

Leptin directly stimulates thermogenesis in skeletal muscle

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Abstract Using a method involving repeated oxygen uptake (MO_2) determinations in skeletal muscle *ex vivo*, the addition of leptin was found to increase MO_2 in soleus muscles from lean mice. These effects were found to be inhibited by phosphatidylinositol 3-kinase inhibitors, absent in muscles from obese Lepr^{db} mice which have the dysfunctional long form of leptin receptor, and blunted in muscles from diet-induced obese mice in the fed state but not during fasting. These findings indicate that leptin has direct thermogenic effects in skeletal muscle, and that these effects require both the long form of leptin receptors and phosphatidylinositol 3-kinase signalling. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Leptin, a hormone encoded by the *ob* gene and produced mainly by adipocytes [1], has been shown to play an important role in the regulation of body weight via its effects on both food intake and energy expenditure [2]. Gene mutations that lead either to a deficiency in the production of functional leptin or to non-functional leptin receptors (OB-R) result in hyperphagia and hypometabolism, both of which contribute to obesity and insulin resistance [3]. In several animal models, the administration of recombinant leptin results in improved insulin sensitivity concomitant to weight losses which are partly due to decreased food intake and partly to counteracting the suppression of thermogenesis (i.e. energy conservation) that occurs in response to the diminished energy intake [4–7]. The mechanisms by which leptin exerts its control over thermogenesis are thought to involve its central effects on hypothalamic OB-R, leading to increased sympathetic outflow [8]. Indeed, leptin administration has been shown to increase sympathetic activation of brown adipose tissue thermogenesis [9], an effect that can be entirely abolished by surgical ablation of this tissue's sympathetic innervation [10]. Whether skeletal muscle, which is considered to be quantitatively the most important tissue for dietary regulation of thermogenesis, is also a site for leptin's action on thermogenesis is unclear, particu-

larly given reports that central sympathetic outflow to skeletal muscle, as judged by norepinephrine turnover rates in individual muscles, is unresponsive to diet [11]. However, the facts that skeletal muscle expresses OB-R [12] and that leptin is now known to activate signal transduction directly in insulin-sensitive organ/tissues [13,14] prompted us to investigate whether leptin could exert direct control on thermogenesis in skeletal muscle. To this end, the direct effect of leptin on skeletal muscle thermogenesis was evaluated by assessing changes in metabolic rate of the soleus muscle *ex vivo* by a method that involves repeated oxygen uptake determinations in indirect microcalorimeter chambers. We investigated the thermogenic responses to leptin in muscles from lean mice, from mice which developed obesity because of mutations either in the production of functional leptin or in leptin receptors, as well as in mice which developed obesity when shifted from chow to a high-fat energy-dense diet.

2. Materials and methods

2.1. Animals and diets

Several strains of mice were studied, all being males and aged 6–8 weeks. BALB/c mice were obtained from CMU, University of Geneva (Switzerland), whereas *ob/ob* mice (C57BL/6OlaHsd-Lepr^{ob}), *db/db* mice (C57BL/KsOlaHsd-Lepr^{db}) and their respective controls were purchased from Harlan (Horst, The Netherlands). Prior to the start of each experiment, the mice were adapted for at least 1 week to a temperature-controlled room (22°C) with a 12-h light/dark cycle. They had free access to tap water, and were maintained on a commercial pelleted laboratory diet (Provimi-Lacta, Cossonay, Switzerland) consisting, by energy, of 24% protein, 66% carbohydrates, and 10% fat. In the study of diet-induced obesity, BALB/c mice were fed for 10–17 days on a high-fat diet providing approximately 50% of energy from lard, 25% from carbohydrates and 25% from protein; the control mice were fed the low-fat chow diet. All diets contained minerals and vitamins at levels recommended by the American Institute of Nutrition. All animals used in the present studies were maintained in accordance with our institute's regulations and guide for the care and use of laboratory animals.

2.2. Muscle tissue preparations

Each mouse was killed by decapitation between 8.30 and 9.00 h, and their soleus muscles were carefully dissected out intact together with their tendons and freed only of loosely attached connective tissue. They were then placed on a stainless steel frame, at physiological resting length, in the test chambers of twin indirect microcalorimeters perfused with a Krebs–Ringer bicarbonate buffer of the following composition (in mmol/l): 116.8 NaCl, 25 NaHCO₃, 5.9 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 1.25 CaCl₂, and 5 glucose. The medium was gassed continuously with a mixture of 95% O₂ and 5% CO₂ and was maintained at a set temperature of 30 ± 0.2°C. At the end of the experiment, the muscles were weighed after removal of their tendons and rapid blotting on filter paper.

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2.3. Measurement of tissue respiration rate

The respiratory rate of skeletal muscle was measured by a method involving repeated O_2 uptake determinations [15], as described by Barde et al. [16]. The O_2 partial pressure (pO_2) of a bubble-free liquid phase enclosed in a thick-walled Lucite chamber was measured by a Clark O_2 electrode connected to a polarographic circuit, whose output voltage is directly proportional to pO_2 . At 10-min intervals, a pump partially exchanged the solution for a fresh one within 2 min. All values for O_2 uptake rate (MO_2) were taken during steady-state respiration. For each drug, this corresponded to 90–120 min after drug administration. The basal steady-state MO_2 was taken 120–150 min after placing the muscle preparations in the calorimeter chambers.

2.4. Chemicals and solutions

All chemicals were of analytical grade and were purchased from Fluka (Buchs, Switzerland). Recombinant murine leptin was purchased from Insight Biotechnology (Middlesex, UK), wortmannin from Calbiochem (Lucerne, Switzerland) and LY294002 from Sigma (St. Louis, MO, USA).

2.5. Statistics

The analysis of data on changes in MO_2 in response to leptin was performed either by paired *t*-test or by analysis of variance (ANOVA) with repeated measures, using the statistical software STATISTIK (St. Paul, MN, USA). Upon detection of significant increases by ANOVA, post-hoc pairwise comparisons were conducted using Tukey's test, with the level of statistical significance taken as $P < 0.05$.

3. Results

3.1. Murine models with intact or mutated leptin receptors

The results of our first experiment, presented in Fig. 1a, indicate that the addition of leptin, at 5 or 10 nM, resulted in significant increases in MO_2 above the baseline rate in muscles from BALB/c mice, namely by 22% and 35%, respectively. In subsequent experiments, we examined the extent to which the thermogenic responsiveness to leptin might be altered in Lep^{ob} and $Lepr^{db}$ mice, murine models which develop obesity because of a specific mutation leading either to the production of non-functional leptin or to dysfunctional leptin receptors, respectively. The results indicate that leptin was effective in increasing muscle MO_2 to the same extent in Lep^{ob} mice and in lean wildtypes, i.e. by about 20% above baseline rates (Fig. 1b). The addition of leptin to muscles from $Lepr^{db}$ mice, however, failed to increase MO_2 , and this contrasts with the 23% increase in MO_2 obtained in muscles from their lean wildtypes (Fig. 1c).

3.2. Pharmacological inhibition of phosphatidylinositol 3-kinase (PI 3-kinase)

Following the above-mentioned experiments which suggest that the direct thermogenic effects of leptin in skeletal muscle require intact leptin receptors, we investigated whether post-receptor signalling by PI 3-kinase – which has been shown to be required for other actions of leptin in many tissues or cell systems – is also required for its direct thermogenic effects in the skeletal muscle. To this end, we utilized two known inhibitors of PI 3-kinases, at doses that we had previously found to have no effect on basal MO_2 of the muscle (data not shown). The subsequent addition of either wortmannin or LY294002 completely abolished the leptin-induced increase in muscle MO_2 (Fig. 2), suggesting that PI 3-kinase is also required for the direct action of leptin in stimulating skeletal muscle thermogenesis.

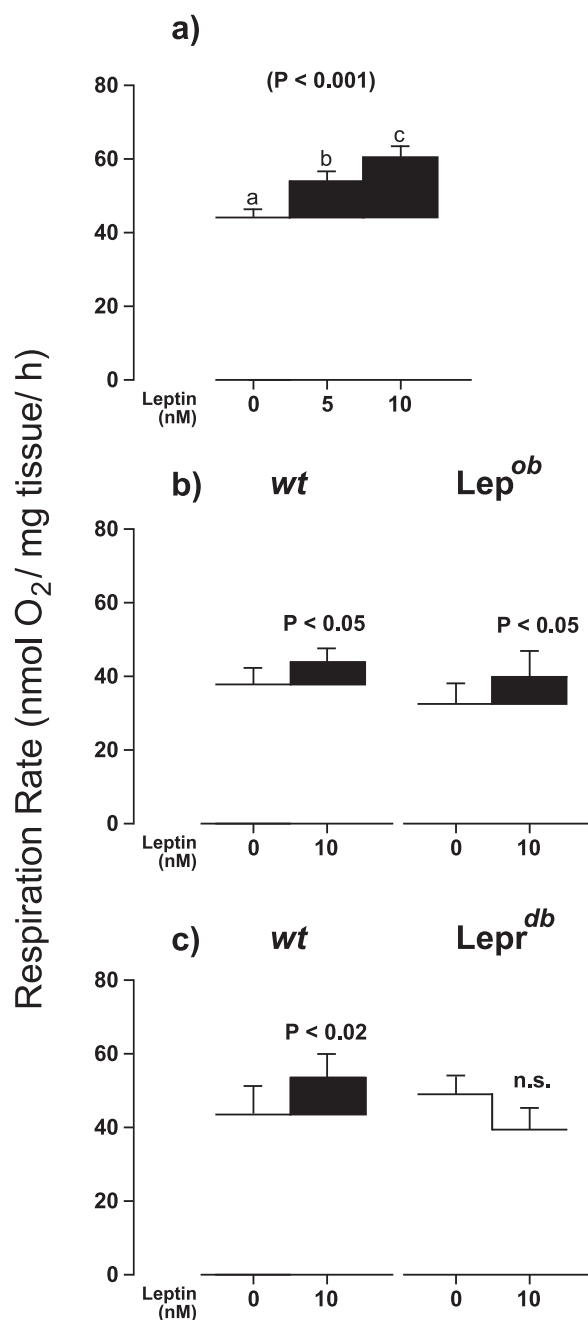


Fig. 1. Respiration rate (MO_2) of skeletal muscle obtained (a) from chow-fed lean BALB/c mice ($n = 16$), (b) from obese Lep^{ob} mice and their lean wildtypes, wt ($n = 5$), and (c) from obese $Lepr^{db}$ mice and their lean wildtypes ($n = 5$). Data are means, with vertical bars representing S.E.M. Significant effects of leptin in increasing MO_2 , shown as dark areas above basal rates, were assessed either by paired *t*-test or by ANOVA with repeated measures. Following ANOVA, post-hoc pairwise comparisons were made by Tukey's test, and values not sharing the same superscript (a, b, c) are significantly different from each other ($P < 0.05$); n.s. = no significant difference.

3.3. Dietary manipulation

Given evidence that high fat feeding can contribute to the development of obesity, not only by increasing energy intake but also by decreasing thermogenesis [17,18], and the general

associations between dietary-induced obesity, hyperleptinemia and leptin resistance [3], we investigated whether the thermogenic effect of leptin in muscles from mice made obese by high fat feeding for about 2 weeks could be impaired. The results, shown in Fig. 3a, indicate that the addition of leptin failed to stimulate MO_2 in muscles from these diet-induced obese (DIO) mice; by contrast, muscles from their age-matched control mice fed chow showed a 26% increase in MO_2 following the addition of leptin. To test whether the lack of thermogenic response to leptin in muscles from DIO mice could be reversed by caloric restriction, a state of hypoleptinemia, another experiment assessed the thermogenic effect of leptin in muscles from DIO mice fasted for 36 h. The results, presented in Fig. 3b, confirm our above-mentioned findings about the absence of leptin-induced increase in MO_2 in muscle from DIO mice, and also indicate that leptin was effective in significantly increasing MO_2 in muscle from DIO mice after prior fasting.

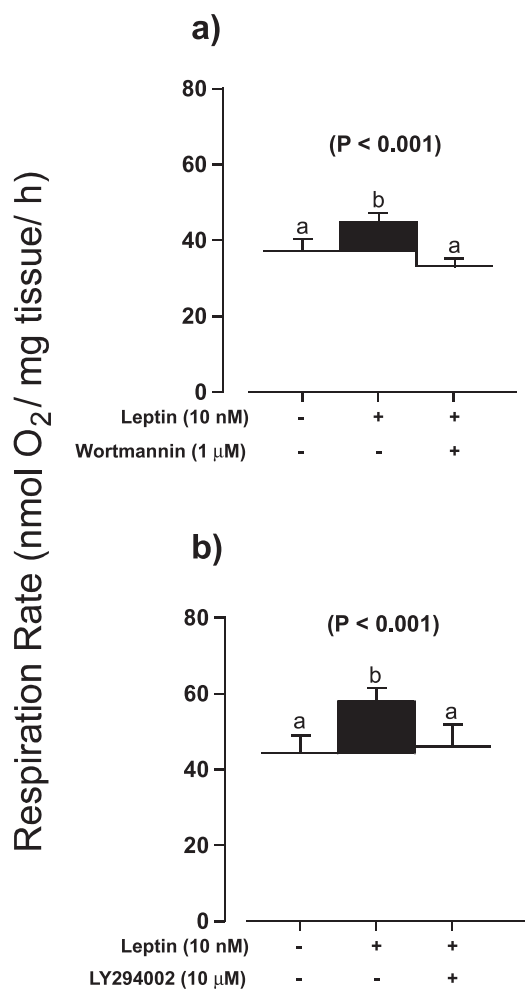


Fig. 2. Respiration rate (MO_2) of skeletal muscles from ad libitum fed BALB/c mice in response to leptin, and the effect of subsequent administration of (a) wortmannin or (b) LY294002, two pharmacological inhibitors of PI 3-kinase. All data are means, with vertical bars representing S.E.M. ($n=5-6$). The significant effects of leptin in increasing MO_2 (shown as dark areas above basal rates) and their inhibition by subsequent addition of wortmannin or LY294002 were assessed by ANOVA with repeated measures. Following ANOVA, post-hoc pairwise comparisons were made by Tukey's test, and values not sharing the same superscript (a, b) are significantly different from each other ($P < 0.05$).

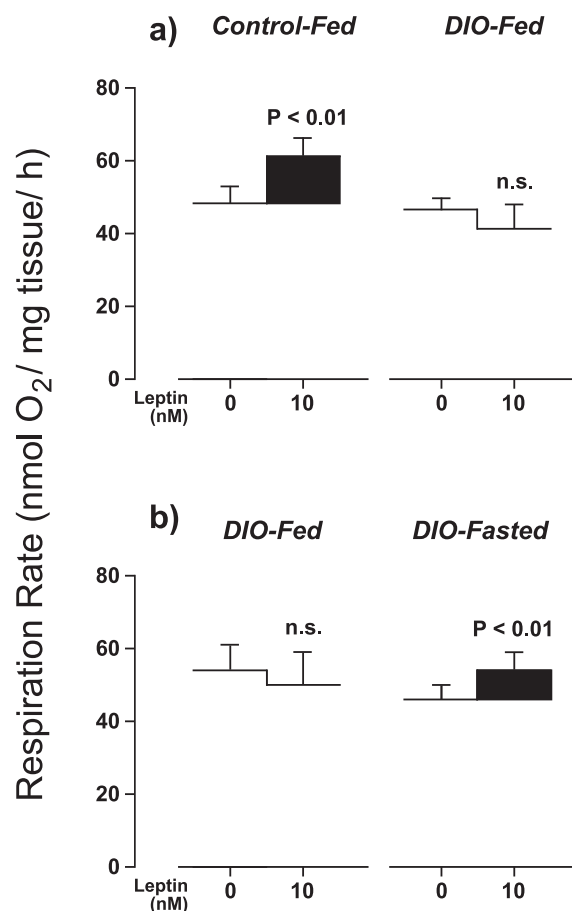


Fig. 3. Respiration rate (MO_2) of skeletal muscles obtained (a) from BALB/c mice fed ad libitum on either a low-fat chow diet or a high-fat diet (DIO mice) for a period of 10–17 days; the percentage body fat in the dietary-induced obese (DIO) mice was significantly higher than that in the chow-fed controls (16.9% versus 7.9%; $P < 0.01$), (b) from DIO mice fed ad libitum or fasted for 36 h. All data are means, with vertical bars representing S.E.M. ($n=5-6$). Significant effects of leptin in increasing MO_2 , shown as dark areas above basal rates, were assessed by paired t -test; n.s. = no significant difference.

4. Discussion

Over much of the past century, the skeletal muscle, by virtue of its large size contributing 30–40% of body mass, has been considered the major tissue that enables mammals to adapt to changes in food availability by turning down the rate of heat production during periods of food scarcity so as to conserve energy, or by turning it up during food abundance so as to burn excess food energy. However, the mechanisms by which skeletal muscle could allow such adaptations remain elusive, in part because of practical difficulties in studying metabolism in intact muscles. Using a method involving repeated oxygen consumption determinations in the whole muscle ex vivo, we report here that leptin, a hormone whose role in the control of whole-body thermogenesis has so far focused on its effect on brain centers that activate the sympathetic nervous system, also stimulates thermogenesis by its direct effects on leptin receptors in the skeletal muscle. We also demonstrate that this peripheral effect of leptin on thermogenesis, which requires PI 3-kinase activity, is blunted in muscles from mice made obese by feeding an energy-dense

high-fat diet, but reversed by caloric restriction. Furthermore, the finding here that the thermogenic responsiveness to leptin is unimpaired in muscles from obese Lep^{ob} mice which are deficient in functional leptin raises the possibility that the direct effect of leptin on skeletal muscle thermogenesis may have contributed to the reversal of obesity in this mutant following chronic leptin replacement therapy.

It is known that alternative splicing of the leptin receptor (OB-R) gene provides several OB-R mRNA isoforms that encode receptors that differ in the length of their intracellular domains and that are expressed in multiple tissues at varying amounts [12]. The OB-R isoform with a long intracellular domain (OB-Rb) is highly expressed in the hypothalamus, and its activation leads to PI 3-kinase signalling [3]. This isoform is also expressed, albeit at lower levels, in skeletal muscle and several other peripheral tissues [12,13]. The present study also shows that both the intact OB-Rb and PI 3-kinase activity are required for the direct effect of leptin on skeletal muscle thermogenesis. Indeed, the increase in muscle MO₂ in response to leptin is abolished in the presence of PI 3-kinase inhibitors, wortmannin or LY294002, and absent in muscles from Lep^{db} mice which are characterized by a mutation leading to dysfunctional OB-Rb signalling. These results raise questions as to whether in skeletal muscles from mice made obese by high fat feeding, the blunted thermogenic response to leptin in the fed state, which is reversed by fasting, occurs by a reversible desensitization of the OB-Rb signalling pathway or is dependent upon inactivation of the final effector mechanisms. Whatever the answer, our data provide evidence that leptin has the potential to exert direct control over skeletal muscle thermogenesis by activating the OB-Rb pathway in the fed state or during caloric restriction. During starvation therefore, the prompt and drastic fall in circulating leptin would lead to energy conservation not only by the withdrawal of its central actions on sympathetically mediated thermogenesis in tissues with a high specific metabolic rate such as brown adipose tissue, liver, heart and kidneys, but also by removal of its direct peripheral action on skeletal muscle thermogenesis.

The skeletal muscle is a major tissue contributing not only to metabolic rate and thermogenesis but also accounting for a large proportion of glucose utilization and whole-body lipid oxidation. Comparison of our data on the direct effects of leptin in stimulating skeletal muscle thermogenesis with those previously reported about the direct effects of leptin in altering muscle substrate metabolism [19] – i.e. increased glucose uptake and fatty acid oxidation at the expense of storage as triglycerides – shows striking similarities concerning PI 3-kinase requirement and in the blunting of the muscle response in animals made obese by high fat feeding [20–22]. These findings suggest that, in the skeletal muscle, the mechanisms by which leptin affects substrate metabolism and thermogenesis are interdependent. In this context, the question arises as to whether stimulation of AMP-activated protein kinase, which has recently been shown to be a principal mediator of the effects of leptin on fatty acid oxidation [23], is also required together with PI 3-kinase signalling for its direct thermogenic effect in skeletal muscle. Since the development of obesity and insulin resistance are associated with decreased lipid oxidation and increased concentration of intramuscular triglycerides, one could entertain the possibility that impaired skeletal muscle thermogenesis to leptin, as observed here dur-

ing the development of diet-induced obesity in mice, may be a primary event which then leads to impairments in substrate metabolism and in insulin resistance.

With evidence in humans implicating a low capacity for diet-induced thermogenesis [24] and marginal leptin deficiency [25] as risk factors for obesity and insulin resistance, our studies provide the impetus for the elucidation of molecular pathways by which leptin exerts direct control on skeletal muscle thermogenesis. Furthermore, it remains to be established whether the direct effect of leptin on skeletal muscle thermogenesis is essentially endocrine, paracrine with leptin being secreted from adipocytes in close vicinity to muscles or indeed autocrine as suggested by evidence for nutritionally regulated leptin gene expression in skeletal muscle [26]. Although the increase in metabolic rate (20–35%) due to the direct effect of leptin on muscle may not, a priori, be considered to be large, it must be emphasized that in terms of overall impact on whole-body energy expenditure, leptin-induced thermogenesis in skeletal muscle could be considerable by virtue of the total proportion of body mass attributable to muscle. From a therapeutic standpoint therefore, our demonstration of a direct effect of leptin on skeletal muscle to activate thermogenesis provides the rationale for the development of peripherally acting anti-obesity drugs that will selectively target leptin signalling in a tissue which is viewed as the major site for impairments in the dissipation of excess fat to heat.

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