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Anatomy

Remodeling of rat stromal-vascular cells to brite/beige adipocytes by prolyl-hydroxyproline

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ABSTRACT. The aim of this study was to determine the effects of prolyl-hydroxyproline (Pro-Hyp) on the proliferation and differentiation of rat stromal-vascular cells (SVCs) being cultured in a medium with (Pro-Hyp group) or without Pro-Hyp (control group). The results showed that there was no significant difference in proliferation rate of SVCs, lipid droplet (LD) diameter or intracellular concentration of triglycerides between two groups. However, the diameter range of LDs in the Pro-Hyp group tended to be smaller than that in the control group. Transmission electron microscopy showed a tendency for increase in the area of mitochondria and decrease in the number of mitochondria in the Pro-Hyp-treated SVCs. The mRNA expression levels of white adipose tissue differentiation markers (*Cbp*, *Fabp* and *Serpina3k*) were significantly lower, but those of the brown adipose tissue differentiation markers (*Dio2*, *Ucp1* and *Ucp3*) were significantly higher in the Pro-Hyp group than in the control group. Our results suggested that Pro-Hyp can facilitate SVCs to differentiate into "brite/beige" adipocytes.

KEY WORDS: brite/beige adipocyte, collagen derived peptide, prolyl-hydroxyproline, stromal-vascular cell

Obesity is a major health problem that increases the risk of diabetes, heart disease, fatty liver and various cancers. An understanding of the biological basis of obesity should contribute to the establishment of methods for prevention and treatment of obesity [9]. Fat is mainly stored as well-defined lipid droplets (LDs) in either white adipocytes of white adipose tissue (WAT) or brown adipocytes of the brown adipose tissue (BAT). Therefore, intracellular accumulation of LDs is widely believed to be the typical characteristic and function of both white and brown adipocytes [26]. A white (unilocular) adipocyte typically contains one large LD with a diameter of up to 100 μm that occupies most of the cytoplasmic space. A few mitochondria with compact mitochondrial cristae are seen in this type of cell [6, 42]. Functionally, WAT is the main storage site of excess energy, primarily in the form of triglycerides. A brown (multilocular) adipocyte is characterized by the presence of multiple and relatively small LDs in its cytoplasm [5, 39, 42]. A large number of mitochondria with regularly arranged cristae in the cytoplasm indicate high mitochondrial activities of brown adipocytes. Brown adipocytes can uncouple mitochondrial electron transport from ATP synthesis to a greater extent than other cells by permeabilizing the inner mitochondrial membrane. This mode allows inter-membranous protons to leak back into the mitochondrial matrix, primarily through uncoupling protein-1 (Ucp1) and secondarily through other mitochondrial proteins [5, 6, 34]. Recently, another brown-type adipocyte, brown in white (brite) or beige, is found dispersed within WAT depots where their development is enhanced by cold adaptation and beta 3 adrenergic stimulation [12, 15, 49]. Despite their nearly identical phenotypes, brite/beige and brown adipocytes have different developmental origins [35, 41]. Because brite/beige adipocytes, like brown adipocytes, have a large amount of mitochondria and express *Ucp1* gene, brite/beige adipocytes specialize in dissipating energy as heat. Therefore, stimulating the development of brite/beige adipocytes in WAT, so called "browning", might reduce adverse effects of WAT and could help to improve metabolic health [1, 31, 32]. In other words, an increase in the number of brite/beige adipocytes has been shown to have anti-obesity and anti-diabetic effects in mice [41].

Collagen is a major structural protein that is distributed throughout the body. The denatured form of collagen, gelatin, is commonly used in foods, pharmaceuticals, photographic films and cosmetics. Collagen peptide (CP) is prepared by partial hydrolysis of gelatin. The molecular bonds between collagen strands can be broken down by combining treatments using heat,

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acids and proteases. CP has been used to improve the functions of tissues, such as skin, bone, cartilage and tendons. Beneficial effects of ingesting gelatin or CP have been shown in various clinical and animal studies. For instance, our recent studies showed that ingestion of CP can improve mechanical properties of an Achilles tendon [24] and suppress tendinitis induced by collagenase [44]. In addition, a decrease of serum triacylglycerides following ingestion of CP was recently reported [32, 48]. After ingestion of CP, 6 types of hydroxyproline (Hyp)-containing oligopeptides appear in the blood, prolyl-hydroxyproline (Pro-Hyp) being the most abundant of the oligopeptides [18]. Pro-Hyp has been shown to stimulate the growth of mouse skin fibroblasts on collagen gels [37] and to modulate osteoarthritis induced by a high phosphorus diet in rat [27]. In addition, we previously reported that ingestion of Pro-Hyp alters the metabolic state of 3T3-L1 cells [25]. 3T3-L1 cells derived from mouse embryo fibroblasts undergo a highly conserved and efficient program of adipogenesis in culture and upon transplantation *in vivo* [17]. These studies suggest that Pro-Hyp is one of the physiologically active oligopeptides that appears in the blood after ingestion of collagen hydrolysate.

Recently, primary stromal-vascular cells (SVCs), which are derived from inguinal, abdominal, perirenal, retroperitoneal and epididymal adipose tissues, have been reported to be capable of differentiating into adipocytes [4, 14, 47]. These cells have therefore been used as *in vitro* models to clarify cellular and molecular events involved in adipocyte differentiation [26]. Thus, the aim of the present study was to determine the effects of Pro-Hyp on proliferation and differentiation of SVCs.

MATERIALS AND METHODS

Cell culture system

A commercial primary culture system (Visceral Adipocyte Culture Kit-1; Primary Cell Co., Ltd., Sapporo, Japan), previously established by Shimizu *et al*. [38], was used. SVCs derived from mesenteric adipose tissue of Sprague-Dawley rats were cultured in a medium based on DMEM/F12 (Gibco BRL, Tokyo, Japan) in accordance with the manufacturer's instructions. The cells were seeded at an approximate concentration of 0.5×10^5 cells/cm^2 on 24-well plastic culture plates. The medium was changed every 2 days until day 8. Viable SVCs were counted using a Cell Counting Kit 8 (Dojindo Co., Kumamoto, Japan) on days 1, 3, 5, 7 and 8 following the manufacturer's instructions. The experiments were performed in triplicate.

Pro-Hyp oligopeptide

Pro-Hyp was purchased from Bachem Co., Ltd (G-3025; Bubendorf, Switzerland). Two hundred ng/ml Pro-Hyp was added to the medium every 2 days according to the method established by Minaguchi *et al*. [25]. The effects of Pro-Hyp on proliferation of cultured cells, morphology and number of LDs and mitochondria, and concentration of triglycerides in SVCs were evaluated on days 1, 3, 5, 7 and 8.

Concentration of triglycerides

The cultured cells were washed three times with Hank's Balanced Salt Solution (HBSS; Gibco BRL) before being harvested on days 1, 3, 5, 7 and 8. The cells were homogenized in HBSS, and the homogenates were used for evaluating the concentration of triglycerides in SVCs. An assay kit (TG-EN Kinos, Kinos Laboratories Inc., Tokyo, Japan) was used according to the manufacturer's instructions.

Morphological analysis of lipid droplets and mitochondria

SVCs were observed on days 1, 3, 5, 7 and 8 by using a phase contrast microscope (CX2; Olympus), and their images were recorded with a digital camera for microscopes (DP20; Olympus). In addition, 500 LDs were randomly selected, and their diameters were measured using Image J software (version 1.47). A box-whisker plot was used for showing the diameter distribution of well-defined intracytoplasmic LDs.

Observation of mitochondria in the SVCs was performed on day 8. The samples were pre-fixed with 2.5% glutaraldehyde in 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco BRL) buffer (pH 7.4) for 1 hr and post-fixed with 1% osmium tetroxide in 30 mM HEPES buffer (pH 7.4) for 1 hr. The samples were dehydrated with a graded series of ethanol and embedded in Quetol 812. Ultrathin sections were stained with 1% uranyl acetate and 2% lead citrate and then observed by a JEM-1220 (JEOL, Tokyo, Japan) or HT-7700 (Hitachi, Tokyo, Japan) electron microscope. Mitochondrial density (number of mitochondria per square μm of the SVC cytoplasm) was analyzed in 10 view fields using Image J software (version 1.47).

Quantification of mRNA of WAT and BAT differentiation markers

TRIzol reagent (Life Technologies Co., Tokyo, Japan) was used according to the manufacturer's instructions for isolating total RNA from SVCs on day 8. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Life Technologies Co.) according to the manufacturer's instructions. cDNA was analyzed using the StepOne Real-time PCR System (Applied Biosystems, Tokyo, Japan). Primers for qPCR are shown in Table 1. *HPRT* was used as a control gene. The relative mRNA levels of WAT differentiation markers (*Cbp*, *Fabp* and *Serpina3k*) and BAT differentiation markers (*Dio2, Ucp1* and *Ucp3*) were quantified for determining the effects of Pro-Hyp. All data are presented as relative mRNA levels.

Statistical analysis

Mann-Whitney's U test and Student's *t*-test were used for determination of statistical differences at $P<0.05$. Mann-Whitney's U test was used to evaluate the diameter of lipid droplet. Student's *t*-test was used to evaluate mitochondrial area, mitochondrial

Table 1. Primers for real-time PCR

Gene	Forward primer	Reverse primer
Cbp	CATGAATGCTAACTTCAACCAGAC	TTGAGCCTGATTCATTAAGCTATG
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
Fabp	CAAGCCCAACATGATCATCAGC	CACGCCCAGTTTGAAGGAAATC
Serpina3k	GGCTGAAGGCAAAGTCAGTGT	TGGAATCTGTCCTGCTGTCCT
Ucp1	TGGCCTCTACGACTCAGTCC	GCTTGCATTCTGACCTTCAC
Ucp3	TGCTGAGATGGTGACCTACG	AGTGACAGGGGAAGTTGTCAG

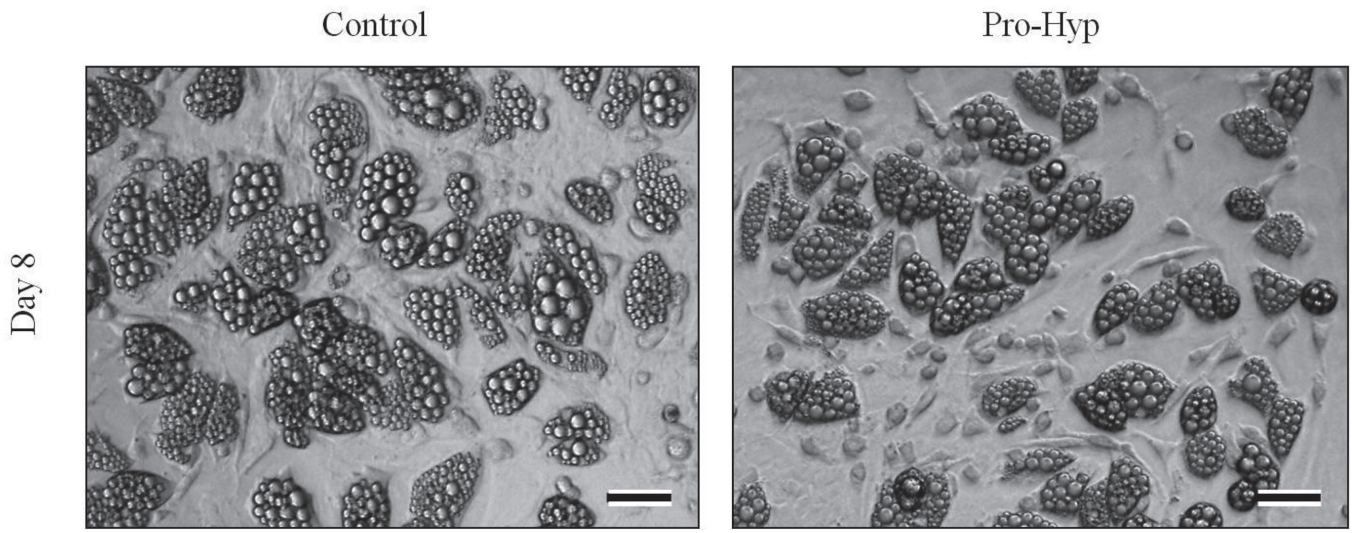

Fig. 1. Phase-contrast micrographs of day 8 SVCs in the control group (left) and Pro-Hyp group (right). Distributions of well-defined lipid droplets in the cells of the two groups are similar. Bar=100 μm.

number, TG concentration and mRNA expression.

RESULTS

Morphology and proliferation rate of SVCs

The morphology of SVCs cultured for 8 days in the medium with Pro-Hyp and the morphology of SVCs cultured for 8 days without Pro-Hyp were not distinctive (Fig. 1). Well-defined LDs were clearly observed in both groups. Proliferation rates of SVCs, with and without Pro-Hyp treatment, were not significantly different on any of the observation days (Fig. 2).

Diameter of lipid droplets

Morphology of LDs in SVCs was determined under an optical microscope. Diameters of well-defined intracytoplasmic LDs were measured and plotted as a box-whisker plot (Fig. 3). The median LD diameter in the control group and Pro-Hyp group was 17.2 μm (first quartile=13.8 μm and third quartile=22.0 μm) and 17.4 μm (first quartile=14.2 μm and third quartile=21.3 μm), respectively. The percentage of LDs under 15 μm in diameter was 29.4% in the Pro-Hyp group, whereas 33.9% in the control group. The percentage of LDs over 30 μm in diameter was 1.4% in the Pro-Hyp group, whereas 6.9% in the control group. The diameter range was found to be slightly narrow in the Pro-Hyp group. However, no significant difference was found by Mann-Whitney's *U* test in median LD diameters between the control group and Pro-Hyp group.

Area and number of mitochondria

Area and number of mitochondria per square micrometer of the SVC cytoplasm were analyzed. The mean (± SD) area of mitochondria in the control group and Pro-Hyp group was 0.12 μm^2(± 0.02) and 0.15 μm^2(± 0.05), respectively, whereas the mean (± SD) number of mitochondria in the control group and Pro-Hyp group was 56.5 (± 16.6) and 47.4 (± 16.9), respectively. The area and number of mitochondria in the Pro-Hyp group tended to be larger and smaller than those in the control group, respectively, but the differences were not significant (Fig. 4).

Intracellular concentration of triglycerides

The concentration of intracellular triglycerides in the SVCs was investigated to determine the effects of Pro-Hyp. Time courses

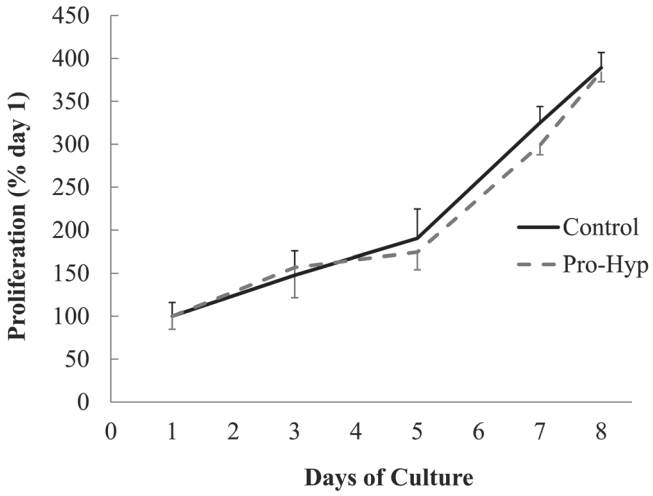

Fig. 2. Effect of Pro-Hyp on proliferation of SVCs in the control group and Pro-Hyp group. Number of viable cells was counted on days 1, 3, 5, 7 and 8 and is shown as mean ± SD. Proliferation rates in the two groups were similar.

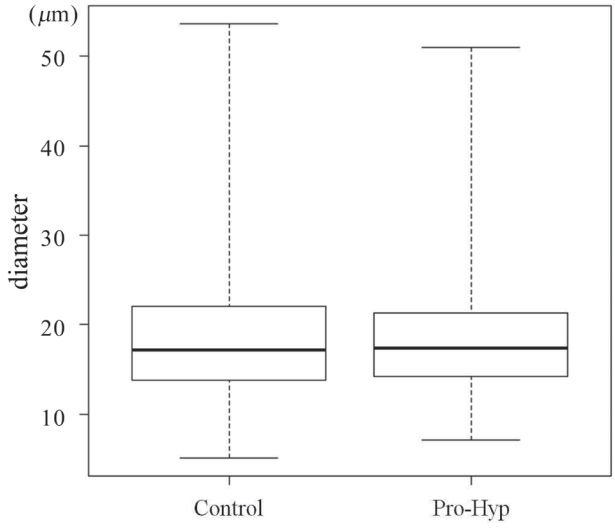

Fig. 3. Effect of Pro-Hyp on morphology of lipid droplets in SVCs. Box plots of diameter distributions of lipid droplets in the control group and Pro-Hyp group are shown. No significant difference in diameter was found.

of changes in the concentrations of triglycerides in SVCs were compared between two groups (Fig. 5). The concentration of triglycerides was low during the first 5 days of culture, but abruptly increased thereafter. However, no significant difference between the control group and Pro-Hyp group was found.

mRNA levels of WAT and BAT differentiation markers

To investigate the effects of Pro-Hyp on gene expression, the relative mRNA levels of WAT differentiation markers (*Cbp*, *Fabp* and *Serpina3k*) and BAT differentiation markers (*Dio2, Ucp1* and *Ucp3*) were quantified. In the Pro-Hyp group, the mRNA levels of *Cbp*, *Fabp* and *Serpina3k* were significantly lower, and those of *Dio2*, *Ucp1* and *Ucp3* were significantly higher than in the control group (Fig. 6).

DISCUSSION

In this study, we investigated the effect of Pro-Hyp on fat metabolism in rat SVCs. The results showed that Pro-Hyp affects the LD diameter, gene expression and mitochondrial morphology. However, effects on cell proliferation and TG accumulation were not observed.

Rather than being static organelles, LDs are recognized as dynamic organelle having an active role in lipid homeostasis [22]. A decrease in the number of large LDs in the Pro-Hyp group suggests that Pro-Hyp can suppress the fusion of existing LDs in SVCs to become multilocular. Moreover, the decrease in the number of small LDs in the Pro-Hyp group suggests that Pro-Hyp has an inhibitory effect on the incorporation of triacylglycerol in LDs.

Mitochondria are also dynamic organelles for which morphology is controlled by fusion and fission activities. Such activities occur to maintain a certain number of functional mitochondria [2]. A recent study has provided evidence that mitochondrial dynamics is crucial for cell differentiation [20]. As we demonstrated here, mitochondrial area tended to be larger, but mitochondrial number tended to be smaller in the Pro-Hyp group than in the control group. This indicates occurrence of enhanced mitochondrial fusion in the SVCs of the Pro-Hyp group, suggesting that the Pro-Hyp has an influence on the fate of adipocytes through the fusion of mitochondria.

The real-time RT-PCR data revealed up-regulation of *Ucp1, Ucp2* and *Dio2* in the Pro-Hyp group. These genes are known to express brown adipocyte-specific proteins [3, 28]. In contrast, the mRNA expression of several genes that are selectively enriched in white adipocytes [10, 11, 19, 40], including *Cbp*, *Fabp* and *Serpina3k*, were suppressed in the Pro-Hyp group. Ucp is a major thermogenic protein that drives heat production in mitochondria through proton conductance from outside the inner mitochondrial membrane into the matrix. An increase in the activity of this protein may directly manifest potential discharging and heat generation in BAT [8]. Since mitochondrial fusion is enhanced under acceleration of mitochondrial metabolism [13, 21], the results of our morphological analysis of mitochondria also indicate that Pro-Hyp can increase mitochondrial function without a change in the number of mitochondria. Dio2 is an essential component of the lipolysis, thyroid-sympathetic synergism and adaptive thermogenesis process [7, 23]. Thus, the up-regulation of *Dio2* in the Pro-Hyp group may reflect an increase in cellular metabolic activity.

In WAT, development of brite/beige adipocytes helps to reduce obesity [1, 29, 30]. The formation of brite/beige adipocytes in

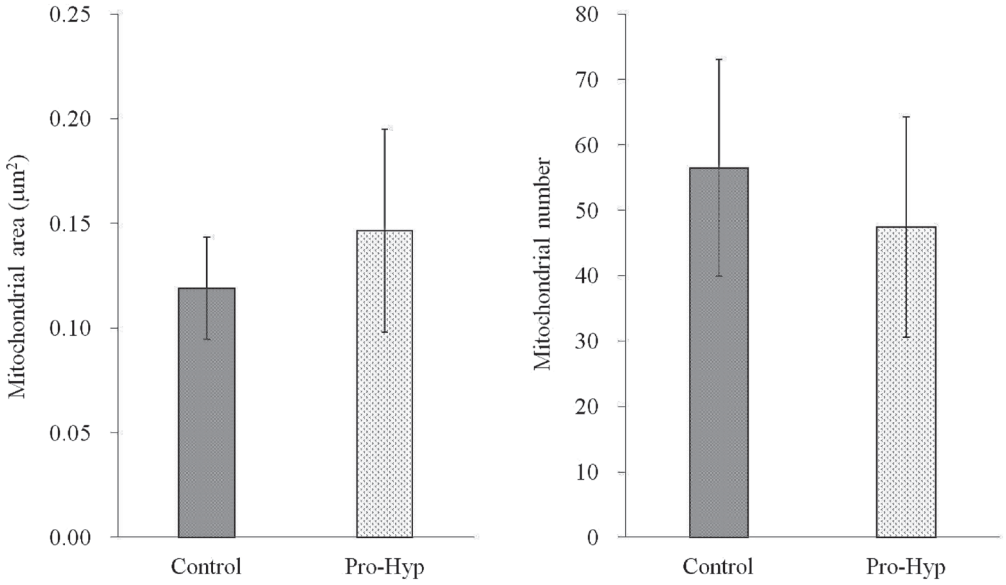

Fig. 4. Effects of Pro-Hyp on mitochondrial area (Left) and mitochondrial number (Right) per square micrometer of the SVC cytoplasm. The mitochondrial area tended to be larger, whereas the mitochondrial number tended to be smaller.

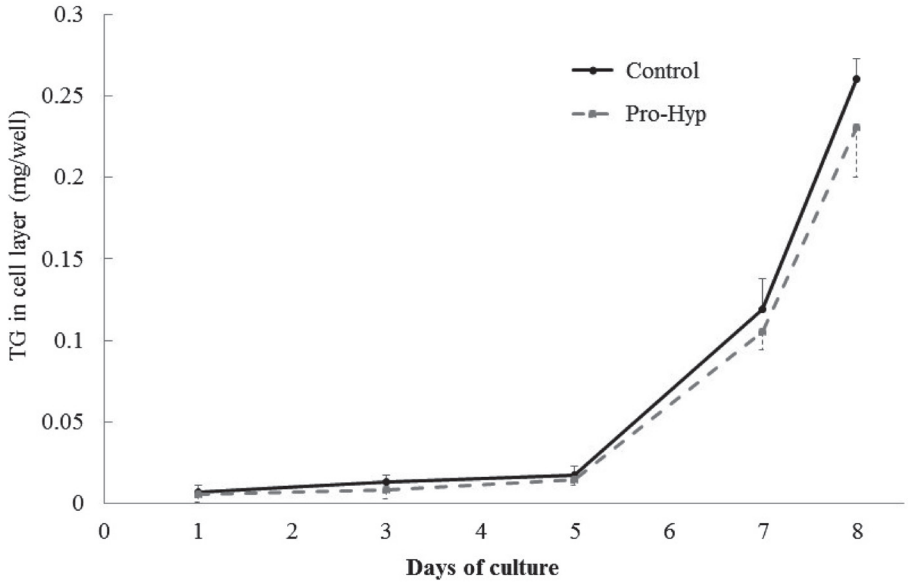

Fig. 5. Effect of Pro-Hyp on the concentration of triglycerides (TG) in SVCs. The investigation was performed on days 1, 3, 5, 7 and 8. There was no significant difference in the concentration of TG between the control group and Pro-Hyp group. Values are presented as means ± SD.

SVCs by increasing expression of genes specific to brown adipocytes contributes to the beneficial effects in metabolism [46]. In the presence of Pro-Hyp, SVCs were found to differentiate into adipocytes having characteristics similar to those of brown adipocytes. Although white and brown adipocytes have a common adipomyocytic precursor, they are not sister cells; brown adipocytes are closely related to myocytes [43, 45]. Two types of classical white adipocytes exist as white adipocytes and brite/beige adipocytes, and the latter expresses Ucp1 like brown adipocytes [16, 31, 33]. In this study, SVCs might have differentiated into brite/beige adipocytes, and Pro-Hyp might have promoted this differentiation.

Many food-derived collagen peptides, such as hydroxyprolil-glycine (Hyp-Gly), are now available. Greater activity of Hyp-Gly than that of Pro-Hyp for fibroblast growth was shown in a previous study [36], suggesting that the biological effects of collagen ingestion may be the combination of various food-derived collagen peptides. Additional research to clarify their roles is needed. The therapeutic and preventive potentials of Pro-Hyp against the global increase of obesity and related disorders should be clinically investigated.

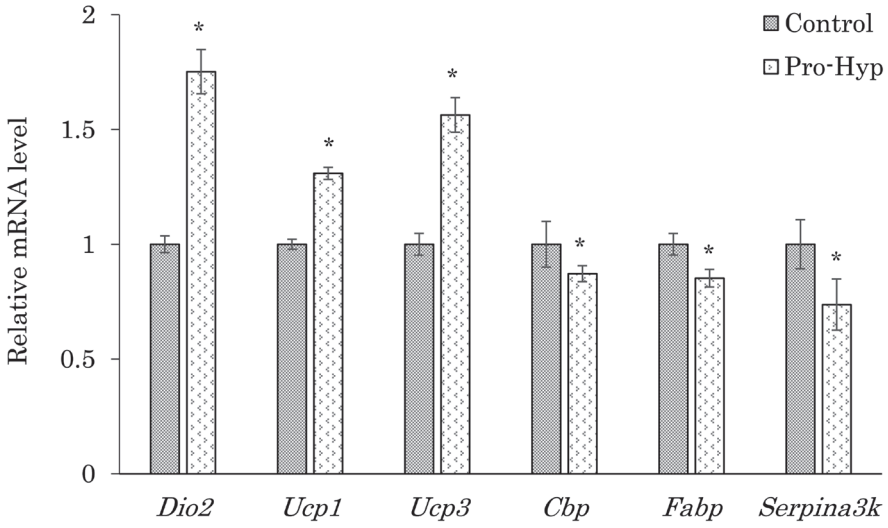

Fig. 6. Relative mRNA expression levels of *Dio2, Ucp1, Ucp3, Cbp, Fabp and Serpina3k* were compared in the control group and Pro-Hyp group. The expression levels of Dio2, Ucp1 and Ucp3 were significantly higher, whereas those of Cbp, Fabp and Serpina3k were significantly lower in the Pro-Hyp group. * $P<0.05$ or a higher degree of significance when compared with the control group.

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