CHICKEN COLLAGEN HYDROLYSATE: VASOPROTECTIVE EFFECT AND MECHANISM OF ACTION

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Abstract—Chicken collagen hydrolysate is obtained by treating chicken feet with enzymes to produce an angiotensin I converting enzyme (ACE)-inhibitory peptide. Administration of this chicken collagen hydrolysate (we call it "C-COP") for 12 weeks at 2.9 g/day reduced brachial-ankle pulse wave velocity (baPWV) in humans. In addition, we investigated the concentrations of food derived-peptides following oral intake of C-COP. "Blood peptides" derived from C-COP (namely Pro-Hyp, Leu-Hyp, Pro-Hyp-Gly, Phe-Hyp, Ala-Hyp, Hyp-Gly, Glu-Hyp-Gly, Ala-Hyp-Gly, and Ser-Hyp-Gly) were detected in human blood. Using these blood peptides, we analyzed the mechanism by which C-COP has a vasoprotective effect in humans. Addition of the blood peptides to human umbilical vein endothelial cells (HUVECs) upregulated endothelial nitric oxide synthase (eNOS) mRNA expression and promoted eNOS phosphorylation, thereby increasing NO production. We hypothesized that these responses occur via BK2, which is the receptor for bradykinin, a bioactive endogenous peptide. These findings suggest that C-COP is transferred to the human body in the form of blood peptides, which act on vascular endothelial cells to promote the production of NO, which has a vasoprotective effect.

Index Terms—chicken collagen hydrolysate, vasoprotective effect, eNOS, NO

I. INTRODUCTION

Collagen is one of the main proteins in living organisms and accounts for approximately one-third of all protein in the living bodies of mammals—in particular, humans. It is an extracellular matrix that plays a role in the formation of connecting tissues and as a scaffold for cells, but its accumulation in the body declines with age. Collagen peptide has therefore been used as a food to boost these reduced internal levels, and there have been a substantial number of reports of feelings of subjective wellbeing in subjects taking it as a supplement. However, there has been a great deal of doubt about these findings, and it has been suggested that they are due to a placebo effect. In recent years, vigorous research has been conducted to elucidate both the mechanism of absorption of collagen peptide from food into the body and the tertiary functions of this protein. It has been shown that orally administered collagen peptide is transferred to the blood in the form of dipeptides or tripeptides, without complete degradation to amino acids. Furthermore, collagen peptide increases the number of free-floating fibroblasts in vitro (Shigemura, Iwai, Morimatsu, Iwamoto, Mori and Sato, 2009). A double-blind placebo-controlled trial has confirmed an increase in the moisture content of the skin as a result of intake of collagen peptide (Ohara, Ito,Iida and Matsumoto, 2009). Therefore, in light of the growing body of evidence on the physiological functions of ingested collagen peptide, we investigated the effects of this compound on the circulatory system.

II. MATERIALS AND METHODS

baPWV

A total of 58 subjects with either mild hypertension or high-normal blood pressure were randomly assigned to two groups: a test food group in which subjects were given a drink containing C-COP at 2.9 g/day, and a placebo group in which subjects were given a dummy drink. The study period comprised a total of 18 weeks: 2 observational weeks before the treatment, a 12-week treatment period, and 4 weeks for post-treatment observation to investigate the parameters of brachial-ankle pulse wave velocity (baPWV).

Blood peptides derived from C-COP

Testing was conducted in five healthy subjects. The day before the test, the evening meal was consumed by 21:00; breakfast was not allowed on the day of the test. The test meal was an aqueous solution containing C-COP at a concentration of 20% (w/v). Subjects received C-COP at 25 g/60 kg body weight. Blood was sampled 2 h after intake of the test meal. Using hydroxyproline (Hyp) (a collagen-specific amino acid) as a marker, the blood peptides derived from C-COP were fractionated by gel-filtration high performance liquid chromatography. The structures of the fractionated peptides were determined by using a protein sequencer.

PCR analysis of eNOS and BK2

Each blood peptide was added to EBM-2 medium at 10 times the concentration at which it was detected in the blood. Human umbilical vein endothelial cells (HUVECs) were incubated in this medium for 24 h at 37°C. Cells were recovered and the RNA was extracted to synthesize cDNA. Using this cDNA as a template, endothelial nitric oxide synthase (eNOS) mRNA expression was examined by real-time PCR with primers specific to the human eNOS sequence. Similarly, the bradykinin receptor BK2 was subjected to RT-PCR.

eNOS phosphorylation

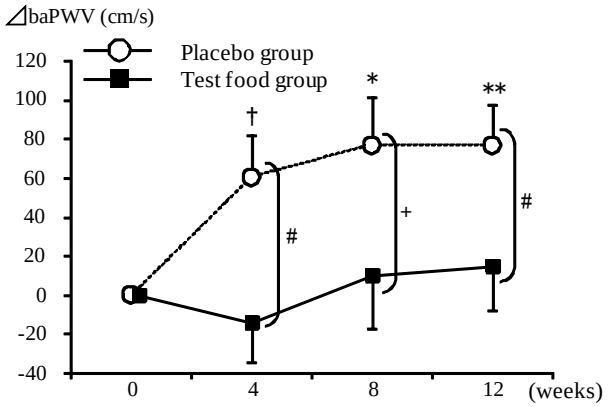
Each blood peptide was added to EBM-2 medium at 10 times the concentration at which it was detected in the blood. HUVECs were incubated in this medium for 3 min at room temperature. The cells were then immediately recovered, lysed in lysis buffer, and subjected to Western blotting by the conventional method. Using eNOS antibody and eNOS phosphorylation antibody, each specific protein was detected and the percent eNOS phosphorylation was analyzed.

NO production

Each blood peptide was added to EBM-2 medium at 10 times the concentration at which it was detected in the blood. HUVECs were incubated in this medium for 24 or 48 h at 37°C. The supernatants were recovered and the NO level was analyzed by using and NO_2/NO_3 assay kit**.**

III. RESULTS AND DISCUSSION

baPWV in the placebo group showed a significant or non-significant increase 4 weeks (1550.5±193.0 cm/s: $P< 0.1$), 8 weeks (1566.8±227.6 cm/s: $P < 0.05$), and 12 weeks (1566.9±182.8 cm/s: $P < 0.01$) after the start of treatment, compared with 1490.0±175.4 cm/s at 0 weeks. The ⊿baPWV in the test food group was significantly less than that in the placebo group during the treatment period (Figure 1). A total of nine kinds of blood peptides derived from C-COP were detected in human blood after intake of C-COP (Iwai, Zhang, Kouguchi, Saiga Egusa, Shimizu & Morimatsu, 2009) (Table 1).



Significantly different from placebo group : +p<0.1, #p<0.05, ##p<0.01

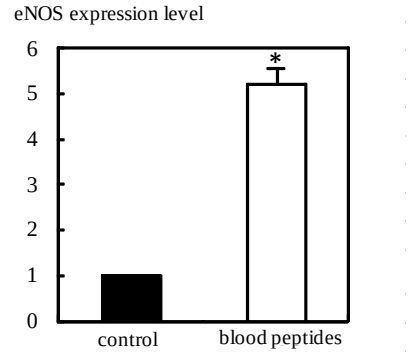
Significantly different from 0wk : †p<0.1, *p<0.05, **p<0.01

Figure 1. Changes in ⊿baPWV of subjects, against the measurements at 0wk, during the treatment period with test food or placebo.

Table 1. Structure and estimated concentration of peptides in blood of healthy volunteers after single oral intake of C-COP.

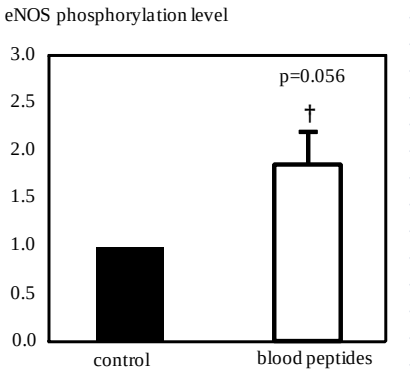
Sequences of amino acid	Concentration (μmol/L)
Pro-Hyp	47.79
Leu-Hyp	28.22
Pro-Hyp-Gly	5.58
Phe-Hyp	3.79
Ala-Hyp	2.27
Hyp-Gly	1.68
Glu-Hyp-Gly	1.02
Ala-Hyp-Gly	0.50
Ser-Hyp-Gly	0.26

Each of these nine kinds of blood peptides was added to HUVECs. After 24 h, the eNOS mRNA expression level was upregulated by about 400% (Figure 2). The eNOS phosphorylation rate was increased 100% by addition of the blood peptides (Figure 3).



Significantly different from control：*p<0.05

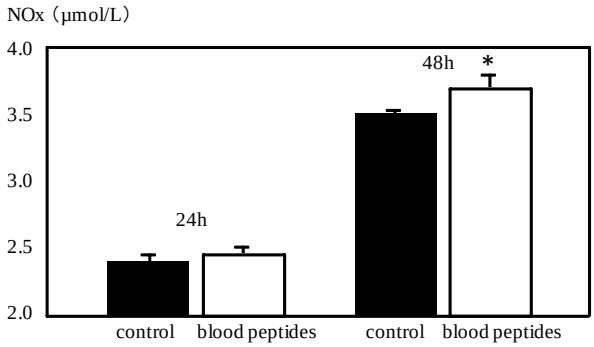
Figre 2. HUVEC eNOS mRNA expression level upregulated by blood peptides.



Significantly different from control：†p<0.1

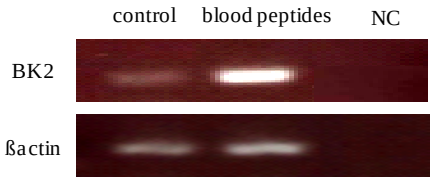
Figure 3. HUVEC eNOS phosphorylation level promoted by blood peptides.

We then examined the release of NO, which is the product of eNOS phosphorylation. As a result of addition of the blood peptides, NO production showed a slight increasing trend after 24 h and by 48 h had significantly increased (Figure 4). Furthermore, we investigated the receptors involved in NO production and confirmed that addition of the blood peptides resulted in upregulation of the expressin of bradykinin receptor BK2 (Figure 5).



Significantly different from control：*p<0.05

Figre 4. HUVEC NOx production increased by blood peptides.



Figre 5. HUVEC BK2 mRNA expression level upregulated by blood peptides.

In humans, C-COP, prepared originally as a collagen peptide with ACE-inhibitory activity (Saiga, Iwai, Hayakawa, Takahata, Kitamura and Morimatsu, 2008), has been confirmed not only to decrease blood pressure but also to improve baPWV, which is an index of vascular flexibility. The improvement in baPWV values demonstrates that C-COP also has a vasoprotective effect. A total of nine kinds of blood peptides derived from C-COP were found in the blood after intake of C-COP. This suggested that incorporated C-COP is transferred to the body in the form of blood peptides, which work on the vascular endothelium to lower blood pressure and exert a vasoprotective effect. Therefore, using HUVECs, we examined the effect of the blood peptides on endothelial cells. Our results showed that the blood peptides upregulated eNOS mRNA expression and promoted eNOS phosphorylation. The body has several factors that stimulate eNOS phosphorylation, including shear stress caused by blood flow, hormones such as acetylcholine, and bioactive peptides such as bradykinin. Bradykinin is a nine-residue peptide that has a vasorelaxing effect and activates BK2 receptors on endothelial cells. Examination of parameters such as the molecular weights of the blood peptides suggested that the blood peptides behaved similarly to bradykinin. We therefore examined BK2 expression levels and found that BK2 expression on endothelial cells was increased by addition of the blood peptides. This suggested that the blood peptides, using the bradykinin receptor, communicate with endothelial cells to enhance NO production.

These findings indicate that C-COP follows a clear pathway to exert a vasoprotective effect in the human body and hence could serve as a useful food.

IV. CONCLUSION

Intake of C-COP improves baPWV values, thus maintaining vascular flexibility. This is probably because blood peptides entering the body as a result of C-COP intake act on vascular endothelial cells via the bradykinin BK2 receptor and increase NO production by increasing eNOS expression and phosphorylation.

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