Changes in Energy-Regulated Molecules in the Trophocytes and Fat Cells of Young and Old Worker Honeybees (Apis mellifera)

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Trophocytes and fat cells of honeybees (Apis mellifera) have been used for cellular senescence studies, but the changes in the expression, concentration, and activity of cellular energy–regulated molecules that occur with aging in worker bees is unknown. In this study, energy-regulated molecules were evaluated in the trophocytes and fat cells of young and old workers. The results showed that (i) adenosine monophosphate–activated protein kinase-α2 (AMPK-α2) expression increased with aging, whereas phosphorylated AMPK-α2 expression, the phosphorylated AMPK/AMPK ratio, and AMPK activity decreased with aging; (ii) adenosine diphosphate and adenosine triphosphate concentrations decreased with aging, the AMP concentration was unchanged, the adenosine diphosphate/adenosine triphosphate ratio did not change with aging, and the AMP/adenosine triphosphate ratio increased with aging; (iii) the cyclic AMP concentration decreased with aging, and cyclic AMP–specific phosphodiesterases activity increased with aging; (iv) silent information regulator 2 (Sir2) expression increased with aging, whereas its activity decreased with aging; and (v) peroxisome proliferator–activated receptor-α expression decreased with aging. These results show that the trophocytes and fat cells of young workers have higher cellular energy status and express higher levels of energy-regulated molecules than those of old workers and that aging results in a decline in the energy status of trophocytes and fat cells in worker honeybees.

Key Words: Aging—AMPK—Sir2—ATP—ADP—AMP—PPAR-α—Honeybee.

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HONEYBEE (Apis mellifera) is a useful model system for aging studies because queen honeybees have a much longer life span than worker bees. In addition, they live in large colonies, are easily handled, and their genome has been sequenced. Recently, trophocytes and fat cells have been used for studies of cellular senescence (1–5). Trophocytes are large and irregularly shaped, and fat cells are small and spherical. They attach to each other to construct a single layer of cells around each abdominal segment. No cell division during adulthood, the ease of isolation from the abdomen, and convenient manipulation make them suitable targets for the studies of cellular senescence (1,2,4,5).

The aging of workers in field hives results from an accumulation of cellular damage due to factors such as oxidation, task performance, radiation, and pollution throughout the lives of the workers. In addition, workers in field hives experience similar life histories. Therefore, the aging level of the trophocytes and fat cells of workers from field hives may correlate with their chronological age. This hypothesis is supported by a recent study showing that the levels of senescence-associated β-galactosidase, lipofuscin, lipid peroxidation, and protein oxidation increase with advancing age in the trophocytes and fat cells of workers reared in the field (1). In addition, to eliminate the influence of task performance and confirm the relationship between chronological age and aging, newly emerged workers were reared in a thermostat at 34°C for their entire life and without task performance. Age was the only difference between these workers. The expression levels of age-related molecules in the trophocytes and fat cells of workers reared in a thermostat at 34°C were similar to those of workers reared in field hives (1,4). In addition, the adenosine triphosphate (ATP) concentration in the trophocytes and fat cells of workers reared in a thermostat at 34°C was similar to that of workers reared in the field (4,5). These studies indicate that the aging level in the trophocytes and fat cells of workers reared in the field correlates with their chronological age, permitting the use of the trophocytes and fat cells of young and old workers reared in the field in studies of cellular senescence. A recent study showed that old workers exhibit higher levels of mitochondrial density and lower levels of mitochondrial membrane potential, NAD+ and ATP concentrations, and NAD+/NADH ratios compared with young workers, indicating that young workers have a higher efficiency of mitochondrial energy utilization than old workers (5). Mitochondrial energy utilization is associated with cellular energy metabolism. In particular, the NAD+ and
AMPK activity and NAD+/NADH ratio are correlated with the activity of adenosine monophosphate–activated protein kinase (AMPK) and SirT1. To use trophocytes and fat cells as a model system to study cellular senescence, it is important to determine the changes in cellular energy–regulated molecules in the trophocytes and fat cells of young and old workers. These data can be further used as reference values to monitor cellular rejuvenation or senescence in future aging studies, particularly for antiaging drug screens. Therefore, in the current study, the expression of AMPK, phosphorylated AMPK (pAMPK), silent information regulator 2 (Sir2), and peroxisome proliferator–activated receptor-α (PPAR-α); the activity of AMPK, phosphodiesterase (PDE), and Sir2; and the concentration of AMP, adenosine diphosphate (ADP), ATP, and cyclic AMP (cAMP) were evaluated in the trophocytes and fat cells of young and old workers reared in a field hive to clarify the relationship between the cellular energy requirement and cellular senescence in worker honeybees.

**METHODS**

**Honeybees**

Honeybees (*A. mellifera*) were bred in an open environment in a bee-breeding room on the 10th floor of our institute in Taiwan. The bees were able to fly freely into or out of hives for foraging as they do in field hives. Sucrose solution and pollen grains mixed with sucrose solution were sometimes added to the hives as dietary supplements. As described in a previous study (1,5), young (1-day-old) and old (50-day-old) workers were selected from the same colony in the hive on the same day for the experiments. The young workers had dense, light yellowish brown fuzz and light yellowish brown epidermises, whereas the old workers had sparse, dark brown fuzz and blackish brown black epidermises.

**Western Blotting**

The abdomens of the workers were dissected with scissors. Trophocytes and fat cells from the abdomens were detached from the cuticles of the workers in phosphate-buffered saline using a knife. Trophocytes and fat cells were isolated from three young or old workers, homogenized with a blue pestle and sonicator in 100 µL of lysis buffer containing protease inhibitors (11697498001; Roche Applied Science), and centrifuged at 5,000g for 10 minutes at 4°C to obtain the supernatant. The supernatant (10 µL) and kinase reaction buffer (90 µL) were applied to each well of a 96-well plate and incubated for 30 minutes at 30°C. After washing, 100 µL of the anti-phospho-mouse IRS-1 S789 monoclonal antibody was added and incubated for 30 minutes at room temperature. After washing, 100 µL of horseradish peroxidase–conjugated anti-mouse IgG was added and incubated for 30 minutes at room temperature. After washing, 100 µL of substrate reagent was added and incubated for 15 minutes at room temperature. Finally, 100 µL of stop solution was added to stop the reaction. The absorbance was measured at 450 nm using an ELISA plate reader (Synergy HT; Bio-TEK, VT). The protein concentration of the supernatant was determined using a protein assay (500-0006; Bio-Rad Laboratories). The AMPK activity was expressed as the absorbance at 450 nm per mg of protein (6). This experiment was performed with 16 biological replicates and used a total of 16 young and 16 old workers.

**AMP, ADP, and ATP Concentrations**

Trophocytes and fat cells were isolated from one young or old worker and placed in an Eppendorf tube. ATP concentration and NAD+/NADH ratio are correlated with the activity of adenosine monophosphate–activated protein kinase (AMPK) and SirT1. To use trophocytes and fat cells as a model system to study cellular senescence, it is important to determine the changes in cellular energy–regulated molecules in the trophocytes and fat cells of young and old workers. These data can be further used as reference values to monitor cellular rejuvenation or senescence in future aging studies, particularly for antiaging drug screens. Therefore, in the current study, the expression of AMPK, phosphorylated AMPK (pAMPK), silent information regulator 2 (Sir2), and peroxisome proliferator–activated receptor-α (PPAR-α); the activity of AMPK, phosphodiesterase (PDE), and Sir2; and the concentration of AMP, adenosine diphosphate (ADP), ATP, and cyclic AMP (cAMP) were evaluated in the trophocytes and fat cells of young and old workers reared in a field hive to clarify the relationship between the cellular energy requirement and cellular senescence in worker honeybees.

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**AMP, ADP, and ATP Concentrations**

Trophocytes and fat cells were isolated from one young or old worker and placed in an Eppendorf tube. Next, 0.5 mL of 80% methanol/10 mM KOH was added to the tube, and the sample was vortexed and centrifuged at 16,000g for 30 minutes at 4°C. The cell suspension was dried under nitrogen gas and redissolved in 100 µL of ddH₂O. Analyses
were performed with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Acquity UPLC; Waters, MA). The samples were kept at 6°C, and aliquots of the supernatants were applied to a 2.1-mm × 10-cm reverse-phase column (Acquity UPLC HSS T3; Waters) at 37°C. The samples were eluted isocratically for 15 minutes with a starting buffer of 25 mM KH$_2$PO$_4$ (pH 6.2) at a flow rate of 0.38 mL/min. A linear gradient from 0% to 15% methanol was then applied over 15 minutes. The absorbance of the effluent was monitored with a photodiode array detector at a wavelength of 260 nm. Peaks were identified by comparing their retention times to standards and analyzing the given peak spectrum from a recorded three-dimensional field with Empower2 software (Waters). The nucleotide concentration was determined from a measurement of peak area at 260 nm (7.8). This experiment was performed with 10 biological replicates and used a total of 10 young and 10 old workers.

**Sir2 Activity**

The abdomens of the workers were dissected with scissors. Trophocytes and fat cells from the abdomens were detached from the cuticles of the workers in phosphate-buffered saline using a knife. Trophocytes and fat cells were isolated from three young or old workers, homogenized with a blue pestle and sonicator in 100 µL of phosphate-buffered saline containing protease inhibitors (11697498001; Roche Applied Science), and centrifuged at 5,000g for 10 minutes at 4°C to obtain the supernatant. The protein concentration of the supernatant was determined using a protein assay (500-0006; Bio-Rad Laboratories). Sir2 activity was evaluated using the CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CY-1151; CycLex) according to the manufacturer’s instructions. Briefly, trophocytes and fat cells were isolated from one young or old worker, homogenized with a blue pestle and sonicator in 50 µL of PB buffer containing protease inhibitors (11697498001; Roche Applied Science), and centrifuged at 5,000g for 10 minutes at 4°C to obtain the supernatant. The protein concentration of the supernatant was determined using a protein assay (500-0006; Bio-Rad Laboratories). Sir2 activity was evaluated using the CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CY-1151; CycLex) according to the manufacturer’s instructions. Briefly, the final reaction mixture (50 µL) contained 30 µL of SirT1 reaction buffer, 5 µL of 10× NAD, 5 µL of dH$_2$O, and 10 µL of the supernatant. The fluorescence intensity was immediately measured every minute for a total of 60 minutes using a ultraviolet/visible spectrophotometer (Spectramax M2; Molecular Devices, NY) with an excitation wavelength of 340 nm and an emission wavelength of 460 nm at room temperature. The fluorescence was normalized by the protein content. The Sir2 activity was reported as the relative fluorescence per milligram of protein. This experiment was performed with seven biological replicates and used a total of 21 young and 21 old workers.

**cAMP Concentration**

The cAMP concentration was assayed using the cAMP Direct Immunoassay Kit (ab65355; Abcam) according to the manufacturer’s instructions. Briefly, trophocytes and fat cells from one young and old worker were treated with 110 µL of 0.1 M HCl, incubated for 20 minutes at room temperature, and centrifuged at 10,000g for 10 minutes at 4°C to obtain the supernatant. The supernatant was used directly in the assay. After recording the background luminescence, 100 µL of the diluted cAMP standard solution (0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, and 10 pmol/µL) or 100 µL of the supernatant was added to the standard reaction solution and measured spectrophotometrically at 450 nm at room temperature using an ELISA plate reader (Synergy HT; BIO-TEK). The protein concentration was determined using a protein assay (500-0006; Bio-Rad Laboratories). The cAMP values, expressed as picomole of cAMP per nanogram of protein, were obtained from the luminescence measurements (standardized to background) by reference to a cAMP standard curve that was generated concurrently. This experiment was performed with 11 biological replicates and used a total of 11 young and 11 old workers.

**PDE Activity**

The PDE activity was assayed using the PDE-Glo Phosphodiesterase Assay Kit (V1361; Promega, WI) according to the manufacturer’s instructions. Briefly, trophocytes and fat cells were isolated from one young or old worker, homogenized with a blue pestle and sonicator in 50 µL of PB buffer containing protease inhibitors (11697498001; Roche Applied Science), and centrifuged at 5,000g for 10 minutes at 4°C to obtain the supernatant. The supernatant (37.5 µL) was incubated with 12.5 µL of 2 µM cAMP, 12.5 µL of 20 µM cyclic guanosine monophosphate, 12.5 µL of termination buffer, and 12.5 µL of detection buffer for 20 minutes at room temperature. Then, 50 µL of kinase-glo reagent was added and incubated for 10 minutes at room temperature. Luminescence was measured using a plate-reading illuminometer (Gloma X; Promega). The protein concentration was determined using a protein assay (500-0006; Bio-Rad Laboratories). The PDE activity was expressed as fluorescence per nanogram of protein. This experiment was performed with nine biological replicates and used a total of nine young and nine old workers.

**Statistical Analysis**

The differences in the mean values between the two age groups were examined using two-sample t tests. A p value of less than .05 was considered statistically significant.

**RESULTS**

**AMPK Expression and Activity**

To understand the cellular energy status of trophocytes and fat cells with aging, we analyzed the expression of AMPK-α2 and pAMPK-α2 and the activity of AMPK in young and old workers. The trophocytes and fat cells of the young workers expressed lower levels of AMPK-α2 than old workers (Figure 1A). The statistical analysis showed that the expression levels of AMPK-α2 were significantly increased with aging in the trophocytes.
and fat cells of workers ($n = 18$, $p < .01$) (Figure 1B). However, the trophocytes and fat cells of young workers expressed higher levels of pAMPK-α2 than old workers (Figure 1C). The statistical analysis showed that the expression levels of pAMPK-α2 were significantly decreased with aging in the trophocytes and fat cells of workers ($n = 18$, $p < .05$) (Figure 1D). The pAMPK/AMPK ratio was used to represent the AMPK activity. The pAMPK/AMPK ratio showed that young workers had higher AMPK activity than old workers ($n = 18$, $p < .05$) (Figure 1E). To further confirm AMPK activity, we assayed AMPK activity with the AMPK Kinase Assay Kit. The mean AMPK activity was $0.62 \pm 0.11$ and $0.14 \pm 0.03$ at 450 nm per mg of protein in the trophocytes and fat cells of young and old workers, respectively ($n = 16$, $p < .01$), indicating that the young workers had higher AMPK activity than the old workers (Figure 1F). This result is consistent with that of the pAMPK/AMPK ratio. Both results showed that the trophocytes and fat cells of young workers have higher AMPK activity, revealing that AMPK activity decreases with aging in the trophocytes and fat cells of workers.

Figure 1. The expression of phosphorylated adenosine monophosphate–activated protein kinase-α2 (pAMPK-α2) and AMPK-α2, the pAMPK/AMPK ratio, and the AMPK activity in the trophocytes and fat cells of young and old workers. (A) AMPK-α2 and (C) pAMPK-α2 were analyzed through western blotting. Tubulin served as the loading control. $Y =$ young workers; $O =$ old workers; $P =$ positive control (rat muscle). The expression levels of (B) AMPK-α2 and (D) pAMPK-α2 were normalized to those in young workers. The results are presented as the means ± standard error of the means (SEMs) and are expressed as percentages ($n = 18$, in panel B; $n = 18$, in panel D). (E) The pAMPK/AMPK ratio was normalized to that in young workers. The results are presented as the means ± SEMs and are expressed as percentages ($n = 18$). (F) The AMPK activity results are presented as the means ± SEMs ($n = 16$). Asterisk indicates a statistically significant difference (*$p < .05$, **$p < .01$; two-sample $t$ test).
ATP, ADP, and AMP Concentrations and the AMP/ATP
and ADP/ATP Ratios

To determine whether young workers have higher cellular energy status than old workers, we assayed the AMP, ADP, and ATP concentration in the trophocytes and fat cells of young and old workers. The mean ATP concentration in the trophocytes and fat cells were 44.39 ± 3.50 and 30.51 ± 2.43 nmol/mg of protein in the young and old workers, respectively (n = 10, p < .01) (Figure 2A). The mean ADP concentration in the trophocytes and fat cells were 4.60 ± 0.39 and 2.78 ± 0.26 nmol/mg of protein in the young and old workers, respectively (n = 10, p < .005) (Figure 2B). The mean AMP concentration in the trophocytes and fat cells were 1.45 ± 0.16 and 1.61 ± 0.28 nmol/mg of protein in the young and old workers, respectively (n = 10, p > .01) (Figure 2C). We also calculated the ADP/ATP ratio and AMP/ATP ratio because fluctuation in the AMP/ATP and ADP/ATP ratios can influence AMPK activity to regulate cellular metabolism. The ADP/ATP ratio was not significantly different between the young and old workers (n = 10, p > .05) (Figure 2D). The young workers had a lower AMP/ATP ratio than the old workers (n = 10, p < .005) (Figure 2E). These results indicated that energy status decreased with aging in the trophocytes and fat cells of workers.

Figure 2. The concentration of (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP), and (C) adenosine monophosphate (AMP) and the ratio of (D) ADP/ATP and (E) AMP/ATP in the trophocytes and fat cells of workers. The values are expressed as the means ± standard error of the means (n = 10, in panel A; n = 10, in panel B; n = 10, in panel C; n = 10, in panel D; n = 10, in panel E). Asterisk indicates a statistically significant difference (**p < .01, ***p < .005; two-sample t test).
**Cyclic Adenosine Monophosphate and Phosphodiesterase**

To evaluate the function of the high ATP concentration observed in the trophocytes and fat cells of the young workers, we assayed the cAMP concentration and PDE activity because cAMP is derived from ATP, used for intracellular signal transduction, and decomposed into AMP by PDE. The mean cAMP concentration in the trophocytes and fat cells was $0.22 \pm 0.04$ and $0.12 \pm 0.03$ pmol/ng of protein in the young and old workers, respectively ($n = 11$, $p < .01$) (Figure 3A), indicating that the cAMP concentration decreased with aging in the trophocytes and fat cells of workers. The mean PDE activity in the trophocytes and fat cells was $2.35 \pm 0.23$ and $3.13 \pm 0.20$ fluorescence/ng of protein in the young and old workers, respectively ($n = 9$, $p < .05$) (Figure 3B), indicating that the PDE activity decreased with aging in the trophocytes and fat cells of workers. These results show that the higher ATP concentration in young workers may be associated with a higher cAMP concentration.

**Sir2 Expression and Activity**

In addition to AMPK, SirT1 also regulates cellular energy metabolism. Therefore, the expression and activity of Sir2 were assayed in the trophocytes and fat cells of young and old workers. The trophocytes and fat cells of young workers expressed lower levels of Sir2 than old workers (Figure 4A). The statistical analysis showed significantly lower Sir2 expression levels in the young workers ($n = 21$, $p < .05$) (Figure 4B), indicating that Sir2 expression increased with aging in the trophocytes and fat cells of workers. We also determined the Sir2 activity. The mean Sir2 activity was $73.2 \pm 0.9$ and $59.7 \pm 3.7$ fluorescence/µg of protein in the trophocytes and fat cells of the young and old workers, respectively ($n = 30$, $p < .01$) (Figure 4C), indicating that the Sir2 activity decreased with aging in the trophocytes and fat cells of workers.

**PPAR-α Expression**

To confirm that Sir2 regulates cellular energy metabolism, we assayed PPAR-α expression in the trophocytes and fat cells of young and old workers. The trophocytes and fat cells of young workers expressed higher levels of PPAR-α than old workers (Figure 5A). The statistical analysis showed significantly higher levels of PPAR-α expression in young workers than in old workers ($n = 18$, $p < .01$) (Figure 5B), indicating that Sir2 may regulate cellular energy metabolism through PPAR-α.

**Discussion**

In this study, we evaluated the expression of AMPK, pAMPK, Sir2, and PPAR-α; the activity of AMPK, Sir2, and PDE; and the concentration of AMP, ADP, ATP, and cAMP in the trophocytes and fat cells of young and old worker bees. The pAMPK/AMPK ratio, AMPK activity, and ATP and ADP concentrations showed that young workers have higher cellular energy status than old workers. This cellular energy status may induce the activation of signal transduction to promote cellular energy metabolism, which involves the activation of Sir2 and PPAR-α.

**AMPK Expression and Activity**

AMPK is a metabolic energy gauge and regulates cellular metabolism (9,10). This enzyme is a heterotrimeric Ser/Thr kinase that contains α, β, and γ subunits (11). The phosphorylation of the Thr172 residue in the α subunits is required for full enzymatic activity (12). In this study, AMPK-α expression increased and pAMPK-α expression decreased with aging, which are consistent with previous studies (13,14). In addition, the pAMPK/AMPK ratio and AMPK activity decreased with aging, which are also in agreement with previous studies (13–16).

Activated AMPK can activate glycolysis, fatty acid oxidation, mitochondrial biogenesis, and autophagy (10,15,17). Higher AMPK activity in young workers may be associated with younger age, mitochondrial biogenesis, and autophagy. AMPK activity increased with aging, which may be associated with decreased mitochondrial biogenesis and autophagy.
with their growth, which needs a lot of energy. In this study, parallel decreases in AMPK activity and mitochondrial function (5) with aging indicate that AMPK activates mitochondrial function in the trophocytes and fat cells of workers. In addition, caloric restriction (CR), which extends the life span of organisms, is associated with an increase in AMPK-α phosphorylation (18,19). Thus, high AMPK activity may play a role in the trophocytes and fat cells of young workers similar to that of CR.

The trophocytes and fat cells of young workers have high AMPK activity and high thioredoxin reductase activity (C.-Y. Hsu, Y.-S. Hsieh, unpublished data), implying that AMPK reduces senescence-associated β-galactosidase activity (1), suggesting that AMPK activates thioredoxin reductase activity to reduce oxidative stress. This speculation is supported by a previous study (20). In addition, the trophocytes and fat cells of young workers have high AMPK activity and low senescence-associated β-galactosidase activity (1).
activity. This inference is supported by a previous study (21).

**ATP, ADP, and AMP Concentrations and the AMP/ATP and ADP/ATP Ratios**

The trophocytes and fat cells of young workers have higher ATP concentrations than those of old workers, which is consistent with previous studies (4,5,13,22). Ambient temperature reduction, which can extend the life span of organisms, is associated with an increase in ATP concentration (23). The *daf-2* *Caenorhabditis elegans* mutant (*e1370*), which has an extended life span, also has a higher ATP concentration than the wild-type nematode (22). These results indicate that a high ATP concentration is associated with life-span extension. Thus, high ATP concentrations may play a role similar to that observed in ambient temperature reduction and the *daf-2* (*e1370*) mutant.

The higher ATP concentration observed in young workers may derive from increasing glycolysis or fatty acid oxidation by activated AMPK (10). NADH from glycolysis or fatty acid oxidation was converted to NAD+ by NADH dehydrogenase 1 to establish mitochondrial membrane potential. Subsequently, ATP was synthesized from ADP by ATP synthase. This inference is supported by higher mitochondrial energy utilization in young workers (5). A high ATP concentration may affect the energy supply, the phosphorylation of proteins and lipids through kinases, and protein ubiquitination for proteasomal degradation.

The trophocytes and fat cells of young workers had a higher ADP concentration than those of old workers, which is consistent with a previous study (22). However, the AMP concentration did not significantly differ with aging. These phenomena indicate that the trophocytes and fat cells of workers primarily used energy from ATP hydrolysis to ADP but not from ADP hydrolysis to AMP. In other words, the energy obtained from ATP hydrolysis to ADP is sufficient for trophocytes and fat cells. The finding that the AMP/ATP ratio increased with aging is in agreement with previous studies (24,25). Previous studies showed that AMPK activity depends on the AMP/ATP ratio, that a very small rise in the AMP level can induce a strong increase in AMPK activity (26) and that the AMP/ATP and ADP/ATP ratios control AMPK activity to regulate cellular metabolism (9,10). In this study, however, the AMP/ATP and ADP/ATP ratios do not seem to be key mediators of AMPK activity in the trophocytes and fat cells of worker honeybees. The most likely explanation is that the cellular energy requirement, not the energy deficiency, induces AMPK activity. Further research is required to clarify this point.

**Cyclic Adenosine Monophosphate and Phosphodiesterase**

In addition to its role in energy storage, ATP can be transformed into cAMP by adenylate cyclase. cAMP is involved in triggering calcium signals by releasing calcium from intracellular stores. In general, the ATP concentration is higher than the ADP concentration, indicating that ATP may have some biological functions, most likely transforming into cAMP as a second messenger in signal transduction. In this study, young workers had a higher cAMP concentration than old workers, which is consistent with a previous study (27). Higher cAMP concentrations resulting from higher ATP concentrations can induce higher levels of signal transduction to activate cellular metabolism. This hypothesis is supported by previous studies (5,28).

In contrast, PDE hydrolyzes cAMP to AMP and inhibits the signal transduction of cAMP. In this study, the PDE activity of young workers was lower than that of old workers, which is consistent with previous studies (29,30). Lower PDE activity leads to higher cAMP concentrations, which can induce higher levels of signal transduction to activate cellular metabolism in the trophocytes and fat cells of young workers. This conclusion is supported by a previous study (28). Conversely, higher PDE activity results in lower cAMP concentrations, which can induce lower levels of signal transduction to slow cellular metabolism. This interpretation is consistent with a previous study (5).

**Sir2 Expression and Activity**

SirT1, a NAD+-dependent deacetylase, regulates a variety of cellular functions, including cellular stress responses and energy metabolism. In this study, Sir2 expression increased with aging. However, Sir2 activity decreased with aging. Previous studies reported similar findings (31,32). Increase in Sir2 expression and decrease in Sir2 activity with aging indicate that Sir2 efficiency decreases with aging. This result is similar to efficiency of NADH dehydrogenase 1 and ATP synthase (5).

SirT1 activity is activated by an increase in the intracellular NAD+ concentration, which results from the induction of AMPK (28,33). In this study, parallel decreases in AMPK activity, Sir2 activity, and NAD+ concentration (5) with aging indicate that activated AMPK induces NAD+ generation, which consequently increases Sir2 activity. This inference is supported by a previous study (33). In addition, CR is associated with an increase in SirT1 activity (34). Thus, a high level of Sir2 activity may play a role similar to that of CR. In addition, SirT1 deacetylates LKB1 kinase, which subsequently increases LKB1 kinase activity. Because LKB1 is an upstream activator of AMPK, this signaling pathway also stimulates AMPK activation (35).

**Sir2 Induces PPAR-α Expression**

PPAR-α, a nuclear receptor transcription factor, increased fatty acid level by lipase and stimulated β-oxidation (36–38). In this study, PPAR-α expression decreased with aging, which is consistent with previous studies (39–41). Previous studies also showed that SirT1 regulates lipolysis by positively regulating PPAR-α (42,43). In this study, parallel
decreases in Sir2 activity, PPAR-α expression, and NAD+ concentration (5) with aging reflect that NAD+ activates Sir2, which consequently induces PPAR-α in the trophocytes and fat cells of workers. This inference is supported by previous studies (42–44). Furthermore, CR is associated with an increase in PPAR-α expression (37,44,45). Thus, high PPAR-α expression may play a role in the trophocytes and fat cells of young workers similar to that in CR. Increase in PPAR-α expression may increase fatty acid level to increase ATP concentration by β-oxidation in young workers.

These findings indicate that young workers have a higher level of cellular energy status than old workers. The fluctuation in the expression, concentration, and activity of energy-regulated molecules in the trophocytes and fat cells of workers can be used to evaluate cellular rejuvenation or senescence in future studies, especially in antiaging drug screens.

Taking this and previous studies into consideration, we propose a short working hypothesis for cellular energy regulation in the trophocytes and fat cells of young workers. Young workers have a higher level of cellular energy status, which derives from higher mitochondrial energy utilization, leading to higher ATP and NAD+ concentrations through the higher efficiency of NADH dehydrogenase I and ATP synthase. The high concentration of ATP is transformed into a high concentration of cAMP under lower PDE activity. Then, the high level of cAMP activates AMPK. Simultaneously, the high NAD+ concentration activates Sir2 and PPAR-α (5,35,46).

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**Conflict of Interest**
The authors have declared that there are no conflicts of interest in relation to the subject of this study.

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