Postprandial Stimulation of Muscle Protein Synthesis in Old Rats Can Be Restored by a Leucine-Supplemented Meal

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ABSTRACT Aging is characterized by a progressive loss of muscle mass. A decrease of muscle protein synthesis stimulation has been detected in the postprandial state and correlated to a decrease of muscle protein synthesis sensitivity to leucine in vitro. This study was undertaken to examine the effect of a leucine-supplemented meal on postprandial (PP) muscle protein synthesis during aging. Adult (8 mo old) and old (22 mo old) rats were fed a semiliquid 18.2% protein control diet for 1 mo. The day of the experiment, rats received no food (postabsorptive group) or either an alanine or leucine-supplemented meal for 1 h (postprandial groups: PP and PP + Leu, respectively). Muscle protein synthesis was assessed in vivo 90–120 min after the meal distribution using the flooding dose method (1-13C phenylalanine). Plasma leucine concentrations were significantly greater in the PP + Leu group compared with the PP group at both ages. Muscle protein synthesis was significantly greater in the adult PP group, whereas it was not stimulated in the old PP group. When supplemented with leucine, muscle protein synthesis in old rats was stimulated and similar to that observed in adults. We conclude that acute meal supplementation with leucine is sufficient to restore postprandial stimulation of muscle protein synthesis in old rats. Whether chronic leucine meal supplementation may limit muscle protein wasting during aging remains to be verified.


KEY WORDS: aging • protein synthesis • leucine • muscle • rats

During aging, a progressive loss of muscle mass has been well described in both humans (1) and rodents (2,3). This loss of proteins results from an imbalance between protein synthesis and degradation rates. Although some authors have shown a decrease of myofibrillar protein synthesis rates in humans (4,5), this imbalance is not clearly apparent when basal rates of protein turnover are measured (6–8). A decrease in muscle protein synthesis stimulation was detected nevertheless in old rats during the postprandial (PP) period (9), suggesting that the “meal signal” was altered during aging. The origin of this alteration remains obscure because muscle protein synthesis responded normally if large amounts of amino acids were perfused continuously in old rats (7) or given orally in aged volunteers (10). Taken together, these results suggest that aged muscle may be less sensitive to the stimulatory effects of amino acids at physiologic concentrations but is still able to respond if the increase of amino acidemia is large enough.

Amino acids play an important role in regulating muscle protein synthesis both in vitro [see (11) for a review] and in vivo (12,13). Among the amino acids, leucine seems to play the major role. Indeed, Anthony et al. (14) showed that orally administered leucine stimulated muscle protein synthesis by itself in vivo and this was independent of insulin. Furthermore, leucine has been shown to act as a real mediator by modulating specifically the activities of intracellular kinases linked to the translation of proteins such as phosphatidylinositol 3’ kinase and mammalian target of rapamycin (mTOR)/70-kDa ribosomal protein S6 (p70S6K) kinases (14–16). We recently demonstrated in vitro that protein synthesis of old rat muscles becomes resistant to the stimulatory effect of leucine in its physiologic concentration range (16). However, when leucine concentration was increased greatly above its postprandial level, protein synthesis was stimulated normally. We hypothesized that the defect in postprandial stimulation of muscle protein synthesis could be overcome by increasing plasma leucine concentration. In the present work, we studied the effect of acute leucine supplementation on protein synthesis in old and adult rats in both gastrocnemius and soleus muscles.

MATERIALS AND METHODS

Animals and experimental design. The animal facilities and protocol were approved by the animal ethics committee of the Institut National de la Recherche Agronomique. Male Wistar rats (Iffa-Credo, Lyon, France) aged 8 mo (adult) and 22 mo (old) were used.

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They were housed individually under controlled environmental conditions (temperature, 22°C; 12-h dark period starting at 0800 h) and fed a semiliquid 18.2% protein control diet (Table 1); water was provided freely. Rats had free access to food between 0830 and 1700 h. Rats were acclimated for 1 mo to their surroundings and food intake was measured daily. Food consumption was stable; adult and old rats ate 8–10 g dry matter during the first feeding hour and 18–20 g dry matter daily. The day of the experiment, rats of each age were divided into three groups. One group did not receive any more food (17 h food deprived or postabsorptive group; PA), one group received the alanine meal for 1 h (postprandial group; PP) and the last group received the leucine meal for 1 h (postprandial group; PP + Leu). The composition of the two experimental meals is given in Table 1. They contained similar amount of protein (17.4 and 17.2%, respectively) and crude energy (17.4 and 17.5 MJ/kg, respectively). The leucine meal was supplemented with leucine to increase plasma leucine to twice the normal postprandial concentration. Pilot experiments, which were carried out to adjust leucine supplementation, showed that plasma valine and isoleucine were decreased. The leucine meal was then supplemented with the two other branched-chain amino acids to maintain their plasma concentrations at normal postprandial levels. To have the same amount of nitrogen in the two experimental meals, the alanine meal was supplemented with alanine, an amino acid that does not affect muscle protein metabolism (17).

**Measurements of in vivo protein synthesis.** Protein synthesis rates were assessed in gastrocnemius and soleus muscles 90–120 min after the feeding hour. Protein synthesis rates were measured using the flooding-dose method (18, 19). The method was adapted to our experimental design because a branched-chain amino acid could not be used. Each rat was injected intravenously with phenylalanine (50 μmol/100 g body) to flood the precursor pools with L-[1-13C]phenylalanine (99%). Blood samples were collected at the time indicated, centrifuged and stored at −20°C. Measurement of free phenylalanine enrichment was done as its t-butyldimethylsilyl derivative under electron impact ionization by gas chromatography/mass spectrometry coupled to an organic mass spectrometer quadrupole. Values are means, n = 3. Three rats were repeatedly sampled at the time indicated.

Free and bound phenylalanine enrichments were determined as follows. Muscles were powdered in liquid nitrogen in a ball mill (Dangoumeau, Prolabo, Paris, France). A 0.6-g aliquot of frozen muscle powder or 0.5 mL of plasma was homogenized in 8 volumes of ice-cold 0.61 mol/L trichloroacetic acid (TCA). Homogenates were centrifuged (5000 × g, 15 min, 4°C) and supernatants, containing free amino acids, were desalted by cation-exchange chromatography (AG 50 × 8, 100–200 mesh, H+ form, Bio-Rad, Richmond, CA) in minidisposable columns. Phenylalanine and other amino acids were eluted with 4 mol/L NH4OH. After evaporation of NH4OH under vacuum, free amino acids were resuspended in 0.01 mol/L HCl for enrichment measurements. TCA-insoluble materials were washed in 4 volumes of cold 0.61 mol/L TCA and 3 times in 4 volumes of 0.2 mol/L perchloric acid. Resultant pellets were resuspended in 0.3 mol/L NaOH and incubated at 37°C for 1 h. Protein concentration was determined using the bicinchoninic procedure. Proteins were precipitated with 1.99 mol/L HClO4 overnight at 4°C, samples centrifuged (10,000 × g, 3 min, 4°C) and RNA content determined spectrophotometrically in the supernatant as described by Manchester and Harris (20). The protein pellet was hydrolyzed in 6 mol/L HCl at 110°C for 24 h. HCl was removed by evaporation and amino acids purified by cation-exchange chromatography as described above.

**Measurement of enrichments.** Measurement of free phenylalanine enrichment was done as its t-butyldimethylsilyl derivative under electron impact ionization by gas chromatography/mass spectrometry, with an HP-5980 gas chromatograph coupled to an HP-5972 organic mass spectrometer quadrupole (Hewlett-Packard, Paris, France). The ions m/z 336 and 337 were monitored. Enrichment of [1-13C]phenylalanine into protein was measured as its N-acetyl-propyl derivative by gas chromatography-combustion-isotope ratio mass spectrometry (Micromass Isocrom II, Fisons Instruments, Middlewich, UK).

**Plasma insulin and amino acid measurements.** Plasma insulin were analyzed using a commercial RIA kit (ERIA Diagnostics Pasteur, Sannois, France). Plasma amino acids were purified by ion-exchange chromatography after protein precipitation, i.e., 500 μL of plasma was added to 125 μL of sulfosalicylic acid solution (1 mol/L in ethanol with 0.5 mol/L thioglycol) previously evaporated completely. Norleucine was added as an internal standard. Samples were incubated on ice for 1 h and centrifuged at 3500 × g for 1 h at 4°C. An aliquot (250 μL) of the supernatant was combined with 125 μL of 0.1 mol/L lithium acetate buffer, pH 2.2. Amino acid concentrations were determined using an automated amino acid analyzer with BTC 2410 resin (Biotronic LC 3000, Roquai, Velizy, France).

**Calculations.** Protein fractional synthesis rate (FSR; in %/d) was calculated from the following formula (18):

\[
FSR = \frac{Sb \times 100}{Sa \times t}
\]

where Sb is the enrichment at time t (minus basal enrichment of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Composition of the diet and experimental meals</th>
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<tbody>
<tr>
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<td>Control diet</td>
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<tr>
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<td>236.2</td>
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<tr>
<td>Wheat starch</td>
<td>649.1</td>
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<tr>
<td>Agar-agar</td>
<td>33.1</td>
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<tr>
<td>Mineral mixture</td>
<td>37.9</td>
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<tr>
<td>Vitamin mixture</td>
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<td>Cotza oil</td>
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<td>Sunflower oil</td>
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<td>Peanut oil</td>
<td>17</td>
</tr>
<tr>
<td>Amino acid supplement</td>
<td>0</td>
</tr>
<tr>
<td>Energy, MJ/kg</td>
<td>17.1</td>
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</table>

1 Composition, mg/100 g mineral mixture: CaHPO4, 73160; NaCl, 70900; K2CO3, 2750; MgCl2·6H2O, 14330; ammonium iron II citrate, 995; MnCO3, 390; CuSO4·5H2O, 183; ZnSO4·7H2O, 613; Al2(SO4)3·K2SO4·330; NaF, 55.7; IK, 1.23; CoCO3, 0.96; SeO2, 0.571.
2 Multivitamin mixture was from Usine d’Alimentation Rationnelle, Epernay-sur-orge, France.
3 Alanine meal = 44.5 g alanine; leucine meal = 44.5 g leucine + 9.8 g isoleucine + 5.7 g valine.

**Figure 1** Plasma free phenylalanine enrichment in rats injected intravenously with phenylalanine (50 μmol/100 g body) to flood the precursor pools with L-[1-13C]phenylalanine (99%). Blood samples were collected at the time indicated, centrifuged and stored at −20°C. Measurement of free phenylalanine enrichment was done as its t-butyldimethylsilyl derivative under electron impact ionization by gas chromatography/mass spectrometry coupled to an organic mass spectrometer quadrupole. Values are means, n = 3. Three rats were repeatedly sampled at the time indicated.
protein) of the protein-bound phenylalanine, $t$ is the incorporation time in days, and $S_t$ is the mean enrichment of tissue free phenylalanine between time 0 and time $t$. The mean $S_t$ enrichment was the $S_r$ ($t = \infty$) value calculated from the linear regression obtained in tissue between the time 0 and time $t$. The absolute synthesis rate (ASR) was calculated from the product of FSR and protein content of the tissue and expressed in milligrams per day. Protein synthetic capacity was estimated as the ratio of RNA to protein (mg RNA/g protein) because most of the RNA in tissues are ribosomal.

**Statistical analysis.** Values presented are means ± SEM. Because studies of adult and old rats were not performed at the same time, statistical evaluation of the data was performed by one-way ANOVA to test the effect of the experimental meals within age groups. When a significant overall effect was detected, differences among experimental groups of the same age, and over the 1-h feeding period, food intake was not affected by the meal (Table 2). Protein concentration and total protein content of muscles were not different among experimental groups of the same age, and over the 1-h feeding period, food intake was not affected by the meal consumed (Table 2).

**RESULTS**

**Rat characteristics.** Body weights of old and adult rats were similar (Table 2). As shown in Table 2, old rats showed the usual muscle atrophy recorded in gastrocnemius and soleus masses compared with adult rats (−15% and −13%, respectively). However, because adult and old rats were not studied at the same time, they were not compared statistically. Protein concentration and total protein content of muscles were not different among experimental groups of the same age, and over the 1-h feeding period, food intake was not affected by the meal consumed (Table 2).

**Amino acids and insulin concentrations.** Amino acid concentrations were determined at the time of the killing (90–120 min after the feeding hour). Pilot experiments showed that plasma amino acid concentrations were similar between 60 and 120 min after the feeding hour in all groups. Feeding the adult rats the alanine meal (PP) increased several essential amino acids such as branched-chain amino acids (isoleucine, leucine, valine) and methionine compared with the postabsorptive (PA) rats (Table 3). When adult rats were fed the leucine meal, plasma leucine concentration was markedly elevated compared with the PP group. The other amino acids were unchanged except lysine, which was slightly lower in PP + Leu rats (Table 3).

In old rats, feeding also increased some essential amino acids (isoleucine, leucine, valine, methionine), and the extent of this increase was similar to that of the adult group (Table 3).

**Muscle protein metabolism.** FSR was significantly increased in both gastrocnemius and soleus muscles when adult rats were fed the alanine meal (Table 4). When the meal was supplemented with leucine, FSR was also increased in both muscles to the same extent as with the alanine supplementation. The total amount of protein synthesized (ASR) was also increased in both muscles in the postprandial state and no significant differences were observed between rats fed the two experimental meals (Table 4). By contrast, old rats did not show a significant stimulation of the FSR when fed the alanine meal (PP) (Table 4) and showed no increase in the total protein synthesized in either muscle (Fig. 3). This is in accordance with the defect in postprandial anabolism usually observed in muscle with age. However, when the meal was supplemented with leucine, the FSR (Table 4) and the ASR (Fig. 3) were significantly greater than in the PP meal-fed rats. As observed in adults, experimental meals had no effect on the muscle protein synthesis capacity in old rats (Table 4).

**TABLE 2**

| Characteristics of adult and old male rats that were food deprived (PA) or refed for 1 h control alanine diet (PP) or control diet supplemented with leucine (PP + Leu)† |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Adult | | | Mean | | Adult | | | Mean | |
| Body weight, g | 592 ± 9 | 599 ± 6 | 605 ± 12 | 599 ± 8 | 610 ± 13 | 597 ± 18 | 625 ± 20 | 612 ± 15 |
| Meal dry matter intake, g | — | 11.3 ± 0.4 | 11.4 ± 0.4 | — | 11.0 ± 0.3 | 10.5 ± 0.6 | — | — | — | — |
| Gastrocnemius | | | | | | | | | | | |
| Weight, g | 2.74 ± 0.09 | 2.64 ± 0.08 | 2.74 ± 0.09 | 2.72 ± 0.05 | 2.36 ± 0.09 | 2.34 ± 0.12 | 2.45 ± 0.05 | 2.38 ± 0.05 |
| Protein concentration, mg/g | 199 ± 6 | 193 ± 4 | 192 ± 5 | 195 ± 4 | 184 ± 4 | 196 ± 4 | 187 ± 4 | 186 ± 4 |
| Total protein, mg | 548 ± 25 | 509 ± 11 | 524 ± 19 | 527 ± 16 | 435 ± 17 | 439 ± 28 | 458 ± 13 | 443 ± 18 |
| Soleus | | | | | | | | | | | |
| Weight, g | 214 ± 7 | 224 ± 12 | 216 ± 6 | 218 ± 6 | 190 ± 6 | 188 ± 8 | 190 ± 3 | 189 ± 5 |
| Protein concentration, mg/g | 185 ± 4 | 188 ± 3 | 186 ± 2 | 186 ± 3 | 180 ± 3 | 182 ± 2 | 182 ± 1 | 181 ± 2 |
| Total protein, mg | 40 ± 1 | 42 ± 2 | 40 ± 1 | 41 ± 1 | 34 ± 1 | 34 ± 2 | 33 ± 1 | 34 ± 1 |

† Values are means ± SEM, $n = 8–10$. 

When supplemented with leucine (PP + Leu), the meal also further increased plasma leucine levels to concentrations similar to that of the PP + Leu adult group.

In both adult and old rats, nonessential amino acid concentrations were not modified by the alanine meal (PP) except alanine, which increased, and glutamate, which decreased significantly (Table 3). In rats fed the leucine meal (PP + Leu), glycine and glutamine remained at the postabsorptive levels in both age groups, whereas other nonessential amino acids were slightly lower than in the postabsorptive groups (adult rats: aspartate, asparagine, glutamate, serine; and old rats: aspartate, glutamate, serine).

In adult rats, feeding the alanine meal (PP) significantly increased insulinemia (Fig. 2). Insulin levels were maximal 60–100 min after the beginning of meal consumption and then decreased to the postabsorptive levels at 150 min. When the leucine meal (PP + Leu) was consumed, no significant differences in insulin levels were recorded between the PP and PP + Leu groups (Fig. 2). Similar insulinemia kinetics were observed in old rats and insulin levels did not differ between those fed the alanine and leucine meals (Fig. 2).
DISCUSSION

Our study clearly showed that the defect in postprandial stimulation of protein synthesis observed in old rat muscle was overcome when the meal was supplemented with leucine. In our experiment, feeding old rats a control meal did not significantly increase muscle protein synthesis in either muscle studied, whereas the leucine meal stimulated muscle protein synthesis to an extent similar to that in adults.

The defect in postprandial anabolism with age has been observed previously in rodents (9) and humans (21) and has been proposed to be one of the mechanisms responsible for the loss of muscle mass during aging. Amino acids play an important role in regulating muscle protein synthesis [see (11) for a review] and it has been hypothesized that during aging, the availability of amino acids is affected. Boirie et al. (22) showed in humans that the first-pass splanchnic uptake of leucine increases with age and may limit the availability of amino acids to the peripheral tissues. Volpi et al. (10) confirmed this observation but showed that the delivery of amino acids to the tissues increased to the same extent in adult and elderly humans. In our study, no differences in dry matter intake or in essential amino acid concentrations in plasma were recorded, indicating that a defect in amino acid availability cannot be responsible per se for the defect in postprandial anabolism.

Postprandial stimulation of muscle protein synthesis in rats originated mainly from absorbed amino acids because this stimulation was observed after feeding a high protein meal but not after an isonenergetic protein-free meal (12). Similar observations have been made in humans (23,24) and suggest that amino acids are essential in the regulation of postprandial muscle protein metabolism. Among amino acids, leucine seems to play the major role. A recent study of Anthony et al. (14) showed that orally administered leucine stimulated muscle protein synthesis independently of insulin in vivo. Furthermore, we showed (16) that leucine alone reproduced the effect...
of total amino acids on muscle protein synthesis in vitro and that this effect occurred in the leucine physiologic range. We also investigated whether a decrease in the sensitivity of muscle protein synthesis to this amino acid with age may explain the defect in postprandial anabolism. We found that muscle protein synthesis in vitro still responded to the leucine signal in old rats but required a two to three times greater leucine concentration than in young or adult rats (16). This indicated that at postprandial amino acid levels, muscle protein synthesis was maximally stimulated in adult rats but poorly stimulated in old rats. In our present experiment conducted in vivo, leucine supplementation had no additional effect on muscle protein synthesis in adults but totally restored its stimulation in old rats, and this occurred in both muscles studied. Only leucine concentrations in plasma reached supraphysiologic levels in both age groups (two times the control postprandial values) and confirmed in vivo our hypothesis that old rat muscles are less sensitive to the “leucine signal” but are still able to respond when the concentration of this amino acid is greatly increased.

Other studies have also explored the direct effect of amino acids on muscle protein synthesis and have shown that protein synthesis responds normally if amino acids are infused continuously in old rats (7). Similarly, Volpi et al. (10) observed that muscle protein synthesis was still stimulated with an increase of amino acid availability in elderly humans after oral amino acid administration. It is important to note that the amount of amino acids perfused or orally given in these two experiments led to a sustained large hyperaminoacidemia (most of the essential amino acids were more than doubled) and was not representative of the plasma amino acid profile observed with normal mixed meal consumption (25, 26). Our study explains why amino acid infusion, which tripled plasma leucine, stimulated muscle protein synthesis to the same extent in adult and old rats (7). A similar conclusion could be made from the study of Volpi et al. (10) who observed a similar effect of oral amino acid administration on muscle protein synthesis in adult and elderly volunteers; leucine concentrations were 2.5–3.5 higher than with a control meal. Recently, Arnal et al. (21) demonstrated that the response of protein turnover was restored in elderly humans if a “pulse-protein feeding” pattern (80% of daily protein intake in one meal) was used instead of “spread-protein feeding” (daily protein equally distributed in four meals). Even if the plasma amino acids were not measured in this experiment, it could be easily assumed that amino acid availability to peripheral tissues (i.e., leucine) after the high protein meal was higher with pulse-protein feeding than with spread-protein feeding. An increase in dietary protein intake was thus beneficial for the maintenance of muscle protein synthesis in an elderly population. However, it required 80% instead of 25% of protein in the meal and it has been shown that high protein diets may have deleterious effects on renal function in the elderly (27). Because leucine alone restored muscle protein synthesis in our experiment, supplementation of this amino acid represents a good alternative to high protein diets. Nevertheless, we cannot exclude the possibility that the increases in amino acids other than leucine may also have had beneficial effects on muscle protein synthesis in old rats.

Leucine has been shown to stimulate insulin secretion; thus the restoration of muscle protein synthesis in old rats could originate indirectly through an increase in insulinemia. This is

### TABLE 4

Fractional synthesis rate (FSR) and protein synthesis capacity (Cs) of adult and old male rats that were food deprived (PA) or refed for 1 h control alanine diet (PP) or control diet supplemented with leucine (PP + Leu)

<table>
<thead>
<tr>
<th>FG</th>
<th>Adult</th>
<th></th>
<th></th>
<th>Old</th>
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<tr>
<td></td>
<td>PA</td>
<td>PP</td>
<td>PP + Leu</td>
<td>PA</td>
<td>PP</td>
<td>PP + Leu</td>
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<tr>
<td>FSR, %/d</td>
<td></td>
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<tr>
<td>Gastrocnemius</td>
<td>3.80 ± 0.14</td>
<td>4.53 ± 0.15</td>
<td>4.54 ± 0.18</td>
<td>4.15 ± 0.11</td>
<td>4.51 ± 0.18</td>
<td>4.94 ± 0.22</td>
</tr>
<tr>
<td>Soleus</td>
<td>6.63 ± 0.28</td>
<td>7.59 ± 0.21</td>
<td>7.72 ± 0.22</td>
<td>7.75 ± 0.23</td>
<td>7.46 ± 0.25</td>
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<tr>
<td>Cs, mg/g protein</td>
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<tr>
<td>Gastrocnemius</td>
<td>3.16 ± 0.21</td>
<td>3.26 ± 0.23</td>
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<tr>
<td>Soleus</td>
<td>6.39 ± 0.43</td>
<td>5.71 ± 0.16</td>
<td>5.77 ± 0.14</td>
<td>5.76 ± 0.25</td>
<td>5.81 ± 0.25</td>
<td>5.92 ± 0.19</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8–10. * P < 0.05 vs. PA of the same age; † P < 0.05 vs. PP of the same age.
2 All measurement were made 90–120 min after the feeding hour.

**FIGURE 3** Effect of leucine supplementation on muscle protein synthesis in adult and old rats that were food deprived (PA) or refed for 1 h control alanine diet (PP) or control diet supplemented with leucine (PP + Leu). Each rat was injected intravenously with phenylalanine (50 μmol/100 g) to flood the precursor pools with L-[1-13C]phenylalanine (99%). The incorporation time was 40 min. Rats were anesthetized with pentobarbital sodium 90–120 min after the meal distribution. Gastrocnemius and soleus muscles were excised, weighed, frozen in liquid nitrogen and stored at −80°C until analysis. Muscle protein synthesis was assessed as described in Materials and Methods. Values are means ± SEM, n = 8–10. Values in a panel not sharing a letter differ, P < 0.05.
not the case in the present experiment because the kinetics of insulinemia were not different in the rats fed the control and the leucine-supplemented meals. Furthermore, insulin levels were not different from those of the adult groups in which stimulation of muscle protein synthesis had nevertheless been noted. However, it is important to emphasize that the presence of insulin seems to be indispensable in the postprandial stimulation of muscle protein synthesis by amino acids. Indeed, the acute decrease in postprandial insulinemia to postabsorptive levels due to diazoxide treatment greatly impaired muscle stimulation of muscle protein synthesis by amino acids. Indeed, the meal signal borne by leucine could not be initiated. In our study, by increasing leucine availability to peripheral tissues because all other amino acids as well as insulin were not significantly different from those in rats fed a control meal. Whether chronic oral leucine supplementation would be beneficial for maintaining muscle protein mass in elderly persons remains to be studied. It could be a good alternative to high protein diets, which have deleterious effects on renal function in the elderly.

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LITERATURE CITED