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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 2 h 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 14 d. *Ob/ob* mice were injected intraperitoneally with sterile PBS or recombinant mouse leptin (0·1 μg/g body weight) for 14 d. 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 leptin and leucine 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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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 μmol 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[3,18,25,31]. 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[8,18,34]. 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 μM-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 myotubes were washed three times with ice-cold sterile PBS, and were dissolved in 1 M-NaOH. Proteins of the myotubes were assayed by the Lowry method using bovine serum albumin as the standard[36].

Mice and diets

All mice used in the present study were humanely managed according to the established guidelines of the China Department of Agriculture. The experimental protocol was approved by the China Agricultural University Animal Care and Use Committee (Beijing, China). C57BL/6 male mice weighing 13–15 g and leptin-deficient *ob/ob* male mice weighing 30–42 g were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). Mice were individually housed in a temperature- and light-controlled room with the temperature set at 21–23°C and the lighting schedule set at 12 h light–12 h dark. Food and water were available *ad libitum*.

A 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 2·04 % (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 the leucine- and alanine-supplemented diets are summarised in Table 1.

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 (isonitrogenous control) diet (*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 μg/g body weight of a solution of recombinant mouse leptin dissolved in PBS. The injection dose of 0·1 μg/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,

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

	+Ala	+Leu
Ala	3·05	1·22
Asp	1·76	1·90
Arg	1·33	1·32
Cys	0·23	0·25
Glu	3·78	3·84
Gly	0·89	0·97
His	0·73	0·69
Ile	0·79	0·76
Leu	1·56	4·49
Lys	1·04	1·03
Met	0·45	0·43
Phe	0·93	0·96
Pro	2·58	2·56
Ser	0·91	0·97
Thr	0·75	0·78
Trp	0·25	0·26
Tyr	0·63	0·62
Val	0·94	0·98

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

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

(Mean values with their pooled standard errors, *n* 10)

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

+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-[$^2H_5$]phenylalanine (150 μmol/100 g body weight) as described previously[1,42]. 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 N_2 and used for the determination of protein synthesis; Western blot and RNA isolate analyses were conducted as described later. The contralateral 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-[$^2H_5$]phenylalanine in the free and protein-bound pools were measured as described previously[32,43]. Ions with mass:charge ratios of 148 and 153 were monitored and converted to the percentage of molar enrichment (mol%) using calibration curves.

Protein degradation of the gastrocnemius and soleus muscles was determined by the release of tyrosine as described previously[6,44]. Briefly, the intact gastrocnemius and soleus muscles were preincubated at 37°C in Krebs–Henseleit bicarbonate buffer supplemented with 5 mM-HEPES, 5 mM-glucose and 0·1 % bovine serum albumin, which was equilibrated with 95 % O_2 and 5 % CO_2, following the isolation of the muscles. After 30 min of preincubation, the muscles were transferred into fresh Krebs–Henseleit bicarbonate buffer containing 0·5 mM-cycloheximide, and further incubated at 37°C. The rate of protein degradation was determined by the release of tyrosine into the buffer containing cycloheximide in a 2 h period. Tyrosine was assayed using an S-433D Amino Acid Analyser (Sykam, GmbH), as described previously[35].

Calculations

The fractional protein synthesis rate (FSR) in myotubes and skeletal muscles was calculated as: FSR (%/d) = $(E_{Bound} \times 1440 \times 100\,\%)/(E_{Free} \times t)$, where E_{Bound} is the isotopic enrichment (%) of the tracer phenylalanine in the protein-bound pool at time t; 1440 is the number of min/d; E_{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].

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 × 2 factorial using the general linear model procedures of the Statistical Analysis System (SAS Institute). The factors in the models included the

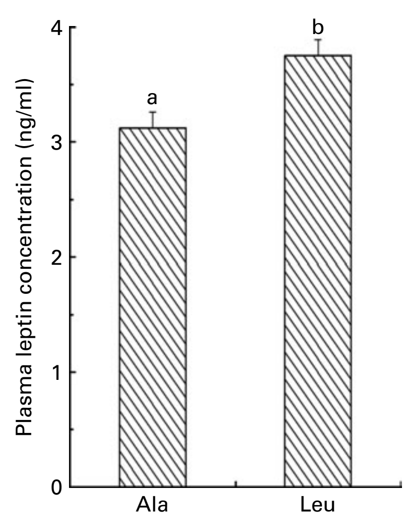

Fig. 1. Effects of dietary leucine (Leu) supplementation on plasma leptin concentration (ng/ml) in C57BL/6 mice. Mice were fed the Leu-supplemented diet or the alanine (Ala)-supplemented (isonitrogenous control) diet for 14 d. After the mice received their diets for 2 h on day 14, blood samples were obtained from the orbital sinus. Plasma leptin concentrations were measured. Values are means (*n* 6), with their standard errors represented by vertical bars. [a,b] 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)

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

+Ala, L-alanine-supplemented diet; +Leu, L-leucine-supplemented diet.
Mean values were significantly different from those of the +Ala group: * $P<0·05$, ** $P<0·01$.

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 leucine 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 5 mM-leucine or 50 ng/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).

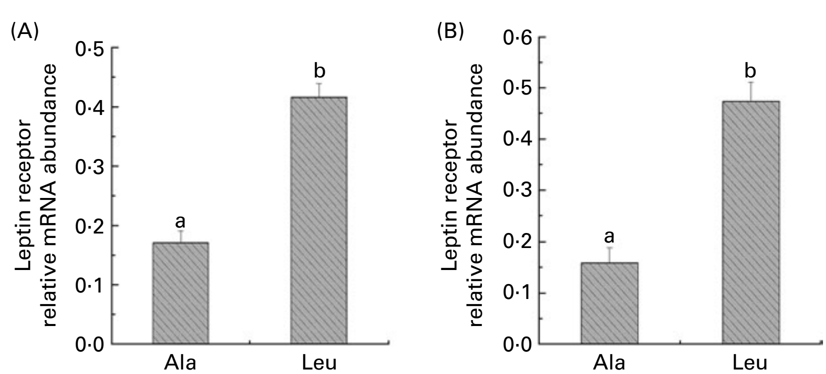

Fig. 2. Effects of dietary leucine (Leu) supplementation on leptin receptor mRNA expression in skeletal muscles. Mice were fed the Leu-supplemented diet or the alanine (Ala)-supplemented (isonitrogenous control) diet for 14 d. After the mice received their diets for 2 h on day 14, the (A) gastrocnemius and (B) soleus muscles were excised and used for quantitative real-time PCR analysis. The relative abundance for the leptin 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 ($P<0·05$).

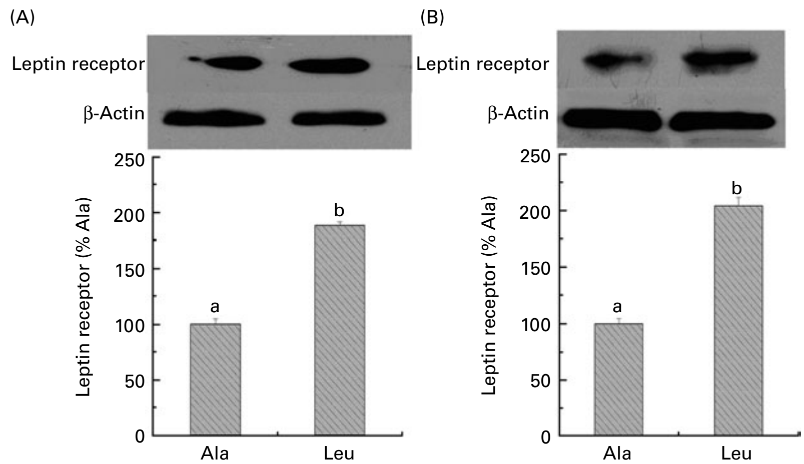

Fig. 3. Effects of dietary leucine (Leu) supplementation on leptin receptor protein levels in skeletal muscles. Mice were fed the Leu-supplemented diet or the alanine (Ala)-supplemented (isonitrogenous control) diet for 14 d. After the mice received their diets for 2 h on day 14, the (A) gastrocnemius and (B) soleus muscles were excised and used for the Western blot analysis. Representative Western blots are shown and results are expressed as the amount of the leptin receptor relative to β-actin in each group as a percentage of the control. 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).

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)

	+Ala Mean	+Leu Mean	SEM
Fractional synthesis rate (%/d)			
Gastrocnemius	12·98	13·85**	0·13
Soleus	13·55	14·46**	0·18
Protein degradation (nmol Tyr/(mg wet weight 2 h))			
Gastrocnemius	0·288	0·221*	0·015
Soleus	0·413	0·346*	0·017

+Ala, L-alanine-supplemented diet; +Leu, L-leucine-supplemented diet.
Mean values were significantly different from those of the +Ala group: * $P<0·05$, ** $P<0·01$.

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 ($P<0·01$) but not in the gastrocnemius 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[37–39]. 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[2,46]. 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 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)

	−Leptin		+Leptin			*P*		
	−Leu Mean	+Leu Mean	−Leu Mean	+Leu Mean	SEM	Leu	Leptin	Interaction
Fractional synthesis rate (%/d)	16·58[c]	19·02[b]	17·45[b,c]	24·55[a]	0·71	<0·01	<0·01	<0·05
Protein degradation (nmol Tyr/(mg protein 6 h))	5·99[a]	3·37[b]	3·19[b]	2·30[c]	0·16	<0·01	<0·01	<0·01

−Leptin, no supplemented leptin in medium; +Leptin, leptin-supplemented in medium; −Leu, no supplemented L-leucine in medium; +Leu, L-leucine-supplemented medium.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

decreased feed intake and body weight in *ob/ob* mice for 7 d when the injection was provided for a 7 d period but not when the treatment lasted for 28 d. Therefore, the difference between the present results and those of others could be due to the differences in the duration of the intraperitoneal injection of leptin. However, Pelleymounter *et al.*[41] also showed that the injection dose of 1·0 and 10·0 μg/g body weight of leptin could continuously decrease feed intake and body weight in *ob/ob* mice for 28 d. These results suggest that when the leptin treatment was provided at a concentration of 0·1 μg/g body weight per d for an extended duration of time, *ob/ob* mice could have developed leptin resistance, which results in the insignificant effects of leptin injection on feed intake and body weight in the later period of the experiment.

Recent studies have shown that chronic leucine administration can dramatically affect plasma leucine levels[11,47]. The present data demonstrated that plasma concentrations of leucine, alanine, glutamate, serine, threonine, tyrosine and valine were significantly altered in mice fed the leucine-supplemented diet compared with mice fed the alanine-supplemented diet (Table 3). In addition, plasma urea concentrations were dramatically lower in leucine-supplemented mice than in the alanine-treated control group (Table 3), which indicates that dietary leucine supplementation may increase the amount of amino acids that are available for tissue growth[48]. Previous studies have demonstrated that acute and chronic leucine administration can stimulate protein synthesis and inhibit the protein degradation of skeletal muscles in rats[6,47,49,50]. 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[51].

Acute leucine treatment could stimulate the production of specific proteins in various tissues and cells, such as leptin in adipose tissue[12,52–56], while chronic leucine administration has been shown to modestly increase the concentration of plasma leptin in rats[52]. In the present study, we found that plasma leptin concentrations were dramatically higher 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 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)

	−Leptin		+Leptin			*P*		
	+Ala Mean	+Leu Mean	+Ala Mean	+Leu Mean	SEM	Leu	Leptin	Interaction
Initial body weight (g)	33·15	33·75	33·93	33·63	2·29			
Final body weight (g)	40·42[a]	40·48[a]	34·68[b]	34·35[b]	1·70	0·94	<0·01	0·91
Feed intake (g)	44·18	43·17	39·92	40·88	1·71	0·99	0·07	0·57
Tissue weight (mg/g body weight)								
Gastrocnemius	4·55[c]	5·71[b]	6·20[a]	6·41[a]	0·12	<0·01	<0·01	<0·01
Soleus	0·44[d]	0·54[c]	0·60[b]	0·74[a]	0·01	<0·01	<0·01	<0·01
Fractional synthesis rate (%/d)								
Gastrocnemius	9·58[c]	10·39[b]	10·04[b]	10·79[a]	0·12	<0·01	<0·01	0·80
Soleus	10·20[c]	10·75[b]	10·30[c]	11·41[a]	0·09	<0·01	<0·01	<0·01
Protein degradation (nmol Tyr/(mg wet weight 2 h))								
Gastrocnemius	0·43[a]	0·37[b]	0·38[b]	0·28[c]	0·014	<0·01	<0·01	0·13
Soleus	0·50[a]	0·31[b]	0·31[bc]	0·26[c]	0·014	<0·01	<0·01	<0·01

−Leptin, intraperitoneal PBS injection; +Leptin, intraperitoneal leptin injection; +Ala, L-alanine-supplemented diet; +Leu, L-leucine-supplemented diet.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

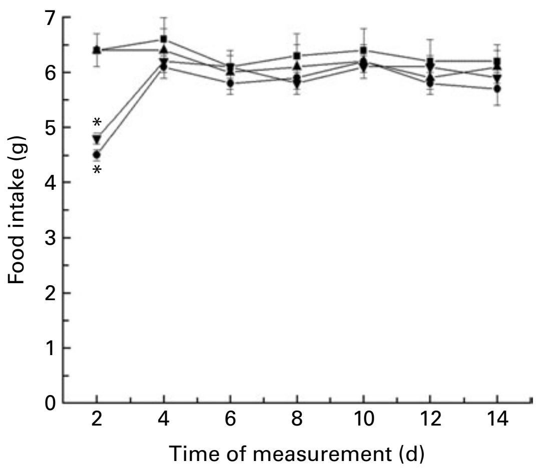

Fig. 4. Effects of dietary leucine supplementation and intraperitoneal leptin injection on feed intake in *ob/ob* mice during 14 d. *Ob/ob* mice were fed the leucine-supplemented diet or the alanine-supplemented (isonitrogenous control) diet. During 14 d, half of the *ob/ob* mice on each diet were intraperitoneally injected with sterile PBS or 0·1 μg/g body weight of leptin per d. During 14 d, the feed intake of *ob/ob* mice was measured per 2 d. Values are means (*n* 6), with their standard errors represented by vertical bars. * Mean values were significantly different from the alanine + PBS (-■-) group ($P<0·05$). -●-, Alanine + leptin; -▲-, leucine + PBS; -▼-, leucine + leptin.

Previous studies conducted in our laboratory 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 *ob/ob* mouse, whose background strain is the C57BL/6J mouse, cannot produce functional leptin on account of a nonsense mutation at the *ob* gene[13]. The use of C2C12 myotubes and *ob/ob* mice may eliminate the effects of endogenous functional leptin on protein metabolism, which also facilitate the investigation of the synergistic effects of leucine and leptin on protein metabolism in skeletal muscles.

Recent reports have shown that leucine and leptin treatments can regulate protein metabolism in skeletal muscles both *in vitro* and *in vivo*[6–8,17–19]. However, little is known about this synergistic action. In the present study, we found that leucine or leptin treatment stimulated protein synthesis and inhibited protein degradation in the C2C12 myotubes and skeletal muscles of *ob/ob* mice, which is the reason why dietary leucine supplementation and intraperitoneal leptin injection could increase the relative weight of the gastrocnemius and soleus muscles in *ob/ob* mice (Tables 5 and 6). These results showed that there was no appearance of leptin 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.

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.

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