Leptin and leucine synergistically regulate protein metabolism in C2C12 myotubes and mouse skeletal muscles

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Abstract
Leucine and leptin play important roles in regulating protein synthesis and degradation in skeletal muscles in vitro and in vivo. However, the objective of the present study was to determine whether leptin and leucine function synergistically in regulating protein metabolism of skeletal muscles. In the in vitro experiment, C2C12 myotubes were cultured for 2h in the presence of 5 mM-leucine and/or 50 ng/ml of leptin. In the in vivo experiment, C57BL/6 and ob/ob mice were randomly assigned to be fed a non-purified diet supplemented with 3% L-leucine or 2.04% L-alanine (isonitrogenous control) for 14d. Ob/ob mice were injected intraperitoneally with sterile PBS or recombinant mouse leptin (0.1 μg/g body weight) for 14d. In C57BL/6 mice, dietary leucine supplementation increased (P<0.05) plasma leptin, leptin receptor expression and protein synthesis in skeletal muscles, but reduced (P<0.05) plasma urea and protein degradation in skeletal muscles. Dietary leucine supplementation and leptin injection increased the relative weight of the gastrocnemius and soleus muscles in ob/ob mice. Moreover, leucine and leptin treatments stimulated (P<0.05) protein synthesis and inhibited (P<0.05) protein degradation in C2C12 myotubes and skeletal muscles of ob/ob mice. There were interactions (P<0.05) between the leucine and leptin treatments with regard to protein metabolism in C2C12 myotubes and soleus muscles of ob/ob mice but not in the gastrocnemius muscles of ob/ob mice. Collectively, these results suggest that leucine and leptine synergistically regulate protein metabolism in skeletal muscles both in vitro and in vivo.

Key words: Leptin: Leucine: Protein metabolism: Mouse skeletal muscles: C2C12 myotubes

As a functional amino acid, leucine can regulate protein metabolism in multiple tissues and cells, including skeletal muscles and myogenic cells, through insulin-dependent and -independent ways(1–10). In addition, leucine treatment increases the expression of specific proteins in some tissues and cells including leptin in adipocytes and adipose tissues(11,12).

Leptin, a product of the obesity (ob) gene, is a 16 kDa hormone(13), and is primarily expressed in the adipose tissue of multiple mammalian species(14). Leptin regulates many important physiological functions, including fatty acid metabolism, body temperature, reproduction, energy consumption, protein metabolism and insulin function(15–19). However, leptin exerts its action via leptin receptors that are a type of transmembrane receptor.

Leptin receptors are the product of the diabetes (db) gene(20). There are at least six isoforms of leptin receptors produced by alternative splicing of the RNA transcript of the db gene(21). Leptin receptors are found in many mammalian tissues. Recent studies have shown that the expression of leptin receptors in specific tissues is affected by various nutrients and hormones(22–24). We have recently shown that leucine promotes leptin receptor expression in C2C12 myotubes(25). However, it has not been determined whether leucine can stimulate the expression of leptin receptors in vivo.

Several recent studies have alluded to potentially synergistic effects of different factors on some physiological functions(4,26–30). Leucine or leptin has been shown to regulate protein metabolism in skeletal muscles. However, it is also possible that leucine and leptin can cross-talk in regulating protein metabolism in skeletal muscles. Therefore, the present study was conducted to test the hypothesis that leptin and leucine could synergistically regulate protein metabolism in mouse skeletal muscles and myogenic cells.

Abbreviations: db, diabetes; FSR, fractional protein synthesis rate; mTOR, mammalian target of rapamycin; ob, obesity.

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Leptin and leucine regulate protein metabolism

Materials and methods

Cell culture

C2C12 myoblasts (American Type Culture Collection) were used as an in vitro model for skeletal muscle and were cultured and differentiated into C2C12 myotubes as described previously\(^{(25)}\). Before the beginning of the treatment, the myotubes were starved for 12 h in serum- and antibiotic-free Dulbecco’s modified Eagle’s medium/F12, and the in vitro experiment was carried out in this starvation medium.

Measurement of protein synthesis and degradation in vitro

After starvation, the myotubes were cultured for 2 h in the presence of 5 mM-leucine and/or 50 ng/ml of leptin, and subsequently 2 \(\mu\)mol L-\([2H_5]\)phenylalanine (Cambridge Isotopes Laboratories) was added to each well without changing the medium. The supplemental levels of 5 mM-leucine and 50 ng/ml of leptin were chosen because they have been shown in previous studies and our preliminary study to regulate the protein metabolism of C2C12 myoblasts or myotubes\(^{(32,33)}\). Following incubation, the isotopic enrichment of L-\([2H_5]\)phenylalanine in the free pool and protein-bound pool of the myotubes was measured according to previously published procedures\(^{(32,33)}\). Ions with mass-charge ratios of 148 and 153 were monitored and converted to a percentage of molar enrichment (mol%) using calibration curves.

Protein degradation in the myotubes was determined by the release of tyrosine as described previously\(^{(8,18,54)}\). Briefly, following starvation, the myotubes in six-well plates were incubated with 5 mM-leucine and/or 50 ng/ml of leptin for 2 h in the starvation medium. Then, the medium was immediately aspirated, and each well was washed two times with ice-cold sterile PBS. The C2C12 myotubes were incubated at 37°C for 6 h in a Krebs–Henseleit–HEPES buffer supplemented with 0.5 mM-pyruvate, 14.5 mM-glucose and 20 \(\mu\)M-cycloheximide. After incubation, the buffer was collected, and tyrosine concentration was analysed using an S-433D Amino Acid Analyser (Sykam), as described previously\(^{(35)}\). The tyrosine concentration was calculated using calibration curves.

In vivo experimental design

After 3 d of acclimatisation, twenty C57BL/6 mice or twenty-four ob/ob mice were assigned on the basis of body weight to be fed the leucine-supplemented diet or the alanine-supplemented diet (isonitrogenous control) (\(n=10\) or \(n=12\)). For 14 d, half of the ob/ob mice (\(n=6\)) on each diet were intraperitoneally injected with sterile PBS, while the other half were intraperitoneally injected with 0.1 \(\mu\)g/g body weight of a solution of recombinant mouse leptin dissolved in PBS. The injection dose of 0.1 \(\mu\)g/g body weight of leptin was chosen for 14 d because it was shown in previous studies to regulate physiological functions in ob/ob mice, but not to significantly affect their feed intake in the longer term\(^{(40,41)}\). The feed was supplied for every 7 d. In each supplying feed, the remaining feed and the supplied feed would be weighed, which was used to measure the feed intake of mice. On the morning of days 0 and 14, the body weight of mice was measured following a 12 h fast. On day 14, the non-purified rodent diet based on maize, soyabean meal, wheat flour and fishmeal was obtained from Science Australia United Efforts Incorporation (Beijing, China; catalogue no. 2005-0007-Ka112). Either 3% (w/w) L-leucine or 204% (w/w) L-alanine (isonitrogenous control) was added to this non-purified rodent diet. Feed mixing was conducted by Science Australia United Efforts Incorporation (Beijing, China). The supplemental level of 3% L-leucine was chosen because it has been shown in previous studies to regulate the protein metabolism of skeletal muscles, but not to affect the feed intake of mice or piglets\(^{(37–39)}\). Nutrient levels of the non-purified rodent diet were digestible energy (13.41 MJ/kg), protein (21.5%, w/w), Ca (1.46%, w/w), total P (0.92%, w/w) and available P (0.75%, w/w). The analysed contents (% w/w) of amino acids in leucine- and alanine-supplemented diets are summarised in Table 1.

### Table 1. Analysed contents of amino acids (g/100 g) in the alanine- and leucine-supplemented non-purified rodent diets

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>+ Ala</th>
<th>+ Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>3.05</td>
<td>1.22</td>
</tr>
<tr>
<td>Asp</td>
<td>1.76</td>
<td>1.90</td>
</tr>
<tr>
<td>Arg</td>
<td>1.33</td>
<td>1.32</td>
</tr>
<tr>
<td>Cys</td>
<td>0.23</td>
<td>0.25</td>
</tr>
<tr>
<td>Glu</td>
<td>3.78</td>
<td>3.84</td>
</tr>
<tr>
<td>Gly</td>
<td>0.89</td>
<td>0.97</td>
</tr>
<tr>
<td>His</td>
<td>0.73</td>
<td>0.69</td>
</tr>
<tr>
<td>Ile</td>
<td>0.79</td>
<td>0.76</td>
</tr>
<tr>
<td>Leu</td>
<td>1.56</td>
<td>4.49</td>
</tr>
<tr>
<td>Lys</td>
<td>1.04</td>
<td>1.03</td>
</tr>
<tr>
<td>Met</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>Phe</td>
<td>0.93</td>
<td>0.96</td>
</tr>
<tr>
<td>Pro</td>
<td>2.58</td>
<td>2.56</td>
</tr>
<tr>
<td>Ser</td>
<td>0.91</td>
<td>0.97</td>
</tr>
<tr>
<td>Thr</td>
<td>0.75</td>
<td>0.78</td>
</tr>
<tr>
<td>Trp</td>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.63</td>
<td>0.62</td>
</tr>
<tr>
<td>Val</td>
<td>0.94</td>
<td>0.98</td>
</tr>
</tbody>
</table>

+ Ala, L-alanine-supplemented diet; + Leu, L-leucine-supplemented diet.
bicarbonate buffer containing 0·5 mM-cycloheximide, and the muscles were transferred into fresh Krebs–Henseleit bicarbonate buffer containing cycloheximide in a 2 h period. Tyrosine was determined by the release of tyrosine into the buffer which was equilibrated with 95 % O2 and 5 % CO2, following the isolation of the muscles. After 30 min of preincubation, the intact gastrocnemius and soleus muscles were preincubated at 37°C for 10 min, and stored at −20°C until analysis. The left gastrocnemius and soleus muscles were excised, quickly frozen in liquid N2 and used for the determination of protein synthesis; Western blot and RNA isolate analyses were conducted as described later. The contra-lateral hindlimb muscles were also excised, weighed and used for the measurement of protein degradation.

Measurements of protein synthesis and degradation in muscles

Protein synthesis in muscle samples and the isotopic enrichment of l-[1-14C]phenylalanine (150 μmol/100 g body weight) as described previously(32,43). At 30 min after the isotope administration, mice were anaesthetised with sodium pentobarbital, and blood samples were taken from the orbital sinus using vacutainer tubes coated with sodium heparin (Greiner Vacuette). Plasma was separated from the whole blood by centrifugation at 3000 g for 10 min, and stored at −20°C until analysis. The left gastrocnemius and soleus muscles were excised, quickly frozen in liquid N2 and used for the determination of protein synthesis, Western blot and RNA isolate analyses were conducted as described later. The contra-lateral hindlimb muscles were also excised, weighed and used for the measurement of protein degradation.

Table 2. Performance of C57BL/6 mice fed diets supplemented with alanine or leucine

<table>
<thead>
<tr>
<th></th>
<th>+ Ala Mean</th>
<th>+ Leu Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>14·02</td>
<td>14·25</td>
<td>0·33</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>18·94</td>
<td>18·91</td>
<td>0·30</td>
</tr>
<tr>
<td>Body-weight gain (g)</td>
<td>4·92</td>
<td>4·67</td>
<td>0·25</td>
</tr>
<tr>
<td>Feed intake (g)</td>
<td>34·07</td>
<td>33·28</td>
<td>2·10</td>
</tr>
<tr>
<td>Feed conversion</td>
<td>0·150</td>
<td>0·146</td>
<td>0·013</td>
</tr>
</tbody>
</table>

+ Ala, l-alanine-supplemented diet; + Leu, l-leucine-supplemented diet.

1·5 h after feeding, mice received an intraperitoneal injection of a flooding dose of l-[1-14C]phenylalanine (150 μmol/100 g body weight) as described previously(32,43). The rate of protein degradation was determined by the release of tyrosine as described previously(32,43). At 30 min after the isotope administration, mice were anaesthetised with sodium pentobarbital, and blood samples were taken from the orbital sinus using vacutainer tubes coated with sodium heparin (Greiner Vacuette). Plasma was separated from the whole blood by centrifugation at 3000 g for 10 min, and stored at −20°C until analysis. The left gastrocnemius and soleus muscles were excised, quickly frozen in liquid N2 and used for the determination of protein synthesis, Western blot and RNA isolate analyses were conducted as described later. The contra-lateral hindlimb muscles were also excised, weighed and used for the measurement of protein degradation.

Calculations

The fractional protein synthesis rate (FSR) in myotubes and skeletal muscles was calculated as: 

$$\text{FSR} = \frac{\text{F}_{\text{Bound}} \times 1440 \times 100\%}{\text{F}_{\text{Free}} \times t}$$

where $\text{F}_{\text{Bound}}$ is the isotopic enrichment (%) of the tracer phenylalanine in the protein-bound pool at time $t$; 1440 is the number of min/d; $\text{F}_{\text{Free}}$ is the enrichment of the tracer phenylalanine in the free pool at time $t$; $t$ is the exact time (min) of incubation with labelled phenylalanine(32,33).

Plasma urea, amino acid and leptin measurement

Plasma urea was measured using an assay kit from Nanjing Jiancheng Biochemistry Institute. Plasma free amino acids were analysed using an S-433D Amino Acid Analyser (Sykam, GmbH) as described previously(35). Leptin levels in plasma were determined using a mouse leptin ELISA kit (R & D Systems, Inc.).

Western blot analysis

Protein levels for β-actin and leptin receptor in skeletal muscles were determined by Western blot analysis as described previously(25,45).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the skeletal muscles with the RNeasy Plus Mini Kit (Qiagen GmbH) according to the manufacturer’s protocol. Then, RT of total RNA and quantitative real-time PCR of the β-actin and leptin receptor genes were conducted as described previously(25,45).

Statistical analysis

Data for the C57BL/6 mouse experiment were analysed using the unpaired $t$ test. Data for the C2C12 myotubes and ob/ob mouse experiments were analysed as a 2 x 2 factorial using the general linear model procedures of the Statistical Analysis System (SAS Institute). The factors in the models included the

Fig. 1. Effects of dietary leucine (Leu) supplementation on plasma leptin concentration (ng/ml) in C57BL/6 mice. Mice were fed the Leu-supplemented diet or the alanine (Ala)-supplemented (isonitrogenous control) diet for 14 d. After the mice received their diets for 2 h on day 14, blood samples were obtained from the orbital sinus. Plasma leptin concentrations were measured. Values are means (n = 6), with their standard errors represented by vertical bars. $^{ab}$Mean values with unlike letters were significantly different (P<0·05).
Table 3. Amino acid and urea concentrations in the plasma of C57BL/6 mice fed diets supplemented with alanine or leucine (Mean values with their pooled standard errors, n 6)

<table>
<thead>
<tr>
<th>Amino acids (µmol/l)</th>
<th>+ Ala Mean</th>
<th>+ Leu Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>1012</td>
<td>741**</td>
<td>56</td>
</tr>
<tr>
<td>Arg</td>
<td>141</td>
<td>139</td>
<td>12</td>
</tr>
<tr>
<td>Asp</td>
<td>40</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>Cys</td>
<td>54</td>
<td>52</td>
<td>3</td>
</tr>
<tr>
<td>Glu</td>
<td>326</td>
<td>236**</td>
<td>11</td>
</tr>
<tr>
<td>Gly</td>
<td>454</td>
<td>425</td>
<td>19</td>
</tr>
<tr>
<td>His</td>
<td>156</td>
<td>121</td>
<td>11</td>
</tr>
<tr>
<td>Ile</td>
<td>149</td>
<td>137</td>
<td>5</td>
</tr>
<tr>
<td>Leu</td>
<td>199</td>
<td>324**</td>
<td>7</td>
</tr>
<tr>
<td>Lys</td>
<td>456</td>
<td>461</td>
<td>28</td>
</tr>
<tr>
<td>Met</td>
<td>62</td>
<td>57</td>
<td>3</td>
</tr>
<tr>
<td>Pro</td>
<td>453</td>
<td>411</td>
<td>24</td>
</tr>
<tr>
<td>Ser</td>
<td>377</td>
<td>302**</td>
<td>6</td>
</tr>
<tr>
<td>Thr</td>
<td>493</td>
<td>308**</td>
<td>6</td>
</tr>
<tr>
<td>Tyr</td>
<td>201</td>
<td>152*</td>
<td>12</td>
</tr>
<tr>
<td>Val</td>
<td>250</td>
<td>155**</td>
<td>14</td>
</tr>
<tr>
<td>Plasma urea (mmol/l)</td>
<td>14-01</td>
<td>10-15*</td>
<td>0-61</td>
</tr>
</tbody>
</table>

+ Ala, L-alanine-supplemented diet; + Leu, L-leucine-supplemented diet.

Main effects of leucine treatment (supplemented or unsupplemented with leucine in the media or the diet) and leptin treatment (leptin or PBS supplementation) as well as their interaction. All analyses were performed using SAS (version 8.1; SAS Institute). Data are expressed as means with their standard errors, or means with their pooled standard errors. P<0.05 was considered to indicate statistical significance.

Results

Effect of dietary leucine supplementation on growth performance and plasma concentrations of leptin, urea and amino acids in C57BL/6 mice

The feed intake, body-weight gain and feed conversion of C57BL/6 mice did not differ between mice fed the alanine- and leucine-supplemented diets (Table 2). However, leucine supplementation increased (P<0.05) the plasma leptin concentration of C57BL/6 mice by 20% (Fig. 1). Plasma urea concentration was 28% lower in mice fed the leucine-supplemented diet compared with the alanine-supplemented diet (P<0.05; Table 3). Moreover, leucine supplementation significantly increased plasma leptin concentration but decreased plasma concentrations of glutamate (P<0.01), serine (P<0.01), threonine (P<0.01), tyrosine (P<0.05) and valine (P<0.01) in C57BL/6 mice (Table 3).

Effect of dietary leucine supplementation on leptin receptor expression and protein metabolism in the skeletal muscles of C57BL/6 mice

Dietary leucine supplementation increased (P<0.01) the mRNA expression of leptin receptors in the gastrocnemius and soleus muscles (Fig. 2), and also increased (P<0.01) leptin receptor protein abundance in the gastrocnemius and soleus muscles (Fig. 3). The FSR of the gastrocnemius and soleus muscles in mice fed the leucine-supplemented diet were significantly higher than those in mice fed the alanine-supplemented diet (P<0.01; Table 4). In addition, dietary leucine supplementation significantly decreased the rate of protein degradation in the gastrocnemius and soleus muscles of C57BL/6 mice (P<0.05; Table 4).

Effect of leucine and/or leptin treatment on protein metabolism in C2C12 myotubes

Protein synthesis was increased and protein degradation was inhibited in C2C12 myotubes treated with 5mm-leucine or 50ng/ml leptin treatment (P<0.01; Table 5). However, there was no significant difference in the protein synthesis of C2C12 myotubes between the control and leptin-only treatments (P>0.10; Table 5). In addition, there was a significant interaction between the leucine and leptin treatments in regulating protein synthesis (P<0.05) and degradation (P<0.01) in C2C12 myotubes (Table 5).

Fig. 2. Effects of dietary leucine (Leu) supplementation on lepton receptor mRNA expression in skeletal muscles. Mice were fed the Leu-supplemented diet or the alanine (Ala)-supplemented (isonitrogenous control) diet for 14 d. After the mice received their diets for 2 h on day 14, the (A) gastrocnemius and (B) soleus muscles were excised and used for quantitative real-time PCR analysis. The relative abundance for the lepton receptor mRNA was normalised to that for β-actin. Values are means (n 6), with their standard errors represented by vertical bars. *a,b Mean values with unlike letters were significantly different (P<0.05).
Effect of dietary leucine supplementation and/or leptin injection on growth performance and relative weights of the gastrocnemius and soleus muscles in ob/ob mice

Dietary leucine supplementation had no significant effect on the final body weight and feed intake of ob/ob mice (P > 0.10; Table 6). Intraperitoneal injection of leptin only decreased the feed intake of ob/ob mice during the first 2 d (P < 0.01; Fig. 4), and tended to decrease the feed intake of ob/ob mice during the whole period of the experiment (P = 0.07; Table 6). In addition, the final body weight of ob/ob mice was significantly reduced by the intraperitoneal injection of leptin (P < 0.01; Table 6). However, there were no significant interactions between dietary leucine supplementation and intraperitoneal leptin injection with regard to the performance of ob/ob mice (P > 0.10; Table 6).

Both dietary leucine supplementation and intraperitoneal leptin injection increased the relative weight of the gastrocnemius and soleus muscles in ob/ob mice (P < 0.01; Table 6). Dietary leucine supplementation and intraperitoneal leptin injection had significant interactions in increasing the relative weight of the gastrocnemius and soleus muscles in ob/ob mice (P < 0.01; Table 6).

Effect of dietary leucine supplementation and/or leptin injection on protein metabolism in ob/ob mice

Both dietary leucine supplementation and intraperitoneal leptin injection stimulated protein synthesis and inhibited protein degradation in the gastrocnemius and soleus muscles of ob/ob mice (P < 0.01; Table 6). In ob/ob mice, there were significant interactions between dietary leucine supplementation and intraperitoneal leptin injection with regard to protein metabolism in the soleus muscles (P > 0.10; Table 6).

Discussion

It has previously been shown that high levels of leucine (>5–6%) in the standard diet depress feed intake and limit growth in rats and pigs while moderate levels of leucine have no influence on either feed intake or growth[57–59]. Moreover, branched-chain amino acids or leucine supplementation in the high-fat diet can decrease the feed intake and growth of rats and mice, but branched-chain amino acids or leucine supplementation in the standard diet do not affect the feed intake and growth of rats and mice[12,40]. In the present study, dietary supplementation with 3% leucine had no effect on feed intake or weight gain in C57BL/6 or ob/ob mice (Tables 2 and 6). Additionally, intraperitoneal injection of leptin at a concentration of 0.1 µg/g body weight per d for 14 d significantly decreased body weight and feed intake in ob/ob mice during the first 2 d (P < 0.01), but not feed intake in ob/ob mice during the whole period of the experiment (Fig. 4 and Table 6). These results are not consistent with previous studies. Picard et al.[40] and Pelleymounter et al.[41] showed that intraperitoneal injection of leptin with the same dose used in the present experiment significantly

Table 4. Fractional synthesis rate and degradation of protein in the gastrocnemius and soleus muscles in C57BL/6 mice fed diets supplemented with alanine or leucine (Mean values with their pooled standard errors, n 6)

<table>
<thead>
<tr>
<th></th>
<th>+ Ala Mean</th>
<th>+ Leu Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractional synthesis rate (%/d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>12.98</td>
<td>13.85**</td>
<td>0.13</td>
</tr>
<tr>
<td>Soleus</td>
<td>13.55</td>
<td>14.46**</td>
<td>0.18</td>
</tr>
<tr>
<td>Protein degradation (nmol Tyr/(mg wet weight 2 h))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.288</td>
<td>0.221*</td>
<td>0.015</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.413</td>
<td>0.346*</td>
<td>0.017</td>
</tr>
</tbody>
</table>

+ Ala, l-alanine-supplemented diet; + Leu, l-leucine-supplemented diet. Mean values were significantly different from those of the + Ala group; * P < 0.05, ** P < 0.01.
ment may increase the amount of amino acids that are 
available for tissue growth. Previous studies have demonstrated that acute and chronic leucine administration can stimulate protein synthesis and inhibit the protein degradation of skeletal muscles in rats. Consistently, the present data indicate that dietary leucine supplementation enhanced protein synthesis and reduced protein degradation in the skeletal muscles of C57BL/6 and ob/ob mice (Tables 4 and 6). In the present study, protein metabolism in the soleus and gastrocnemius muscles was determined. The soleus muscle contains primarily slow-twitch oxidative muscle fibres, but the gastrocnemius muscle contains primarily fast-twitch glycolytic muscle fibres. The results showed that the effect of leucine on protein metabolism in the soleus muscles was larger than that in the gastrocnemius muscles, which could be due to the differences in the type of primary muscle fibres present in these muscles.

Acute leucine treatment could stimulate the production of specific proteins in various tissues and cells, such as leptin in adipose tissue, while chronic leucine administration has been shown to modestly increase the concentration of plasma leptin in rats. In the present study, we found that plasma leptin concentrations were dramatically lower in the leucine-supplemented group than those in the alanine-supplemented control group (Fig. 1), which might be due to the dose of leucine in the present study being much higher than that used in previous studies.

<table>
<thead>
<tr>
<th>Table 5. Effects of leucine and recombinant mouse leptin treatment on the fractional synthesis rate and degradation of protein in C2C12 myotubes (Mean values with their pooled standard errors, n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Fractional synthesis rate (%/d)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Protein degradation (nmol Tyr/(mg protein 6 h))</td>
</tr>
</tbody>
</table>

Table 6. Effects of dietary leucine supplementation and intraperitoneal leptin injection on performance, relative tissue weights and protein metabolism in the skeletal muscles of ob/ob mice (Mean values with their pooled standard errors, n = 6)

<table>
<thead>
<tr>
<th></th>
<th>− Leptin</th>
<th>+ Leptin</th>
<th>− Leptin</th>
<th>+ Leptin</th>
<th>SEM</th>
<th>Leu</th>
<th>Leptin</th>
<th>Interaction</th>
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<tbody>
<tr>
<td>Fractional synthesis rate (%/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>4.44b</td>
<td>5.71b</td>
<td>6.20a</td>
<td>6.41a</td>
<td>0.12</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</tr>
<tr>
<td>Protein degradation (nmol Tyr/(mg wet weight 2 h))</td>
<td>4.36a</td>
<td>0.37b</td>
<td>3.85b</td>
<td>2.8b</td>
<td>0.14</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Recent reports have shown that leucine promotes leptin receptor expression in mouse C2C12 myotubes through the mammalian target of rapamycin signalling pathway and leptin receptor gene expression\(^{25}\). Consistent with these findings, the results of the present study demonstrate that dietary leucine supplementation significantly stimulated both mRNA expression and protein levels of leptin receptor in the skeletal muscles of mice (Figs 2 and 3).

In the present study, we utilised C2C12 myotubes and ob/ob mice as in vitro and in vivo models to study the synergistic effect of leucine and leptin on the protein metabolism of skeletal muscles. C2C12 myotubes, which are generated from the differentiation of C2C12 myoblasts derived from the skeletal muscle of mice, cannot produce or secrete leptin, but can express both the long and short forms of leptin receptor (31). In addition, the present results also demonstrate that leucine and leptin treatments had significant interactions in regulating protein synthesis and degradation in the C2C12 myotubes and soleus muscles of ob/ob mice (Tables 5 and 6), but not in the gastrocnemius muscles of ob/ob mice (Table 6). The difference between the soleus and gastrocnemius muscles could be due to the differences in the type of primary muscle fibres present in these muscles. Moreover, previous studies as well as the present study have all demonstrated that leptin can regulate protein metabolism in vivo and in vitro, but there are discrepancies among the results of these studies, which are due to the differences in leptin dose, treatment duration, cell types, animal health status and animal species. Furthermore, in the present study, we found that after leptin injection, protein metabolism in ob/ob mice was still worse than that in C57BL/6 mice, indicating that there are other factors involved in the regulation of protein metabolism in ob/ob mice.

In conclusion, the results of the present study indicate that leptin and leucine synergistically regulate protein metabolism in skeletal muscles both in vivo and in vitro, and that leucine treatment stimulates the expression of leptin receptors in vivo. These findings provide evidence for a possible pathway whereby leucine regulates protein metabolism in skeletal muscles.

Acknowledgements

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5. Norton LE, Layman DK, Bunpo P, et al. (2009) The leucine content of a complete meal directs peak activation but not resistance during intraperitoneal leptin injection at a concentration of 0.1 μg/g body weight per d for an extended duration of time regulating the muscular protein metabolism of ob/ob mice. In addition, the present results also demonstrate that leucine and leptin treatments had significant interactions in regulating protein synthesis and degradation in the C2C12 myotubes and soleus muscles of ob/ob mice (Tables 5 and 6), but not in the gastrocnemius muscles of ob/ob mice (Table 6). The difference between the soleus and gastrocnemius muscles could be due to the differences in the type of primary muscle fibres present in these muscles. Moreover, previous studies as well as the present study have all demonstrated that leptin can regulate protein metabolism in vivo and in vitro, but there are discrepancies among the results of these studies, which are due to the differences in leptin dose, treatment duration, cell types, animal health status and animal species. Furthermore, in the present study, we found that after leptin injection, protein metabolism in ob/ob mice was still worse than that in C57BL/6 mice, indicating that there are other factors involved in the regulation of protein metabolism in ob/ob mice.

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