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Short-term effects of leptin on skeletal muscle protein metabolism in the rat

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We have examined the short-term effects of leptin on protein metabolism in the rat. Indeed, an intravenous leptin administration (100 μg/kg body weight), which resulted in no changes in circulating insulin in the time interval studied, induced a decrease in the incorporation of ^{14}C-leucine to ^{14}C-skeletal muscle protein. No changes were observed in relation to muscle protein degradation (either measured in vivo following isotope preloading or in vitro as tyrosine released into the incubation medium) and gene expression associated with the different proteolytic systems (cathepsin B, m-calpain and ubiquitin-proteasome system). The effects of leptin on amino acid incorporation into muscle protein do not seem to be direct because incubation of isolated EDL muscles in the presence of 10 μg/ml of leptin did not modify either the protein incorporation or the oxidation of ^{14}C-leucine. It may, therefore, be suggested that leptin is able to influence protein synthesis in skeletal muscle through the action of an unknown mediator. (J. Nutr. Biochem. 11:431–435, 2000)

Keywords: leptin; skeletal muscle; proteolysis; protein turnover; ubiquitin; ATP-dependent proteolysis

Introduction

Leptin (a 16-kDa protein also known as the product of the *ob* gene) is a protein synthetized and secreted by adipose tissue in proportion to fat stores that has been suggested as having a role as a ponderostat signal informing the brain of the adipose tissue mass and, therefore, involved in the regulation of energy balance.[1–4] Leptin levels increase in the fed state, when triglyceride is being stored in adipose cells, and decrease during nutritional deprivation, when adipose triglyceride is being depleted.[5–7] Leptin and obesity seem to be clearly associated. In fact, *ob/ob* mice are obese and manifest great similarities to animals with lessions in the ventromedial hypothalamus, leading to the prediction that leptin acts on the central nervous system to suppress appetite. This hypothesis was supported by the demonstration that intraventricular infusion of leptin is more effective than intraperitoneal administration in causing weight loss in mice.[8] Indeed, infusion of leptin decreases hypothalamic levels of neuropeptide Y,[9] a neurotransmitter that increases food intake.[10] Administration of recombinant leptin reduces food intake and produces weight loss,[2,3,8,11] increases energy expenditure,[3,11] and lowers plasma insulin and glucose[12] in *ob/ob* like: mice. Leptin also decreases food intake and body weight in nonobese mice;[2,3] therefore, a failure to produce adequate amounts of leptin or resistance to its central actions would result in the development of obesity. Studies on the effects of leptin on lean body mass have shown that leptin seems to participate in the control of even the earliest stages of fat deposition after birth.[13] It does not have a major role in the lean body mass loss observed in stressed rats fed a high-fat diet.[14] Administration of leptin for 7 days caused an important reduction in fat pad size and decreases in liver weight, lipid, and glycogen in *ob/ob* mice.[15] Following treatment, the animals also developed tissue-specific changes in insulin sensitivity; thus, adipocytes became insulin-resistant, whereas muscle was more responsive to insulin.[16] In addition to appetite suppression, leptin seems to have specific effects on lipid metabolism and mobilization that are different from the metabolic compensations that normally occur with food deprivation.[17,18]

Concerning muscle metabolism, previous studies have shown that leptin seems to act on this tissue where it

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increases glycogen[19] and induces fatty acid oxidation.[20] This latter effect was observed in incubating muscle, thereby demonstrating that the leptin effects are direct. In addition, leptin decreases lipoprotein lipase (LPL) activity in skeletal muscle, thus contributing to hyperlipemia.[18] Recent work has shown that, in spite of producing anorexia and weight loss, leptin administration for 5 days does not induce an acute-phase response or protein wasting in rats.[21] However, the short-term effects of leptin administration on muscle protein turnover have not been studied. Bearing this in mind, it was the aim of the present investigation to see if an acute administration of leptin to rats had any effects on skeletal muscle protein metabolism.

Methods and materials

Animals

All animals (female Wistar rats weighing 60–80 g) were fed ad libitum on a chow diet consisting (by weight) of 54% carbohydrate, 17% protein, and 5% fat (the residue was nondigestible material) with free access to drinking water, and were maintained at an ambient temperature of 22 ± 2°C with a 12-hr light/dark cycle (lights on from 08:00 AM).

Leptin administration

Animals were intravenously injected with human recombinant leptin (100 μg/kg body weight) dissolved in a 0.9% NaCl solution). Three and a half hours after leptin administration, the animals were sacrificed and samples of arterial blood and soleus, gastrocnemius, and extensor digitorum longus muscle (EDL) were rapidly collected for further processing.

Biochemicals

All biochemicals used were reagent grade and obtained either from Boehringer-Mannheim S.A. (Barcelona, Spain) or from Sigma Chemical Co. (St. Louis, MO USA). Radioactive tracers, [^{3}H]phenylalanine (specific activity: 52 Ci/mmol), [^{14}C]phenylalanine (specific activity: 453 mCi/mmol), and [1-^{14}C]leucine (specific activity: 60 mCi/mmol) were purchased from Amersham Int. (Amersham, Bucks., UK).

Circulating leptin and insulin

Plasma leptin was determined by means of a rat radioimmunoassay kit (Linco Research Inc., St. Charles, MO USA). Plasma insulin was quantified with a rat radioimmunoassay kit from Amersham Int. (Amersham, Bucks., UK).

Leucine oxidation in vivo

Ninety min following administration of leptin, the animals received 3 μCi of [1-^{14}C]leucine dissolved in 0.15 ml of a 0.9% NaCl solution intravenously, without anaesthetic but with minimal stress to the animal. Expired CO_2 was then collected every 30 min for 2 hr by absorption in *Lumasorb* (Lumac, Holland) and the rate of $^{14}CO_2$ production was estimated by counting radioactivity in a sample of *Lumasorb*. After the collection period, the animals were killed after pentobarbital anaesthesia, and samples of arterial blood were taken by means of a heparinized syringe. The gastrointestinal tract (plus contents) was homogenized in 70 ml of 6% (w/v) $HClO_4$. [1-^{14}C]leucine absorption was calculated by subtracting total gastrointestinal radioactivity from that administered. In addition, the total amount of tracer incorporated into the protein fraction was assessed in soleus muscles. The specific activity of the tracer was assessed at the time of sacrifice and there were no significant differences between the control and leptin-treated groups (results not shown).

Muscle preparations and incubations

The dissection and isolation of the soleus and EDL muscles was carried out under pentobarbital anesthesia as previously described.[22] The isolated muscles were fixed to a stainless-steel clip in order to maintain the muscle under slight tension (making it comparable to resting length) during the incubation. Such muscles are able to maintain normal ATP and phosphocreatine concentrations during a 3-hr incubation period. The muscles were incubated in a shaking-thermostatized water bath at 35°C, for 1 hr in 3 ml of Krebs-Henseleit physiological saline, pH 7.4, containing 5 mM glucose, 4 pg/ml insulin, and 20 mM HEPES. After the addition of the muscles to the vials, these were stoppered and the incubation started at a shaking rate of 45 cycles/min. Vials were gassed with O_2/CO_2 19:1 during the incubation period.

Leucine utilization by isolated muscles

Muscles were incubated in the presence or absence of leptin (10 μg/ml) and 0.2 mM [1-^{14}C]leucine (0.625 μCi/ml) in specially designed flasks in a total volume of 1.5 ml of Krebs-Henseleit saline. Flasks were sealed with rubber stoppers equipped with hanging wells and were incubated for 30 min in a thermostatically controlled bath (35°C) with a shaking device (45 cycles/min). At the end of the incubation, hyamine hydroxide (0.2 ml) was added to the hanging well and the reaction was stopped by the addition of 30% (w/v) perchloric acid solution (0.15 ml) to the incubation medium. The wells were counted for radioactivity in order to assess the amount of the substrate that was oxidized to CO_2 during the incubation time. After sonication of muscles, proteins were precipitated with 30% trichloroacetic acid, centrifuged, and [^{14}C]protein was estimated following 0.3 N NaOH + 0.4% sodium deoxicholate solubilization and later mixing with liquid scintillation fluid before counting.

Proteolytic rate in isolated muscles

Total protein degradation by the isolated muscles was calculated as the rate of tyrosine released into the medium in the presence of 500 nM cycloheximide to block the reincorporation of tyrosine into tissue protein. Tyrosine was measured fluorimetrically as previously described.[23]

Protein turnover

Protein turnover was estimated as previously discussed,[24,25] using a double-isotope method and the accumulation induced by bestatin of peptide intermediates in protein degradation.[25] Basically, at the time of leptin administration, the animals received an intraperitoneal injection of 200 μCi/100 per g body weight of [^{3}H]phenylalanine. Ninety min later, the animals were injected intraperitoneally with 9 μCi/100 per g body weight [^{14}C]phenylalanine. Later (3 hr after leptin administration), the animals were intraperitoneally injected with bestatin (20 mg/kg) dissolved in absolute ethanol:NaCl 0.9% (2:3) and sacrificed 30 min later. Tissue protein was determined by the method of Bradford,[26] using bovine serum albumin as working standard.

RNA isolation and Northern blot analysis

Total RNA from gastrocnemius muscle was extracted using the acid guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi.[27] RNA samples (40 μg)

Table 1 Plasma leptin and insulin levels following leptin administration

Experimental group	Leptin (ng/ml)	Insulin (ng/ml)
Control	0.74 ± 0.13 (5)	0.60 ± 0.03 (3)
Leptin	5.78 ± 1.63 (7)*	0.63 ± 0.07 (4)

For more details, see the Methods and materials section. The values are mean ± SEM for the number of animals indicated in parentheses. Statistical significance of the results (Student's *t*-test; leptin vs. control): $^{*}P < 0.001$.

were denaturated, subjected to 1.2% agarose gel electrophoresis, and transferred to Hybond H membrane (Amersham, Amersham, Bucks., UK). RNA was fixed to membrane by illuminating with UV light for 4 min.

Prehybridization was done in 50% formamide/5× SSC (0.3 M NaCl, 65 mM sodium citrate)/5× Denhardt's solution (1× Denarth's solution is 0.1% polyvinylpyrolidone, 0.1% Ficoll, 0.1% BSA)/20 mM sodium phosphate, pH 6.8/0.1% SDS/100 mg/ml denaturated salmon sperm DNA overnight at 42°C. Membranes were hybridized with appropriate probes (10^6–10^7 cpm/ml) at 42°C for 18 hr. Nonspecifically bound probe was removed by successive washes in 2× SSC (15 min at 55°C, twice), 2× SSC + 0.1% SDS (30 min at 55°C) and 0.1× SSC + 0.1% SDS (15 min at 55°C, twice). Specific hybridization was then detected by autoradiography (for more details, see Llovera et al.[28]).

Radiolabeled probes were prepared by the random priming method (Boehringer-Mannheim, Barcelona, Spain). The ubiquitin probe used was a cDNA clone containing 12 base pairs of the second ubiquitin coding sequence plus a complete third and fourth ubiquitin coding sequence, and 120 base pairs of the 3′-untranslated region of the chicken polyubiquitin gene UB1.[29] The C8 proteasome subunit probe used was a cDNA clone containing 850 base pairs of the rat C8 proteasome gene.[30] In addition, rat 80-kDa m-calpain and rat preprocathepsin B cDNAs were used as specific probes. An 18S ribosomic probe was used as a control of loading. Filters were exposed to X Omat AR-5 films (Eastman Kodak Co., Rochester, NY USA) at −70°C for 2–4 days.

Statistical analysis

Statistical analysis of the data was performed by means of both the Student's *t*-test and two-way analysis of variance.

Results and discussion

The majority of studies on the effects of leptin on intermediate metabolism have analyzed chronic long-term effects of the peptide (see Cawthorne et al.[31] for review). Although this approach is interesting, the effects of leptin on food intake in the chronic administration model may hinder some other metabolic effects of leptin, independent of anorexia. The results presented here constitute the first study on the short-term effects of leptin administration on skeletal muscle protein metabolism in the rat. Using our short-term administration model, we have shown that leptin decreases LPL activity in skeletal muscle, thus contributing to hyperlipemia.[18] The main objective of this investigation, therefore, was to analyze the effects of exogenous leptin administration on protein turnover in skeletal muscle.

Following leptin administration, the levels of the protein increased 7.8-fold in relation to the nontreated controls (Table 1). Interestingly, leptin administration did not cause any changes in circulating insulin (Table 1). It has previously been reported that leptin impairs metabolic actions of insulin in adipose tissue[32] and muscle.[20] In addition, the circulating levels of leptin seem to be correlated with those of circulating insulin.[33] However, non-insulin-dependent diabetics show leptin levels that do not differ from those of nondiabetic humans of the same body mass index.[34] Interestingly, short-term insulin administration does not increase leptin secretion in humans.[35] Our data seem to indicate that the contrary is also true, at least in experimental animals.

Table 2 Protein turnover in leptin-treated rats

Experimental group	dpm ^{14}C/dpm ^{3}H	dpm ^{14}C/mg protein
Control	0.071 ± 0.006 (3)	1,230 ± 164 (3)
Leptin	0.074 ± 0.013 (3)	1,041 ± 100 (4)

For more details, see the Methods and materials section. The values are mean ± SEM for the number of animals indicated in parentheses.

The data on protein turnover (Table 2) indicate that leptin treatment did not significantly affect either protein degradation (calculated as the dpm ^{14}C/dpm ^{3}H ratio) or synthesis (calculated as the dpm ^{14}C/mg protein ratio). However, in this last parameter, there was a tendency ($P < 0.2$) for lower values in the leptin-treated group. In view of this, we decided to perform further experiments to see if leptin could indeed influence protein synthesis. We administered [1-^{14}C]leucine to the animals and calculated the amount of tracer incorporated in the muscle protein fraction. The specific activity of the tracer was assessed at the time of sacrifice and there were not significant differences between the control and leptin-treated groups (results not shown). As can be seen in Table 3, leptin treatment resulted in a decreased (33%) incorporation of the tracer in the protein fraction. In addition, leptin treatment induced an increase in the oxidation of [1-^{14}C]leucine to CO_2 by the whole animal (Figure 1).

We also considered the possibility that leptin treatment could affect protein degradation and, therefore, we incubated isolated soleus muscles from animals that were leptin treated, and analyzed the rate of tyrosine release into the incubation medium as a measure of protein degradation. No changes in proteolytic rate were observed in the muscles of

Table 3 Incorporation of [^{14}C]leucine into soleus protein following leptin administration

Experimental group	Incorporation	
	dpm/mg muscle tissue	dpm/total muscle weight
Control	71.3 ± 7.04 (3)	2,469 ± 140 (3)
Leptin	60.3 ± 3.27 (5)*	1,655 ± 105 (4)**

For more details, see the Methods and materials section. The values are mean ± SEM for the number of animals indicated in parentheses. Muscle weights (g) were expressed as percentage of initial body weight. Statistical significance of the results (Student's *t*-test; leptin vs. control): $^{*}P < 0.05$, $^{**}P < 0.01$.

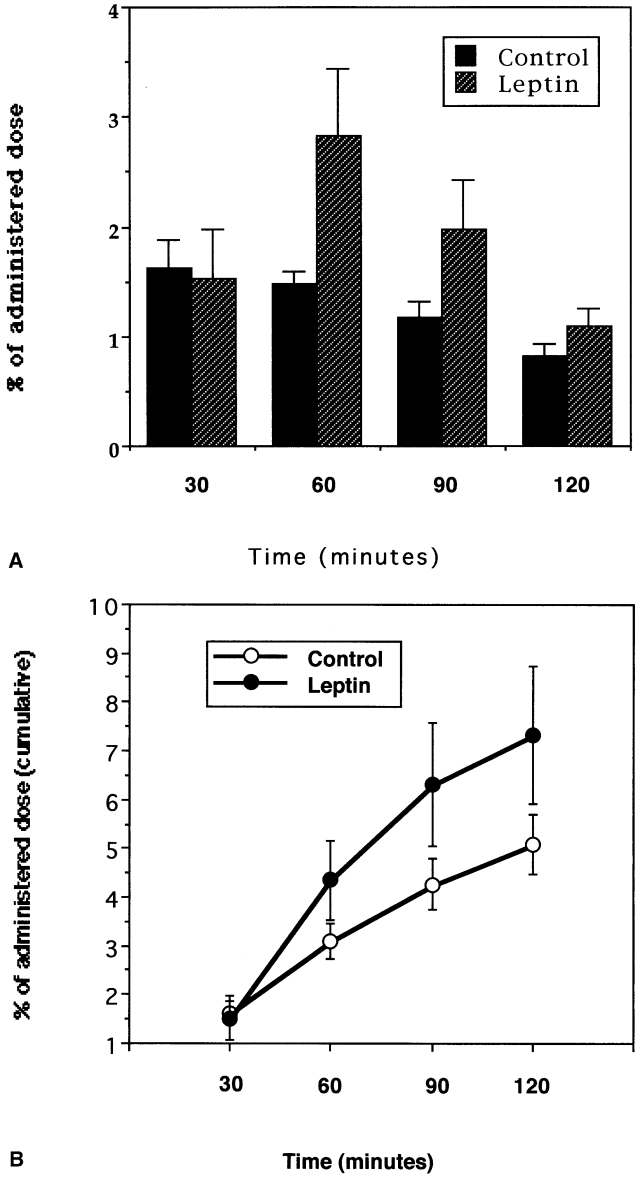

Figure 1 Time-course of the oxidation of [1-^{14}C] leucine to $^{14}CO_2$. For full details, see the Methods and materials section. Statistical differences were assessed using a two-way analysis of variance test. [1^{14}C] leucine oxidation to $^{14}CO_2$ is expressed both as a percentage of the administered dose (A) and as a percentage of the cumulative dose (B). Cumulative $^{14}CO_2$ was significantly higher (vs. treatment) $P = 0.05$ in the leptin-treated group.

the leptin-treated animals in relation to the control group (Table 4). Furthermore, leptin-treated animals did not present any alterations in the expression of the different genes involved in muscle proteolysis, as compared with the control group (Table 5).

Finally, we decided to investigate if the observed effects upon skeletal muscle protein synthesis were direct. Bearing this in mind, we incubated isolated EDL muscles in the presence of leptin and evaluated the effects of the polypeptide on [1-^{14}C]leucine oxidation and incorporation into protein. No changes were observed (Table 6) and we can, therefore, suggest that the short-term effects of leptin

Table 4 Proteolytic rate in incubated soleus muscles from leptin-treated rats

Experimental group	Tyr release (nmoles tyr/total muscle weight/2 hr)
Control	13.5 ± 0.90 (5)
Leptin	12.4 ± 0.76 (5)

For more details, see the Methods and materials section. The values are mean ± SEM for the number of animals indicated in parentheses. Muscle weights (mg) were expressed as percentage of initial body weight.

administration of skeletal muscle protein synthesis are not direct and must be mediated by an unknown molecule. Investigations are currently being undertaken in our laboratory to identify this mediator.

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Table 5 Induction of proteolytic systems following leptin administration

Proteolytic system	Experimental group	
	Control (5)	Leptin (7)
Cathepsin B	1.01 ± 0.22	1.05 ± 0.1
m-Calpain	0.25 ± 0.05	0.22 ± 0.03
Ubiquitin		
1.2 kB	0.30 ± 0.12	0.33 ± 0.08
2.4 kB	0.20 ± 0.02	0.17 ± 0.01
C8	0.38 ± 0.11	0.36 ± 0.04

For more details, see the Methods and materials section. The values (arbitrary units) are mean ± SEM for the number of animals indicated in parentheses.

Table 6 Effects of leptin on incubated extensor digitorum longus muscles

Parameter	Experimental group	
	Control (9)	Leptin (7)
Incorporation of [^{14}C]leucine into protein (nmoles/g of muscle/30 min)	38.9 ± 4.20	39.7 ± 3.48
Oxidation of [^{14}C]leucine to $^{14}CO_2$ (nmoles/g of muscle/30 min)	59.3 ± 5.74	59.0 ± 7.43
Oxidation/incorporation ratio	1.64 ± 0.19	1.65 ± 0.32
Tyr release (nmoles/g muscle/2 hr)	155 ± 9.68	160 ± 10.8

For more details, see the Methods and materials section. The values are mean ± SEM for the number of experiments indicated in parentheses.

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