Dietary protein decreases exercise endurance through rapamycin-sensitive suppression of muscle mitochondria

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Submitted 13 March 2013; accepted in final form 18 July 2013

Mitsuishi M, Miyashita K, Muraki A, Tamaki M, Tanaka K, Itoh H. Dietary protein decreases exercise endurance through rapamycin-sensitive suppression of muscle mitochondria. Am J Physiol Endocrinol Metab 305: E776–E784, 2013. First published July 23, 2013; doi:10.1152/ajpendo.00145.2013.—Loss of physical performance is linked not only to decreased activity in daily life but also to increased onset of cardiovascular diseases and mortality. A high-protein diet is recommended for aged individuals in order to preserve muscle mass; however, the regulation of muscle mitochondria by dietary protein has not been clarified. We investigated the long-term effects of a high-protein diet on muscle properties, focusing especially on muscle mitochondria. Mice were fed a high-protein diet from the age of 8 wk and examined for mitochondrial properties and exercise endurance at the ages of 20 and 50 wk. Compared with normal chow, a high-protein diet significantly decreased the amount of muscle mitochondria, mitochondrial activity, and running distance at 50 wk, although it increased muscle mass and grip power. Inhibition of TORC1-dependent signal pathways by rapamycin from 8 wk suppressed the decline in mitochondria and exercise endurance observed when mice were fed the high-protein diet in association with preserved AMPK activity. Collectively, these findings suggest a role for dietary protein as a suppressor of muscle mitochondria and indicate that the age-associated decline in exercise endurance might be accelerated by excessive dietary protein through rapamycin-sensitive suppression of muscle mitochondria.

skeletal muscle mass; muscle mitochondria; physical performance; exercise endurance; TORC1; AMPK; rapamycin

MAINTENANCE OF PHYSICAL PERFORMANCE is essential for a healthy life, especially among the elderly. The loss of muscle mass associated with aging, termed sarcopenia, is known to predict a wide range of diseases and morbidity. Sarcopenic individuals suffer from increased risk of falls and fractures and, as a result, a 1.5 to 4.6-fold higher risk of loss of independence compared with those with a normal volume of muscle mass (19, 35). Moreover, inactive individuals are at higher risk of coronary heart diseases and all-cause mortality (4, 5, 7, 22, 33, 38), and the relative risk for coronary heart diseases is reported to be similar between inactivity and smoking, hypertension, or dyslipidemia (16).

The age-associated change in muscle is not limited to a decrease in muscle volume; the amount of muscle mitochondria decreases with aging (30, 37). Because skeletal muscle consumes 40% of total energy intake and plays a vital role in whole body energy homeostasis (12, 47), the age-associated reduction in mitochondrial amount and function in skeletal muscle is likely to contribute to an age-associated decline in physical activity, and the development of obesity and diabetes in the elderly, through dysregulation of energy homeostasis (31, 32, 34). Unlike obesity or osteoporosis, however, muscle aging is not recognized as a pathological condition, and there are few effective approaches to prevent it apart from diet therapy and exercise. In addition, a decrease in muscle mitochondria is not considered to be an important factor in muscle aging, although sarcopenia is regarded as a useful marker of muscle aging.

Several longitudinal studies have revealed that a high-protein diet increases skeletal muscle mass (17, 40). Dietary protein intake varies according to geographical location, ranging from 10 to 30% of total energy intake, but a high-protein diet of up to 25 %kcal protein (corresponding to 2 g/kg body wt protein/day) has been shown to increase muscle mass. A high-protein diet is therefore generally recommended for elderly individuals to maintain muscle mass and function. A low-carbohydrate high-fat high-protein diet is also recommended for patients with obesity, because this diet decreases caloric intake and body weight without loss of muscle mass (43). However, the regulation of muscle mitochondria by dietary protein has not been sufficiently clarified, and the ideal protein intake is still under debate. In addition, excessive protein intake is known to be associated with negative effects on renal function, especially in aging individuals, as well as with carcinogenesis and osteoporosis (11).

In these contexts, we examined the long-term effects of a high-protein diet on age-related changes in muscle properties and physical performance, focusing especially on muscle mitochondria. Mice were assigned to protein-adjusted diets with various ratios of protein (10, 20, 30, and 50%) and fat (10 and 45%) from the age of 8 wk. Muscle weight, mitochondrial properties, muscle strength, and exercise endurance were examined at the ages of 20 and 50 wk in mice chronically exposed to the diets. Target of rapamycin complex 1 (TORC1) and AMPK activity, which are essential determinants of muscle mass and mitochondrial amount, respectively, were evaluated by the phosphorylation ratio of these molecules. Rapamycin, an agent that inhibits protein-induced activation of TORC1, was used to clarify the significance of TORC1-dependent signaling in the effects of dietary protein on mitochondria.

MATERIALS AND METHODS

Materials, animals, and cell culture. C57BL/6 mice were purchased from Charles River Laboratories (Tokyo, Japan), and mouse-derived cultured C2C12 myocytes were obtained from RIKEN Bio-Resource Center, (Tsukuba, Japan). C2C12 cells were grown to near

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Table 1. Details of diets and mean body weight and food intake of a mouse at the age of 50 wk

<table>
<thead>
<tr>
<th>Diets</th>
<th>Protein, %</th>
<th>Fat, %</th>
<th>Carbohydrate, % kcal/g</th>
<th>Rapamycin, mg/kg diet</th>
<th>Mean BW, g</th>
<th>Mean Food Intake, kcal/day per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% kcal Protein (P10)</td>
<td>10</td>
<td>10</td>
<td>80</td>
<td>3.8</td>
<td>0</td>
<td>33.93</td>
</tr>
<tr>
<td>20% kcal Protein (P20 = NC)</td>
<td>20</td>
<td>10</td>
<td>70</td>
<td>3.8</td>
<td>0</td>
<td>39.36</td>
</tr>
<tr>
<td>30% kcal Protein (P30)</td>
<td>30</td>
<td>10</td>
<td>60</td>
<td>3.8</td>
<td>0</td>
<td>36.87</td>
</tr>
<tr>
<td>50% kcal Protein (P50 = HP)</td>
<td>50</td>
<td>10</td>
<td>40</td>
<td>3.8</td>
<td>0</td>
<td>33.65</td>
</tr>
<tr>
<td>HF</td>
<td>20</td>
<td>45</td>
<td>35</td>
<td>4.7</td>
<td>0</td>
<td>53.61</td>
</tr>
<tr>
<td>HFHP</td>
<td>45</td>
<td>45</td>
<td>10</td>
<td>4.7</td>
<td>0</td>
<td>46.78</td>
</tr>
<tr>
<td>NCrp</td>
<td>20</td>
<td>10</td>
<td>70</td>
<td>3.8</td>
<td>100</td>
<td>36.05</td>
</tr>
<tr>
<td>HPrap</td>
<td>50</td>
<td>10</td>
<td>40</td>
<td>3.8</td>
<td>100</td>
<td>30.38</td>
</tr>
</tbody>
</table>

NC, normal chow; HP, high protein; HF, high fat; rap, with rapamycin; BW, body weight; n = 8 in each group.

The method to estimate muscle strength of rodents was originally developed by Dr. Meyer and colleagues (26). A mouse was put on a metal mesh and pulled horizontally. The power of traction when the mouse released the mesh was defined as the grip power. Measurements were repeated three times, and the data were averaged. Running distance was measured on a mouse treadmill (LE8170M; PanLab, Barcelona, Spain) according to a previously described protocol (2). Mice were forced to run on the motor-driven treadmill until they were completely exhausted, which was defined as the point at which they remained on the electrical shocker plate for more than 30 s. The treadmill was set at a 10% incline, and the speed was 18 cm/s at the beginning and was increased by 3 cm/s every 2 min, following 3 days of acclimation running at 18 cm/s for 5 min. The average running time until exhaustion for wild-type mice on normal chow was ~30 min.

Examination of mitochondrial amount, enzyme activity and gene expression, and histological analysis in muscle. Tissue samples from quadriceps, gastrocnemius, extensor digitorum longus, and soleus muscles were obtained at the ages of 20 and 50 wk for histological, physiological, and molecular biological analysis. Mice were fed ad libitum and were anesthetized by carbon dioxide (CO2) and euthanized in accordance with domestic law on the protection of laboratory animals.

Examination of physical performance. Grip power reflecting the total power of four limbs of mice was measured using a standard dynamometer for mice (MK-380M; Muromachi Kikai, Tokyo, Japan).
nized at 1800 for tissue collection. Mitochondria were isolated from tissue samples by a modified density gradient centrifugation method using a commercially available kit (no. KC010100, Biochain’s Mitochondria Isolation Kit; Biochain Institute, Hayward, CA), according to the manufacturer’s instructions. Tissue samples were homogenized in mitochondria isolation buffer to rupture cells and centrifuged at 600 g for 10 min to remove debris and large cellular organelles. The supernatant was collected and centrifuged at 12,000 g for 15 min to isolate mitochondria. The pellet was collected and resuspended, and the centrifugation steps were repeated once more before recovery of the isolated mitochondria in the final pellet. Mitochondrial amount in fixed volume of muscle was quantified using a commercially available fluorescent mitochondrial dye (M-7514 Mito Tracker Green FM; Invitrogen Life Technologies, Grand Island, NY). Activity of cytochrome c oxidase (COX, a key enzyme of electron transport complex) was quantified by a commercially available assay kit [no. KC310100, Mitochondria Activity Assay (Cytochrome C Oxidase Assay Kit, Biochain Institute)], according to the manufacturer’s instructions. The kit is designed for measuring mitochondria-specific COX activity, which mediates the chemical reaction: 4Fe2⁺-cytochrome c (ferrocytochrome c) + 4H⁺ + O₂ → 4Fe3⁺-cytochrome c (ferricytochrome c) + 2H₂O. COX has a sharp absorption band at 550 nm in the reduced state, ferrocyanochrome c. The assay is based on observation of the decrease in absorbance at 550 nm of cytochrome c caused by its oxidation by COX. Isolated mitochondria and ferrocyanochrome c were mixed, and absorption at 550 nm was measured to determine COX activity of the samples. Activity of β-hydroxyacyl-CoA dehydrogenase (β-HAD, a key enzyme of β-oxidation of fatty acids) was determined by a previously established method (3). Activity of citrate synthase (CS, a key enzyme of the tricarboxylic acid cycle) was measured by Srere’s method (39), using a commercially available assay kit (Citrate Synthase Assay Kit; Sigma-Aldrich, St. Louis, MO). The muscle fiber diameter and fiber type (I, IIa, or IIb) of superficial gastrocnemius muscle were determined by use of succinate dehydrogenase (SDH) staining, which is an indicator of muscle mitochondrial oxidative activity and fiber type. The fiber types were determined by the density of the staining digitized by gray scale images of the tissue sections. The amount and morphology of muscle mitochondria were examined using electron microscopy (supported by Applied Medical Research Laboratory, Taipei, Taiwan) according to the manufacturer’s instructions.

Experiments using C2C12 cultured myocytes treated with amino acids or rapamycin. C2C12 cells were grown and differentiated in 25-cm² cell culture flasks and treated with glucose-free galactose medium (no. 20383L15 medium; Gibco, Gaithersburg, MD) with or without a commercially available amino acid cocktail (mixture of 2.55–31.6 mg/l each amino acid in final concentration, no. 11140–050 and no. 11130–051, Gibco) and a standard dose of rapamycin (10 nmol/l) for 24 h before the measurements. Oxygen utilization of C2C12 cells was measured using a BD Oxygen Biosensor plate (no. 355830; Becton-Dickinson and Biosciences, Bedford, MA), in which an oxygen utilization-sensitive fluorescent agent is embedded in the 96-well plate. The presence of oxygen in the plate suppresses the fluorescent signal, and the signal increases as oxygen level decreases through cellular respiration. The cell number of each group was counted using an automated counter (no. C10227 Countess; Invitrogen, Tokyo, Japan) according to the manufacturer’s instructions. We applied 4 × 10⁵ cells to each well of the plate. The fluorescent signal (excitation 485 nm, emission 630 nm) was read for 120 min using a microplate reader (Synergy4; BioTek Instruments, Winooski, VT). ATP concentration in C2C12 cells was measured using an ATP Bioluminescence Assay kit CLS II (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer’s instructions.

Estimation of protein levels by Western blotting. Western blotting was performed by standard methods to evaluate the protein levels and phosphorylation status of ribosomal protein S6 kinase 1 (S6K1) and AMPK. TORC1 phosphorylates and activates S6K1, and the axis is known to control fundamental cellular processes, such as protein synthesis and cell growth by sensing nutrient availability (25). TORC1 activity is generally evaluated by phosphorylation status of S6K1, since that of TORC1 molecule itself does not reflect its activity adequately. The TORC1-S6K1 axis is recognized as a master regulator of muscle hypertrophy, and allosteric activation of S6K1 is provoked by the phosphorylation at Thr389 by TORC1 complex is known to activate the process (24). The phosphorylation status of S6K1 was therefore analyzed as a surrogate marker for TORC1 activity and muscle hypertrophy. Total protein extracts (10 µg) from quadriceps muscle or C2C12 cells were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes using a dry blotting system (Trans-Blot Turbo; Bio-Rad, Hercules, CA), and incubated with the antibodies [no. 9205 phospho-p70S6K (Thr389) antibody, no. 9202 p70S6K antibody, no. 2531 phospho-AMPKα (Thr172) antibody, and no. 2532 AMPKα antibody]. Immunolabeled proteins were detected using a chemiluminescence kit (ECL Plus; GE Healthcare, Tokyo, Japan). The density of the blot was estimated by imaging software (MultiGauge; Fujifilm, Tokyo, Japan).

Statistical analysis. All data are expressed as means ± SE. Comparison of means between the two groups was performed by Student’s t-test. When more than two groups were compared, analysis of variance was used to evaluate significant differences among groups, and if significant differences were confirmed, each difference was further examined by Fisher’s protected least significant difference method. P < 0.05 was considered statistically significant.

RESULTS

Long-term administration of high-protein diet reduces exercise endurance and muscle mitochondrial activity. To clarify the effect of dietary protein on physical performance and muscle property, mice were assigned to the diets with different ratios of protein content from 10 to 50% and from the age of 8 wk till 50 wk, and grip power and running distance were examined at the ages of 20 and 50 wk. Grip power was lower in the mice fed the 10% protein (low protein, P10) diet relative to those fed the 20% protein (normal protein, P20) diet at 20 wk and 50 wk. Conversely, grip power in the mice fed the 30% protein (high protein, P30) and 50% (super high protein, P50) diets was significantly augmented at 50 wk. The reduction of grip power from the age of 20 to 50 wk was prominent in the mice fed the lower-protein diet (Fig. 1A). Running distance was lower in both the P30 and P50 diets compared with the P20 diet. The reduction of running distance was prominent in the mice fed higher-protein diet (Fig. 1B).

We next prepared the four diets [namely, normal chow (NC = P20), high-protein (HP = P50), high-fat (HF), and high-fat high-protein (HFHP)] and examined the effect of the dietary

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conditions on physical performance and muscle properties. Grip power at 50 wk was significantly increased in HP compared with NC (Fig. 1C). Conversely, running distance at 50 wk was significantly decreased in HP compared with NC (Fig. 1D). HP and HFHP also increased grip power; however, the diets decreased running distance compared with NC. The reductions in running distance were particularly striking when mice were fed HP or HFHP (Fig. 1D).

The mitochondrial electron transport activity of quadriceps muscle, estimated by COX activity, was significantly reduced in HP and HFHP compared with NC and HF, respectively (Fig. 1E). The weight of gastrocnemius muscle was increased in both HP and HF compared with NC, and in HFHP compared with HF (Fig. 1F). The mitochondrial electron transport activity of quadriceps muscle, estimated by COX activity, was significantly reduced in HP and HFHP compared with NC and HF, respectively (Fig. 1F).

To dissect differences in the effect of HP and HF diets on physical performance, muscle properties, and muscle mitochondria, we examined muscle weight, mitochondrial amount, enzyme activity, expression of mitochondria-related genes, histological findings, and electron micrograph at the age of 50 wk. Activities of TORC1 and AMPK were evaluated by Western blot analysis; they are the representative regulatory molecules of muscle mass and mitochondrial activity, respectively. The muscle weights of gastrocnemius, EDL, and soleus were increased in HP (Fig. 2A); conversely, muscle mitochondrial amount (Fig. 2B) and activity of mitochondrial enzymes COXIV, β-HAD, and CS were severely decreased in HP diet (Fig. 2C). TORC1 activity, as determined by phosphorylation status of S6K1 (p-S6K1/S6K1), was increased, and AMPK activity, as determined by phosphorylation status of AMPK (p-AMPK/AMPK), was decreased in HP (Fig. 2D). Expressions of PGC-1α and PGC1α, Tfam, COXIV, ATPsyn, COX, beta-HAD, CS, and activity of mitochondrial enzymes.

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**Fig. 2.** HP diet suppresses AMPK and decreases muscle mitochondria, although HF diet does not affect them. To dissect differences in the effect of HP and HF diets on physical performance, muscle properties, and muscle mitochondria, we examined muscle weight, mitochondrial amount, enzyme activity, expression of mitochondria-related genes, histological findings, and electron micrograph at the age of 50 wk. Activities of TORC1 and AMPK were evaluated by Western blot analysis; they are the representative regulatory molecules of muscle mass and mitochondrial activity, respectively. The muscle weights of gastrocnemius, EDL, and soleus were increased in HP (Fig. 2A); conversely, muscle mitochondrial amount (Fig. 2B) and activity of mitochondrial enzymes COXIV, β-HAD, and CS were severely decreased in HP diet (Fig. 2C). TORC1 activity, as determined by phosphorylation status of S6K1 (p-S6K1/S6K1), was increased, and AMPK activity, as determined by phosphorylation status of AMPK (p-AMPK/AMPK), was decreased in HP (Fig. 2D). Expressions of PGC-1α and PGC1α, Tfam, COXIV, ATPsyn, COX, beta-HAD, CS, and activity of mitochondrial enzymes.
Tfam, major regulators of mitochondrial biogenesis, were significantly decreased in HP, and that of COXIV and ATP synthase, components of electron transport chain, were also decreased (Fig. 2E). SDH staining revealed that muscle fiber size was increased in HP (Fig. 2, F and G). Proportion of red muscle fibers, types I and IIA, were decreased and that of white muscle, type IIB, was increased in HP (Fig. 2H). A decrease in mitochondrial amount in HP was confirmed by the observation using electron microscope (Fig. 2f).

Similarly to HP, muscle weight was increased in HF (Fig. 2J); however, mitochondrial amount and activity (COX and CS) showed no significant change (Fig. 2, K and L). TORC1 and AMPK activity were unchanged in HP (Fig. 2M). Expressions of genes related to mitochondrial amount and electron transport activity were also unchanged in HP (Fig. 2N).

These findings indicate that dietary protein decreases mitochondrial activity through the decrease in mitochondrial amount associated with changes in muscle fiber type, which is compatible with the decrease in the AMPK activity observed in the mice fed an HP diet.

Chronic activation of TORC1 accelerates age-associated declines in mitochondria and exercise endurance. To elucidate the role of a representative signaling pathway that mediates the TORC1 signal pathways in the regulation of muscle properties, mice were fed the NC or HP diet with or without rapamycin until 50 wk of age. Grip power was significantly decreased in rapamycin-treated mice on either NC or HP diet at the age of 50 wk (Fig. 3A). Running distance was significantly increased in rapamycin-treated mice on either NC or HP diet (Fig. 3, B and H). Muscle weight was decreased by treatment with rapamycin in both NC and HP (Fig. 3C). Mitochondrial amount was increased by rapamycin in HP (Fig. 3D). TORC1 activity was significantly increased in HP and decreased by rapamycin in both diet groups. Conversely, AMPK activity was decreased in HP and reversed by rapamycin (Fig. 3E). The plasma ammonia level was significantly elevated in HP and was not affected by rapamycin (Fig. 3F). The level of fumarate in quadriceps muscle, an intermediate of the tricarboxylic acid cycle, was also increased in HP and was not affected by rapamycin (Fig. 3G).

Fig. 3. Chronic activation of TORC1 (target of rapamycin complex 1) accelerates age-associated declines in mitochondria and exercise endurance. A–G: mice were fed NC or HP diet with or without rapamycin until 50 wk of age. NC, normal chow; NCrap, NC + rapamycin (100 mg/kg diet); HP, high-protein; HPrap: HP + rapamycin. *P < 0.05, **P < 0.01 compared between NC and NCrap or HP and HPrap, *P < 0.05, **P < 0.01 compared between NC and HP or NCrap and HPrap, n = 8. A: grip power. B: running distance. C: weight of gastrocnemius muscle. D: mitochondrial amount in quadriceps muscle. E: Western blot densitometry (left) and blots (right). F: plasma ammonia level. G: fumarate concentration in quadriceps muscle. H–L: mice were fed HP diet with or without rapamycin until 50 wk of age. *P < 0.05, **P < 0.01 between HP and HPrap; *P < 0.05, **P < 0.01 compared between 20 and 50 wk of age in mice fed HP diet; n = 8. H: running distance at 20 and 50 wk. I: mitochondrial amount in quadriceps muscle. J: activity of mitochondrial enzymes. K: Western blot densitometry (left) and blots (right). L: expressions of mitochondria-related genes estimated by quantitative PCR analysis.
We next investigated the time course of the changes in these parameters. In the HP diet group, running distance was unaltered by rapamycin at 20 wk; however, the decrease in running distance with aging was significantly diminished by rapamycin (Fig. 3H). Mitochondrial amount and enzyme activities were significantly increased by rapamycin in HP (3, I and J). TORC1 activity was inhibited by rapamycin in both 20- and 50-wk-old mice; however, AMPK activity was recovered only at 50 wk (Fig. 3K). Expressions of genes related to mitochondrial amount and electron transport activity were significantly reversed by rapamycin (Fig. 3L).

In summary, inhibition of TORC1 in muscle by long-term administration of rapamycin decreased muscle weight and grip power but increased running distance. TORC1 inhibition was associated with muscle AMPK activation that would lead to mitochondrial activation and improvement of exercise endurance.

Amino acids alter energy consumption so as to suppress AMPK in myocytes. To clarify the effects of TORC1-dependent signaling on muscle energy metabolism, cultured C2C12 myocytes were treated with amino acids and rapamycin for 24 h. Amino acids reduced O2 consumption (Fig. 4A) and increased ATP content (Fig. 4B). Mitochondrial amount was significantly decreased by amino acids (Fig. 4C). TORC1 was increased, but AMPK activity was suppressed by amino acids (Fig. 4D). On the other hand, when TORC1 was activated and AMPK activity was increased by rapamycin (Fig. 4F). Mitochondrial amount was reversed by rapamycin (Fig. 3L). Expressions of genes related to mitochondrial amount and electron transport activity were significantly reversed by rapamycin (Fig. 3L).

DISCUSSION

In the present study, we focused on the regulation of muscle mitochondria by dietary protein and found that long-term feeding of a high-protein diet to mice from 8 to 50 wk of age decreased the amount of muscle mitochondria and the mitochondrial activity, which accelerated the decline in exercise endurance, although the diet increased muscle mass and strength. Because chronic suppression of TORC1 by rapamycin recovered the muscle AMPK activity and mitochondrial amount that were reduced by dietary protein, TORC1-dependent suppression of AMPK was considered to explain the effects of dietary protein on muscle mitochondria.

Whereas the regulatory mechanism of muscle volume has been intensively investigated, that of muscle mitochondria is not well understood. AMPK is a representative regulator of energy consumption of the myocytes so as to increase unused ATP. The suppression of AMPK by amino acids would be caused by the increase in ATP.
muscle mitochondria, which promotes mitochondrial biogenesis, a fiber type switch toward slow-twitch fibers and ATP production in muscle (6, 10) through allosteric activation by phosphorylation at Thr172 (15). It is well known that exercise activates AMPK through an increase in AMP associated with ATP consumption (45); however, regulation of AMPK by dietary factors has not been clarified. Previous reports demonstrated that muscle subsarcolemmal mitochondria were decreased in obesity and type 2 diabetes (34) and that overeating could affect mitochondrial function (9). In addition, our previous results demonstrated that a combination of glucose and insulin, which mimics the conditions of obesity, induced mitochondrial dysfunction in C2C12 cultured myocytes (27). On the other hand, calorie restriction has been reported to promote mitochondrial biogenesis and might retard aging (23). However, the effects of dietary protein on muscle AMPK and mitochondria have not been investigated.

We therefore examined the effects of dietary protein on the amount of muscle mitochondria, enzyme activity, and gene expression and found that a high-protein diet strongly decreased the mitochondrial amount and enzyme activities, which were associated with changes in muscle fiber type. In addition, we showed that long-term activation of TORC1 by a high-protein diet could suppress AMPK activity. Suppression of AMPK might explain the decline in exercise endurance, associated with a decrease in muscle mitochondria and slow-twitch fibers. These findings in the present study support the view that dietary protein accelerates the age-associated decline in exercise endurance through TORC1-dependent suppression of muscle AMPK and mitochondria. As described above, calorie restriction has been demonstrated to activate mitochondrial function; in the present study, however, the mice fed a high-protein diet showed deteriorated mitochondrial activity despite the lower calorie intake. In addition, in the treadmill experiments there was a negative correlation between body weight and running distance in general; however, mice fed a high-fat diet showed decreased running distance despite the fact that they had a lighter body weight. On the other hand, a high-fat diet did not change TORC1, AMPK, or mitochondrial activity, although exercise endurance deteriorated under this diet. Therefore, other factors that were related to obesity, rather than mitochondrial dysfunction, probably caused the deterioration in exercise endurance. Although many factors are involved in the regulation of mitochondrial function, the results of the present study at least indicate that dietary protein, rather than fat, contributes to muscle mitochondrial dysfunction.

TORC1/S6K1-dependent signal pathways, which mediate protein synthesis and increased muscle volume under a high-protein diet (8), are known to modulate various physiological processes (28). Suppression of TORC1 signaling renders mice resistant to obesity and diabetes (42) and prolongs lifespan (36). On the other hand, activation of TORC1 has been shown to contribute to the progression of age-related diseases, including cancer, type 2 diabetes, atherosclerosis, hypertension, cardiac hypertrophy, and osteoporosis (18, 41). Whereas the biological significance of TORC1 has been well characterized in these contexts, the mechanism by which it promotes age-related diseases and aging itself has not been clarified. The rapamycin-sensitive suppression of AMPK and mitochondria might provide a basis for the deteriorative effects of TORC1 on the age-related diseases described above.

Experiments using C2C12 myocytes were performed to confirm the in vivo effects of dietary protein on AMPK activity and mitochondria. The in vitro results supported the in vivo data in that excessive amino acids, which represent the characteristics of mice fed a high-protein diet, could reduce mitochondrial function. The effect of rapamycin to increase AMPK activity and mitochondria was also reproduced in the myocytes. Because oxygen consumption was dramatically decreased in the amino acid-treated cells, it seems that the amino acids suppressed energy metabolism in the myocytes, thereby increasing ATP and suppressing AMPK. Although we found that blockade of TORC1/S6K1 signaling by rapamycin treatment in the myocytes increased oxygen consumption, AMPK activity, and mitochondrial amount, which is compatible with a previous report showing that deletion of S6K1 increased oxygen consumption (42), the mechanism by which rapamycin or blockade of TORC1/S6K1 signaling affects energy metabolism needs to be further clarified. The increases in AMPK activity and mitochondria in the present study were easily observed in myocytes 24 h after rapamycin treatment; in mice fed a rapamycin-mixed diet from 8 wk of age, however, the increases were not evident at 20 wk, although they could be observed at 50 wk. Although the reason for the difference in the time course of the effects of rapamycin between the in vitro experiments and in vivo experiments was not clear in the present study, we speculate that the complex homeostatic system that regulates energy metabolism, AMPK activity, and mitochondria in mice delays the stage when the effects of rapamycin become apparent. In previous studies, loss of cellular ATP was shown to activate AMPK and subsequently suppress TORC1/S6K1 signaling (13) independently of rapamycin treatment.

Because prevention of the detrimental effects of a high-protein diet by rapamycin on muscle mitochondria and exercise endurance in the present study was partial, we explored other factors that might mediate the residual part of the effects. A high-protein diet would cause hyperammonemia, and ammonia has been reported to cause deterioration of mitochondria through the production of reactive oxygen species (1, 29), and it has been known to provoke central and peripheral fatigue (14) and would alter energy consumption. Fumarate, which is a degradation product of protein and amino acids, has been reported to cause impairment of muscle contractility (21) and fatigue (20). In this study, the plasma ammonia level and fumarate concentration in quadriceps muscle were elevated by dietary protein in mice; however, the elevations were not affected by rapamycin. Therefore, the increased degradation products from a high-protein diet were considered to be partially responsible for the detrimental effects of dietary protein on muscle mitochondria and exercise endurance, which were not dependent on rapamycin.

The present study has some limitations, which should be interpreted carefully. The decline in muscle mass and physical performance was apparent and significant in mice before 50 wk of age, which corresponds to ~40 yr of age in humans, an age when sarcopenia and loss of physical performance are not apparent. Therefore, a fundamental difference in the time course and mechanism of muscle aging might exist between mice and humans. Although previous reports have used mice aged 50 wk as models of muscle aging (44, 46), this difference should be taken into account when findings in mice are applied
to humans. In the present study, we demonstrated that exercise endurance decreased in parallel with mitochondrial amount and enzyme activity in response to dietary protein; however, exercise endurance is determined by many factors involving multiple organs. Therefore, several factors other than muscle mitochondria are likely to be involved in the decline in exercise endurance associated with a high-protein diet.

In summary, the present study revealed that long-term exposure to a high-protein diet decreases muscle mitochondria through TORC1-dependent suppression of AMPK. In addition, the diet was found to decrease exercise endurance despite an increase in muscle mass. We suggest that a decrease in muscle mitochondria caused by dietary protein provides a basis for accelerated decline in exercise endurance. Because activation of muscle mitochondria seems to be an indispensable factor to accelerated decline in exercise endurance, given the fatigue mechanisms of rats submitted to prolonged physical exercise. 

**REFERENCES**