Methionine mitigates protein deficiency by regulating the expression of translation initiation factors, even with sufficient sulfur, in rats fed a protein-deprived diet

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Abstract

Dietary supplementation with methionine and threonine mitigates protein deficiency in rats fed a protein-deprived diet, an effect that is not observed for other essential amino acids. However, the precise mechanisms are not fully understood. The aim of this study was to explore whether the activation of translation initiation factors in the mammalian target of rapamycin pathway in skeletal muscle contributes to protein retention by supplementation with methionine and/or threonine. Male Sprague-Dawley rats were freely fed 0% protein diets for 2 weeks, and furthermore fed for 12 days 14.5 g of a restricted diet containing 12% soy protein supplemented with methionine and threonine (MT), methionine (M), threonine (T), or neither (NA) (n = 8), while two additional groups were fed 0% protein or 20% casein protein diets as controls (n = 6). Body weight and gastrocnemius muscle weight were higher, and blood urea nitrogen and urinary nitrogen excretion were lower, in the M and MT groups than in the T and NA groups, respectively. p70 S6 kinase 1 abundance and protein expression levels were higher, and eukaryotic translation initiation factor 4E-binding protein 1 abundance and mRNA levels were lower, in the skeletal muscles of rats fed with methionine (M and MT groups). These results suggest that methionine regulates the mass of protein via translation initiation machinery in the skeletal muscle, retaining whole-body protein in protein-deprived rats.

Introduction

Methionine is an essential amino acid (EAA) containing sulfur that contributes to the biosynthesis of antioxidants, non-essential amino acids and other nitrogen-containing substances, methyl donors, phospholipids, and vasodilatory intermediates (Sbodio et al. 2019; Martínez et al. 2017; Lauinger et al. 2021).

Methionine and threonine, but not other EAAs, mitigate protein deficiency when these sulfur-containing amino acids are added to casein in rodent chow (Yoshida et al. 1957; Yokogoshi et al. 1976). The improved reutilization of endogenous amino acids for protein synthesis by adding methionine and threonine appears to be the main cause for the protein retention effect in protein-malnourished rats (Yokogoshi et al. 1977; Yokogoshi et al. 1979). However, rodents have a dense covering of fur consisting of keratin whose main components are sulfur-containing amino acids (Powell et al. 1991; Marshall et al. 1976; Humeniuk et al. 1978). It is unclear whether the positive effect of methionine on protein deficiency is the result of meeting the high requirements for sulfur-containing amino acids for keratin, or if it contributes to some other mechanism of protein metabolism.

Methionine also methylates protein phosphatase 2A, resulting in the stimulation of mammalian target of rapamycin complex 1 (mTORC1) (Kitada et al. 2020; Zhang et al. 2018; Lauinger et al. 2021), which activates protein synthesis via the phosphorylation of the translation initiation factors eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and p70 S6 kinase 1 (S6K1) (Liu et al. 2020; Cafferkey et al. 1993; Sabatini et al. 1994). However, the involvement of mTORC1 in the development of protein retention by methionine has not been clarified in protein-deprived rats.
In this study, we tested the hypothesis that methionine increases mTORC1-dependent translation initiation factors in skeletal muscle, even when 0.3% cysteine is added to satisfy the sulfur-containing amino acid requirements, resulting in protein-retention effects such as increased muscle mass, nitrogen sufficiency, and body weight gain.

Materials And Methods

Animals and housing

Forty-four male Sprague-Dawley rats (age, 5 weeks) were purchased from Charles River Japan (Yokohama, Japan) and housed in a conventional animal room under controlled conditions (temperature, 23 ± 3°C; 12:12-h light:dark cycle, lights on at 07:00). The Animal Care and Use Committee of Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan) approved the experimental protocol.

Experimental Design

All rats acclimated for 6 days on a rodent diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) were first given a 0% protein diet for 2 weeks (Table 1). After this, on day 0, all rats were assigned to six groups: the methionine and threonine supplementation (MT) group (n = 8), the methionine supplementation (M) group (n = 8), the threonine supplementation (T) group (n = 8), the no supplementation (NA) group (n = 8), the 0%P (no protein) group (n = 6), and the 20%P group (n = 6) equal to their body weight. About 56 kcal of the experimental diets (14.5 g of each test diet) were individually given each day for 12 days. The body weights (BWs) of the rats were measured every other day, and their food intake was measured daily before feeding. Urine pooled for three days was collected (on days 10–13). On the morning of day 13, we confirmed that all rats had completely eaten the test diets given on the last day. Each group of rats was anesthetized with isoflurane (Intervet Inc., Tokyo, Japan) after 5 h of fasting to minimize the effects of feeding, and blood from the abdominal aortas, the right gastrocnemius muscles, and both epididymal white adipose tissues were immediately collected and weighed, then quickly frozen in liquid nitrogen.

Preparation Of Experimental Diets

The compositions of the experimental diets are shown in Table 1.

Preparation Of Protein Extracts From Tissues

The frozen muscles were homogenized in ice-cold protein extraction reagent (T-PER™ Tissue Protein Extraction Reagent, ThermoFisher Scientific, USA) containing one tablet per 10 mL solution of phosphatase-inhibitor cocktail (PhosSTOP™, Roche Diagnostics, Germany) and protease inhibitor cocktail (cOmplete, Roche Diagnostics). The homogenates were then centrifuged at 10,000 ×g at 4°C for 10 min,
and the protein concentrations of supernatants were determined (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific).

**Western Blot Analysis**

Western blot analysis
Simple western analysis was performed using an automated capillary electrophoresis immunoassay system according to the manufacturer’s recommendations with slight modifications (Wes, Protein Simple, CA, USA). These protein samples (4 µg/µL) were denatured by heating to 95°C for 5 min. Compass software (Protein Simple) was used to quantify indicated peak areas by a Gaussian fitting protocol, and the raw data were converted to images. Primary antibodies against target proteins were from Cell Signaling Technology (Beverly, MA, USA) and Proteintech (Tokyo, Japan) and were used at the following dilutions: phospho-p70 S6 kinase (Thr421/Ser424) (#9204), 1:50; p70 S6 kinase (49D7) (#2708), 1:500; phospho-4E-BP1 (Thr37/46) (#9459), 1:100; 4E-BP1 (#9452), 1:50; phospho-FOXO1 (Ser256) (E1F7T) (#84192S), 1:250; FOXO1 (#18592-1-AP), 1:2000. Each protein level was normalized to the total proteins. Protein phosphorylation levels were normalized to the total corresponding protein level.

**Real-time Pcr**

The total RNA was prepared using ISOGEN II (NIPPON GENE CO., LTD. Tokyo, Japan) and purified with the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) according to the manufacturers’ instructions. Relative mRNA expression was determined by the 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and Luna Universal One-Step RT-qPCR Kit (New England Biolabs Japan, Tokyo, Japan). The primer sequences used were as follows: forward, 5'-CACAGCAGTCAGGCCTTGTA-3', reverse, 5'-CAGGGAGGGGTAGGTGAGA-5' for EIF4EBP1; forward, 5'-ATGCTGCTGCTTCTCGTCT, reverse, 5'-TTGAGTCATCTGGGCTGT-3' for p70S6K; and forward, 5'-GCAAGAGAGGCCCTCAG-3', reverse, 5'-TGTGAGGGAGATGCTAGTG-3' for GAPDH (Takara Bio Inc., Otsu, Japan). The mRNA expression was quantified using the comparative ΔCT method, with GAPDH as the reference gene.

**Measurement Of Amino Acid Concentrations**

Plasma samples were deproteinized by precipitation with acetonitrile and centrifuged twice for 10 min at 12,000 rpm at 4°C. The content in the fractions was determined by a liquid chromatograph-mass spectrometer (LC-MS2020; Shimadzu, Kyoto, Japan).

**Measurement Of Other Biochemical Markers**
Plasma blood urea nitrogen (BUN) and urine creatinine were determined using an automatic biochemistry analyzer (7180; Hitachi High-Technologies Co., Ltd., Tokyo, Japan). Urinary nitrogen levels were measured with a total nitrogen analyzer (TN-100; Dia Instruments Co., Kanagawa, Japan). Urine samples for 3-methylhistidine (3-MetHis) were measured by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS: ACQUITY UPLC, Waters Corporation, Milford, MA, USA; API4000, AB SCIEX, Tokyo, Japan).

Statistical analysis

Values for each group are presented as means ± SD. Data were analyzed using EXSAS Ver. 10 software (EPS CO., LTD, Tokyo, Japan). Differences between the normal (20%P) and positive control (0%P) groups were assessed by Student’s t test. Comparisons between the 0%P group and the four test groups were assessed by Dunnett’s test. The results of four test groups were analyzed by a two-way ANOVA followed by Tukey’s test when appropriate. Differences were considered statistically significant at P<0.05.

Results

Body weight, gastrocnemius muscle weight, epididymis fat weights, and biological parameters

In the four test groups, the final BW and gastrocnemius muscle weights of the MT and M groups were significantly higher than those of the T and NA groups (Fig. 1 (b, c)). In contrast, there was no significant difference in the epididymis fat weights in these groups (Fig. 1 (d)). For nitrogen metabolism-related biomarkers, BUN levels were lower in the test four groups than in the 0%P group. Furthermore, the MT and M groups showed lower BUN levels compared to the T group. The MT group also showed significantly lower BUN levels than the NA group (Fig. 1 (e)). In the four tested groups, the MT and M group showed lower levels of urinary nitrogen excretion than the T and NA groups, even though equal protein amounts in the diet were given in each group (Fig. 1 (f)). Total urinary 3-MetHis excretion did not differ between the 0%P group and the four tested groups. However, the MT group showed significantly lower levels of urinary 3-MetHis excretion than the NA group (Fig. 1 (g)).

Plasma Essential Amino Acid Concentrations

The concentration of methionine was lower in the T and NA groups than in the 0%P group, and threonine was higher in the MT and T groups than in the 0%P group (Table 2). In the four tested groups, the concentration of methionine was higher in the MT and M groups than in the T and NA groups, and threonine was higher in the MT and T groups than in the M and NA groups.

Changes In Protein And Mrna Expression Of S6k1 And 4e-bp1 Proteins And Their Phosphorylation Status
Total protein and mRNA expression levels of S6K1 were significantly higher in the MT, M, and T groups than in the 0%P group (Fig. 2 (a, d)). The phosphorylation ratio of S6K1 was significantly higher in the M group than in the 0%P group (Fig. 2 (b)). In the four tested groups, protein levels of S6K1 were significantly higher in the MT and M groups than in the NA group, and in the MT group it was significantly higher than in the T group.

There was no difference between the 0%P group and the four tested groups in protein levels of 4E-BP1, but the phosphorylation ratio of 4E-BP1 was higher in the four tested groups than in the 0%P group (Fig. 3). mRNA expression levels of 4E-BP1 were significantly lower in the MT and M groups, and significantly higher in the NA group, than in the 0%P group (Fig. 3 (d)). In the four tested groups, protein levels of 4E-BP1 were significantly lower in the MT and M groups than in the NA group, although there was no difference in the phosphorylation ratios (Fig. 3 (a, b)). mRNA expression levels of 4E-BP1 were significantly lower in the MT and M groups than in the T and NA groups, and they were significantly lower in the T group than in the NA group (Fig. 3 (d)).

Changes In Foxo1 In The Gastrocnemius Muscle

Although protein levels of total FOXO1 were higher in the NP group than in the four test groups, there was no difference among these groups (Fig. 4 (a, c)). However, the phosphorylation level of FOXO1 at Ser256 was lower in the 0%P group than in the four test groups, but there was no difference among these groups (Fig. 4 (b, c)).

Discussion

In this study, we made the following novel findings: 1) even with 0.3% cysteine added, further methionine supplementation retained body protein, and 2) methionine supplementation regulated the expression of translation initiation factors downstream of the mTORC1 pathway.

The standard diet for rats contains 20% casein supplemented with 0.3% sulfuric amino acids (Cys for AIN-93G and Met for AIN-76) (Reeves et al. 1993). Although supplementation with methionine and threonine has been reported to retain protein in rats (Yoshida et al. 1957; Yokogoshi et al. 1976), it is important whether this is a secondary consequence of adequate sulfur supply for keratin or if methionine is directly supplied to other body proteins. Therefore, 0.3% cysteine was added in all tested diets except 0%P in an amount sufficient to meet the requirements.

In this study, we used soy protein as the protein source of the test diets. This is because adding methionine to soy protein has been reported to have a protein retention effect (Fujisawa et al. 1995), and we wanted to investigate whether supplementation with methionine would have further protein retention effects even under conditions of adding cysteine. The amount of protein in the tested diets was set to 12%, because a higher protein retention effect of both methionine and threonine was found with lower
protein contents in test diets in a pilot study in which several protein levels (12%, 16%, 20%, and 24%) were tested (data not shown).

We showed that further supplementation of methionine mitigated body protein loss, even though the requirement for sulfur-containing amino acids was satisfied. Furthermore, to test whether the effect of methionine supplementation on body protein retention in this study was due to methionine itself or to the supply of sulfur, several kinds of sulfur-containing amino acids (methionine, cysteine, taurine) were compared in the same model. The protein retention effect was observed only when methionine was added and not for the other sulfur-containing amino acids (data not shown). So we conclude that methionine itself, and not sulfur in general, is important for protein retention. It was clear from the protein metabolism results that nitrogen was retained in rat bodies by adding methionine.

Regarding the involvement of amino acids in protein synthesis, the regulation of mTORC1 by amino acids such as leucine is well known (Hara et al. 1998; Anthony et al. 2000), but there are few reports on mTORC1 regulation by specific amino acids under conditions of protein malnutrition. Interestingly, the protein expression level of S6K1 was increased while the protein expression level of 4E-BP1 was decreased in the methionine-added groups (MT and M). These alternations may act favorably on protein synthesis when mTORC1 is activated. This is consistent with the methionine-added groups showing a protein retention effect accompanied with an increase in gastrocnemius muscle weight.

More interestingly, the effects of methionine supplementation on the regulation of S6K1 and 4E-BP1 were not exactly the same; 4E-BP1 not only had a decreased protein expression level but also had a decreased gene expression level, but for S6K1, an increase in the protein expression was observed without any change in gene expression. Recently, evidence has accumulated that 4E-BP1 plays an important role in protein translation, while deletion of S6K1 in mouse liver and muscle cells does not reduce translation (Mieulet et al. 2007; Pende et al. 2004). Thus, it has been reported that 4E-BP1 plays a more important role than S6K1 in protein translation in muscle, but it is unclear which transcription factor predominates under malnutrition. In this study, the effect of methionine supplementation was observed not only on the protein expression but also on the gene expression of 4E-BP1 under malnutrition. These findings strongly suggest that the body protein retention effect observed by methionine supplementation under malnutrition involved 4E-BP1.

In this study, there was no difference in gene expression of S6K1, and only the protein expression level was increased. Although there are few reports about the gene expression of S6K1, especially under malnutrition, it has been reported that calorie restriction decreases the protein expression level of S6K1 along with decreased skeletal muscle weight (Margolis et al. 2016). Therefore, we speculate that the regulation of 4E-BP1 was more prioritized than S6K1 in order to maintain protein synthesis under protein malnutrition. These results showed that further supplementation of methionine retained body protein under conditions in which sulfur-containing amino acid requirements were satisfied, and this effect was mediated by the protein expression levels of 4E-BP1 and S6K1 downstream of mTORC1.
Conclusion

In this study, we investigated the effects of supplementation with methionine and/or threonine to a soy protein diet on retaining body protein in 0% protein diet-induced malnourished rats. Further supplementation with methionine mitigated body protein loss under protein malnutrition. The protein expression levels of S6K1 and 4E-BP1 were altered in the methionine-supplemented groups, suggesting that methionine retained body protein by regulating translation initiation factors downstream of mTORC1.

Declarations

Acknowledgments

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Competing interests

None declared except that all authors are employed by Otsuka Pharmaceutical Factory, Inc.

Authors’ contributions

All authors conceived the idea of the study. FY and IY developed the statistical analysis plan and conducted the statistical analyses. All authors contributed to the acquisition and analysis of the data and the interpretation of the results. FY drafted the original manuscript. IY reviewed the manuscript draft and revised it critically for intellectual content. All authors approved the final version of the manuscript to be published.

Funding

Not applicable.

Compliance with ethical standards

All animals were treated in accordance with the guidelines established by the Animal Care and Use Committee of Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). The experimental protocol was approved by the Animal Experimentation Committee of Otsuka Pharmaceutical Factory, Inc.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.
References


24. Zhang Y et al. (2018) mTORC1 signaling-associated protein synthesis in porcine mammary glands was regulated by the local available methionine depending on methionine sources. Amino Acids 50:105115. https://doi.org/10.1007/s00726-017-2496-0

**Tables**
<table>
<thead>
<tr>
<th>Ingredient, g/kg</th>
<th>MT</th>
<th>M</th>
<th>T</th>
<th>NA</th>
<th>0%P</th>
<th>20%P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>Soy protein isolate</td>
<td>110</td>
<td>117</td>
<td>118</td>
<td>120</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>3.95</td>
<td>3.87</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>L-Threonine</td>
<td>3.39</td>
<td>3.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Corn starch</td>
<td>480.16</td>
<td>476.61</td>
<td>476.37</td>
<td>477.49</td>
<td>600.49</td>
<td>397.49</td>
</tr>
<tr>
<td>a-Corn starch</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
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<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
<td>50</td>
<td>50</td>
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<td>50</td>
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<tr>
<td>Mineral mix</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
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</tbody>
</table>

Cysteine (0.3%) was added to all diets except the 0% protein diet. As supplementation with methionine and threonine to 12% soy protein showed a protein improvement effect in preliminary studies, the four tested diets were prepared with 12% soy protein, and the total amount of methionine and threonine in the four tested diets was calculated to equal to the amount present in the AIN-93G diet in this study (Reeves et al. 1993). Soy protein amounts were adjusted to match the total amino acid amounts among the four tested diets. The mineral and vitamin mixtures were prepared according to the AIN-93G prescription.
Table 2
Plasma essential amino acid concentrations

<table>
<thead>
<tr>
<th></th>
<th>MT</th>
<th>M</th>
<th>T</th>
<th>NA</th>
<th>0% Protein</th>
<th>20% Protein</th>
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</thead>
<tbody>
<tr>
<td>µmol/L</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>His</td>
<td>45.65 ± 10.42&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>46.39 ± 9.79&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>66.41 ± 6.96&lt;sup&gt;b+++&lt;/sup&gt;</td>
<td>55.55 ± 7.40&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>137.85 ± 28.19</td>
<td>63.17 ± 6.07&lt;sup&gt;§§§&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ile</td>
<td>30.99 ± 6.90</td>
<td>26.00 ± 7.07&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>31.63 ± 6.04</td>
<td>28.33 ± 6.45&lt;sup&gt;+&lt;/sup&gt;</td>
<td>40.60 ± 10.16</td>
<td>51.53 ± 6.41&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leu</td>
<td>51.69 ± 8.69&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>52.93 ± 12.20&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>55.91 ± 12.14&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>54.61 ± 10.24&lt;sup&gt;+&lt;/sup&gt;</td>
<td>78.48 ± 12.78</td>
<td>85.72 ± 7.59</td>
</tr>
<tr>
<td>Lys</td>
<td>142.00 ± 95.51&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>226.53 ± 63.08&lt;sup&gt;ac+++&lt;/sup&gt;</td>
<td>356.04 ± 84.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>294.04 ± 64.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>360.82 ± 41.56</td>
<td>476.70 ± 67.48&lt;sup&gt;§§§&lt;/sup&gt;</td>
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<tr>
<td>Met</td>
<td>24.81 ± 2.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.29 ± 6.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.33 ± 3.68&lt;sup&gt;b+++&lt;/sup&gt;</td>
<td>12.55 ± 2.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.43 ± 3.07</td>
<td>46.18 ± 8.36&lt;sup&gt;§§§&lt;/sup&gt;</td>
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<tr>
<td>Cys</td>
<td>16.08 ± 5.62</td>
<td>14.28 ± 3.81</td>
<td>16.86 ± 4.09</td>
<td>17.88 ± 2.49&lt;sup&gt;+&lt;/sup&gt;</td>
<td>18.73 ± 3.76</td>
<td>14.77 ± 2.88</td>
</tr>
<tr>
<td>Phe</td>
<td>34.81 ± 5.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.60 ± 4.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.71 ± 7.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.73 ± 3.92&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>Tyr</td>
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<td>64.20 ± 11.16&lt;sup&gt;§§§&lt;/sup&gt;</td>
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<td>Thr</td>
<td>1264.36 ± 243.00&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>59.48 ± 37.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1507.49 ± 363.01&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>289.78 ± 82.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.92 ± 10.36</td>
<td>353.25 ± 62.28&lt;sup&gt;§§§&lt;/sup&gt;</td>
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<tr>
<td>Trp</td>
<td>76.46 ± 20.47&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>64.04 ± 15.69&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>84.31 ± 12.63&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>82.13 ± 11.54&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>29.95 ± 4.03</td>
<td>67.63 ± 7.83&lt;sup&gt;§§§&lt;/sup&gt;</td>
</tr>
<tr>
<td>Val</td>
<td>79.86 ± 11.36&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>72.24 ± 11.60&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>79.38 ± 14.62&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>74.14 ± 11.12&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>102.37 ± 14.29</td>
<td>124.77 ± 12.07&lt;sup&gt;$&lt;/sup&gt;</td>
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<tr>
<td>EAA total</td>
<td>1924.38 ± 234.19&lt;sup&gt;a+++&lt;/sup&gt;</td>
<td>763.31 ± 138.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2480.24 ± 331.33&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>1146.98 ± 160.76&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1086.83 ± 103.92</td>
<td>1483.02 ± 107.52&lt;sup&gt;§§§&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEAA total</td>
<td>2866.33 ± 217.14&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>2735.96 ± 194.96&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>2910.79 ± 178.79&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>2726.73 ± 221.56&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>3619.70 ± 301.40</td>
<td>2380.27 ± 76.80&lt;sup&gt;§§§&lt;/sup&gt;</td>
</tr>
<tr>
<td>AA total</td>
<td>4790.70 ± 403.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3499.28 ± 318.75&lt;sup&gt;b+++&lt;/sup&gt;</td>
<td>5391.03 ± 446.09&lt;sup&gt;+++&lt;/sup&gt;</td>
<td>3873.70 ± 343.75&lt;sup&gt;b+++&lt;/sup&gt;</td>
<td>4706.53 ± 379.01</td>
<td>3863.28 ± 164.07&lt;sup&gt;§§§&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values not sharing a common letter are significantly different at p < 0.05 among MT, M, T, and NA (no addition) groups, Tukey's test.
†: $p < 0.05$, ††: $p < 0.01$, †††: $p < 0.001$, significantly different from the 0%P group, Dunnett’s test

§: $p < 0.05$, §§: $p < 0.01$, §§§: $p < 0.001$, significantly different 0%P vs. 20%P group, Student’s $t$ test

Figures

(a) Body Weight (g) over days.

(b) Final Body Weight (g).

(c) Gastrocnemius (g).

(d) Epididymis (g).

(e) Blood Urea Nitrogen (mg/dL).

(f) Urinary Nitrogen Excretion.

(g) Total 3-Methyl-u-Cr (mg/dL).

Figure 1
Characteristics and biological parameters of rats. Rats were fed 56 kcal/day of each experimental diet for 12 days. On day 13, after feeding, blood samples were collected, and gastrocnemius and epididymis were excised under anesthesia. (a): Body weight. (b): Final body weight. (c): Gastrocnemius. (d): Epididymis. (e): Blood urea nitrogen. (g): Total urea 3-methyl histidine. Values are means ± SD (n=8 or 6). *: p<0.05, **: p<0.01, ***: p<0.001, Tukey's test. †: p<0.05, † †: p<0.01, † † †: p<0.001 vs. 0%P group, Dunnett's test. §: p<0.05, §§: p<0.01, §§§: p<0.001 vs. 0%P vs. 20%P group, Student's t test.

**Figure 2**

Changes in the gastrocnemius S6K1 content, phosphorylation ratio, and gene expression. (a): Quantitative ratios of S6K1 to total protein. (b): Quantitative ratios of phospho-S6K1 (T421/S424) to S6K1. (c): Representative western blots of S6K1 and phospho-S6K1 (T421/S424). (d): mRNA expression of S6K1 adjusted to GAPDH levels. mRNA expression levels are expressed relative to the mean expression observed in the 20%P group. Values are means ± SD (n=8 or 6). *: p<0.05, **: p<0.01, ***: p<0.001, Tukey's test.
Changes in the gastrocnemius 4E-BP1 content, phosphorylation, and gene expression. (a): Quantitative ratios of 4E-BP1 to total protein. (b): Quantitative ratios of phospho-4E-BP1 (T37/46) to 4E-BP1. (c): Representative western blots of 4E-BP1 and phospho-4E-BP1 (T37/46). (d): mRNA expression of 4E-BP1-adjusted to GAPDH levels. mRNA expression levels are expressed relative to the mean expression observed in the 20%P group. Values are means ± SD (n=8 or 6). *: p<0.05, **: p<0.01, ***: p<0.001, Tukey’s test. †: p<0.05, ††: p<0.01, †††: p<0.001 vs. 0%P group, Dunnett’s test. §: p<0.05, §§: p<0.01, §§§: p<0.001, 0%P vs 20%P group, Student’s t test.
Figure 4

Changes in gastrocnemius FOXO1 content and phosphorylation. (a): Quantitative ratios of FOXO1 to total protein. (b): Quantitative ratios of phospho-FOXO1 (S256) to FOXO1. (c): Representative western blots of FOXO1 and phospho-FOXO1 (S256) in gastrocnemius. Values are means ± SD (n=8 or 6). *: p<0.05, **: p<0.01, ***: p<0.001, Tukey’s test. †: p<0.05, ††: p<0.01, †††: p<0.001 vs. 0%P group, Dunnett’s test. §: p<0.05, §§: p<0.01, §§§: p<0.001 vs. 0%P vs. 20%P group, Student’s t test.