

CALL FOR PAPERS | *Endocrine and Metabolic Dysfunction During Aging and Senescence*

Late-onset caloric restriction alters skeletal muscle metabolism by modulating pyruvate metabolism

Chiao-nan (Joyce) Chen,^{1,2} Shang-Ying Lin,¹ Yi-Hung Liao,³ Zhen-jie Li,¹ and Alice May-Kuen Wong⁴

¹Department of Physical Therapy, Medical School, Chang Gung University, Tao-Yuan, Taiwan; ²Healthy Aging Research Center, Chang Gung University, Tao-Yuan, Taiwan; ³Department of Exercise and Health Science, College of Human Development and Health, National Taipei University of Nursing and Health Sciences, Taipei, Taiwan; and ⁴Department of Physical Medicine and Rehabilitation, Chang Gung Memorial Hospital, Taipei, Taiwan

Submitted 7 November 2014; accepted in final form 7 April 2015

Chen CN, Lin SY, Liao YH, Li ZJ, Wong AM. Late-onset caloric restriction alters skeletal muscle metabolism by modulating pyruvate metabolism. *Am J Physiol Endocrinol Metab* 308: E942–E949, 2015. First published April 14, 2015; doi:10.1152/ajpendo.00508.2014.—Caloric restriction (CR) attenuates age-related muscle loss. However, the underlying mechanism responsible for this attenuation is not fully understood. This study evaluated the role of energy metabolism in the CR-induced attenuation of muscle loss. The aims of this study were twofold: 1) to evaluate the effect of CR on energy metabolism and determine its relationship with muscle mass, and 2) to determine whether the effects of CR are age dependent. Young and middle-aged rats were randomized into either 40% CR or ad libitum (AL) diet groups for 14 wk. Major energy-producing pathways in muscles, i.e., glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), were examined. We found that the effects of CR were age dependent. CR improved muscle metabolism and normalized muscle mass in middle-aged animals but not young animals. CR decreased glycolysis and increased the cellular dependency for OXPHOS vs. glycolysis in muscles of middle-aged rats, which was associated with the improvement of normalized muscle mass. The metabolic reprogramming induced by CR was related to modulation of pyruvate metabolism and increased mitochondrial biogenesis. Compared with animals fed AL, middle-aged animals with CR had lower lactate dehydrogenase A content and greater mitochondrial pyruvate carrier content. Markers of mitochondrial biogenesis, including AMPK activation levels and SIRT1 and COX-IV content, also showed increased levels. In conclusion, 14 wk of CR improved muscle metabolism and preserved muscle mass in middle-aged animals but not in young developing animals. CR-attenuated age-related muscle loss is associated with reprogramming of the metabolic pathway from glycolysis to OXPHOS.

dietary restriction; muscle; glycolysis; mitochondrial pyruvate carrier; lactate dehydrogenase

IMPROVEMENT OF HEALTH DURING AGING has long been a widely studied topic. To date, caloric restriction (CR) is the only nonpharmaceutical and nongenetic strategy that increases the lifespan of animals and provides health benefits (14). Regarding skeletal muscle, an organ that is critical for movement and

fuel metabolism, studies have reported that CR attenuates age-related muscle loss (32). The mechanism underlying the beneficial effects of CR on muscles has not been fully elucidated; however, increased antioxidant capacity, decreased free radical generation (23, 26), enhanced stem cell function (9), and improvement in mitochondrial function (25) are proposed mechanisms.

Cellular metabolism is determined primarily by glycolysis and oxidative phosphorylation (OXPHOS). Caloric-restricted animals were previously found to have improved mitochondrial function characterized by increased oxygen consumption (16, 25). The benefits of CR for glycolysis and the cellular dependency on OXPHOS or glycolysis are not known. However, perturbed metabolism (metabolic transition from OXPHOS to glycolysis) has been found to be associated with diseases such as diabetes (35), cancer (11), and accelerated aging (18, 27). Such perturbed metabolism may also play a role in muscle atrophy because high glycolytic activity is associated with an accumulation of methylglyoxal, which glycates proteins and increases the formation of intracellular reactive oxygen species (19, 20, 43) that could result in increased protein degradation. A new technology was recently developed where mitochondrial OXPHOS and glycolysis can be quantified in real time (21). The simultaneous determination of OXPHOS and glycolysis enables the elucidation of the shift between these two pathways with aging and after interventions. Using this new technology, we tested the first hypothesis of this study, that the CR-induced positive effects on muscle mass are associated with an improvement in cellular metabolism.

To date, most studies have focused on examining the effects of “life-long” CR, where CR was initiated when animals were still growing and the effects were evaluated when the animals were at an advanced age (16, 25, 34). Recently, it has been suggested that the effects of CR are age dependent. For instance, a study comparing the effects of CR on immunity among animals with different ages found benefits in adult animals but not in animals in puberty or at an advanced age (33). This finding suggests that age-related biological changes in animals may influence the effects of CR. The second hypothesis of this study was that the beneficial effects of CR on muscle mass and cellular metabolism are age dependent. We hypothesized that the beneficial effects of CR on muscles are more significant in older animals because cellular metabolism

Address for reprint requests and other correspondence: C. N. (Joyce) Chen, Dept. of Physical Therapy, Medical School, Chang Gung University, 259 Wen-Hwa 1st Rd., Kweishan, Taoyuan, Taiwan (e-mail: cnchen@mail.cgu.edu.tw).

is impaired in aging muscles (24), and the effects of CR are associated with metabolic reprogramming (9).

EXPERIMENTAL PROCEDURES

Animals and diet intervention. Young (4 mo old; $n = 20$) and middle-aged (16 mo old; $n = 40$) Sprague-Dawley rats purchased from the Laboratory Animal Center of Chang Gung University were housed in the research animal facility at Chang Gung University. These rats were randomized into either ad libitum (AL; $n = 10$ in the young group and $n = 20$ in the middle-aged group) or CR ($n = 10$ in the young group and $n = 20$ in the middle-aged group) groups. Rats in the CR group received 10% CR in the first week of intervention and 25% in the second week. CR rats were maintained at 40% restriction after the second week. The diet intervention lasted for 14 wk. Daily food intake and body weight were measured during the experiment period. Food was removed from the animals' cages 12 h before euthanization. Young rats in both AL and CR groups all survived through the 14 wk of intervention. However, we lost eight and five middle-aged rats in the AL and CR groups, respectively, during the intervention period. Thus, by the end of intervention, there were 10 rats in the young AL group, 10 rats in the young CR group, 12 rats in the middle-aged AL group, and 15 rats in the middle-aged CR group. Animals were anesthetized with an intraperitoneal injection of ketamine (37.5 mg/kg) and xylazine (12.5 mg/kg). The soleus muscle was dissected, and ~40 mg of muscle tissue was freshly prepared for an energy metabolism assay. The remaining parts of the soleus muscles were frozen in liquid nitrogen and stored at -80°C for further analysis. The protocol for this study was approved by the Institutional Animal Care and Use Committees of Chang Gung University.

Energy metabolism assay. The basal state energy metabolism of the soleus muscles was analyzed using a Seahorse XF-24 analyzer (Seahorse Bioscience, North Billerica, MA), with which the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells were determined simultaneously. The OCR reflects the level of mitochondrial OXPHOS, and the ECAR reflects the level of lactic acid generated during glycolysis (13, 46). Studies have shown that the ECAR determined by the XF24 analyzer is a reliable surrogate for the glycolytic rate of cells (48, 49). The ratio of OCR to ECAR indicates the relative rate of OXPHOS and glycolysis in the cell. Briefly, fresh soleus muscle (40 ± 0.5 mg) was torn into small pieces with tweezers in a dish filled with incubation medium that contained Dulbecco's modified Eagle's medium (DMEM) and 2% FBS on ice. The muscle pieces were then triturated gently, using a 1,000- μl pipette. The sample was then collected into a tube and centrifuged at 350 g for 10 min at 4°C . The pellet was resuspended with 100 μl of DMEM. Resuspended fibers (100 μl) were dispersed into the XF24 tissue microplate. Before measurement, 600 μl of prewarmed DMEM was added into each sample well; the four control wells received 700 μl . The microplate was incubated at 37°C for 20 min to enable the temperature and pH values to equilibrate. The microplate was then placed into the Seahorse XF24 analyzer, which was prewarmed at 37°C overnight. The OCR (pmol/min) and ECAR (mpH/min) of muscle cells were recorded with 15 repetitions of a measurement cycle that consisted of 5 min of mixing, a 2-min waiting period, and 3 min of measurement (40).

Western blot. Muscle tissue was homogenized in ice-cold buffer (wt/vol = 1:12; 20 mM HEPES, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM Na_3VO_4 , pH = 7.4) using a Teflon pestle. The homogenate was subsequently centrifuged at 14,000 g for 10 min at 4°C . Protein concentrations were determined in triplicate using a bicinchoninic acid assay (Thermo Fisher, Rockford, IL). Proteins [7 μg for sirtuin 1 (SIRT1), 10 μg for lactate dehydrogenase B (LDHB), 20 μg for peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and lactate dehydrogenase A (LDHA), 30 μg for AMP-activated protein kinase (AMPK), and 35 μg for

cytochrome *c* oxidase subunit IV (COX-IV) and mitochondrial pyruvate carrier (MPC)] were electrophoresed on 10 (SIRT1, PGC-1 α , AMPK, LDHA, and LDHB) or 13% (COX-IV and MPC) polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). After resolution, the proteins were transferred to polyvinylidene difluoride membranes at 90 V for 90 min (SIRT1, PGC-1 α , AMPK, LDHA, and LDHB) or 0.4 A for 60 min (COX-IV and MPC) at 4°C (Bio-Rad Laboratories). Ponceau S staining was used to ensure equal loading and the quality of transfer.

Protein-bound membranes were blocked with 5% low-fat dried milk in Tris-buffered saline containing 0.01% Tween 20 (TBST) for 1 h at room temperature. After blocking, membranes were washed with TBST and incubated with primary antibodies diluted in blocking solution with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) overnight at 4°C . Specifically, primary antibodies against SIRT1 (Cell Signaling Technology, Boston, MA), AMPK (Cell Signaling Technology), phospho-AMPK (Thr¹⁷²) (Cell Signaling Technology), COX-IV (Cell Signaling Technology), PGC-1 α (GeneTex, San Antonio, TX), and MPC (GeneTex) were diluted 1:500. Primary antibodies against LDHA (GeneTex) and LDHB (GeneTex) were diluted 1:800 and 1:6,000, respectively. After overnight incubation with the primary antibodies, the membranes were washed in TBST and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) (1:5,000 in 2.5% low-fat dried milk blocking buffer) at room temperature for 1 h. Electrochemiluminescence (Invitrogen, Carlsbad, CA) was used to detect immuno-bound antibodies, and images were captured using a ChemiDoc imaging system (Bio-Rad Laboratories). Quantity One software (Bio-Rad Laboratories) was used to quantify the optical density (OD) of each sample. To compare samples across multiple membranes, the OD of the samples was normalized to that of a standard sample on the same membrane.

Statistics. Statistical analysis was conducted using SPSS (Armonk, NY). Differences between the AL and CR groups in each age group and differences between young AL and middle-aged AL groups were determined by Student's *t*-test. Data for MPC and SIRT1 were logarithmically transformed because of the skewed distribution of the nontransformed values. The relationship between outcome variables was examined by Pearson product-moment correlation coefficients. Data were reported as means \pm SE. $P < 0.05$ was considered to be statistically significant.

RESULTS

Muscle weight. To determine how age influences the effects of CR on muscle mass, we weighed the soleus, plantaris, and gastrocnemius muscles of rats on CR or AL diets. As expected, the muscle mass of middle-aged animals was lower than that of young animals. Fourteen weeks of CR decreased the muscle mass of young rats ($P < 0.05$ for all muscle types). However, it did not significantly alter the muscle mass of middle-aged rats. Normalized muscle weight, calculated as muscle weight divided by body weight, is an indicator of how well the muscle will perform in weight-bearing tasks (12) and was also found to be associated with metabolic function of muscles (41). We found that the normalized muscle weight of middle-aged rats was lower than that of young rats ($P < 0.05$ for all muscle types). Late-onset CR reversed the normalized muscle weight back to the level of young control rats. However, CR did not show positive effects on normalized muscle weight in young rats (Table 1).

Energy metabolism in muscle tissue. The basal OCR, basal ECAR, and ratio of OCR to ECAR in muscles of young and middle-aged rats on CR or AL diets were determined. We found that the basal OCR and ratio of OCR to ECAR were lower in the middle-aged rats than in young rats (Fig. 1, A and

Table 1. Effects of CR on BW, abdominal fat mass, and muscle weight

Diet	BW, g	Abdominal Fat Mass, g	MW, g			Normalized MW [MW (mg)/BW (g)]		
			Sol	Pl	Gas	Sol	Pl	Gas
Young								
AL	562.3 ± 13.7	10.34 ± 1.36	0.25 ± 0.01	0.42 ± 0.02	2.42 ± 0.03	0.45 ± 0.02	0.77 ± 0.05	4.43 ± 0.13
CR	384 ± 20.7*	2.45 ± 0.52*	0.18 ± 0.02*	0.27 ± 0.02*	1.59 ± 0.07*	0.48 ± 0.02	0.72 ± 0.05	4.19 ± 0.18
Middle-aged								
AL	579.6 ± 37.6	11.91 ± 4.01	0.21 ± 0.01†	0.30 ± 0.02†	1.82 ± 0.03†	0.36 ± 0.01†	0.54 ± 0.06†	3.21 ± 0.28†
CR	397.8 ± 16.3*	1.47 ± 0.41*	0.19 ± 0.01	0.29 ± 0.02	1.55 ± 0.07	0.47 ± 0.01*	0.73 ± 0.03*	3.89 ± 0.08*

Data are means ± SE. CR, caloric restriction; BW, body weight; MW, muscle weight; Sol, soleus; Pl, plantaris; Gas, gastrocnemius; AL, ad libitum. * $P < 0.05$ CR compared with AL at the same age level; † $P < 0.05$ compared with young animals fed AL.

C). CR significantly decreased the basal ECAR and increased the OCR-to-ECAR ratio in middle-aged rats but not in young rats (Fig. 1, B and C). We found that normalized muscle weight was highly correlated with the OCR-to-ECAR ratio ($r = 0.965$, $P = 0.035$). Soleus muscles that had a greater OCR-to-ECAR ratio had a greater normalized muscle weight (Fig. 1D).

Lactate dehydrogenase. The LDHA content of muscle tissue was not different between young and middle-aged rats ($P = 0.853$; Fig. 2A). CR decreased the LDHA content in muscles of middle-aged rats ($P = 0.003$), but the changes in young rats were not significant. The LDHB content in muscles was similar between young and middle-aged rats (Fig. 2B). CR did not change the LDHB content in muscles regardless of the age of the animals. There was a positive relationship between the ECAR and the LDHA content in muscles ($r = 0.382$, $P = 0.018$; Fig. 2C).

Mitochondrial pyruvate carrier. The MPC content in muscles of middle-aged rats was similar to that of young rats (Fig. 2D). CR significantly increased the MPC content in middle-aged rats ($P < 0.05$). The MPC content in the muscles of middle-aged animals with CR was 62% greater than that in

animals fed AL. CR did not significantly change the MPC content in muscles of young animals.

AMP-activated protein kinase. The total AMPK and phosphorylated AMPK content were greater in muscles of middle-aged rats than in muscles of young rats ($P < 0.05$; Fig. 3, A and B). The ratio of phosphorylated to total AMPK was not different between young and middle-aged rats (Fig. 3C). The effects of CR on total AMPK and phosphorylated AMPK levels depended on the age of animals. Specifically, late-onset CR decreased total AMPK and phosphorylated AMPK content ($P < 0.05$; Fig. 3, A and B) and increased the ratio of phosphorylated to total AMPK ($P < 0.05$; Fig. 3C). Late-onset CR reversed the total AMPK content and phosphorylation level back to that of young rats. CR did not significantly change the total AMPK or phosphorylated AMPK levels in the muscles of young animals.

Proteins associated with mitochondrial biogenesis. SIRT1 is a key regulator of metabolism (21). We found that the SIRT1 content in muscle was not different between young and middle-aged rats. The effect of CR on SIRT1 content depended on the age of the animals. CR elevated SIRT1 content in muscles of middle-aged animals ($P < 0.05$) but not young animals (Fig.

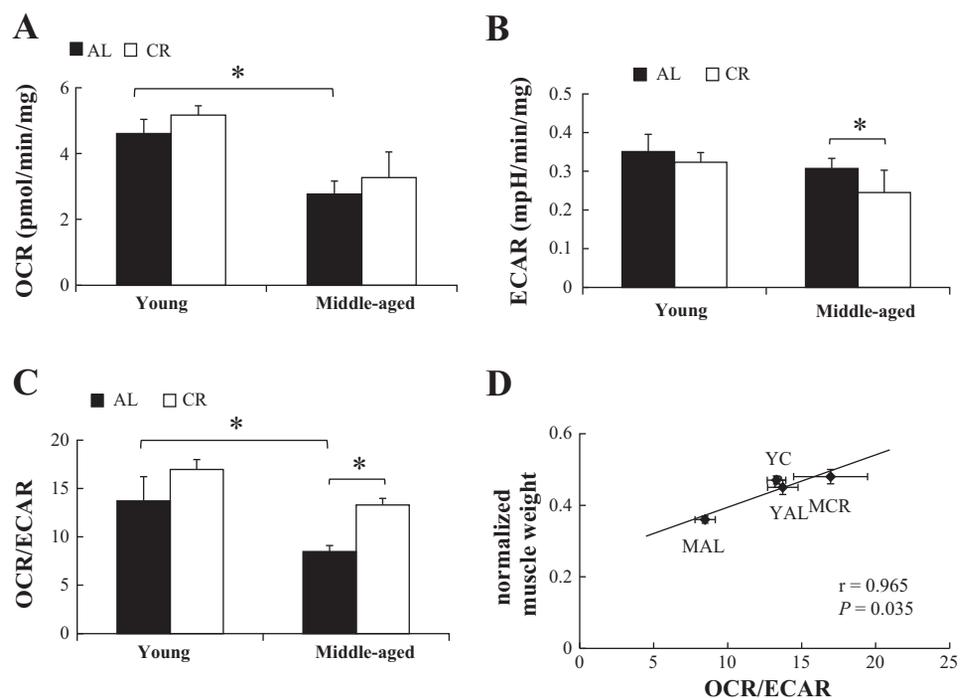


Fig. 1. Effect of age and caloric restriction (CR) on energy metabolism in soleus muscles. A: oxygen consumption rate (OCR). B: extracellular acidification rate (ECAR). C: ratio of OCR to ECAR. D: correlation between the normalized muscle weight and ratio of OCR to ECAR; 6–14 rats in each group. AL, ad libitum; YAL, young rats fed AL; YCR, young rats with CR; MAL, middle-aged rats fed AL; MCR, middle-aged rats with CR. Values are expressed as means ± SE. * $P < 0.05$.

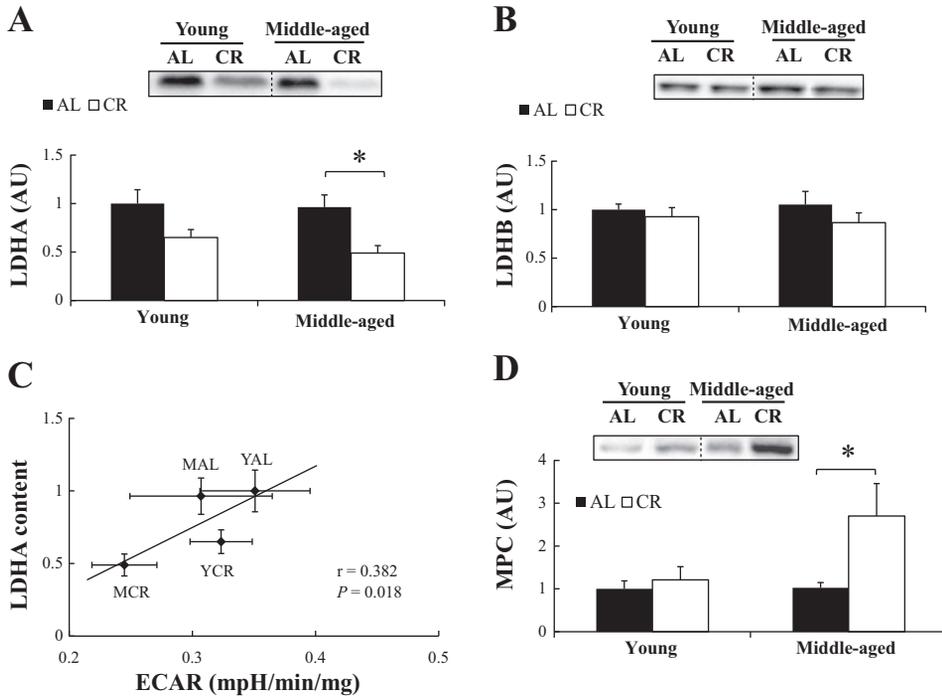


Fig. 2. The effect of age and CR on proteins that catalyzes the conversion/transportation of pyruvate in soleus muscles of rats. *A*: relative content of lactate dehydrogenase A (LDHA). *B*: relative content of lactate dehydrogenase B (LDHB). *C*: relationship between LDHA content and ECAR. *D*: relative content of mitochondrial pyruvate carrier (MPC); 9–14 rats in each group. Values are expressed as means \pm SE. * $P < 0.05$. AU, arbitrary units relative to young AL.

4A). SIRT1 content was strongly correlated with ECAR ($P = 0.01$; Fig. 4B). Higher SIRT1 expression was associated with a lower ECAR value.

PGC-1 α is the key transcriptional coactivator of mitochondrial biogenesis. We found that the PGC-1 α content in muscle did not change significantly with age or CR (Fig. 4C). COX-IV is the terminal enzyme complex in the electron transport chain. We found that the COX-IV content in muscles of young and middle-

aged rats was not different. (Fig. 4D). CR upregulated COX-IV in muscles of middle-aged rats ($P < 0.05$) but not young rats.

DISCUSSION

We investigated whether CR reprogrammed muscle metabolism and whether this improvement was associated with the observed increase in muscle mass. In addition, we examined

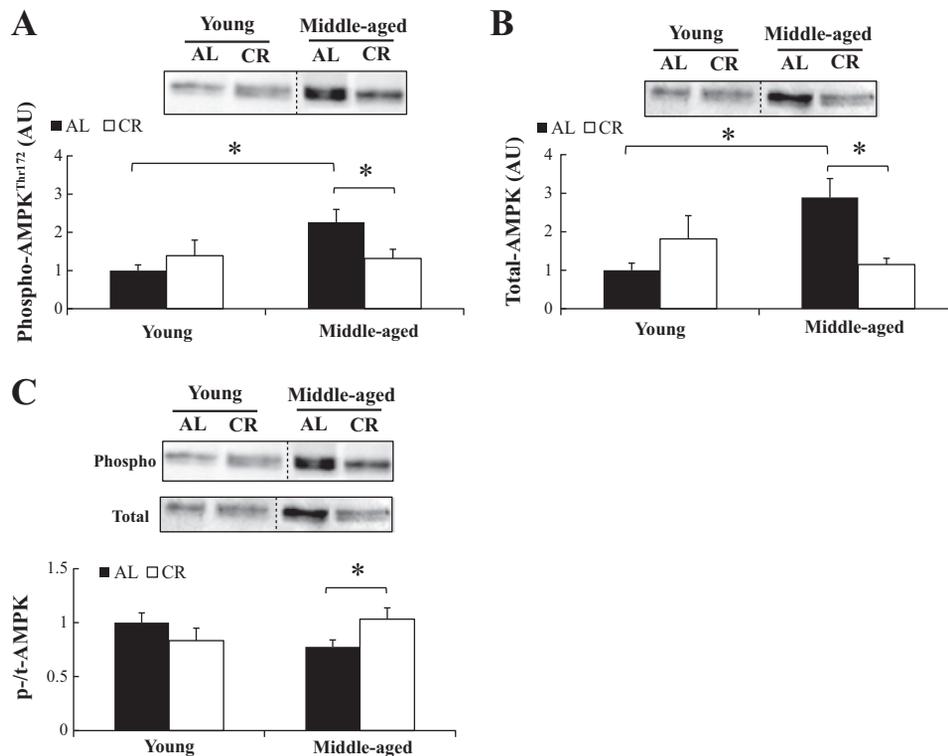


Fig. 3. Effect of age and CR on AMP-activated protein kinase (AMPK) in soleus muscles of rats. *A*: relative content of phosphorylated AMPK Thr¹⁷². *B*: relative content of total AMPK. *C*: ratio of phosphorylated to total AMPK (p-/t-AMPK); 9–14 rats in each group. Values are expressed as means \pm SE. * $P < 0.05$.

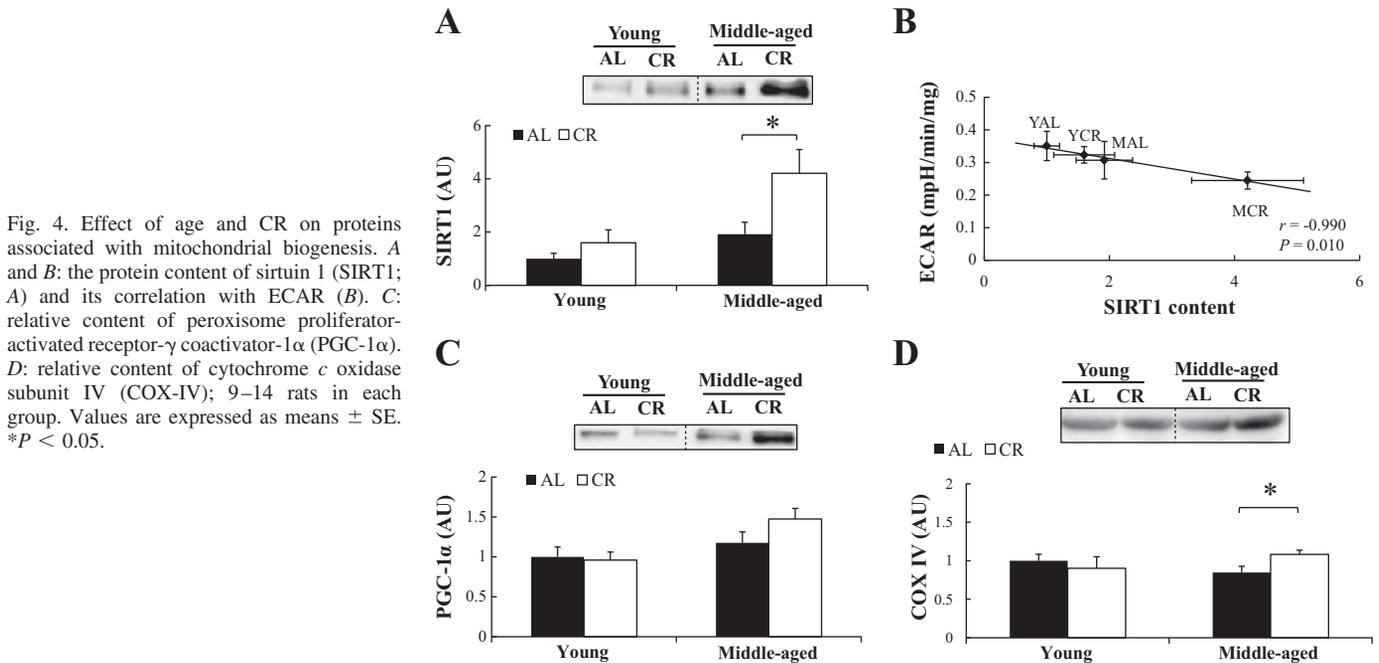


Fig. 4. Effect of age and CR on proteins associated with mitochondrial biogenesis. *A* and *B*: the protein content of sirtuin 1 (SIRT1; *A*) and its correlation with ECAR (*B*). *C*: relative content of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α). *D*: relative content of cytochrome *c* oxidase subunit IV (COX-IV); 9–14 rats in each group. Values are expressed as means \pm SE. * $P < 0.05$.

whether the CR-induced changes were age dependent. To our knowledge, the current study is the first to examine the effects of CR on glycolysis and OXPHOS in muscle cells simultaneously. The measurement of both OXPHOS and glycolysis in muscle tissues enables us to determine the relative contribution of these dominant energy-yielding pathways to energy production in muscle cells. Intact muscles were used in this study because they are in a more native state than isolated mitochondria or cultured cells (21, 40). We found that 14 wk of CR reprogrammed cellular metabolism, where the relative contribution of OXPHOS and glycolysis in muscles of middle-aged rats with CR was similar to that in muscles of young rats. This metabolic reprogramming by CR is associated with pyruvate metabolism. CR upregulated MPC and proteins associated with mitochondrial biogenesis and downregulated LDHA (Fig. 5). The improvement in cellular metabolism was strongly corre-

lated with the positive effects of CR on normalized muscle mass. Importantly, we found that short-term CR improved normalized muscle mass and cellular metabolism in middle-aged rats but not in young developing rats.

Effects of age and CR on muscle mass and cellular metabolism. We found that cellular metabolism in the soleus muscle was different between young and middle-aged rats. The relative dependency of energy production on OXPHOS vs. glycolysis was lower in muscles of middle-aged rats than in muscles of young rats. Mitochondrial dysfunction likely contributed to this metabolic shift. Consistent with previous reports that mitochondrial function declines with aging (16, 21, 25), we found that the OCR in muscles of older rats was only 60% of that in young rats under basal conditions. Compared with OXPHOS, glycolysis is a less efficient way to generate ATP; thus, aging muscles may experience ATP deficiency when they have impaired mitochondrial function and depend more on glycolysis as the energy source. ATP deficiency can inhibit protein synthesis and facilitate protein degradation in cells (39). Similarly, we found a strong correlation between cellular metabolism and normalized muscle mass, where muscles that depend more on OXPHOS over glycolysis had greater normalized muscle mass. Collectively, cellular metabolism is perturbed in muscles of middle-aged rats, which is associated with the loss of muscle mass.

CR is a potential intervention to prevent muscle atrophy in middle-aged rats by bringing metabolism back to conditions that are seen in young animals. We found that CR normalized the metabolism pattern of muscles of middle-aged rats by decreasing glycolysis and increasing the ratio of OXPHOS to glycolysis. This CR-induced metabolic reprogramming is not a novel event. For example, CR-induced reprogramming was also found in muscle stem cells. Cerletti et al. (9) found that satellite cells from mice with 12 wk of CR had greater mitochondrial OXPHOS and less glycolytic lactate production than those from mice fed ad libitum. Additionally, they found

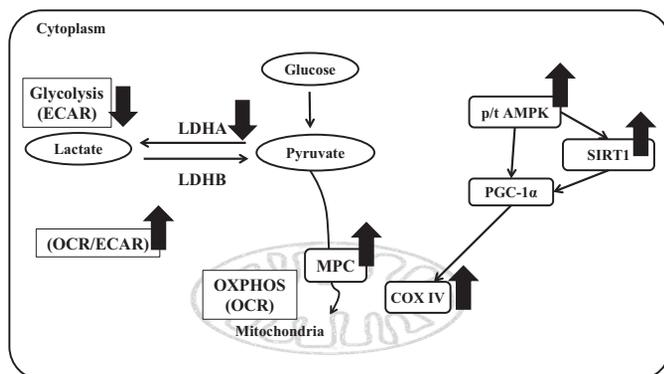


Fig. 5. CR reprograms metabolic pathways in muscles of middle-aged rats. Fourteen weeks of CR decreased the cellular dependency on glycolysis for energy generation and increased the cellular dependency for oxidative phosphorylation (OXPHOS) vs. glycolysis as an energy source. The shift in dependency for energy metabolism pathways was associated with the downregulation of LDHA and the upregulation of MPC and proteins associated with mitochondrial biogenesis.

that CR-induced metabolic reprogramming was associated with greater satellite cell availability and better regenerative capacity (9). The CR-induced metabolic reprogramming also occurs in other tissues and at the whole body level. Livers from mice with 24 h of fasting showed an upregulation of genes related to fatty acid oxidation and a downregulation of genes related to glycolysis (47). McCarter and Palmer (31) determined the primary fuel source used by animals and found that rats with CR used more percentage of fat and a lower percentage of carbohydrate as their energy source compared with rats fed ad libitum. Taken together, CR may be an effective intervention to shift cellular metabolism from glycolysis to OXPHOS.

Reduced reliance on glycolysis is considered a benefit because high glycolytic activity contributes to the accumulation of the glycolytic byproduct methylglyoxal (38). Methylglyoxal is a toxic glyating agent that glycates and cross-links proteins (19, 20). Studies have indicated that methylglyoxal induces oxidative damage, causes mitochondrial dysfunction (43), and is involved in many age-related diseases such as diabetes and cancer (38, 45). Indeed, decreased glycolysis has been proposed as a strategy for cancer treatment and retardation of aging (11, 19).

A novel finding of this study is that the CR-induced decrease in glycolysis in muscles of middle-aged rats occurred through changes in the metabolism of pyruvate. Aberrant pyruvate metabolism (e.g., increased glycolysis and lactate production along with decreased oxidation of pyruvate in mitochondria) has been found in diseases such as cancer, neurodegeneration, and heart diseases (15). Pyruvate is the product of glycolysis and is either converted to lactate or transported into mitochondria, where it is converted to acetyl-coenzyme A. The conversion between pyruvate and lactate is catalyzed by LDH. The LDH isoform LDHA catalyzes the conversion of pyruvate to lactate; LDHB catalyzes the conversion of lactate to pyruvate (44). Pyruvate is transported from the cytosol into the mitochondrial matrix by MPC, a small transmembrane protein in the mitochondrial inner membrane (4, 17). We found that CR downregulated LDHA and upregulated MPC in muscles of middle-aged rats. This finding is similar to that of Zhang et al. (47), where MPC expression was found to be increased in the liver of mice after 24 h of fasting. The CR-induced changes in pyruvate metabolism reprogrammed cellular metabolism from glycolysis toward OXPHOS. Indeed, decreased levels of TCA cycle intermediates and lactic acidosis were found in animals and patients with MPC mutations (4, 5). In addition, animals with lower LDHA content showed less blood lactate accumulation during exercise compared with control littermates (44). Taken together, MPC and LDHA are critical for the metabolism pattern of cells and can be modulated by CR.

Effects of age and CR on proteins related to mitochondrial biogenesis and oxidative phosphorylation. Mitochondrial biogenesis determines the mitochondrial content in cells and thus is important in cellular metabolism. Mitochondrial biogenesis is triggered by energy deprivation (30, 36). Increased AMP levels activate the energy-sensing enzyme AMPK by phosphorylation (8). Phosphorylated AMPK facilitates the activation of SIRT1 and, together with activated SIRT1, activates PGC-1 α , the key regulator of mitochondrial biogenesis (8). We found that both the total AMPK and phosphorylated AMPK content were greater in muscles of middle-aged rats than that in

muscles of young rats. CR reduced the expression of total and phosphorylated AMPK in muscles of middle-aged rats to the levels of young rats. A high basal level of phosphorylated AMPK has been found to be deleterious in yeast and mammalian tissues (2, 6). Burwinkel et al. (6) indicated that an increased basal level of phosphorylated AMPK predisposes individuals to the development of cardiomyopathy. Our finding is similar to a previous report (28) where a higher basal level of phosphorylated AMPK was found in the brain tissue of old rats. The elevated AMPK content in old animals may be a compensatory adaptation to blunted AMPK activation upon stimulation (8). The increased basal level of phosphorylated AMPK may be a cellular response to the energy deficiency or AMPK resistance of downstream signaling. Our data and previous studies have shown energy deficiency in muscles of older animals by demonstrating decreased mitochondrial function and ATP generation (24, 36). Further studies are needed to examine the changes in the signal transduction downstream of AMPK in the muscle tissue with aging.

Similarly to AMPK, SIRT1 responds to changes in nutrient availability and energy expenditure (22). We found that CR increased the ratio of phosphorylated to total AMPK and the expression of SIRT1 in muscles of middle-aged rats. The results are consistent with the findings of Quintas et al. (37) in that CR prevented a significant reduction in SIRT1 protein levels in the rat hippocampus with aging. The important role of SIRT1 in the effects of CR has been reported extensively in the literature; however, to our knowledge, this study is the first to report that SIRT1 content is regulated by changes in NAD⁺ abundance and the NAD⁺/NADH ratio. SIRT1 is upregulated when NAD⁺ abundance and the NAD⁺/NADH ratio increase (22). Increased AMPK activation and decreased lactate levels have been found to be associated with an increased NAD⁺ abundance and the NAD⁺/NADH ratio (7, 22). Suchankova et al. (42) examined glucose-induced changes in AMPK activity and found that increased lactate release decreased SIRT1 content and activity in mammalian cells. Our finding that AMPK, SIRT1, and COX-IV are upregulated in the muscles of middle-aged rats with CR is consistent with previous studies where life-long CR was applied to animals (13, 16, 25). Collectively, CR increased mitochondrial biogenesis signaling in muscles of middle-aged rats, which is associated with decreased glycolysis.

The change in PGC-1 α content did not agree with the observed changes in AMPK activation, SIRT1 content, or COX-IV content in the current study. AMPK and SIRT1 are upstream regulators of PGC-1 α , and COX-IV is a downstream target of PGC-1 α and is a commonly used marker for mitochondrial abundance (29). We observed that CR activated AMPK and upregulated SIRT1 and COX-IV without a significant change in PGC-1 α content in middle-aged rats. These inconsistent changes with CR may arise because the role of PGC-1 α in mitochondrial biogenesis may not be fully explained by its abundance. The subcellular localization of PGC-1 α is another critical factor that determines its function. For example, PGC-1 α has been found in both the nucleus and the cytoplasm under basal conditions. Under stress conditions, PGC-1 α translocates to the nucleus (1). Similarly, Hempenstall et al. (16) found that although total PGC-1 α content did not differ between old animals with CR and those fed ad libitum, the PGC-1 α content in the nucleus was significantly greater in

old rats with CR than in rats fed ad libitum. Thus, additional experiments are warranted to investigate the causality of the association between upregulation of COX-IV following late-onset CR and nuclear PGC-1 α content.

Effects of CR on muscle mass and metabolism are age dependent. This study is the first study to examine whether the effects of CR on muscle health are age dependent. Our results show that all of the observed benefits induced by CR in muscles of middle-aged animals were not significant in young animals. Young animals with CR even had a lower muscle mass than the control group. The nonsignificant changes in cellular metabolism in young animals with CR likely result from a ceiling effect because the cellular metabolism in muscles of young animals is normal, thus reducing the benefits that animals can obtain from CR. A ceiling effect is also observed in exercise training studies, where training-induced positive adaptations in muscle metabolism were found less frequently in fit or well-trained individuals (3, 10).

There are two limitations of this study. First, we measured only the basal oxygen consumption. Thus, it is unknown whether 14 wk of CR improves maximal oxygen consumption in the muscle of animals. Second, we analyzed the cellular PGC-1 α content rather than PGC-1 α levels in the nucleus, where it executes its function. Therefore, the current study is unable to delineate the role of PGC-1 α in CR-induced mitochondrial biogenesis. Future study is needed to clarify this role.

In conclusion, the effects of CR on muscle mass and energy metabolism in muscles were age dependent. CR decreased glycolysis and increased the cellular dependency for OXPHOS vs. glycolysis as an energy source in middle-aged rats but not young rats. The metabolic reprogramming by CR occurs through modulation of the metabolism of pyruvate. These findings are important in the application of CR.

GRANTS

This work was funded by the Healthy Aging Research Program, Chang Gung University (EMRPD1E1651); Chang Gung Memorial Hospital (CMRPD3D0211); and the Ministry of Science and Technology (NSC 101-2314-B-182-006-MY3).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.-N.C. conception and design of research; C.-N.C., S.-Y.L., and Z.-J.L. performed experiments; C.-N.C., S.-Y.L., and Z.-J.L. analyzed data; C.-N.C., S.-Y.L., Y.-H.L., and A.-M.W. interpreted results of experiments; C.-N.C. and S.-Y.L. prepared figures; C.-N.C. and S.-Y.L. drafted manuscript; C.-N.C., S.-Y.L., and Y.-H.L. edited and revised manuscript; C.-N.C., S.-Y.L., Y.-H.L., and A.-M.W. approved final version of manuscript.

REFERENCES

- Anderson RM, Barger JL, Edwards MG, Braun KH, O'Connor CE, Prolla TA, Weindruch R. Dynamic regulation of PGC-1 α localization and turnover implicates mitochondrial adaptation in calorie restriction and the stress response. *Aging Cell* 7: 101–111, 2008.
- Ashrafi K, Lin SS, Manchester JK, Gordon JL. Sip2p and its partner snf1p kinase affect aging in *S. cerevisiae*. *Genes Dev* 14: 1872–1885, 2000.
- Breen L, Philp A, Witard OC, Jackman SR, Selby A, Smith K, Baar K, Tipton KD. The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. *J Physiol* 589: 4011–4025, 2011.
- Bricker DK, Taylor EB, Schell JC, Orsak T, Boutron A, Chen YC, Cox JE, Cardon CM, Van Vranken JG, Dephousse N, Redin C, Boudina S, Gygi SP, Brivet M, Thummel CS, Rutter J. A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, *Drosophila*, and humans. *Science* 337: 96–100, 2012.
- Brivet M, Garcia-Cazorla A, Lyonnet S, Dumez Y, Nassogne MC, Slama A. Impaired mitochondrial pyruvate importation in a patient and a fetus at risk. *Mol Genet Metab* 78: 186–192, 2003.
- Burwinkel B, Scott JW, Buhner C, van Landeghem FK, Cox GF, Wilson CJ, Grahame Hardie D, Kilimann MW. Fatal congenital heart glycolipidosis caused by a recurrent activating R531Q mutation in the gamma 2-subunit of AMP-activated protein kinase (PRKAG2), not by phosphorylase kinase deficiency. *Am J Hum Genet* 76: 1034–1049, 2005.
- Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J. AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature* 458: 1056–1060, 2009.
- Canto C, Auwerx J. Calorie restriction: is AMPK a key sensor and effector? *Physiology* 26: 214–224, 2011.
- Cerletti M, Jang YC, Finley LW, Haigis MC, Wagers AJ. Short-term calorie restriction enhances skeletal muscle stem cell function. *Cell Stem Cell* 10: 515–519, 2012.
- Costford SR, Bajpeyi S, Pasarica M, Albarado DC, Thomas SC, Xie H, Church TS, Jubrias SA, Conley KE, Smith SR. Skeletal muscle NAMPT is induced by exercise in humans. *Am J Physiol Endocrinol Metab* 298: E117–E126, 2010.
- DeBerardinis RJ, Thompson CB. Cellular metabolism and disease: what do metabolic outliers teach us? *Cell* 148: 1132–1144, 2012.
- Edström E, Ulfhake B. Sarcopenia is not due to lack of regenerative drive in senescent skeletal muscle. *Aging Cell* 4: 65–77, 2005.
- Ferrick DA, Neilson A, Beeson C. Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov Today* 13: 268–274, 2008.
- Fontana L, Partridge L, Longo VD. Extending healthy life span—from yeast to humans. *Science* 328: 321–326, 2010.
- Gray LR, Tompkins SC, Taylor EB. Regulation of pyruvate metabolism and human disease. *Cell Mol Life Sci* 71: 2577–2604, 2014.
- Hempstead S, Page MM, Wallen KR, Selman C. Dietary restriction increases skeletal muscle mitochondrial respiration but not mitochondrial content in C57BL/6 mice. *Mech Ageing Dev* 133: 37–45, 2012.
- Herzig S, Raemy E, Montessuit S, Veuthey JL, Zamboni N, Westermann B, Kunji ER, Martinou JC. Identification and functional expression of the mitochondrial pyruvate carrier. *Science* 337: 93–96, 2012.
- Hipkiss AR. Does chronic glycolysis accelerate aging? Could this explain how dietary restriction works? *Ann NY Acad Sci* 1067: 361–368, 2006.
- Hipkiss AR. On the mechanisms of ageing suppression by dietary restriction—is persistent glycolysis the problem? *Mech Ageing Dev* 127: 8–15, 2006.
- Hipkiss AR. Dietary restriction, glycolysis, hormesis and ageing. *Biogerontology* 8: 221–224, 2007.
- Horan MP, Pichaud N, Ballard JW. Review: quantifying mitochondrial dysfunction in complex diseases of aging. *J Gerontol A Biol Sci Med Sci* 67: 1022–1035, 2012.
- Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* 13: 225–238, 2012.
- Jang YC, Liu Y, Hayworth CR, Bhattacharya A, Lustgarten MS, Muller FL, Chaudhuri A, Qi W, Li Y, Huang JY, Verdin E, Richardson A, Van Remmen H. Dietary restriction attenuates age-associated muscle atrophy by lowering oxidative stress in mice even in complete absence of CuZnSOD. *Aging Cell* 11: 770–782, 2012.
- Johnson ML, Robinson MM, Nair KS. Skeletal muscle aging and the mitochondrion. *Trends Endocrinol Metab* 24: 247–256, 2013.
- Lanza IR, Zabielski P, Klaus KA, Morse DM, Heppelmann CJ, Bergen HR 3rd, Dasari S, Walrand S, Short KR, Johnson ML, Robinson MM, Schimke JM, Jakaitis DR, Asmann YW, Sun Z, Nair KS. Chronic caloric restriction preserves mitochondrial function in senescence without increasing mitochondrial biogenesis. *Cell Metab* 16: 777–788, 2012.
- Li XD, Rebrin I, Forster MJ, Sohal RS. Effects of age and caloric restriction on mitochondrial protein oxidative damage in mice. *Mech Ageing Dev* 133: 30–36, 2012.
- Lisanti MP, Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Pestell RG, Howell A, Sotgia F. Accelerated aging in the tumor microenvironment. *Cell Cycle* 10: 2059–2063, 2014.

28. Liu F, Benashski SE, Persky R, Xu Y, Li J, McCullough LD. Age-related changes in AMP-activated protein kinase after stroke. *Age (Dordr)* 34: 157–168, 2012.
29. Ljubicic V, Hood DA. Diminished contraction-induced intracellular signaling towards mitochondrial biogenesis in aged skeletal muscle. *Aging Cell* 8: 394–404, 2009.
30. Lopez-Lluch G, Irueta PM, Navas P, de Cabo R. Mitochondrial biogenesis and healthy aging. *Exp Gerontol* 43: 813–819, 2008.
31. McCarter RJ, Palmer J. Energy metabolism and aging: a lifelong study of Fischer 344 rats. *Am J Physiol Endocrinol Metab* 263: E448–E452, 1992.
32. McKiernan SH, Bua E, McGorray J, Aiken J. Early-onset calorie restriction conserves fiber number in aging rat skeletal muscle. *FASEB J* 18: 580–581, 2004.
33. Messaoudi I, Fischer M, Warner J, Park B, Mattison J, Ingram DK, Totonchy T, Mori M, Nikolich-Zugich J. Optimal window of caloric restriction onset limits its beneficial impact on T-cell senescence in primates. *Aging Cell* 7: 908–919, 2008.
34. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* 310: 314–317, 2005.
35. Oberbach A, Bossenz Y, Lehmann S, Niebauer J, Adams V, Paschke R, Schön MR, Blüher M, Punkt K. Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care* 29: 895–900, 2006.
36. Peterson CM, Johannsen DL, Ravussin E. Skeletal muscle mitochondria and aging: a review. *J Aging Res* 2012: 1–20, 2012.
37. Quintas A, de Solís AJ, Díez-Guerra FJ, Carrascosa JM, Bogónez E. Age-associated decrease of SIRT1 expression in rat hippocampus. *Exp Gerontol* 47: 198–201, 2012.
38. Ramasamy R, Yan SF, Schmidt AM. Methylglyoxal comes of AGE. *Cell* 124: 258–260, 2006.
39. Sandri M. Signaling in muscle atrophy and hypertrophy. *Physiology* 23: 160–170, 2008.
40. Schuh RA, Jackson KC, Khairallah RJ, Ward CW, Spangenburg EE. Measuring mitochondrial respiration in intact single muscle fibers. *Am J Physiol Regul Integr Comp Physiol* 302: R712–R719, 2012.
41. Srikanthan P, Karlamangla AS. Relative muscle mass is inversely associated with insulin resistance and prediabetes. Findings from the third National Health and Nutrition Examination Survey. *J Clin Endocrinol Metab* 96: 2898–2903, 2011.
42. Suchankova G, Nelson LE, Gerhart-Hines Z, Kelly M, Gauthier MS, Saha AK, Ido Y, Puigserver P, Ruderman NB. Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells. *Biochem Biophys Res Commun* 378: 836–841, 2009.
43. Suh KS, Choi EM, Rhee SY, Kim YS. Methylglyoxal induces oxidative stress and mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. *Free Radic Res* 48: 206–217, 2014.
44. Summermatter S, Santos G, Pérez-Schindler J, Handschin C. Skeletal muscle PGC-1 α controls whole-body lactate homeostasis through estrogen-related receptor α -dependent activation of LDH B and repression of LDH A. *Proc Natl Acad Sci USA* 110: 738–743, 2013.
45. Walker G, Houthoofd K, Vanfleteren JR, Gems D. Dietary restriction in *C. elegans*: from rate-of-living effects to nutrient sensing pathways. *Mech Ageing Dev* 126: 929–937, 2005.
46. Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol* 292: C125–C136, 2007.
47. Zhang F, Xu X, Zhou B, He Z, Zhai Q. Gene expression profile change and associated physiological and pathological effects in mouse liver induced by fasting and refeeding. *PLoS One* 6: e27553, 2011.
48. Zhang J, Khvorostov I, Hong JS, Oktay Y, Vergnes L, Nuebel E, Wahjudi PN, Setoguchi K, Wang G, Do A, Jung HJ, McCaffery JM, Kurland IJ, Reue K, Lee WN, Koehler CM, Teitell MA. UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. *EMBO J* 30: 4860–4873, 2011.
49. Zhang J, Nuebel E, Wisidagama DR, Setoguchi K, Hong JS, Van Horn CM, Imam SS, Vergnes L, Malone CS, Koehler CM, Teitell MA. Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nat Protoc* 7: 1068–1085, 2012.