c, total cholesterol and nitrate/nitrite (NOx), or number of cigarettes between the two groups ($p > 0.2$).

Methods

Subjects' Characteristics and the Evaluation of Endothelial Function

Twenty-five Japanese men with mild hypertension defined as a systolic blood pressure (SBP) of 140–159 mmHg or diastolic blood pressure (DBP) of 90–99 mmHg were enrolled in the study. Their ages ranged from 40 to 65 years (54.1 ± 8.1 [mean $\pm$ SD] years). Those who suffered from clinical disorders in liver function, or in the cardiovascular, respiratory, or endocrine systems were excluded from the study. None of the patients were receiving any medication for systemic hypertension, hypercholesterolemia/hypertriglyceremia, or diabetes mellitus. The ethics committee of Soiken Clinic approved the study protocol. Written informed consent for participation was obtained from each subject.

First, the vasodilator response to either reactive hyperemia, an index of endothelium-dependent vasodilatation, or the sublingual administration of nitroglycerin (NTG), an index of endothelial-independent vasodilatation, was evaluated in each subject at baseline and 1 week after the continuous intake of casein hydrolysate containing both VPP and IPP (test sample) or placebo. Each subject had fasted for at least 12 h before the measurement. They were kept in the supine

Fig. 2. The maximum blood flow after the sublingual administration of nitroglycerin following the intake of the test food. No significant difference was detected between the two groups. n.s., not significant.

position in a quiet and air-conditioned room (a constant temperature of 24°C to 27°C) throughout the study. After the subjects had rested for 30 min in that position, we measured forearm blood flow (FBF).

Secondly, the effects of reactive hyperemia on FBF were evaluated by inflating a cuff over the left upper arm to a pressure of 50 mmHg above the SBP for 5 min. After the cuff occlusion was released, FBF was measured for 3 min. After all of the parameters returned to the baseline, we administered 0.3 mg of NTG sublingually using an NTG spray (TOA EIYO Co., Tokyo, Japan), and FBF was again measured for 10 min.

Thirdly, the body weight was measured after the subjects had fasted from the last 9:00 PM and both blood pressure and heart rate were measured during a 30-min rest in the supine position. The fasting serum or plasma concentrations of total cholesterol, triglyceride, glucose, insulin, nitrate/nitrite (NOx), angiotensin II, thromboxane B2, brain natriuretic peptide (BNP), adiponectin, total plasminogen activator–plasminogen activator inhibitor-I (PA–PAI-I) complex, high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor- α (TNF- α), and plasma renin activity (PRA) were measured with serum or plasma obtained after a 30-min rest period.

Test Material

The casein hydrolysate containing both VPP and IPP was prepared using *Aspergillus oryzae* protease (9), and these peptides were measured by liquid chromatography–mass spectrometry as described previously. Subjects ingested 1.25 g of casein hydrolysate powder containing 3.42 mg of VPP and 3.87 mg of IPP per day, and this daily dose was divided into 4 capsules. An equivalent dose of sodium caseinate, which is the starting material for casein hydrolysate, was used as a placebo.

Effects of the Intake of Casein Hydrolysate Containing Both VPP and IPP on Endothelial Function of the Forearm Resistance Arteries

The study protocol is summarized in Fig. 1. One of the 25 subjects dropped out of the study for personal reasons. The other 24 subjects were randomly divided into two groups of 12 each. We conducted a double-blind crossover study with these two groups. First, subjects took either the test sample or the placebo every morning for a week. Then, those who had taken the test sample took the placebo, and those who had taken the placebo took the test sample for 1 week after a washout period of 2 weeks. Each subject was asked to avoid making any changes in lifestyle or dietary habits during the study.

FBF Measurement Using a Strain-Gauge Plethysmograph

The measurement of FBF using a mercury-filled Silastic strain-gauge plethysmograph (EC-6; D. E. Hokanson, Inc., Bellevue, USA) was described previously (11). Briefly, a strain gauge was attached to the lower part of the left arm, connected to a plethysmography device, and supported above the right atrium. A wrist cuff was inflated to a pressure of 50 mmHg above SBP to exclude the hand circulation from measurements 1 min before each measurement and throughout the measurement of FBF. The upper arm cuff was inflated to 40 mmHg for 7 s in each 15-s cycle to occlude venous outflow from the arm using a rapid cuff inflator (E20; D. E. Hokanson, Inc.). The output signal of FBF was transmitted in a recorder. FBF is expressed as mL per min per 100 mL of forearm tissue volume.

Analytical Methods

The baseline FBF was obtained as the average of five measurement values of FBF. The endothelial function of the forearm resistance arteries was evaluated by 1) the maximum blood flow after releasing the occlusion of a cuff over the left upper arm, 2) the change in the pattern of blood flow measured every 15 s, and 3) the area under the curve (AUC) of the time and blood flow relation from 0 s to 60 s.

Samples of venous blood were placed in plastic tubes with or without ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) (1.5 mg/mL). After the plastic tubes were kept at room temperature for 30 min, the serum was separated by centrifugation at 3,000 rpm at 4°C for 10 min. Samples were stored at -80°C until assayed. The plastic tubes containing EDTA-2Na were immediately centrifuged at 3,000 rpm at 4°C for 10 min to separate the plasma. Routine measurement methods were used to determine the serum or plasma concentrations of total cholesterol, triglyceride, glucose, insulin, hsCRP, NOx, and PRA. Angiotensin II and PRA were assayed by radioimmunoassay (RIA), and BNP was assayed by chemiluminescent enzyme immunoassay (CLEIA). The total PA-PAI-I complex was assayed by latex photometric immunoassay (LPIA); adiponectin and TNF- α were assayed by enzyme-linked immunosorbent assay (ELISA); and thromboxane B2 was assayed by radioimmunoassay-thromboelastography (RIA-TEG).

Statistical Analysis

Results are presented as the mean $\pm$ SD. The paired *t*-test was used to compare the clinical parameters between the test group and the placebo group both before and after the administration of capsules, and between before and after the administration of capsules was also compared. Time-course curves of the FBF during reactive hyperemia were compared by two-way ANOVA for repeated measures. A *p* value less than 0.05 was accepted as statistically significant. Data were processed using the software package SPSS ver.11.5 (SPSS Japan Inc., Tokyo, Japan).

Results

Clinical Characteristics and the Evaluation of Endothelial Function

The clinical characteristics of all eligible subjects are presented in Table 1. In the comparison of the two groups before the intake of test materials, both triglyceride and total PA-PAI-I complex levels were higher in the test group than in the placebo group. However, the endothelial functions measured by plethysmography, *i.e.*, the baseline FBF, maximum FBF and AUC, did not differ significantly between the two groups (Table 2). The maximum blood flow after an administration of NTG did not differ between the two groups (Fig. 2).

Table 1. Clinical Characteristics

<i>n</i>	24
Sex	Male
Age (years old)	54.1 $\pm$ 8.1
Height (cm)	169.2 $\pm$ 4.7
Body weight (kg)	68.4 $\pm$ 8.3
Body mass index (kg/m ²)	23.9 $\pm$ 2.5
Systolic blood pressure (mmHg)	144.3 $\pm$ 6.4
Diastolic blood pressure (mmHg)	86.8 $\pm$ 8.1
Heart rate (bpm)	70.7 $\pm$ 11.8
Serum glucose (mg/dL)	98.0 $\pm$ 10.8
HbA1c (%)	4.8 $\pm$ 0.4
Total cholesterol (mg/dL)	220.4 $\pm$ 50.2
NOx (μ mol/L)	23.5 $\pm$ 10.8
Number of smokers	10
Baseline forearm blood flow (mL/min/100 mL tissue)	3.4 $\pm$ 1.2
Maximum forearm blood flow (mL/min/100 mL tissue)	21.5 $\pm$ 7.6

All results are presented as mean $\pm$ SD. Blood pressure was measured at the sitting position. NOx, nitrate/nitrite.

Effects of Casein Hydrolysate Containing Both VPP and IPP on Endothelial Function of the Forearm Resistance Arteries

Table 2 shows the effects of administration of the test sample for 1 week. Although the baseline FBF did not change, the maximum FBF increased from 21.5 $\pm$ 8.3 mL/min/100 mL tissue to 30.0 $\pm$ 10.4 mL/min/100 mL tissue in the test group, which was significantly higher than the value in the placebo group. The AUC increased from 11.4 $\pm$ 4.7 mL/100 mL tissue to 12.9 $\pm$ 4.5 mL/100 mL tissue. In the plotting of the blood flows, the values of blood flow in the test group after 0 and 15 s were significantly higher than the values in the placebo group, and a significant difference was also detected in the comparison of the changing patterns in the ANOVA analysis (Fig. 3). In contrast, no significant difference was detected in FBF after the administration of NTG between the two groups (Fig. 2).

Effects of the Other Markers of Endothelial Function

Both body weight and BMI decreased after the intake of the test sample, but similar tendencies were also observed in the placebo group. SBP did not change after the intake of the test sample but DBP was higher in the test group than in the placebo group. Among the hematological markers, TNF- α was decreased in both groups although the decrease was more remarkable in the test group. Other markers, including the levels of total cholesterol, triglyceride, glucose, insulin, NOx, PRA, angiotensin II, thromboxane B2, BNP, adiponectin,

Table 2. Result by Treatment in Each Group

	Test sample (n=24)		Placebo (n=24)	
	Before	After	Before	After
Body weight (kg)	68.5±8.5	68.1±8.2**	68.3±8.4	67.9±8.4**
Body mass index (kg/m ²)	23.9±2.6	23.8±2.5**	23.9±2.5	23.7±2.6**
Systolic blood pressure (mmHg)	136.2±12.3	137.1±13.6	135.9±13.0	133.8±11.5
Diastolic blood pressure (mmHg)	86.9±9.2	87.2±7.1 ^{##}	84.3±8.5	83.4±7.3
Heart rate (bpm)	68.2±8.5	68.5±6.9	66.8±8.9	68.3±7.5
Total cholesterol (mg/dL)	207.8±45.6	207.0±52.0	204.1±45.4	206.0±45.0
Triglyceride (mg/dL)	167.7±146.6 [#]	135.9±75.2	112.0±60.2	138.1±94.0
Serum glucose (mg/dL)	91.8±9.9	91.0±9.6	94.0±10.3	93.3±10.0
Serum insulin (μU/mL)	4.0±2.2	3.9±1.6	4.4±3.1	4.8±4.8
HOMA-R	0.9±0.6	0.9±0.4	1.1±0.9	1.1±1.2
PRA (ng/mL/h)	1.5±1.2	1.7±1.5	1.5±1.1	1.3±1.0
NOx (μmol/L)	22.2±12.9	21.3±8.4	19.7±8.9	22.2±10.6
Angiotensin II (pg/mL)	11.5±7.5	11.5±12.2	15.5±17.2	10.6±8.0
Thromboxane B2 (pg/mL)	22.0±20.7	14.4±4.6	15.3±7.8	14.1±5.7
BNP (pg/mL)	20.6±27.3	19.9±20.7	22.7±24.8	21.1±22.3
Adiponectin (μg/mL)	6.7±2.7	6.7±2.5	6.7±3.2	6.9±3.4
Total PA-PAI-I complex (ng/mL)	58.7±37.6 [#]	50.7±36.1	44.5±19.4	41.4±17.1
hsCRP (ng/mL)	0.1±0.0	0.1±0.1	0.1±0.2	0.1±0.3
TNF-α (pg/mL)	0.8±0.2	0.5±0.2***	0.7±0.2	0.6±0.2*
Basal FBF (mL/min/100 mL tissue)	3.4±1.1	3.1±0.8	3.4±1.3	3.3±1.2
Maximum FBF (mL/min/100 mL tissue)	21.5±8.3	30.0±10.4***,###	21.5±7.1	20.8±6.7
AUC (mL/100 mL tissue)	11.4±4.7	12.9±4.5*	11.5±4.5	11.4±3.9

Results are presented as mean±SD. Probability value refers to comparison to placebo group ([#] $p<0.05$, ^{##} $p<0.01$, ^{###} $p<0.001$) and to the value before the intake (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) validated by paired t -test. HOMA-R, Homeostasis model assessment insulin resistance; PRA, plasma renin activity; BNP, brain natriuretic peptide; PA-PAI-I, plasminogen activator-plasminogen activator inhibitor-I; hsCRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α; FBF, forearm blood flow; AUC, area under the curve.

total PA-PAI-I complex, and hsCRP, were not changed (Table 2).

Discussion

In this study, we investigated the effect of casein hydrolysate containing both VPP and IPP on the vascular endothelial function in male subjects with mild hypertension, and we observed that it remarkably improved physiological vascular endothelial function. Thus, continuous intake of VPP and IPP could help to prevent cardiovascular diseases in hypertensive subjects, because endothelial dysfunction is believed to be linked to either atherosclerosis or plaque rupture.

Importance of Endothelial Function in the Prevention of Cardiovascular Diseases

According to the report published by the Ministry of Health, Labour and Welfare in 2003 on the causes of death in the Japanese, about one-third of deaths were attributable to atherosclerosis, and thus both the pathophysiology and etiology of atherosclerosis has become a subject of keen interest among

cardiovascular researchers. Recently, the many lines of evidence on the causes of atherosclerosis have culminated in the hypothesis that the deterioration of vascular endothelial function plays a major role (12, 13). Indeed, both cerebral infarction and myocardial infarction have been attributed to the rupture of instable plaques formed by vascular endothelial insufficiency (14). While hypertension is a significant risk factor for deterioration of vascular endothelial function (15), other factors, such as smoking (16), obesity (17), aging (18), menopause (19), and lifestyle-related diseases including diabetes mellitus (20) and hyperlipidemia (21), have also been shown to impair endothelial function. Therefore, the substances that improve vascular endothelial function such as antioxidants (22), substances containing statin (22), estrogen-like substances (23) and ACEIs are expected to decrease the incidence of cardiovascular diseases. In the present study, we found another novel substance containing both VPP and IPP that improves the vascular endothelial insufficiency seen in subjects with mild hypertension. In this study, only men were enrolled to avoid increasing the variance caused by the gender difference. We should test whether the improvement of endothelial function is also observed in women in the future.

Fig. 3. The effects of intake of casein hydrolysate containing both Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) on the plethysmographic changes in the blood flow after the release of the cuff occlusion. In comparison with intake of the placebo, the maximum forearm blood flow after intake of the casein hydrolysate containing both VPP and IPP significantly increased ($p < 0.001$ and $p < 0.05$), and a significant change was detected in the ANOVA analysis between 0 and 45 s after the cuff occlusion is released ($p < 0.001$).

Possible Mechanisms by Which VPP and IPP Improve Vascular Endothelial Dysfunction

Next, we will consider the cellular mechanisms by which the combination of VPP and IPP improves vascular endothelial functions. Since both tripeptides inhibit ACE, there are several possible mechanisms by which they may improve vascular endothelial functions. First, ACE inhibitors increase the blood concentration of bradykinin, and bradykinin increases nitric oxide (NO) production (24), and thus improves endothelial function. Secondly, it is also known that angiotensin II activates NADH/NADPH oxidase at cell membranes and increases superoxide derived from vascular endothelial cells, resulting in the deactivation of NO. Inhibition of these processes is also considered to be a possible mechanism.

Both VPP and IPP may alter the hemodynamic parameters and secondarily modulate the vascular endothelial functions because they inhibit ACE and may decrease systemic blood pressure. However, since intake of the test sample did not significantly alter the hemodynamic parameters in this study, this mechanism is unlikely. The discrepancy between the improvements of vascular endothelial function and the lack of change in blood pressure may be attributable to the fact that the decreases in vascular resistance due to both VPP and IPP would be compensated for by other endogenous vasoconstrictive substances. If we were to administer the test sample for a longer period than used in the present study, the blood pressure may decrease and further improve the vascular endothelial function, because hypertension is believed to impair the vascular endothelial function. However, we should note that not all antihypertensive agents used in clinical practice at present improve endothelial function, and many reports state that short acting-calcium channel blockers and diuretics lack such effects (25).

Thus, it is inconceivable that the change in blood pressure

brought by the antihypertensive therapy has a direct effect on the vascular endothelial function and, in fact, it has been reported that the ACEIs show a reducing effect on the left ventricular hypertrophy that is superior to that of other antihypertensive agents (26).

Assessments of Vascular Endothelial Function

While various approaches have been attempted to evaluate the improvement of the vascular endothelial function, this study employed reactive hyperemia determined by plethysmography as a physiological parameter of vascular endothelial function (27). Plethysmography, which calculates the capability of the level of reactive vasodilatation due to shear stress after stimulation of the vascular endothelium, is a highly reproducible measure requiring no special techniques (28). In this study, the maximum FBF increased significantly in comparison to the baseline and to the placebo group as well as the time-course curve of blood flow.

Flow-mediated vascular dilation is another, physiological method that can be used to evaluate vascular endothelial function. This method measures the dilation in conduit arteries of 2 to 5 mm in diameter, whereas plethysmography measures the endothelial function in the resistance vessels. In comparison to conduit arteries, which are largely dependent on the activity of NO, the resistance vessels are affected by physiologically active substances other than NO that are derived from vascular endothelial cells (29). It is considered that the entire value measured by plethysmography is not necessarily regulated by NO, because NO is involved in the vasodilative ability after 30–60 s rather than being involved in the maximum blood flow (30). Thus, the dilation of the resistance vessels is a reflection of not only NO but also the other substances released from endothelial cells, suggesting that the reactive hyperemia measured by plethysmography may indi-

cate the entire vasodilative function of endothelial cells. This may be one of the reasons that the values of NOx were not different between the two groups, while the vascular reactivity assessed by plethysmography in the test sample group was superior to that in the control group. However, the NOx value was assessed before the measurement of plethysmography. If we had measured the NOx value during reactive hyperemia, an increase of NOx value may have been observed. We should consider how casein hydrolysate affects endothelial function in a future study. Since the vasodilatory actions following the administration of NTG were not different between the test sample and placebo groups, the flow changes in the test sample group were attributable to the endothelial cells, but not to the smooth muscle cells.

On the other hand, there are many physiologically active substances that are considered to reflect the vascular endothelial function, but they have not been established as markers for the vascular endothelial function due to the instability of the methods used for their measurement or the fluctuation of markers for secondary action. When these physiologically active substances were measured, a remarkable decrease in TNF- α would be noted from the baseline. TNF- α is an adipocytokine that contributes to the progression of arteriosclerosis by inducing insulin resistance and accelerating the production of various adhesion factors and growth factors by acting on the vascular endothelial cells. It has been found that ACEIs inhibit the increase in TNF- α (31), and in the present study, the test sample containing both VPP and IPP was also shown to inhibit the increase in TNF- α . Although other markers did not show a significant change after the test sample intake, it is possible that trends in the changes in these highly fluctuating markers were not captured due to the short measurement period of only 1 week. Examination over a longer period is thus considered necessary.

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