Effects of Collagen Peptide Administration on Visceral Fat Content in High-Fat Diet-Induced Obese Mice

Ran WATANABE1, Mana YAMAGUCHI1, Kyosuke WATANABE1, Muneshige SHIMIZU2, Azusa TAKAHASHI1, Hideyuki SONE1 and Shin KAMIYAMA1,*

1Department of Health and Nutrition, Faculty of Human Life Studies, University of Niigata Prefecture, 471 Ebijagase, Higashi-ku, Niigata 950–8680, Japan
2Department of Fisheries, Faculty of Marine Science and Technology, Tokai University, 3–20–1 Orido, Shimizu, Shizuoka 424–8610, Japan

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Summary Collagen peptides (CPs) are bioactive molecules that have beneficial effects on bone metabolism and against joint disorders. In the present study, we investigated the effect of CP supplementation on visceral fat mass and plasma lipid concentrations in high-fat diet (HFD)-induced obese mice. Male ddY mice were fed a normal diet or HFD for 3 wk, and assigned to N or NCP groups and to F or FCP groups, respectively. The NCP and FCP group mice were administered experimental diets containing 25 mg/g CPs for 3 wk further. During the experimental period, CP supplementation affected neither the food consumption nor the body weight of the mice. No significant differences in the plasma triglyceride, non-esterified fatty acid, and cholesterol concentrations were observed among all the groups. In contrast, the weight of testicular fat mass was significantly decreased in the FCP group as compared with that in the F group. The expression levels of leptin and tumor necrosis factor (TNF)-α genes in the adipose tissue correlated with the visceral fat mass, although these differences were not significant. These findings indicate that CPs may have a reducing effect on visceral fat content but are less effective in reducing body weight.

Key Words chicken collagen hydrolysate, obesity, visceral fat mass, fat-reducing effect, tumor necrosis factor α

Collagen is the primary protein found in various types of connective tissues in the body. The denatured form of collagen, gelatin, is widely used in food, cosmetics, and biomedical industries. Ingestion of gelatin has been traditionally used for therapeutic purposes to reduce pain in joint disorders, although its nutritional value is low. Since collagen is insoluble in cold water, collagen hydrolysates prepared by enzymatic degradation of gelatin, namely collagen peptides (CPs), are used instead in food supplements and pharmaceutical preparations. Many studies have reported that oral administration of CPs has beneficial effects on bone metabolism and against joint disorders (1–6). We previously reported that administration of CPs derived from chicken cartilage could partially suppress the rheumatoid arthritis score and levels of plasma inflammatory cytokines in SKG mice (7). Clinical trials suggested that CP supplementation may have positive therapeutic effects against osteoarthritis and other joint disorders (4–6).

Unlike other proteins, collagen is comprised of a repeated triplet sequence (glycine (Gly)-X-Y) and contains the specific modified amino acids hydroxyproline (Hyp) and hydroxylysine (Hyl). These unique features of collagen render it resistant to brush border peptidases and allow it to be partially absorbed into the blood as small peptides (8–11). These oligopeptides found in plasma, particularly prolyl-hydroxyproline (Pro-Hyp), are known to exert various bioactive effects, such as stimulation of skin fibroblast growth (12) and chondroprotective effects in articular cartilage (13). Furthermore, biofunctional peptides derived from CPs exhibit an inhibitory effect on angiotensin I-converting enzyme (14). CP supplementation also exerts modulatory effects on the human circulation system, as seen in patients with mild hypertension or high-normal blood pressure (15).

Recent studies also reveal the potential of CPs to improve glucose and lipid metabolism. A high dose (4.5 g/kg body weight/d) of CPs derived from marine fishes could improve glucose metabolism and reduce insulin resistance in type-2 diabetes rats (16). CPs also inhibit dipeptidyl peptidase-IV and stimulate glucagon-like-peptide-1 (GLP-1) secretion. Administration of CPs enhances glucose tolerance through both GLP-1-dependent and independent mechanisms in mice (17). In humans, nutritional supplementation with CPs reduces fasting blood glucose and HbA1c levels and improves insulin sensitivity in type-2 diabetic patients (18). Furthermore, oral administration of fish skin CPs suppressed lipid absorption and reduce the levels of plasma total lipids and triglycerides in rats, although the body, liver, and fat weights of rats are not affected (19). Thus, although CPs have a variety of beneficial health effects, their efficacy against obesity and obesi-

*To whom correspondence should be addressed.
E-mail: kammy@unii.ac.jp
ty-related diseases remains unclear.

Obesity is associated with a higher risk of metabolic syndromes such as diabetes, heart disease, and hypertension. We recently reported that the administration of chicken CPs in combination with exercise has a suppressive effect on food intake in high-fat diet (HFD)-induced obese mice (20). In the present study, we further investigated the effect of CP administration on obesity and its metabolism in mice. We administered CPs to HFD-induced obese mice and evaluated their effects on visceral fat mass and plasma lipid concentrations.

MATERIALS AND METHODS

**Animals and experimental diet.** Male ddY mice (4 wk old) were obtained from Japan SLC Inc. (Shizuoka, Japan). The mice were housed individually in an air-conditioned room, which was maintained at 12 h light–dark cycle. C-LAP, a low-molecular weight collagen hydrolysate derived from chicken feet, was purchased from NH Food Ltd. (Osaka, Japan). Casein was purified from Oriental Yeast Co. Ltd. (Tokyo, Japan). Experimental diets for groups were prepared using a commercial powdered diet (CE-2-powder, CLEA Japan). The mice were housed individually in an air-conditioned room, which was maintained at 12 h light–dark cycle.

**Preparation of cDNA samples from testicular fat tissue.** Total RNA was extracted from the tissue samples using TRIzol reagent (Thermo Fisher Scientific, MA, USA). Frozen tissue samples were homogenized in 1 mL TRIzol, and total RNA was prepared according to the manufacturer’s protocol. cDNA was synthesized from random primers using the PrimeScript first strand

| Gene              | Forward primer (5′-3′) | Reverse primer (5′-3′) | Accession number |
|-------------------|------------------------|------------------------|------------------|
| β-Actin           | ctgggtgatagatcttgcttg  | gtacttgctgctaggaggg   | NM_007393        |
| ACC1              | gcacgtcagaggaagatgg    | tggagaagtagcatcagtg    | NM_133360        |
| ACS1              | agatgctgtgtagtaacgcgg  | ttagctcaactgctgggta   | NM_001302163     |
| Adiponectin       | cccagctctgcaagtaagtg   | agtgcacacctgctgctcag  | NM_009605        |
| ATGL              | caacpcctacactctacgc    | accagtgtaagggaggtg    | NM_001166389     |
| CD36              | gtttaacccagatgcgctg    | tccaaacagctgaagctgctg | NM_001159558     |
| DGAT-1            | atatccccgtgcacaagtgg   | agaatcggcccacatcagct  | NM_010046        |
| DGAT-2            | ggcgtacctccagactacac   | tcggagttaccgaccaac    | NM_026384        |
| FAS               | tcgtgcacgctgtctctac    | ggagttgctgcctgctcct   | NM_007988        |
| IL-6              | eggcctctcctactcacaactc| caagtgctgctgtgcttc    | NM_009605        |
| MCP-1             | gtctggtcatgaccccaagaa | lgcgtgggggcttgctgaga | NM_011333        |
| HSL               | tggattttggggtgctctggt  | gtacccctctgtctgcttc   | NM_010719        |
| Leptin            | ccattttacacacagctgctg  | agcccagtagaaccctgag   | NM_008493        |
| PPAR-γ            | agggcgcgtgagaggagaaga | cggccatccgcccttgaaga  | NM_001127330     |
| TNF-α             | ccacggtagatgtcagcgg    | actgtgagagggagggccat  | NM_001278601     |
| SREBP-1c          | cccttcacaaacctggtactc  | aacgacaggagagggccat   | NM_011480        |
cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan).

Quantitative analysis of gene expression by real-time PCR. The primers used in this study are listed in Table 1. The relative amount of transcripts in the cDNA samples was determined by real-time PCR using SYBR Premix Ex Taq II (Perfect Real Time; Takara Bio Inc.) and the PicoReal 96 real-time PCR system (Thermo Scientific). The reaction was performed through 40 cycles of denaturation at 95°C for 5 s and extension at 60°C for 30 s and the results analyzed using the delta-delta Ct method. The specificity of the amplification was confirmed by melting curve analyses. The relative amounts of transcripts were normalized to those of 18S/H9252-actin transcripts present in the same cDNA sample.

Statistical analysis. Statistical significance was evaluated using analysis of variance (ANOVA) and multiple comparisons Holm test. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the R-software (version 3.6.2).

RESULTS

Body weight and food intake

The ddY strain mice are known to easily gain body weight and show hypertriglyceridemia in response to dietary fat (21). Figure 1A shows the change in body weight of the mice in each group during the obesity-inducing period and the subsequent experimental diet-feeding period. After 3 wk of the obesity-inducing period, mice fed the HFD showed significantly higher body weight compared with those fed a normal diet. During the following 3 wk of the experimental diet-feeding period, the mice fed HFD with or without CPs (F and FCP groups, respectively) retained significantly higher body weight than those fed the corresponding normal diet (N and NCP groups). However, no difference was observed between the N and NCP groups, or between those in the F and FCP groups (Fig. 1A).

The cumulative food intake of the mice in each group during the experimental period is shown in Fig. 1B. Mice fed the HFD showed significantly lower daily food intake throughout the experimental period. In the FCP group, the amount of CPs ingested by the mice was estimated to be approximately 120 mg CPs per day. Supplementation with CPs had no effect on the daily food intake in both the normal diet- and the HFD-fed groups.

Weight of organs and visceral fat tissues

Table 2 shows the body weight and weights of liver, kidney, and visceral fat tissues of the mice in each group at the end of the experimental period. The mice in the FCP groups showed significantly higher liver weights than those in the N and NCP groups; however, no difference was observed between the mice in the N and NCP groups, or between those in the F and FCP groups. The

![Fig. 1. Body weight changes (A) and cumulative food intake (B) of mice in each group during the experimental period. (A) Body weight changes. Values obtained from 10 (2 groups, 0 to 2 wk) or 5 (4 groups, 3 to 6 wk) mice are shown as mean ± SE. The initial (0) and the last (6) week of the experimental period correspond to 5 and 11 wk of age, respectively. a,b Mean values indicated by dissimilar letters are significantly different (p < 0.05). (B) Cumulative food intake was determined from the daily food intake. Values obtained from 5 mice are shown as the mean ± SE. The first (1) and the last (6) week of the experimental period correspond to 6 and 11 wk of age, respectively. a,b Mean values indicated by dissimilar letters are significantly different (p < 0.05).]

|                      | N       | NCP     | F       | FCP     |
|----------------------|---------|---------|---------|---------|
| Body weight (g)      | 43.5 ± 1.7a | 43.9 ± 1.9a | 56.7 ± 3.7b | 56.0 ± 2.7b |
| Liver (g)            | 1.53 ± 0.05a | 1.57 ± 0.11a | 1.88 ± 0.12ab | 2.09 ± 0.19b |
| Kidney (g)           | 0.61 ± 0.02a | 0.69 ± 0.03ab | 0.67 ± 0.03ab | 0.75 ± 0.04b |
| Mesenteric fat (g)   | 0.39 ± 0.10a | 0.38 ± 0.10 | 0.86 ± 0.16 | 0.73 ± 0.14 |
| Perirenal fat (g)    | 0.25 ± 0.04a | 0.24 ± 0.06a | 0.81 ± 0.18b | 0.82 ± 0.12b |
| Testicular fat (g)   | 0.71 ± 0.24a | 0.88 ± 0.24ae | 2.58 ± 0.21b | 1.67 ± 0.27c |
| Total visceral fat (g)| 1.34 ± 0.34a | 1.50 ± 0.39a | 4.25 ± 0.52b | 3.22 ± 0.40b |

Values obtained from 5 mice are shown as the mean ± SE.
a,b,c Mean values indicated by dissimilar letters are significantly different (p < 0.05).
Expression of genes involved in triglyceride metabolism in terms of total liver lipid concentration.

No significant differences were observed among the groups in the concentrations of total liver lipid, plasma lipids, and total liver lipid of the mice in each group. Regarding the adiponectin, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) gene expression levels, no significant differences were observed among the groups. Similarly, no significant differences were observed among the groups in terms of total liver lipid concentration.

Expression of genes involved in triglyceride metabolism in adipose tissue

The expression levels of genes involved in triglyceride metabolism and catabolism in testicular adipose tissue are shown in Table 3. The mice in the F group showed significantly higher expression levels than those in the N and NCP groups. Interestingly, the weight of testicular fat mass in the FCP group was significantly decreased as compared with that in the F group.

Blood glucose, plasma lipids, and total liver lipid concentrations

The concentrations of fasting blood glucose, plasma lipids, and total liver lipid of the mice in each group are shown in Table 3. The mice in the F group showed significantly higher blood glucose concentrations than those in the N group. In the FCP group, the blood glucose level was similar to that in the NCP group. Regarding the levels of plasma triglycerides, non-esterified fatty acids, and total cholesterol, no significant differences were observed among the groups. Similarly, no significant difference was observed among the groups in terms of total liver lipid concentration.

Expression of genes involved in triglyceride metabolism in adipose tissue

The expression levels of genes involved in triglyceride synthesis and catabolism in testicular adipose tissue are shown in Table 4. Among the 11 genes tested (3 for fatty acid and acyl CoA synthesis, 1 for fatty acid translocase, 2 for diacylglycerol esterification, 3 for triglyceride lipases, and 2 for regulatory transcription factors), the sterol regulatory element binding protein-1c (SREBP1c) gene in the NCP group showed significantly higher expression levels than those in the F and FCP groups. Regarding other genes, no significant difference was observed. These results suggest that CP administration may have little impact on the expression of genes involved in triglyceride metabolism.

DISCUSSION

In the present study, the administration of chicken feet-derived CPs resulted in a decrease in visceral fat, particularly testicular fat, in mice with HFD-induced obesity.
obesity. However, CPs did not affect the total body weight or plasma and liver lipid concentrations. These findings indicate that CPs may be more effective at reducing visceral fat content than at reducing body weight.

We previously reported that the administration of 100 mg CPs after low-intensity exercise has a suppressive effect on food intake and on the expression of some fat synthesis-associated genes in adipose tissues in mice with HFD-induced obesity (20). In the present study, mice in the NCP and FCP groups were allowed free access to experimental feed containing 25 mg/g CPs, which was equivalent to 10% of the total protein content. The mass of supplemental CP protein in mice reflects 5 g of CPs in humans who ingest 50 g of protein per day. Here, the CP dose had little effect on food consumption and on the expression of fat metabolism-associated genes in fat metabolism in adipose tissues, possibly reflecting differences in administration method between our two studies. We previously reported that the chicken feet-derived CPs could be absorbed partially as di- or tripeptide forms by rats (9). In humans, Pro-Hyp and other small oligopeptides are detectable in plasma after collagen ingestion; they remain undegraded because of their resistance to peptidase (8, 11, 23). Nevertheless, the precise levels of these bioactive peptides required to exert fat-reducing effects in the plasma remain unknown. To confirm the effectiveness of CPs, it is crucial that fat-reducing peptides be identified and that their efficacy be evaluated by intraperitoneal or intravenous administration.

In the present study, the fat-reducing mechanism of CPs was unclear, but it is possible that CPs increased total energy consumption in the skeletal muscles. It has been reported that the collagen-derived dipeptide Hyp-Gly promotes myoblast differentiation and myotube hypertrophy in skeletal muscle cells by activating the PI3K/Akt/mTOR signaling pathway (24). A randomized controlled trial has shown that the combination of CP supplementation and resistance training increases muscle strength and improves body composition in elderly men with sarcopenia by increasing fat-free mass, muscle strength, and loss of fat mass (25). In the present study, we did not assess the skeletal muscle mass of mice in each group; therefore, body composition and factors involved in muscle energy metabolism should be analyzed in future studies. Additionally, details on the expression status of regulators of muscular protein synthesis and degradation are needed to know the action of CPs.

Another possible underlying mechanism is that CPs affect the absorption of nutrients in the gut. It has been suggested that CPs suppress lipid absorption and reduce plasma lipid levels in rats (19). CPs is also known to improve glucose tolerance by inhibiting intestinal glucose uptake and enhancing insulin secretion (17). We measured fasting blood glucose and plasma lipid concentrations in mice, but further studies on postprandial blood glucose and plasma lipid levels are needed to yield clues for determining the action of CPs. In addition, the effects of CP supplementation might be attributable to differences in protein sources. In the present study, we used casein as the reference protein for CP supplementation. It is also important to confirm the effectiveness of CP administration in obese mice by comparing the administration of CP with that of various reference proteins, including protein hydrolysates and amino acid mixtures. Further investigation is required to elucidate the significance of CP supplementation in obesity and obesity-related diseases.

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