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Leptin Inhibits Insulin Secretion and Reduces Insulin mRNA Levels in Rat Isolated Pancreatic Islets

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The ob gene product leptin over the concentration range 0.1–100nM demonstrated a U-shaped dose–response inhibition of glucose-stimulated insulin secretion by rat pancreatic islets. Thus, leptin (1 and 10nM) produced a significant inhibition whereas 100nM was ineffective. The inhibitory effect of leptin was glucose dependent, had a rapid onset and was readily reversed upon removal of leptin. Sub-chronic exposure of islets to leptin (10nM) reduced both insulin secretion and the level of insulin transcript. These findings support the hypothesis that excessive production of leptin by adipose tissue could play a role in the development of non-insulin dependent diabetes in obese subjects.

Leptin is the product of the ob gene expressed in adipose tissue and acts at the hypothalamus to reduce food intake and increase energy expenditure (1-4). In leptin-deficient *ob*/*ob* mice, the chronic administration of leptin reduces the circulating insulin concentration and this effect cannot be entirely accounted for by the reduction in food intake and body weight (1,2,5). These findings suggested that leptin might regulate insulin secretion directly and we have shown recently that the long form of the leptin receptor, Ob-Rb, which is the putative functional receptor (6,7), is present in pancreatic islets of *ob*/*ob* and wild-type mice (8).

Furthermore, leptin (1-100nM) inhibited glucose-induced insulin secretion in a dose-dependent manner (8). The inhibitory effect of leptin on insulin release in islets from *ob*/*ob* has recently been confirmed (9) and it does not occur in islets from *db*/*db* mice that lack a functional Ob-Rb receptor (8).

Although both the generic form of the leptin receptor mRNA (10) and the isoform that contains the signal transduction motif in the cytoplasmic domain (11 and present study) have both been identified in rat pancreatic islets, the former authors (11) failed to demonstrate an effect of leptin on insulin secretion and argued against a role for leptin in regulating insulin secretion. In order to determine whether there are species differences in leptin action on insulin secretion we have now investigated the effect of leptin on glucose-stimulated insulin secretion and insulin transcript levels in isolated pancreatic islets from rats.

MATERIALS AND METHODS

Isolation of islets. Male Wistar rats (200-250g) were obtained from Harlan-Olac (Bicester, UK) and housed on a 12h light/12h dark cycle, with free access to water and diet (Beekay rat and mouse toxicology diet, Bantin and Kingman, Hull, UK). Islets were isolated from excised pancreata of ad-lib fed rats by collagenase digestion. The isolation medium was physiological saline solution (12) gassed with O_2/CO_2 (95:5) to pH 7.4, supplemented with $CaCl_2$ (1mM) and glucose (4mM). Islets were selected using a binocular microscope and used within 1h of isolation.

Secretion studies. Recombinant murine leptin, having a purity of greater than 95% by SDS-PAGE and N-terminal sequence analysis, was obtained from Peprotech, UK and in parallel experiments was identical in action to leptin obtained previously from SmithKline Beecham and used in an earlier study (8). It contained less than 0.1ng endotoxin per μg murine leptin. For static incubations, groups of 10 islets were incubated in 0.5ml of the isolation medium containing glucose, bovine serum albumin (1mg/ml) for 2h at 37°C. The insulin content of the incubation medium was determined by radioimmunoassay (13). For perifusion studies, groups of 80 islets per channel were perifused at 37°C (flow rate 1ml/minute) with isolation medium supplemented with bovine serum albumin (1mg/ml) and continuously gassed with O_2/CO_2 (95:5) pH 7.4. Leptin was introduced at a flow rate of 10μl/min to give a final concentration in the perfusate of 10nM. Samples of medium were collected at 2 minute intervals and the insulin concentration determined by radioimmunoassay (13).

Identification of leptin-receptor transcripts. Total RNA was isolated from 100 pancreatic islets from Wistar rats using RNaid plus kit (BIO 101 Inc., USA) and the RNA treated immediately with DNase I (Gibco/BRL, UK). The integrity of the treated RNA was examined by detection of ribosomal RNA bands (28S and 18S) in ethidium bromide stained agarose gels. RNA from 25 islets was used to generate cDNA with Superscript II reverse transcriptase (RT) (Gibco/BRL, UK) and oligo $(dT)_{18}$. cDNA from approximately 0.3 to 3 islets was used for polymerase-chain reaction (PCR) amplification

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of Ob-R transcript isoforms. The primers used for the PCR amplification of the common extracellular domain of OB-R (465bp; 275-740; GenBank U60151) were, 5′-GGAATGAGCAAGGTCAA-AAC-3′ (sense), 5′-GTGACTTCCATACGCAAACC-3′ (antisense), and the cytoplasmic domain of the long Ob-Rb isoform (399bp; 2761-3160; GenBank U60151), 5′-AGTGTCGATACAGCTTGG-3′ (sense), 5′-CTGAGAATGAAAGTTGTGG-3′ (antisense). As a control for the RT-PCR, a 1kb glyceraldehyde 3 phosphate dehydrogenase fragment was amplified also using commercially available primers (Clontech, USA). PCR products were then sequenced directly or cloned first into a PCR-TRAP cloning system (GeneHunter Corp., USA) and the identity then confirmed by sequencing using ThermoSequenase terminator cycle sequencing kit (Amersham Life Sciences, UK). Expression of insulin transcripts is normalized to the expression of poly(A) mRNA as assessed by hybridization with oligo $(dT)_{21}$ at 25°C on slot blots.

Sub-chronic effect of leptin on insulin secretion and the level of insulin transcript in rat islets. Islets were isolated as described above, washed and pre-incubated for one hour in serum-free RPMI-1640 supplemented with bovine serum albumin (1mg/ml), 5.6mM glucose, 2mM L-Glutamine, 100U/ml penicillin, 100μg/ml streptomycin at 37°C in a 5% CO_2/air cabinet. Batches of 100 islets were then transferred to serum-free medium with bovine serum albumin (1mg/ml), 16.7mM glucose containing 10nM leptin or an equal volume of 10mM Tris control vehicle. At the end of incubation, islets were collected by gentle centrifugation and put on ice. Supernatant was removed and used to determine insulin secretion by radioimmunoassay. Islets were gently resuspended in 100μl Hanks Balanced Salt Solution containing 2mM dithiothreitol and 150 units/ml Human Pancreatic Ribonuclease Inhibitor (Amersham, UK) before RNA isolation was performed. Standard northern blotting was performed by capillary action using Hybond N^+ membranes (Amersham, UK) with a digoxigenin-labelled PCR product amplified from a pGEMZ vector containing the Rat I insulin cDNA, kindly provided by Dr. S. J. Chan (University of Chicago). Detection was carried out according to manufacturers guidelines using digoxigenin-antibody system (Boehringer-Mannheim) and CDP-STAR chemiluminescent substrate (Tropix, USA).

RESULTS

Effect of leptin on glucose-stimulated insulin secretion. Leptin (0.1-100nM) inhibited glucose-stimulated (20mM) insulin secretion by isolated islets, but not in a dose-dependent manner. Thus, both 1nM and 10nM leptin produced a significant inhibitory response ($P<0.05$) but at the higher concentration of leptin (100nM), there was not a significant inhibition (Fig. 1). Perifusion studies demonstrated that leptin (10nM) inhibited insulin secretion significantly within 5 minutes and removal of leptin from the perifusate led to an equally rapid reversal of the inhibitory response (Fig. 2). The glucose dependency of the inhibitory effect of leptin was assessed in static incubations using glucose concentrations from 4-24mM. Leptin only inhibited insulin secretion significantly at glucose concentrations $\geqslant$ 16mM (Fig. 3).

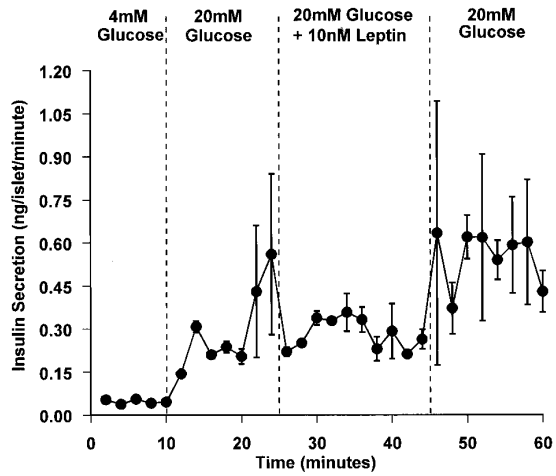

FIG. 2. Kinetics of the effect of leptin (10nM) on glucose-stimulated (20mM glucose) insulin secretion from perifused isolated rat islets. (n = 2). Data are expressed as insulin secretion (ng/islet/min.).

Expression of Ob-R isoforms in rat islets. The mRNA expression of the common Ob-R and the Ob-Rb

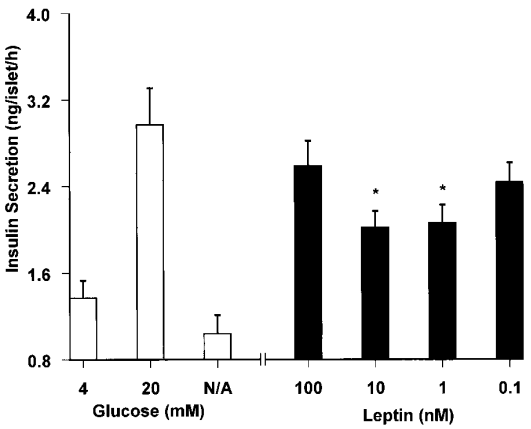

FIG. 1. Dose dependent effect of leptin L (100nM–0.1nM) on glucose-stimulated (20mM) insulin secretion from isolated rat islets of langerhans. Noradrenaline (N/A) was used as a positive control. Data are expressed as insulin secretion (ng/islet/h). n = 15–17 observations, *$p<0.05$.

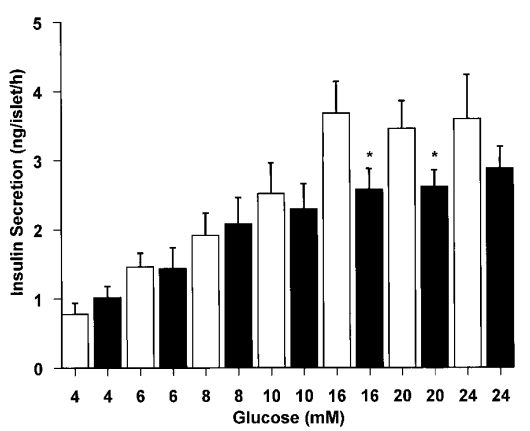

FIG. 3. Effect of leptin (10nM) on glucose dependent insulin secretion. Insulin release in the presence (solid bars) or absence (open bars) of leptin (10nM) was determined using glucose concentrations ranging from 4mM to 24mM. Data are expressed as insulin secretion (ng/islet/h). *$p<0.05$.

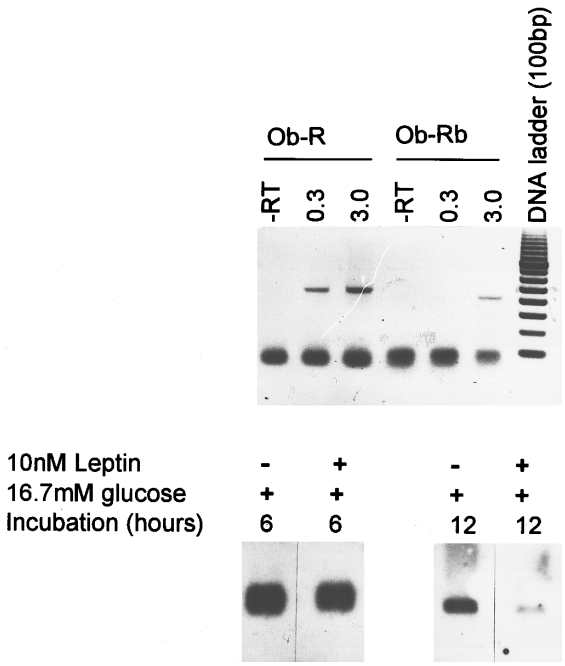

FIG. 4. Identification of the Ob-R and Ob-Rb mRNA expression and leptin-induced inhibition of insulin gene transcription in rat pancreatic islets. Upper panel: RT-PCR amplification of the common extracellular domain Ob-R and the Ob-Rb isoform from 0.3 and 3 islets. Lower panel: Northern blot analysis of the effect of 10nM leptin on the accumulation of insulin gene transcript at different time (hours) of incubation, together with untreated control.

isoform was readily detected by RT-PCR amplification of cDNA from approximately 3 islets, but was undetectable in controls (-RT) (see Fig. 4). When 10-fold less cDNA was used, the Ob-Rb is not detectable whilst the common Ob-R extracellular domain was still readily visualized by ethidium bromide staining (Fig. 4). Both PCR products were sequenced and found to be identical to the corresponding domain of the rat leptin receptor (GenBank U60151).

Sub-chronic effect of leptin on insulin secretion and levels of insulin mRNA. Isolated islets were incubated in high glucose (16.7mM) medium with or without leptin (10nM) for 6 and 12h. At 6h, the leptin-treated islets expressed lower levels of insulin transcript compared to high glucose controls, however, this difference was not significant. At the 12h time point, islets treated with 10nM leptin exhibited a 3 fold reduction in insulin transcript levels compared to high glucose controls ($p<0.001$, Figs. 4 & 5). Furthermore, inhibition of insulin secretion by 10nM leptin was enhanced 2 fold during exposure to leptin from 2 to 6 hours (Fig. 5).

DISCUSSION

There is clear evidence that chronic administration of leptin to leptin-deficient *ob/ob* mice produces a reduction in hyperinsulinaemia that cannot be entirely accounted for by changes in food intake and body weight (1-4), suggesting that leptin might inhibit insulin secretion directly. In an earlier study evidence to support this notion was provided (8). We showed that leptin inhibited basal insulin secretion by the perfused pancreas of the *ob/ob* mouse and that leptin induced a dose-related inhibition of glucose-stimulated insulin secretion in islets from *ob/ob* mice. This latter finding has recently been confirmed (9). In contrast, Leclercq-Meyer *et al* (11) failed to demonstrate an effect of leptin on insulin secretion by the perfused rat pancreas raising the possibility of a species difference in leptin action. In the present study, leptin (1 and 10nM) rapidly inhibited glucose-induced insulin secretion by isolated rat pancreatic islets, at glucose concentrations greater than 16mM (Figs. 1 and 3). It is noteworthy that the earlier study (11) examined only a single concentration of recombinant leptin (1nM) at a glucose concentration of 8.3mM.

We found that the acute inhibitory effect of leptin was rapidly reversed upon withdrawal of leptin suggesting that the inhibitory action was not via a toxic mechanism (Fig. 2). Sub-chronic treatment of islets with leptin (10nM) for a 6h period led to a more pronounced percentage decrease in insulin secretion relative to that occurring during a 2h incubation.

In the present study we show that the Ob-Rb isoform, which contains the conserved box 1 and box 2 motifs responsible for leptin-induced signalling, is expressed in islets and we find that leptin exhibits a U-shaped dose-response in inhibiting glucose-stimulated insulin secretion. The leptin receptor belongs to the class 1 cytokine receptor family and shows the highest similarity to gp 130 (6). Receptors of this class lack intrinsic tyrosine kinase activity and are activated by ligand-induced receptor homo- or hetero-dimerization or oligomerization prior to activation of receptor associated kinases of the Janus family (JAK) (14). The leptin receptor oligomerizes with itself (15,16,17) and not with gp 130. Thus high concentrations of leptin (100nM) may prevent oligomerization inducing desensitization. A similar shaped dose response curve was noted in studies (18) to examine leptin action on luteinizing hormone release from rat anterior pituitary.

We have shown also that leptin at a high physiological concentration (10nM) reduces the level of insulin mRNA. In most circumstances, stimulation of the JAK/STAT signalling pathway leads to DNA binding and positive transcriptional activation (19). However, there are examples of STAT binding leading to reduced transcription (20). Alternatively, leptin treatment may lead to post-transcriptional effects such as increased insulin mRNA turnover.

In man, the circulating leptin concentration is positively correlated with body fat mass (21). It is well established that the risk of developing non-insulin de-

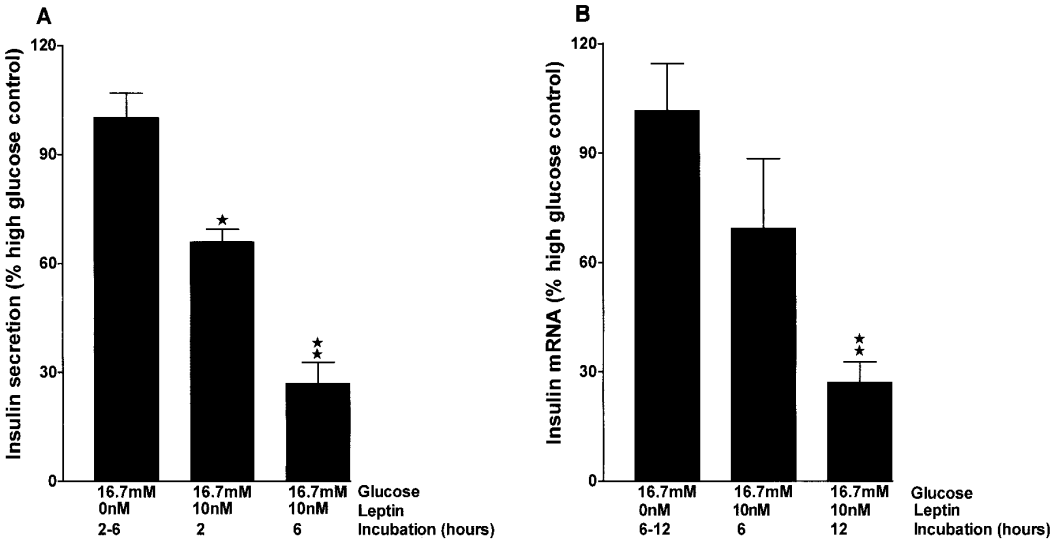

FIG. 5. Quantitation of the sub-chronic effect of 10nM leptin on insulin secretion and insulin mRNA levels in rat islets. A. Sub-chronic effect of 10nM leptin on insulin secretion for the indicated time of incubation (hours). Results are expressed as mean±S.E.M; (n=3) *p<0.05, **p<0.001. B. Effect of 10nM leptin on insulin mRNA level at different times of incubation (hours). Results (arbitrary units) are expressed as mean±S.E.M; n=3. **p<0.001.

pendent diabetes is strongly linked to increased adipose tissue mass. Here we show that leptin concentrations in the high physiological range both inhibit insulin secretion and insulin transcript levels. Thus excessive release of leptin from adipose tissue might be a trigger that precipitates diabetes in obese subjects.

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