Rapid Publications

Expression of the Functional Leptin Receptor mRNA in Pancreatic Islets and Direct Inhibitory Action of Leptin on Insulin Secretion

Valur Emilsson, Yong-Ling Liu, Michael A. Cawthorne, Nicholas M. Morton, and Michelle Davenport

Leptin, encoded for by the mouse *ob* gene, regulates feeding behavior and energy metabolism. Its receptor (Ob-R) is encoded by the mouse diabetic (*db*) gene and is mutated in the *db/db* mouse so that it lacks the cytoplasmic domain. We show that the full-length leptin receptor (Ob-Rb), which is believed to transmit the leptin signal, is expressed in pancreatic islets of *ob/ob* and wild-type mice, as well as in hypothalamus, liver, kidney, spleen, and heart. Recombinant leptin inhibited basal insulin release in the perfused pancreas preparation from *ob/ob* mice but not in that from Zucker *fa/fa* rats. Leptin (1–100 nmol/l) also produced a dose-dependent inhibition of glucose-stimulated insulin secretion by isolated islets from *ob/ob* mice. In contrast, leptin at maximum effective concentration (100 nmol/l) did not inhibit glucose-stimulated insulin secretion by islets from *db/db* mice. These results provide evidence that a functional leptin receptor is present in pancreatic islets and suggest that leptin overproduction, particularly from abdominal adipose tissue, may modify directly both basal and glucose-stimulated insulin secretion. *Diabetes* 46:313–316, 1997

The *ob* gene product, leptin, is produced exclusively in adipose tissue. In *ob/ob* mice, a mutation in the *ob* gene prevents normal leptin production and results in obesity and diabetes (1). The diabetic (*db*) gene product has been identified as the receptor for leptin (2). Daily injections of recombinant leptin inhibit food intake and reduce body weight and fat mass in *ob/ob* mice, but not in *db/db* mice (3,4), and normalize blood glucose concentrations (4,5). In fact, changes in glycemia precede changes in body weight (5). Furthermore, in pair-feeding studies in *ob/ob* mice, infusion of leptin produces not only a greater reduction of body fat than in the pair-fed mice but a dramatic reduction in plasma insulin concentration (3). These findings suggested that leptin may have direct effects on a number of peripheral tissues, including the endocrine pancreas.

The leptin receptor has several alternatively spliced variants (7). One of these spliced variants is expressed at a high level in hypothalamus and is believed to be the functional receptor (Ob-Rb). In *db/db* mice, there is a point mutation that results in alternative splicing of the receptor coding region and the addition of a 106-bp insert containing a premature termination signal. Thus, in *db/db* mice, the cytoplasmic region of the receptor that interacts with the Jak/STAT pathway is missing (7–9). In *fa/fa* rats, a single-base substitution results in an amino acid change affecting dimerization of the receptor (10). Unlike *ob/ob* mice, *db/db* mice do not respond to the administration of exogenous leptin (6). Although there is some evidence that *fa/fa* rats are not totally insensitive, they are very resistant to leptin relative to control rats (11).

The present study provides evidence that, in addition to hypothalamus, the transcript for the predicted functional leptin receptor is expressed in pancreatic islets and several other tissues in the mouse. Furthermore, recombinant leptin directly inhibits insulin secretion in *ob/ob* mice and wild-type mice but not in either Zucker *fa/fa* rats or *db/db* mice. These findings indicate that leptin acts through the functional leptin receptor to inhibit insulin secretion and provides evidence that hyperleptinemia might be part of a link between obesity and diabetes.

RESEARCH DESIGN AND METHODS

Animals. Female *ob/ob* mice and +/+ mice of the Aston strain were bred in house and were housed on a 12-h light/12-h dark cycle (8:00 A.M. to 8:00 P.M.) with free access to water and standard laboratory diet (Beekay rat and mouse toxicology diet, Bantin and Kingman, Hull, U.K.). Zucker (*fa/fa*) rats were obtained from St. George's Hospital Medical School and *db/db* mice from Harlan-Olac (Bicester, U.K.).

Identification of leptin receptor (Ob-Rb) mRNA. Tissues were obtained from 7- to 8-week-old female *ob/ob* and +/+ mice. RNA was isolated using an RNaid Plus kit (BIO 101), treated with DNase I, and reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) and oligo(dT). Polymerase chain reaction (PCR) amplification (37 cycles) of a 0.68-kb fragment (bases 2786–3464) in the cytoplasmic section of Ob-Rb cDNA was performed with a sense primer (5′-TCTTCTGGAGCCTGAACCCATTTC-3′) and an antisense primer (5′-TTCTCACCAGAGGTCCCTAAACTC-3′) by AmpliTaq (95°C, 42-s denaturation; 55°C, 30-s annealing; 72°C, 1-min extension). The amplified fragment from hypothalamus was then cloned into a pCR-TRAP cloning system (GeneHunter) and identified with a specific digoxygenin (DIG)-labeled 34-mer oligonucleotide probe ([DIG]5′-GGGCTGGGAATGTGCACAGGATTCCTGCCTCACC-3′) that hybridizes to bases 3329–3363 in the Ob-

From the Clore Laboratory, University of Buckingham, Hunter Street, Buckingham, U.K.

Address correspondence and reprint requests to Professor M.A. Cawthorne, Clore Laboratory, University of Buckingham, Hunter Street, Buckingham, MK18 1EG U.K. E-mail: mac@buck.ac.uk.

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DIG, digoxygenin; KRB, Krebs-Ringer buffer; Ob-R, leptin receptor; Ob-Rb, full-length leptin receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcriptase.

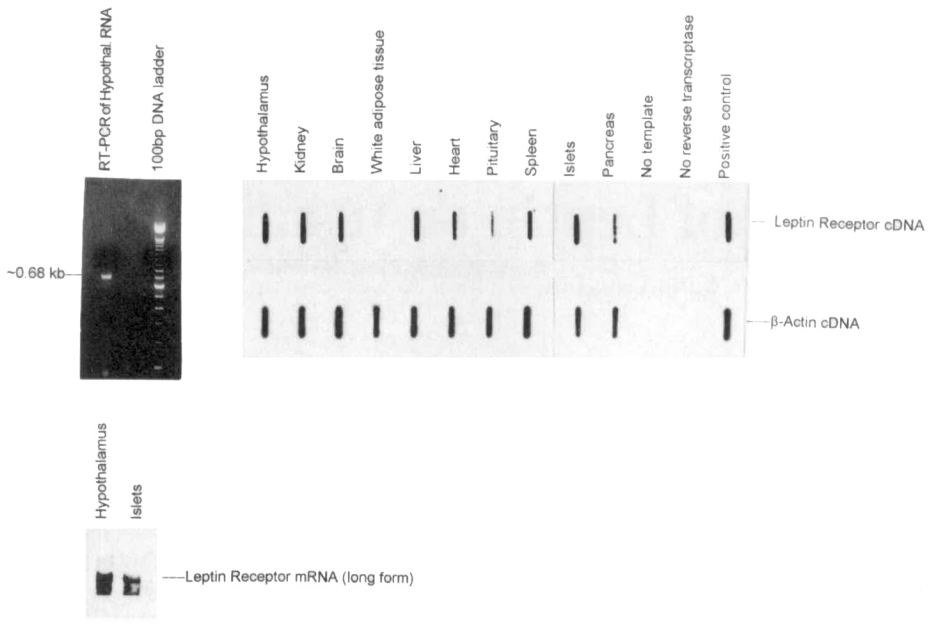

FIG. 1. ***A:*** **the RT-PCR product, a ~0.68-kb fragment (bases 2786–3464), from hypothalamus (left) together with ~0.68-kb products from other tissues of the wild-type mouse was hybridized with an internal DIG-labeled 34-mer oligodeoxyribonucleotide probe (right). Loading was assessed by a 0.54-kb β-actin cDNA probe.** ***B:*** **Northern blot analysis of Ob-Rb mRNA expression in hypothalamus and pancreatic islets of *ob/ob* mice using the ~0.68-kb fragment as probe, showing hybridization to a single band between 3.6 and 3.9 kb in size. No cross-hybridization to the 28S and 18S rRNA bands was detected.**

Rb cDNA. This probe is specific for the Ob-Rb sequence and has no significant homology with any other gene sequence, including the other leptin receptor isoforms. RT-PCR–amplified products from other tissues were also identified by the 34-mer probe. The mouse β-actin sequence was amplified under similar conditions (30 cycles) and used as a loading control. The Ob-Rb 0.68-kb cDNA and 0.54-kb β-actin cDNA probes were labeled with DIG-UTP by PCR amplification. Hybridization against the DIG 5′-end–labeled oligodeoxyribonucleotide probes was carried out (12), and DIG-labeled hybrids were detected with a polyclonal antibody conjugated to alkaline phosphatase together with the chemiluminescent substrate CDP-Star [(disodium 2-chloro-5-(4-methoxyspiro (1,2-dioxetane-3,2′-(5′-chloro)-tricyclo(3.3.1.1)decan)-4-yl)-1-phenyl phosphate] (Tropix).

Preparation of leptin. Murine leptin expressed in *Escherichia coli* with NH_2-terminal tags was extracted, purified, and refolded, and the NH_2-terminal tags were removed by treatment with enterokinase followed by size-exclusion chromatography. Murine leptin was reformulated by buffer exchange into Dulbecco's A phosphate-buffered saline (PBS) and sterilized through a 0.2-μm Millipore filter. The amino acid sequence was confirmed by trypsin digestion followed by peptide mapping and identification of individual peptides using automated Edman sequencing, electrospray mass spectrometry, and matrix laser desorption ionization time and flight spectrometry. The material was ascertained to be endotoxin-free by the Limulus amoebocyte lysate test (11). The purity was >95%. This leptin preparation had previously been shown to inhibit food intake when given either centrally or peripherally (11).

Perfused pancreas. Female *ob/ob* mice (aged 10–16 weeks) were anesthetized by an intraperitoneal injection of sodium pentobarbitone (60 mg/kg body wt). The pancreas was ligated essentially, as described previously for rat pancreas (13), except that the coeliac trunk was ligated rather than the superior mesenteric artery and a catheter was placed in the aorta, which was connected to two syringe-type infusion pumps via a T-piece. The pancreases were perfused with 5.6 mmol/l glucose at 37°C with a flow rate of 0.6 ml/min. A 10- to 15-min postsurgery stabilization period was allowed before collecting fractions. After a further 15-min control perfusion period, the perfusion medium was switched to include either 100 nmol/l recombinant murine leptin (endotoxin-free) dissolved in PBS in perfusion buffer or perfusion buffer containing an equivalent amount of PBS for a 15-min period. The perfusion medium was then switched back to the basal medium for another 5 min. Portal vein samples were collected at 1-min intervals into tubes on ice containing 240 KIU aprotinin (Traysylol, Bayer, U.K.). The insulin content of fractions was determined by radioimmunoassay (13). Zucker (*fa/fa*) rats (aged 24–28 weeks) were perfused as described previously (13).

Isolated islets studies. Female *ob/ob* and +/+ mice aged 7–8 weeks were fasted overnight. Pancreatic islets were isolated after collagenase (2 mg Sigma type V plus 2 mg Worthington type IV in 10 ml Hanks' balanced salt solution) digestion of chopped pancreases. Islets were cultured for 24 h in RPMI 1640 (Gibco/BRL) supplemented with 5.6 mmol/l glucose, 10% fetal calf serum (TCS biologicals, Buckingham, U.K.), and 1% penicillin/streptomycin. Batches of 10 islets were preincubated for 30 min in Krebs-Ringer buffer (KRB) containing 5.6 mmol/l glucose and then incubated for 2 h in either KRB containing 5.6 mmol/l glucose (basal) or KRB plus 16.7 mmol/l glucose (glucose-stimulated) with or without murine recombinant leptin (1–100 nmol/l). After removal of medium, the islets were washed and reincubated in KRB containing 5.6 mmol/l glucose and 20 mmol/l arginine for a further 2 h. Similar studies were undertaken on male and female *db/db* mice, aged 6–8 weeks, except they were not fasted before the preparation of islets.

Statistical analysis. Student's *t* test was used to test the significance of differences between control and leptin-treated parameters.

RESULTS

Expression of leptin receptor (Ob-Rb) in lean and *ob/ob* mice. A ~0.68-kb cDNA sequence that specifically encodes the cytoplasmic domain of the leptin receptor was used to assess expression of Ob-Rb mRNA in tissues of lean and obese (*ob/ob*) mice. The transcript was expressed at a high level in hypothalamus, in agreement with previous studies (7,14). By the use of RT-PCR followed by DNA-specific hybridization, we detected Ob-Rb expression in several tissues, including pancreatic islets, but not in white adipose tissue (Fig. 1). Using the less sensitive RNA blot hybridization, the Ob-Rb transcript was found to be present in liver, brain, kidney, and pancreatic islet, weakly expressed in spleen and heart, but not detected in white adipose tissue, pituitary, or whole pancreas (data not shown). These findings on tissue expression are in general agreement with previous studies (14). Our failure to detect expression in whole pancreas could be due to the fact that the RNase protection assay used previously (14) is several-fold more sensitive than RNA blot hybridization. However, isolation of islets (1–2% of total pancreas) from pancreas allowed us to readily detect expression of the Ob-Rb transcript. Northern blot hybridization shows expression of the Ob-Rb transcript in hypothalamus and islets from *ob/ob* mice (Fig. 1).

Effect of leptin on basal insulin secretion in hyperinsulinemic rodents. Leptin (100 nmol/l) significantly inhibited the rate of insulin release in the perfused pancreas of *ob/ob* mice (Fig. 2), but had no effect in the perfused pancreas of hyperinsulinemic Zucker *fa/fa* rats (Fig. 3).

Effect of leptin on glucose-stimulated insulin secretion by isolated islets. Leptin (1–100 nmol/l) inhibited in a dose-dependent manner the stimulatory effects of 16.7 mmol/l glu-

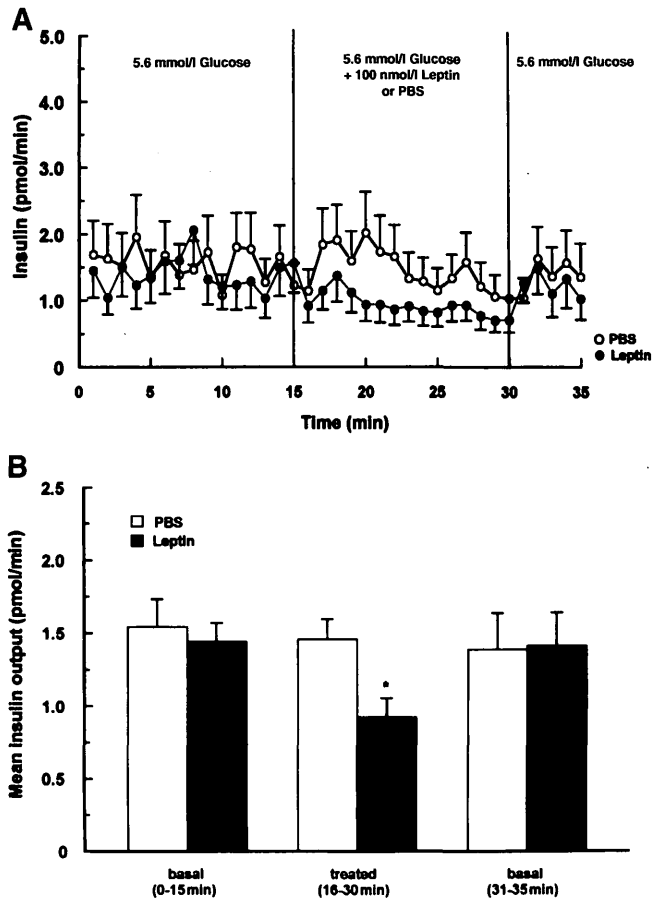

FIG. 2. Effects of leptin (100 nmol/l) on basal insulin release from the isolated perfused pancreas of *ob/ob* mice. The mean insulin output per minute was calculated as the mean value of each perfusion period (n = 4 for PBS-treated; n = 5 for the leptin-treated group). *P < 0.05 (unpaired Student's t test).

cose on insulin secretion by isolated islets from *ob/ob* mice (Fig. 4). The dose of leptin producing a half-maximal inhibitory response was ~10 nmol/l. This concentration of leptin produced a similar inhibitory effect on glucose-stimulated insulin secretion in islets from wild-type mice (basal insulin secretion, 0.25 ± 0.07 pmol/2 h from 10 islets; glucose-stimulated, 0.68 ± 0.02; glucose-stimulated in presence of 10 nmol/l leptin, 0.46 ± 0.04; P < 0.005; 29% inhibition) as it did in islets from *ob/ob* mice. However, leptin (100 nmol/l), which induces a maximal inhibitory effect in *ob/ob* islets, had no inhibitory effect on glucose-stimulated insulin secretion in islets from *db/db* mice. Irrespective of genotype, there was no significant difference in the arginine-stimulated insulin secretion (four- to sixfold relative to basal) in relation to whether or not the mice had been exposed previously to leptin.

DISCUSSION

Previous studies have demonstrated that daily intraperitoneal injections of recombinant leptin for 28 days produced a significant dose-dependent reduction in serum insulin and blood glucose in *ob/ob* but not lean mice (5). Murine leptin also reduces body weight and plasma insulin when given by intracerebroventricular injection to rats fasted for 24 h (11). These studies did not indicate whether the effect on insulin was secondary to an effect on food intake and body weight or to a direct effect on the endocrine pancreas. However, further studies using pair-fed mice have demonstrated that leptin induces a significantly greater weight loss than that produced in pair-fed mice and that plasma insulin concentrations are normalized (*ob/ob* control, 30.6 ± 6.2 ng/ml; leptin-treated *ob/ob*, 0.09 ± 0.08 ng/ml; pair-fed mice, 14.2 ± 4.2 ng/ml) (6). These latter results strongly indicate that the effect on plasma insulin is not solely through a reduction in food intake and body weight.

In the present study, we have provided evidence that leptin can directly reduce insulin secretion. First, we demonstrate that mRNA encoding the putative functional form of the receptor is present in isolated islets of lean and *ob/ob* mice. Second, we have shown in perfused pancreas studies that leptin inhibits basal insulin secretion in hyperinsulinemic *ob/ob* mice but not in Zucker *fa/fa* rats. Third, leptin inhibits glucose-stimulated insulin secretion in isolated islets from *ob/ob* and wild-type mice but not in those from *db/db* mice. It is unlikely that these effects on insulin secretion are through a toxic contamination, since the leptin used was endotoxin-free and both the perfused pancreas preparation and the islets showed a normal functional response on removal of leptin. The identification of the long-form Ob-Rb mRNA expression in pancreatic islets together with a lack of activity of exoge-

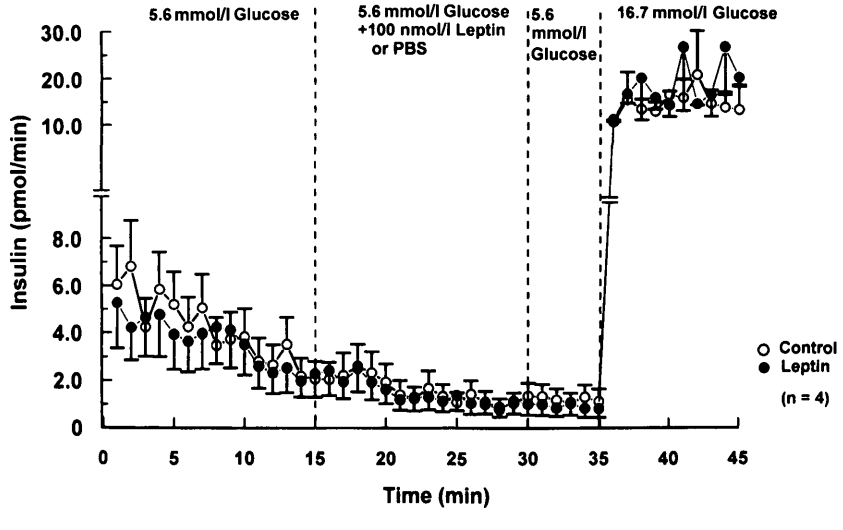

FIG. 3. Effects of leptin (100 nmol/l) on basal insulin release from the isolated perfused pancreas of obese Zucker *fa/fa* rats. Results are expressed as means ± SE (n = 4).

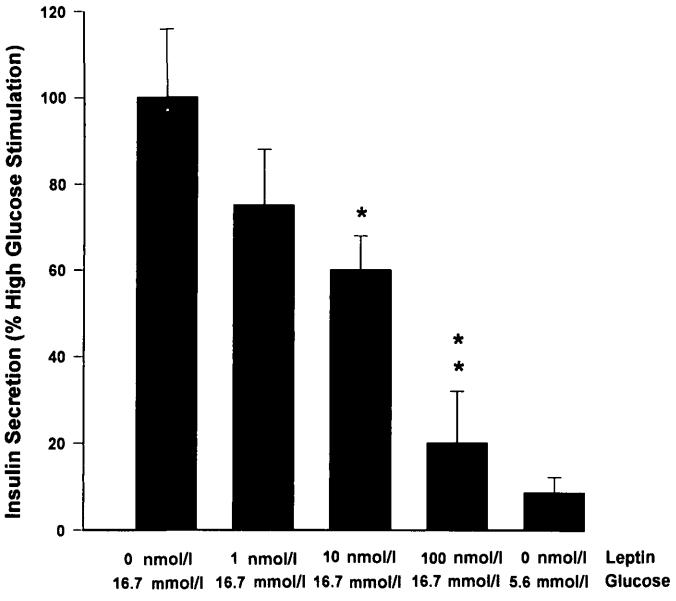

FIG. 4. Dose-dependency of inhibitory effects of recombinant leptin on glucose-stimulated (16.7 mmol/l) insulin secretion from isolated pancreatic islets of *ob/ob* mice. Data are expressed as percentages of mean high glucose control ± SE (n > 10). Basal rate of insulin secretion is 0.45 ± 0.19 pmol insulin/2 h from 10 islets, and the glucose-stimulated rate was 5.24 ± 1.57 pmol/2 h from 10 islets. Islet viability after test conditions was confirmed by washing and then restimulating with 20 mmol/l arginine. *P < 0.05; **P < 0.01.

nous leptin on the perfused pancreas from Zucker *fa/fa* rats and islets from *db/db* mice strongly suggests that these effects are receptor mediated.

The concentration of leptin (1–100 nmol/l) used in these in vitro experiments is somewhat higher than, although overlapping with, that measured in the plasma (0.1–5 nmol/l). Such an apparent discrepancy is found in the work of others (15,16). It is possible that recombinant leptin does not have the same potency as that produced endogenously, possibly as a result of posttranslational modification (15). In addition, there are indications that fetal calf serum contains leptin (16), and hence some downregulation of the receptor may occur under culture conditions. Finally, it is possible that portal vein concentrations of leptin are considerably higher than peripheral concentrations.

Obesity is strongly associated with NIDDM. The basis of this association has largely been assumed to relate to the increase in insulin resistance that occurs with developing adiposity. Insulin resistance results in glucose intolerance, but it is commonly believed that the development of diabetes requires the additional independent development of a pancreatic lesion. These data suggest that leptin overproduction, particularly in abdominal fat, may modify insulin secretion directly and could be involved in the development of the diabetic syndrome.

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