Leptin inhibits glycogen synthesis in the isolated soleus muscle of obese (ob/ob) mice

Yong-Ling Liu, Valur Emilsson, Michael A. Cawthorne*

Clore Laboratory, University of Buckingham, Hunter Street, Buckingham MK18 1EG, UK

Received 1 June 1997

Abstract The *ob* gene product, leptin, causes significant and dose-dependent inhibition of basal and insulin-stimulated glycogen synthesis in isolated soleus muscle from *ob/ob* mice, and a smaller, non-significant inhibition in muscle from wild-type mice. Leptin had no inhibitory effect on glycogen synthesis in soleus muscle from the *diabetic* (*db/db*) mice, which lack the functional leptin receptor. The full-length leptin receptor (Ob-Rb), is expressed in soleus muscle of both *ob/ob* and wild-type mice, however with no detectable differences in expression level. These results suggest that hyperleptinaemia may attenuate insulin action on glucose storage in skeletal muscle.

Key words: Leptin; Leptin receptor; Skeletal muscle; Glycogen synthesis; Insulin; *ob/ob* mouse

1. Introduction

Leptin, encoded by the *ob* gene, is produced exclusively by adipocytes. Reduction in the production of leptin or the sensitivity to leptin in rodents results in a phenotype of obesity and diabetes. Thus, leptin deficiency in the *ob/ob* mouse [1] or the lack of a functional leptin receptor (Ob-Rb) in the *diabetic* (*db/db*) mouse [2] or Zucker fa/fa rat [3] leads to obesity, hyperglycaemia and insulin resistance, resembling non-insulin dependent diabetes mellitus (NIDDM) in humans. Administration of leptin into *ob/ob* mice causes a significant reduction of food intake and body weight as well as normalization of the metabolic status, but has no effects in the *db/db* mice [4–6]. However, the mutations responsible for the *ob/ob*, *db/db* and *fa/fa* phenotypes in rodents have not been detected in obese humans. In fact, plasma leptin levels in obese humans are elevated and there is a positive correlation with body mass index, thus arguing against a simple leptin deficiency as the cause of human obesity [7,8]. These findings have led to the suggestion that leptin resistance may be part of the pathogenesis of human obesity.

The leptin receptor (Ob-R) is expressed as alternatively spliced variants [2]. Only the Ob-Rb isoform, predominantly expressed in hypothalamus, contains the full-length cytoplasmic domain and is believed to be the functional receptor. A point mutation within the Ob-R gene of *db/db* mice results in the lack of the cytoplasmic domain that is thought to interact with the Jak/STAT pathway and is therefore unable to mediate a functional leptin signal [3,9,10]. Ob-Rb mRNA expression has also been detected in peripheral tissues such as pancreatic islets, liver, kidney and lung [2,11,12], indicating that leptin may have other physiological functions in addition to regulating feeding behaviour. In fact, we have demonstrated that leptin can inhibit insulin secretion from pancreatic islets [12], and Levin et al. [13] have shown that infusion of leptin into *ob/ob* mice causes depletion of their liver glycogen content as well as a reduction in the plasma concentrations of glucose, insulin and cholesterol.

Skeletal muscle is the major site of insulin mediate glucose storage. The aim of the present study was to investigate for the first time the effects of leptin on skeletal muscle glucose metabolism.

2. Materials and methods

2.1. Animals

Female *ob/ob* mice and wild-type (+/+) mice of the Aston strain were bred in our laboratory. The *db/db* mice were obtained from Harlan-Olac (Bicester, Bucks, UK). Animals were housed on a 12 h : 12 h dark cycle (08:00–20:00 h) with free access to water and standard laboratory chow (Beekay rat and mouse toxicology diet, Bantin and Kingman, Hull, UK).

2.2. Measurement of glycogen synthesis in isolated soleus muscle

Non-fasted mice were killed by cervical dislocation. Intact soleus muscles weighing 4–6 mg were dissected and tendons were tied onto a stainless steel clip under slight tension to maintain each muscle at resting length. The muscles were immediately placed in individual flasks containing 3 ml of Krebs-Ringer bicarbonate (KRB) buffer with 5.5 mM glucose, 0.14% bovine serum albumin and 10 mM HEPES equilibrated with 95% O_2 : 5% CO_2 (pH 7.4). All flasks were immediately sealed and transferred to a shaking water bath (36°C) and gassed continuously with 95% O_2 : 5% CO_2. After 30 min pre-incubation, the muscles were transferred to fresh incubation flasks with 3 ml of identical buffer, but containing 0.25 μCi/ml of [U-^{14}C]glucose (Amersham International, Amersham, UK) and bovine insulin (Sigma Chemical Co, Poole, UK) at 0, 10, 50, 100, 1000 and 10 000 μU/ml with recombinate murine leptin dissolved in phosphate buffered saline (PBS) or an equivalent volume of PBS [12]. The muscles were then incubated for another 60 min in a shaking water bath with continuous gassing. At the end of the incubation period, muscles were removed, trimmed of tendons, blotted and rapidly frozen in liquid N_2. [U-^{14}C]glucose incorporation into glycogen (glycogen synthesis) was measured following potassium hydroxide digestion and ethanol precipitation [14] and results are expressed as μmol glucosyl units/h/g wet weight. The concentration of lactate in the incubation medium was determined spectrophotometrically [14].

2.3. Expression of the full-length leptin receptor (Ob-Rb) mRNA in soleus muscle

Total RNA was isolated from tissues of lean (+/+) and obese (*ob/ob*) mice by the use of RNaid plus kit (BIO 101 Inc., USA). RNA samples (~4 μg) were treated with DNAse I for 15 min and then reverse transcribed, using $(dT)_{15}$ Superscript reverse transcriptase II together with a biological ribonuclease inhibitor (Amersham Int., Amersham, UK), according to the manufacturers guidelines (GibcoBRL, Life Technol., Paisley, UK). Expression of the full-length leptin receptor mRNA, Ob-Rb, and the common extracellular domain mRNA, Ob-R, were established by RT-PCR using domain-specific primers (Ob-Rb, sense, 5′-TCTTCTGGAGCCTGAACCCATTTC-

*Corresponding author. Fax: (44) (1280) 820261.
E-mail: mac@buck.ac.uk

PII S0014-5793(97)00732-1

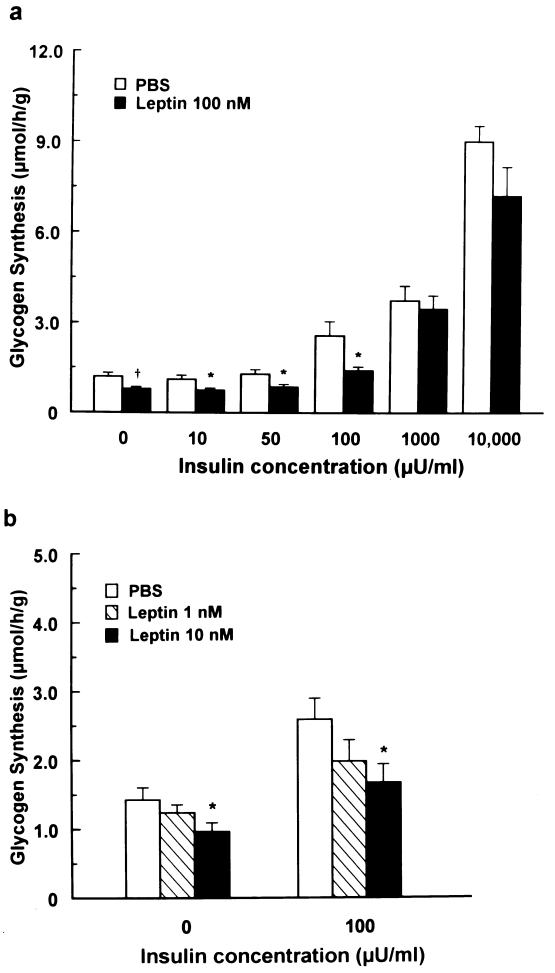

Fig. 1. (a) Effects of leptin (100 nM) on glycogen synthesis in soleus muscle of 8–10 week old *ob/ob* mice ($n = 7$–9). (b) Dose-dependent effects of leptin on basal and insulin-stimulated (100 μU/ml) glycogen synthesis in soleus muscle of 8–11-week-old *ob/ob* mice ($n = 8$–10). $^*P < 0.05$, †$P < 0.01$, leptin-treated versus control (Student's unpaired *t*-test).

3′, antisense, 5′-TTCTCACCAGAGGTCCCTAAACTC-3′, −0.68 kb; and Ob-R, sense 5′-GGAATGAGCAGGTCAAAAC-3′; anti-

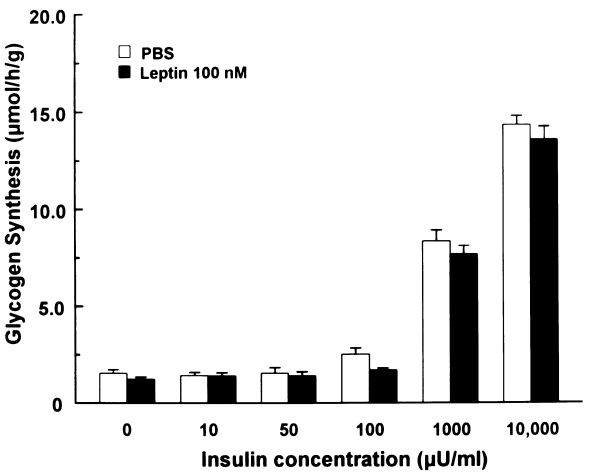

Fig. 2. Effects of leptin (100 nM) on basal and insulin-stimulated glycogen synthesis in soleus muscle of 8–10-week-old wild-type (+/+) mice ($n = 7$–10).

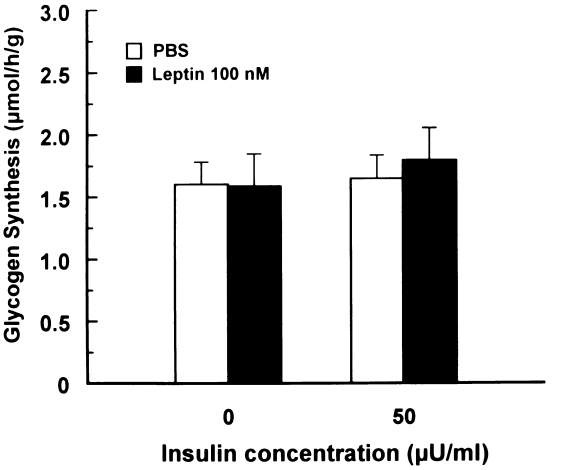

Fig. 3. Effects of leptin (100 nM) on glycogen synthesis in soleus muscle of 6–10-week-old *db/db* mice ($n = 8$).

sense, 5′-GTGACTTCCATATGCAAACC-3′, −0.47 kb). For quantitative PCR a shorter fragment of the Ob-Rb was used to relate it better to the kinetic performances of the Ob-R and β-actin sequences amplified. Thus, all cDNA samples were diluted serially (3–5-fold) then split equally and used either for PCR amplification of the Ob-Rb cDNA isoform (37 cycles, using primers; sense, 5′-ACACTGTTAATTTCACACCAGAG-3′; antisense, 5′-TGGATAAACCCTTGCTCTTCA-3′ −0.45 kb), or all leptin receptor (Ob-R) cDNA isoforms (37 cycles, using the primers listed above) and finally the mouse β-actin cDNA (30 cycles, using Clontech, USA, primers). Polymerase chain reaction (PCR) amplification was performed with AmpliTaq (Perkin Elmer, UK) in a thermocycler (Techne, Cambrdige, UK), each cycle as: 95EC 45 s, 55EC 30 s and 72EC 1 min. After completion (30–37 cycles) the mixture was incubated at 72EC for 10 min. PCR products were either fractionated on agarose gels or spotted on Hybond N+ membranes (Amersham, UK) and the amount assessed quantitatively by Southern blot hybridization using short digoxygenin (DIG-5′) labelled antisense probes. Thus, the Ob-Rb was detected with DIG5′-GGGCTGGGAATGTGCACAGGATTCCTGCCTCACC-3′, and the Ob-R (all isoforms); with DIG5′-GCGAGTCGGTGGAACGTGGCTGAT-3′, and finally the β-actin sequence was detected with a commercial probe (Clontech, USA). Hybridization was in a Rapid-hyb buffer (Amersham, UK) and conditions of hybridization and chemiluminescent detection performed as described previously [12].

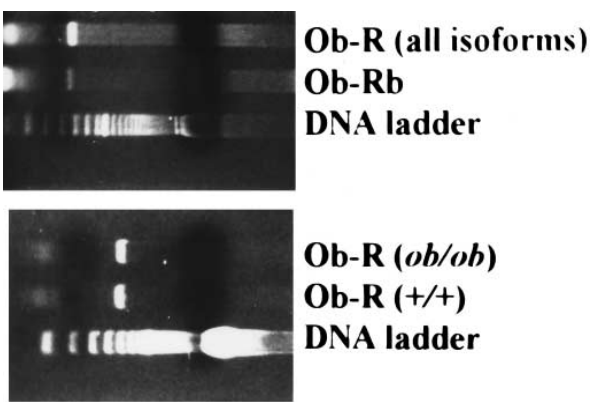

Fig. 4. Expression of Ob-R and Ob-Rb mRNAs in soleus muscle of +/+ and *ob/ob* mice as determined by RT-PCR and ethidium bromide staining, using primers as described in Section 2. PCR amplification of samples (DNase I-treated) in which the reverse transcriptase was omitted, during cDNA synthesis, resulted in non-detectable PCR production (data not shown). A 100-bp DNA ladder was used as a molecular weight marker.

3. Results

Recombinant murine leptin at 100 nM inhibited glycogen synthesis in soleus muscle of *ob/ob* mice (Fig. 1a), with 35% inhibition at basal ($P < 0.01$), and 28%, 30% and 45% at insulin concentrations (10, 50 and 100 μU/ml, respectively, $P < 0.05$) in the physiological range. However, the maximal response following treatment with insulin (10 000 μU/ml) was not significantly affected by leptin (Fig. 1a). The effects of lower concentrations of leptin (1 and 10 nM) on glycogen synthesis in the soleus muscle of *ob/ob* mice were examined under basal condition and in the presence of 100 μU/ml of insulin. Leptin at 10 nM caused significant inhibition of both basal and insulin-stimulated glycogen synthesis (32% and 35%, respectively; $P < 0.05$), whereas at 1 nM the inhibition (13% and 24%) was not significant (Fig. 1b). These results demonstrate that the effect of leptin on glycogen synthesis in muscle from *ob/ob* mice is dose-dependent (i.e. 13%, 32% and 35% inhibition respectively of the basal rate and 24%, 35% and 45% inhibition respectively of the 100 μU/ml insulin-stimulated rate, at 1, 10 and 100 nM of leptin, respectively). The rate of lactate formation in the muscle incubation media of *ob/ob* mice was not affected by leptin (data not shown). Also, leptin (100 nM) had no effect on either basal or insulin-stimulated 2-deoxy-[^{3}H]glucose uptake and phosphorylation in the soleus muscle of *ob/ob* mice (percentage of the paired control values: 95.6 ± 9.6% of basal rate; 105.1 ± 5.6% of 1000 μU/ml of insulin-stimulated rate; $n = 6$).

In lean (+/+) mice, there was a trend towards inhibition of glycogen synthesis by leptin (100 nM), with 20% inhibition at basal (non-significant) and 32% inhibition at 100 μU/ml of insulin ($P = 0.06$; Fig. 2). Studies were also undertaken using soleus muscles of *db/db* mice, which lack the functional Ob-Rb receptor. Leptin (100 nM) did not affect either the basal rate of glycogen synthesis or the rate in the presence of 50 μU/ml of insulin (Fig. 3).

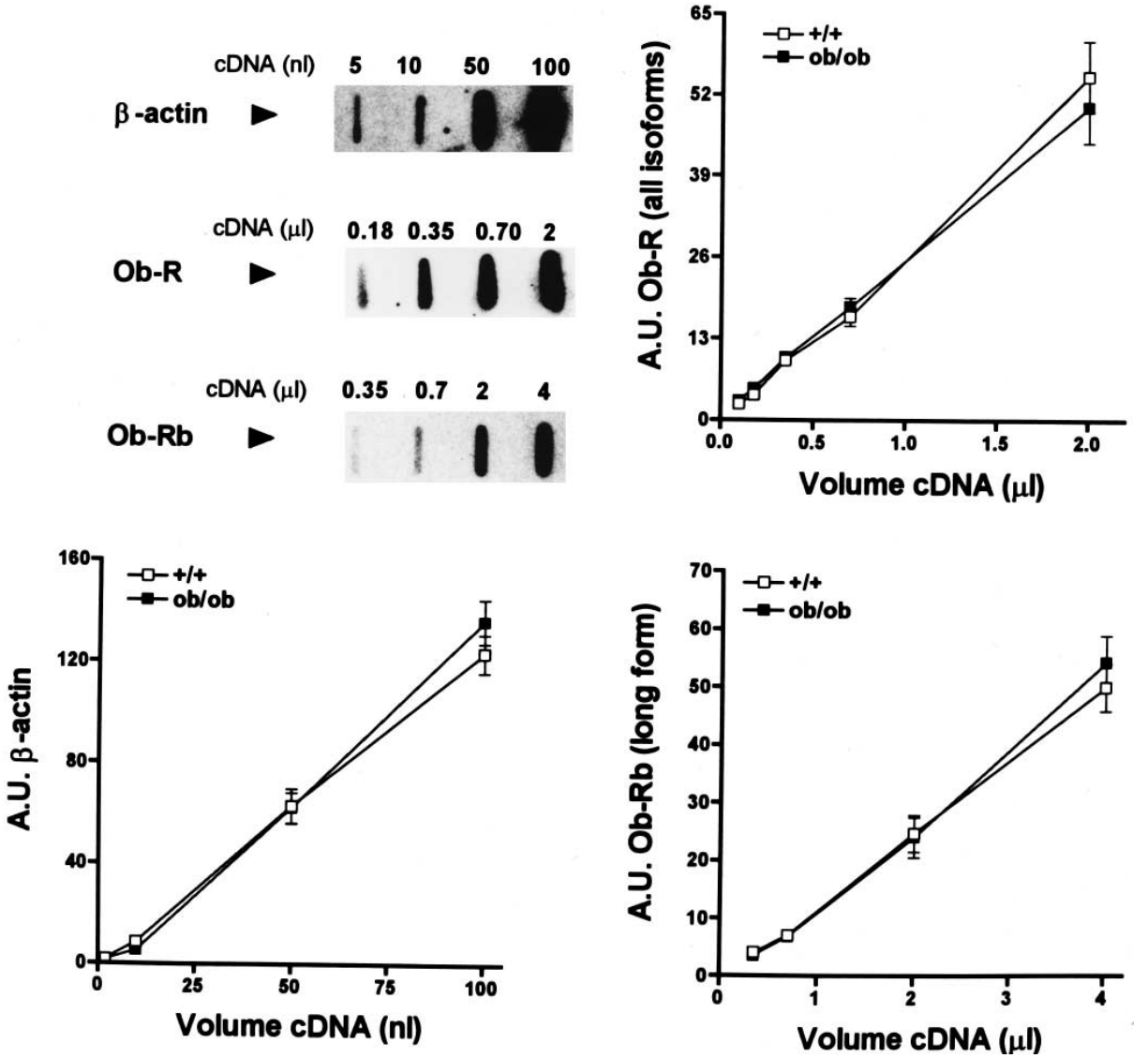

Fig. 5. Expression of Ob-R, Ob-Rb and β-actin mRNA species in soleus muscle of +/+ and *ob/ob* mice, measured ($n = 6$) by quantitative PCR amplification. These are the optimized linear ranges of PCR amplification of different cDNAs specified by hybridization with short DNA probes. The top left panel shows hybridization blots of PCR products when different volumes of cDNA from +/+ mice are used, whilst the other panels show the quantitation of gene expression between the +/+ and *ob/ob* mouse samples. Data are expressed as ± S.E.M. ($n = 3$), in arbitrary units (A.U.).

The expression of the Ob-Rb transcript isoform is detected readily in the soleus muscle by RT-PCR and ethidium bromide staining (Fig. 4). We have also investigated whether there were any differences in the expression level of the leptin-receptor (Ob-R and Ob-Rb) mRNA in soleus tissues of wild-type and *ob/ob* mice, since any differences in receptor number might indicate variation in the potency of leptin action. As the expression of the Ob-Rb transcript is low, quantitative PCR was used to determine the relative abundances of the Ob-Rb mRNA expression in soleus muscle, in a linear range of PCR conditions [15]. This method resolves changes in expression that are less than 2-fold. Thus RNA samples were diluted serially and PCR amplification performed at a fixed number of cycles. β-Actin was used as endogenous control in a separate reaction to assess loading. When the ratios of PCR products from the leptin receptor cDNA and the endogenous actin standard of the wild-type and *ob/ob* mice were compared, no differences in abundances were detected (see Fig. 5).

4. Discussion

The *ob* gene product leptin has been shown to be a regulator of food intake and energy expenditure and these effects are mediated through leptin receptors in the hypothalamus [1]. However, leptin receptors have been found also in many peripheral tissues including lung, ovary, kidney [11,16] and pancreatic islets [12] suggesting that leptin might induce a range of cellular effects in tissues other than brain.

In vivo studies have shown that the administration of leptin to *ob/ob* mice results in a depletion in hepatic glycogen content [13]. Furthermore, leptin impairs the first steps of the insulin signalling chain, i.e. auto-phosphorylation of the insulin receptor and tyrosine phosphorylation of insulin receptor substrate-1 in rat-1 fibroblasts overexpressing human insulin receptors, NIH3T3 cells [17] and hep G2 cells [18].

Skeletal muscle is the major site of insulin-mediated glucose uptake. Furthermore, defective glycogen synthesis in muscle is one of the earliest manifestations of insulin resistance. Since insulin resistance is associated with obesity, which has in turn been associated with increased secretion of leptin, we determined the direct effect of leptin on basal and insulin-stimulated glycogen synthesis using the isolated soleus muscle preparation. Our results provide the first evidence that leptin directly inhibits glycogen synthesis in muscle. This action of leptin was more marked in muscles from *ob/ob* mice than in muscles from lean animals. This latter finding is consistent with the finding of others that *ob/ob* mice are more sensitive to the effects of leptin than lean littermates [4–6]. The reason for these differences in efficacy is not known, but as there were no detectable differences in the expression level of the Ob-Rb in soleus muscle of lean and *ob/ob* mice, it is possible that the leptin receptor or the post-receptor machinery of *ob/ob* mice has increased signalling efficiency to exogenous leptin.

In humans, the serum leptin concentration correlates with percentage body fat [7,8], but insulin resistance has been reported to be associated with elevated plasma leptin levels independent of body fat mass [19]. Leptin concentrations in humans are usually in the range 0.1–5 nM [20], whereas in rodents plasma concentrations up to 20 nM [21] have been recorded. The concentration of leptin found to be effective in reducing insulin mediated-glycogen synthesis, in the present study (10–100 nM), is somewhat higher than that measured in most human obese subjects. However, such an apparent discrepancy between exogenous doses of leptin used in vivo and in vitro in rodents studies and the plasma concentration in man is a common finding [22,23]. It is possible that recombinant leptin does not have the same potency as that produced endogenously, possibly as a result of post-translational modification [22]. Thus the present findings raise the possibility that high circulating leptin concentrations in obese subjects might partially inhibit insulin-mediated muscle glycogen synthesis. Taken together with our earlier finding [12] that leptin can inhibit glucose-induced and basal insulin secretion, it is proposed that excessive leptin secretion might potentiate impaired glucose tolerance in obese subjects.

Acknowledgements: We thank D. Hislop and P. Randall for their technical assistance. We are grateful to Dr. Jonathan Arch and SmithKline Beecham Pharmaceuticals for providing us with the recombinant leptin.

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