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Note

Effects of Collagen-Derived Oligopeptide Prolylhydroxyproline on Differentiation of Mouse 3T3-L1 Preadipocytes

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Beneficial effects of collagen peptide ingestion to reduce serum triacylglycerides have been reported, suggesting that the physiological conditions of adipocytes are modulated following collagen peptide ingestion. In this study, the effects of prolylhydroxyproline, a major digestive product of ingested collagen peptide in the blood, on the differentiation of mouse 3T3-L1 preadipocytes *in vitro* were investigated. 3T3-L1 preadipocytes were induced to differentiate to adipocytes and treated with prolylhydroxyproline, or with an amino acid mixture of proline and hydroxyproline as a control. The amount of lipid was not affected by these treatments. However, the size of the lipid droplet was significantly smaller when treated with prolylhydroxyproline compared to the amino acid mixture or the non-treated control. Proton-coupled oligopeptide transporters were expressed in non-differentiated and/or differentiated 3T3-L1 cells. These results suggest that prolylhydroxyproline might modulate the morphology of lipid droplets by incorporation into adipocytes through the transporters.

Keywords: collagen peptide, prolylhydroxyproline, adipocyte, 3T3-L1 cell, proton-coupled oligopeptide transporter

Abbreviations: collagen peptide (CP); hydroxyproline (Hyp); prolylhydroxyproline (Pro-Hyp); Dulbecco's modified Eagle's medium (DMEM); fetal bovine serum (FBS); proline (Pro); adipocyte differentiation-related protein (ADRP)

Introduction

Collagen is the most abundant protein in animal tissues. Collagen in skin and bone is heat-denatured when boiled in hot water and becomes water-soluble as gelatin. Native collagen is highly resistant to hydrolysis by proteases other than collagenase, but gelatin is susceptible to digestion by various proteases. Collagen peptide (CP) is prepared by partial hydrolysis of gelatin with proteases and is used as a common ingredient to improve the functions of tissues such as skin, bone or cartilage. Beneficial effects of the ingestion of gelatin or CP have been reported in clinical and animal studies. For example, ingestion of CP decreases joint pain in athletes (Clark *et al.*, 2008) and increases bone mineral density in animals experiencing protein malnutrition (Koyama *et al.*, 2001). CP ingestion also improves the mechanical properties of the Achilles tendon (Minaguchi *et al.*, 2005) and the skin (Matsuda *et al.*, 2006). Skin damage induced by repeated UVB irradiation in mice is suppressed by CP ingestion (Tanaka *et al.*, 2009). Brittle nails are improved (Rosenburg *et al.*, 1957), and hair diameter increases following the ingestion of gelatin (Scala *et al.*, 1976). In addition, it was reported recently that serum triacylglycerides are decreased following ingestion of CP (Wu *et al.*, 2004; Saito *et al.*, 2009), suggesting the possibility that ingested CP modulates lipid metabolism by altering the function of the liver and/or adipocytes.

Collagen contains a collagen-specific amino acid, hydroxyproline (Hyp). In 1962, Prockop *et al.* reported that a Hyp-containing peptide appeared in the blood and urine after ingesting gelatin. However, the amino acid sequence of this Hyp-containing peptide remained unknown. In 2005, Iwai

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et al. reported that 6 types of Hyp-containing oligopeptide appear in the blood following ingestion of CP. Prolylhydroxyproline (Pro-Hyp) was the most abundant of these oligopeptides. In 2009, it was reported that Pro-Hyp promotes migration of skin cells from an explanted skin piece *in vitro*, and that Pro-Hyp accelerated proliferation of the migrated skin cells on collagen gel (Shigemura *et al.*, 2009). On the other hand, Nakatani *et al.* (2009) reported that ingestion of Pro-Hyp inhibited the loss of chondrocytes and thinning of the articular cartilage layer caused by the ingestion of a large amount of phosphorus. They also showed that Pro-Hyp inhibited mineralization of chondrocytes *in vitro* and modulated the expression of the Runx1 and osteocalcin genes in the murine chondrocytic cell line, ATDC5. These studies suggest that Pro-Hyp is one of the physiologically active oligopeptides derived from ingested CP.

3T3-L1 is a mouse preadipocyte line cell frequently used for studies on the differentiation of preadipocytes *in vitro*, since they differentiate and mature to produce triacylglycerides upon stimulation with insulin, dexamethasone and isobutylmethylxanthine (Pinent *et al.*, 2005). In this study, we investigated the effects of Pro-Hyp on differentiation of 3T3-L1 cells *in vitro* in order to examine whether a digested product of ingested CP modulates the functions of adipocytes.

Methods

Cells A mouse 3T3-L1 preadipocyte cell line (IFO50416) was obtained from Health Science Research Resources Bank (Osaka, Japan). 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, MO, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich, MO, USA). Cells were seeded in a 6- or 24-well plate at a density of 5×10^4 cells/mL. Two days after confluence (day 0), the medium was changed to DMEM + 10% FBS supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Invitrogen, CA, USA), 5 μM insulin (Wako Chemicals, Osaka, Japan), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, MO, USA) and 0.1 μM dexamethasone (Sigma-Aldrich, MO, USA). The medium was changed on day 2 to DMEM+10% FBS with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B and 5 μM insulin. The medium was changed every 2 days until day 14.

Oligopeptide and amino acids Pro-Hyp was purchased from Bachem Co. Ltd (G-3025; Bubendorf, Switzerland). Amino acids L-proline (Pro) and L-Hyp were purchased from Wako Chemicals, Osaka, Japan. 200 ng/mL Pro-Hyp (the Pro-Hyp group) or a mixture of Pro and Hyp (200 ng/mL each; the Pro/Hyp group) was added to the medium every 2 days according to the method of Pinent *et al.* (2005) and Kim *et al.* (2006).

Measurement of lipid amount The amount of lipid was determined by the Oil Red O method. Cells were washed twice with 0.1 M phosphate buffer (pH 7.4) and fixed in 10% formaldehyde solution for 1 h. Lipid in the cells was stained with 0.1 mg/mL Oil Red O solution (Wako Chemicals, Osaka, Japan) for 2 h. After discarding the Oil Red O solution, stained lipid was extracted with 100% isopropylalcohol and measured using a UV-visible spectrophotometer V-530 (JASCO, Tokyo, Japan) at a wavelength of 510 nm. Values were expressed in logarithmic scale.

Morphometric analysis of lipid droplets Lipid droplets in 3T3-L1 cells stained with Oil Red O were observed under a phase contrast microscope (CX2; Olympus, Tokyo, Japan), and their images were recorded with a digital camera for microscopes (DP20; Olympus, Tokyo, Japan). One hundred cells were selected at random, and the diameters of the lipid droplets were measured using the software package Image J (version 1.30, NIH, USA).

In order to observe the ultrastructure of the cells on day 14, the specimens were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), followed by postfixation for 1 hr in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). Samples were then dehydrated with ethanol and embedded in Quetol 812. Ultrathin sections were stained with 1% uranyl acetate and 2% lead citrate and observed under a JEM-1220 electron microscope (JEOL, Tokyo, Japan).

Quantitative real time-PCR RNA was isolated from cells on days 0, 8 and 14 with ISOGEN (Nippon Gene Co., Toyama, Japan). Total RNA from each sample was used to synthesize cDNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. To perform real-time PCR, specific primers and probes for mouse proton-coupled oligopeptide transporter PEPT1, PEPT2, PHT1 and Ci1 (mouse homologue of PHT2 in human), lipid droplet-associating proteins perilipin and adipocyte differentiation-related protein (ADRP), and GAPDH as an endogenous control, were all purchased from Applied Biosystems, CA, USA (TaqMan® Gene Expression Assays; ID: Mm01244861_m1, Mm00453524_m1, Mm00451610_m1, Mm00505709_m1, Mm00491666_m1, Mm00558672_m1, Mm00475794_m1, Mm0330249_g1, respectively). PCR was performed using a 7300 Real-time PCR System (Applied Biosystems, CA, USA) following the manufacturer's protocol. The reaction solution for PCR was added at 20 μL/well and contained 10 μL TaqMan® Universal PCR Master Mix, 1 μL primer and probe, and 9 μL cDNA + RNase-free water. Each PCR performed used 2 wells/sample. Amplification conditions were 2 min at 50°C, 10 min at 95°C and then 40 cycles each con-

sisting of 15 sec at 95°C and 1 min at 60°C. The Ct value obtained by amplification of each target was compared among the samples after normalization using GAPDH expression levels.

Statistical analysis Statistical differences were detected using the Tukey-Kramer method. The difference was considered to be significant at $p < 0.05$.

Results

Amount of lipid Upon induction of differentiation, lipid was observed as small lipid droplets after day 4. The amount of lipid increased during the culture period until day 14 in all three groups. However, no significant difference in the amount of lipid was detected among the three groups (Fig. 1).

Size of lipid droplets The size of the lipid droplets increased during the culture period in all three groups, probably due to the fusion of small droplets into larger droplets. However, smaller droplets were observed more frequently in the Pro-Hyp group (Fig. 2B) than in the control group (Fig. 2A) or the Pro/Hyp group (Fig. 2C). This was confirmed by observation with a transmission electron microscope: smaller diameter droplets were observed in the Pro-Hyp group (Fig. 3B) more frequently than in the control group (Fig. 3A) or the Pro/Hyp group (Fig. 3C). This effect on droplet size seemed specific to Pro-Hyp since larger droplets were frequently found in the Pro/Hyp group (Fig. 3C).

The temporal change of the mean droplet size from day 4 through day 14 is shown in Fig. 4. Droplet size in the control group increased gradually during the 14 days of culture. In

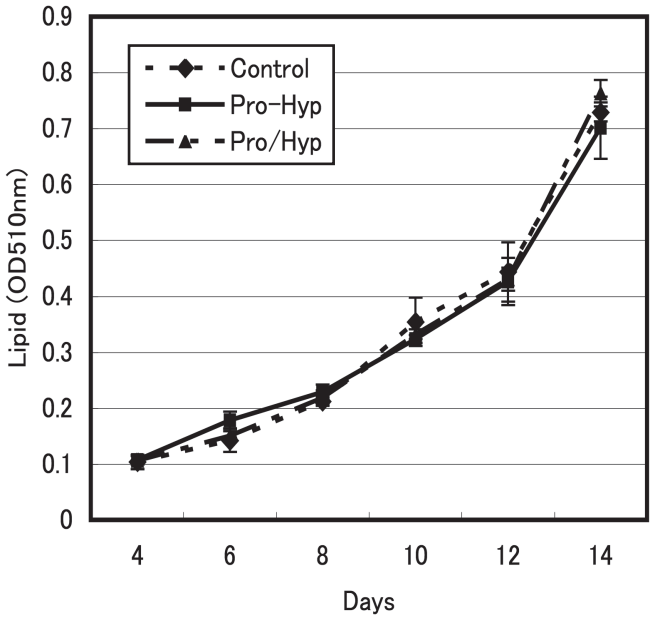

Fig. 1. Changes in the amount of lipid during differentiation of 3T3-L1 cells.

The amount of lipid was determined on days 4, 6, 8, 10, 12 and 14. Mean ± SD.

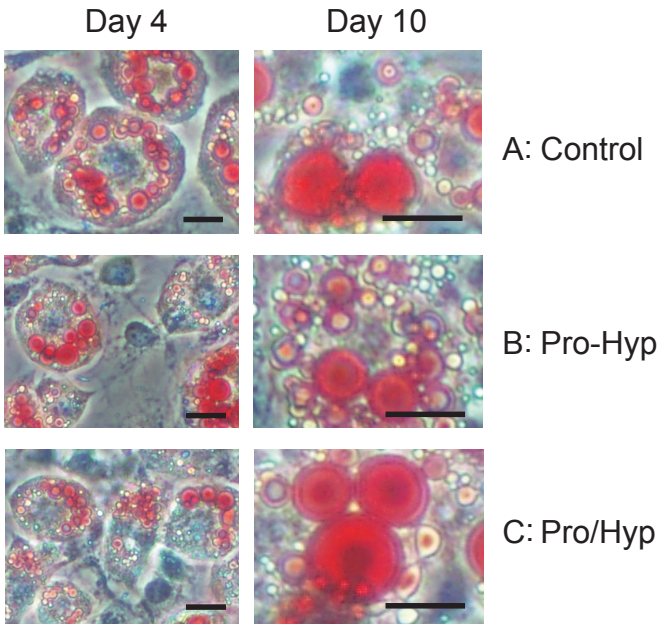

Fig. 2. Light microscopy images of differentiated 3T3-L1 cells stained with Oil Red O.

The control group (A: Control), the Pro-Hyp group (B: Pro-Hyp) and the Pro/Hyp group (C: Pro / Hyp) on day 4 (left) and day 10 (right). Scale bar is 25 μm.

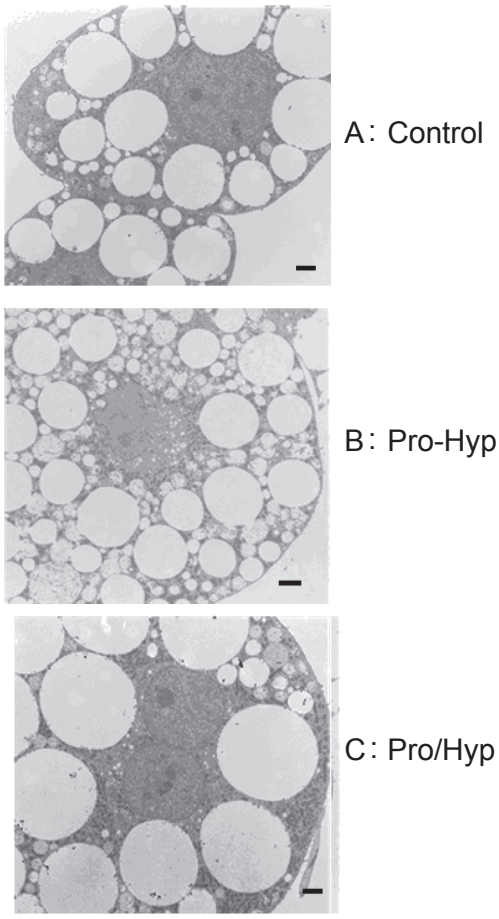

Fig. 3. Transmission electron microscopy images of differentiated 3T3-L1 cells.

The control group (A: Control), the Pro-Hyp group (B: Pro-Hyp) and the Pro/Hyp group (C: Pro/Hyp) on day 14. Scale bar is 5 μm.

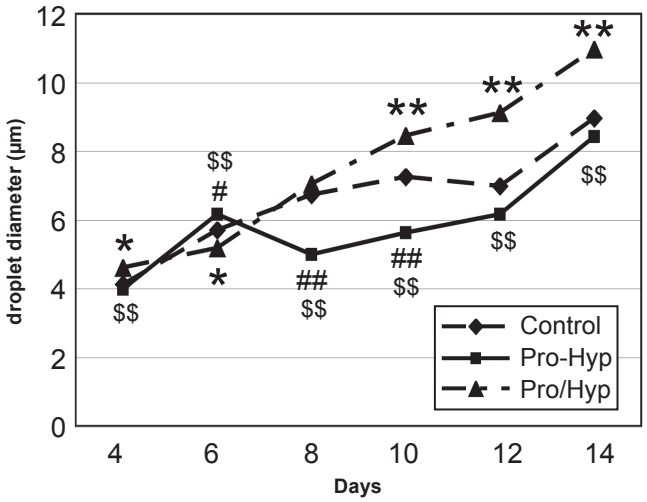

Fig. 4. Changes in lipid droplet size in differentiating 3T3-L1 cells.

The mean of lipid droplet size of the control group (Control), the Pro-Hyp group (Pro-Hyp), and the Pro/Hyp group (Pro/Hyp) is shown. ** and * indicate that the Pro/Hyp group differs at $p < 0.01$ and 0.05, respectively, compared to the control group. $$ indicate that the Pro-Hyp group differs at $p < 0.01$ compared to the Pro/Hyp group. ## and # indicate that the Pro-Hyp group differs at $p < 0.01$ and 0.05, respectively, compared to the control group.

the Pro/Hyp group, droplet size was significantly larger than that of the control group, particularly beyond day 10. In contrast, droplet size was clearly smaller in the Pro-Hyp group than in the control group and the Pro/Hyp group beyond day 8.

Gene expression of proton-coupled oligopeptide transporters Oligopeptides such as Pro-Hyp can be transported into a cell by proton-coupled oligopeptide transporter PEPT1, PEPT2, PHT1 and Ci1 (PHT2 in human). We examined whether PEPT1, PEPT2, PHT1 and Ci1 are expressed in 3T3-L1 cells on days 0, 8 and 14, since Pro-Hyp modulates the differentiation of 3T3-L1 cells by reducing the size of the lipid droplets. As shown in Fig. 5A, PEPT1 expression, which was minimal on day 0, increased by day 8 and even more by day 14. However, no significant difference was detected among the three groups either on day 8 or day 14. In contrast, PEPT2 was highly expressed in 3T3-L1 cells on day 0 (Fig. 5B). PEPT2 mRNA decreased by day 8 in all three groups, but increased again by day 14. No significant difference was detected among the three groups either on day 8 or day 14. PHT1 was expressed on day 0 but decreased by day 8 and decreased further by day 14 (Fig. 5C). Cil mRNA did not show an evident change following differentiation (Fig. 5D).

Gene expression of lipid droplet-associating proteins

Expression of the perilipin gene was not detected in undifferentiated 3T3-L1 cells. On the induction of differentiation, the perilipin gene was highly expressed by day 8 in control cells but decreased again by day 14. This was also the case for the other two groups treated with the amino acid mixture or with Pro-Hyp (Fig. 5E). Expression of another lipid droplet-associating protein, ADRP, was detected in undifferentiated 3T3-L1 cells, which increased by day 8 but decreased again to the initial level by day 14 in all three groups (Fig. 5F).

Discussion

In the present study we investigated the effects of the CP-derived oligopeptide, Pro-Hyp, on the differentiation of mouse 3T3-L1 preadipocytes and found that Pro-Hyp reduced the size of lipid droplets although the total amount of lipid did not change. This effect seems specific to the peptide-form Pro-Hyp, since the addition of an amino acid mixture of Pro and Hyp did not produce this effect on droplet size.

Effects of the ingestion of CP on lipid metabolism in animal studies have been reported. Wu *et al.* (2004) reported that the ingestion of CP at a dose higher than 0.166 g/kg body weight for 4 weeks resulted in a significantly lower concentration of plasma triacylglycerides in rats. Saito *et al.* (2009) reported that the ingestion of 0.4 g CP in combination with 0.5 mL soybean oil induced significantly lower serum triacylglyceride levels 2 h after ingestion. It is noteworthy that the serum level of VLDL was significantly reduced by the ingestion of CP for 14 days. They also reported that single ingestion of CP alone resulted in a significantly lower serum concentration of triacylglycerides compared to normal serum levels. This observation suggests that the serum triacylglyceride level is reduced not by inhibiting oil absorption in the intestine, but by modulating lipid metabolism in the body. In the present study, it was found that Pro-Hyp modulates the morphology of lipid droplets in differentiating 3T3-L1 preadipocytes by reducing droplet size. Since Pro-Hyp is one of the physiologically active factors derived from ingested CP (Iwai *et al.*, 2005; Shigemura *et al.*, 2009; Nakatani *et al.*, 2009), the present study suggests the possibility that ingested CP modulates the functions of adipocytes *in vivo* by reducing the size of lipid droplets.

Oligopeptides such as Pro-Hyp can be transported into a cell via the solute carrier family known as proton-coupled oligopeptide transporters, namely, PEPT1, PEPT2, PHT1 and Ci1 (PHT2 in human) (Gilbert *et al.*, 2008). Therefore, we examined the expression of these transporter genes in 3T3-L1 cells. As is shown in Fig. 5, two patterns of expression were observed. Expression of PEPT1 and Ci1 was induced by differentiation, while the expression of PEPT2 and PHT1 was suppressed. Although no significant difference was observed between the three groups, it is possible that proton-

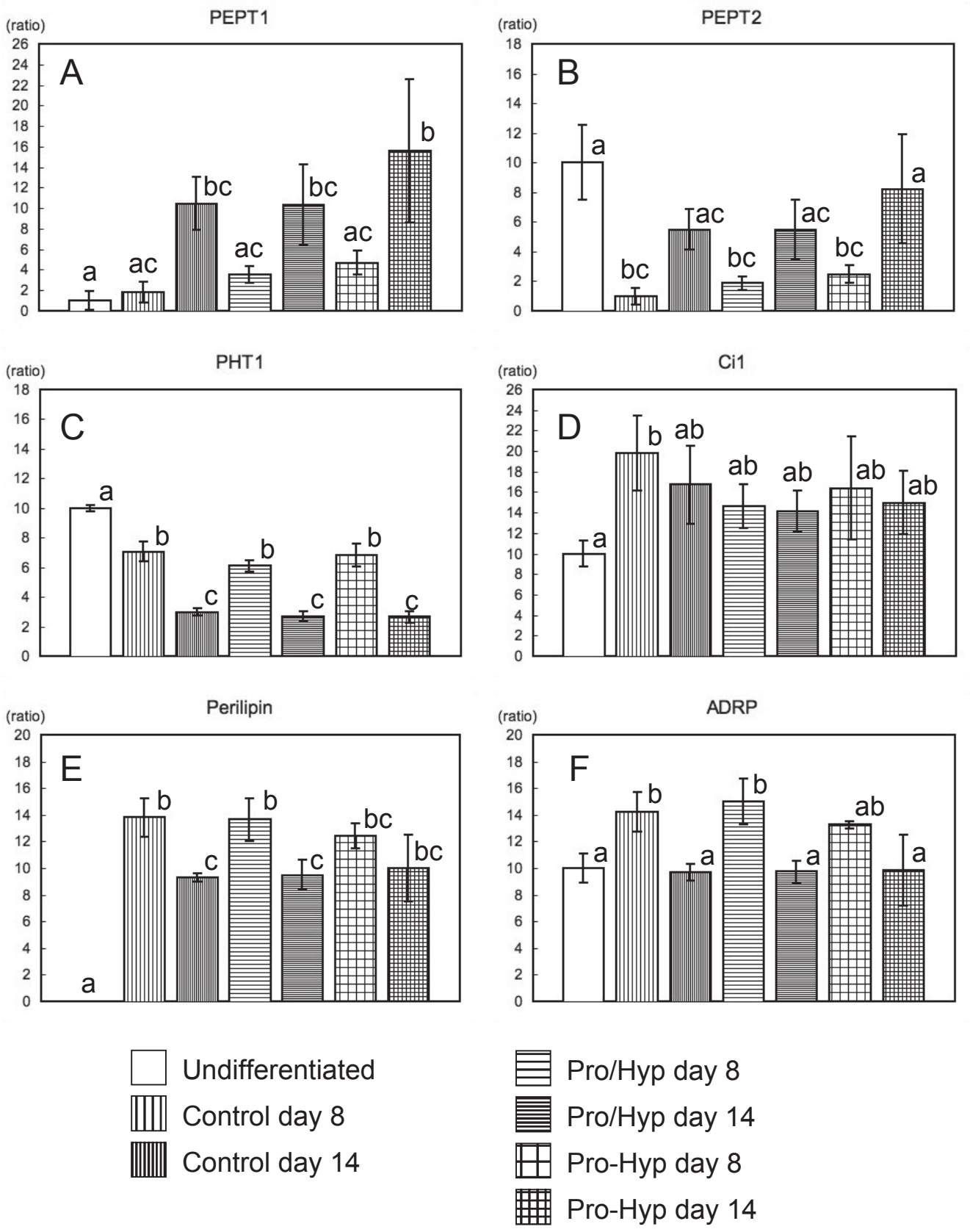

Fig. 5. Changes in the mRNA of the proton-coupled oligopeptide transporter, perilipin and ADRP genes.

The amount of mRNA for PEPT1 (A), PEPT2 (B), PHT1 (C), Ci1 (D), perilipin (E) and ADRP (F) on days 0, 8 and 14 was determined by real-time PCR and standardized using GAPDH as a standard. Values in the same figure not sharing a common letter above the bar are significantly different from one another at $p < 0.05$.

coupled oligopeptide transporters are involved, at least in part, in the effect of Pro-Hyp on droplet size, since they are expressed either in undifferentiated 3T3-L1 cells or in differentiated 3T3-L1 cells.

Triacylglycerides are synthesized in the liver and transported to tissue adipocytes, where they are stored in the form of lipid droplets. Lipid droplets contain a core of triacylglycerides surrounded by a phospholipid monolayer and coated with specific proteins, including proteins of the 5 members of the PAT family (perilipin, adipose differentiation-related protein (ADRP), tail-interacting protein of 47 kDa (TIP47), S3-12 and OXPAT). The PAT family plays a critical role in the dynamic changes in morphology and intracellular location that lipid droplets undergo according to the metabolic state or developmental stage of the adipocytes (Bickel *et al.*, 2009). It is therefore possible that Pro-Hyp reduces droplet size in differentiated 3T3-L1 cells by changing the functions of the PAT family members. However, in the present study, no change was observed in the Pro-Hyp group with regard to the expression of the perilipin and ADRP genes, or the expression of perilipin protein as examined by western blotting (data not shown). Further studies on lipid droplet-associating proteins are necessary to understand the mechanism of action of Pro-Hyp, since perilipin function is regulated not only by its amount but also through phosphorylation (Marcinkiewicz *et al.*, 2006).

Adipose tissue produces bioactive products called adipocytokines, which include inflammatory mediators (IL-6, IL-8), angiogenic proteins (VEGF), and metabolic regulators (adiponectin, leptin). Since adipocytokines are among the most important factors related to obesity (Matsuzawa, 2010), it is tempting to speculate that Pro-Hyp derived from ingested CP affects the production of adipocytokines and results in a reduced level of triglycerides in the blood. How the reduced size of lipid droplets is associated with an altered lipid metabolism should be examined further. Although it is unknown at present whether ingested CP acts solely on adipocytes or also on the liver, this study suggests the possibility that a digestive product of CP alters the metabolic state of adipocytes. Ingestion of CP results in the appearance of a number of Hyp-containing oligopeptides, including Pro-Hyp, in the blood (Iwai *et al.*, 2005). Further studies on these CP-derived oligopeptides on adipocytes as well as on lipid metabolism in the liver may elucidate a novel aspect of lipid metabolism in the body.

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