

Enhanced leptin synthesis and secretion *In vitro* by human adipocytes from type 2 diabetic donors occurs only under hyperinsulinaemic conditions

Abstract

Leptin is 16kDa, 146 amino acid residue peptide hormone that is thought to play a central role in the regulatory role of adipose tissue on energy metabolism. Leptin is synthesised by adipocytes and released to the circulation, where it acts as an afferent signal to the central nervous system, relaying information about the degree of adiposity to the hypothalamus. Hyperthalomic leptin resistance is associated with obesity and an increased risk of type 2 diabetes and hyperinsulinemia. However, the direct effects of hyperinsulinaemia on leptin secretion by adipocytes remain unclear. The aim of this study was to examine leptin secretion *In vitro* by adipocytes from type 2 diabetic and normal donors under normoinsulinaemic and hyperinsulinaemic conditions. The results of this study showed that for normal adipocytes, exposure to 10 and 100ng/ml insulin for 72hours caused an increased leptin secretion of 5.68 ± 0.82 pg/ml/ 10^4 cells ($P < 0.001$) and 16.21 ± 2.72 pg/ml/ 10^4 cells ($P < 0.001$) respectively. For type 2 diabetic adipocytes, however, 72hours insulin exposure at 10 and 100ng/ml produced non-significant and 3.10 ± 0.36 ng/ml/ 10^4 cells ($P < 0.039$) levels of leptin secretion. Following exposure for 120 hours diabetic adipocytes exposed to 10 and 100ng/ml insulin showed increased leptin secretion of 25.92 ± 1.74 ng/ml/ 10^4 cells ($P < 0.001$) and 38.56 ± 2.48 ng/ml/ 10^4 cells ($P < 0.001$) respectively. These values were significantly greater than those for normal adipocytes ($P < 0.003$ and $P < 0.013$ respectively). These results suggest that both normal and supraphysiological insulin concentrations directly stimulate secretion of leptin by adipocytes from both normal and type 2 diabetic donors, but the effect is greatest for type 2 diabetic adipocytes following extended periods of supraphysiological insulin treatment. The relationship between insulin and leptin concentrations in obesity and type 2 diabetes warrants further investigation to uncover potential therapeutic targets.

Keywords: adipocyte, type 2 diabetes, insulin, leptin, adipokines, hormones, steroidogenesis

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Abbreviations: AP, alkaline phosphatase; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PTEN, phosphatase and tensin homolog; TBS, tris buffered saline

Introduction

Prior to the last two decades adipose tissue was often regarded as simply a storage site for excess energy. More recently, however, it has emerged as a complex endocrine organ; the full complexity of which is yet to be understood. Research has shown that adipocytes are capable of secreting many varied bioactive peptides and proteins called adipokines. These act as autocrine, paracrine and endocrine signalling molecules.^{1,2,3}

A greater understanding of the importance of adipose tissue in whole body homeostasis as well as its effects on particular physiological processes has occurred with the discovery of roles for adipose tissue in inflammation, blood pressure regulation, lipid metabolism, insulin sensitivity and coagulation.⁴ In particular, irregularities in adipokine secretion may be associated with weight gain since adipokine dysfunction has been shown to cause dysfunction in the energy regulatory mechanisms of the body.^{2,5,6}

The adipokine leptin contributes to an endocrine circuit that regulates energy homeostasis.^{7,8} It activates receptors in the hypothalamus to modulate the release of the anorexigenic and

exogenic peptides, and in this regard leptin acts as an afferent signal in a negative feedback loop from adipocytes to the hypothalamus that regulates adiposity in mammals.⁹ Changes to the normal physiological regulation of leptin may therefore be expected to lead to weight gain and obesity, and may have also other physiological consequences in other hormonal circuits. Understanding the causative factors that may affect the production, availability and function of leptin is therefore important in order to understand and potentially treat obesity and its consequences.

The role of leptin in obesity has been shown to involve complex interactions with other adipokines and insulin resistance.^{10,11} Kolaczynski et al.¹² found serum leptin concentrations to be correlated with body mass index (BMI) and also to be raised in response to long-term hyperinsulinaemia *in vitro*. Additional rodent studies have suggested that high leptin concentrations result in decreased hypothalamic sensitivity to leptin.¹³ Such changes to the insulin and leptin system are characterized as *obesity-associated insulin resistance*, a condition that is associated to the onset of type 2 diabetes.¹⁴

Although the development of high circulatory leptin levels may be affected by decreased hypothalamic sensitivity, no examination of leptin synthesis and secretion by adipocytes taken from diabetic donors has been undertaken under hyperinsulinaemic conditions. Such an examination is important because it may show a direct influence of insulin on leptin secretion by adipocytes, and this may contribute

to the high circulatory levels seen in obesity. Furthermore, the identification of raised insulin as a therapeutic target for the reversal of the raised levels of leptin seen in obesity could consequently provide a mechanism to reverse hypothalamic leptin insensitivity. We therefore examined the interaction that may exist between insulin and leptin at the level of the adipocyte by measuring the effects of insulin treatment on leptin secretion by adipocytes derived from normal and from diabetic donors which have been shown to exhibit insulin resistance *in vitro*.¹⁵ A study *in vitro* was designed to avoid potential confounding influences from other factors that have been shown to enhance leptin secretion from adipocytes such as angiotensin II,¹⁶ ghrelin,¹⁷ oleic acid¹⁸ and Wnt pathway activation.¹⁹

Materials and methods

Cell culture

Murine 3T3-L1 cells (kindly donated by Nottingham University, UK) and pooled human preadipocytes from normal and type 2 diabetic donors (Lonza) were maintained in 25cm² tissue culture flasks at 37°C in RPMI 1640 medium supplemented with 10% foetal bovine serum and with a gas phase of 3% CO₂ in air. Cell suspensions were prepared by trypsinisation and viable cells (those excluding 0.2% trypan blue) were counted in a Neubauer haemocytometer. Normal and type 2 diabetic human adipocytes were compared for glucose uptake and differentiation following passage to ensure the phenotype was retained. Murine 3T3-L1 cells cultured in medium supplemented with 10ng/ml insulin¹⁶ were used as positive controls. For leptin secretion assays 5x10³ normal or type 2 diabetic human preadipocytes were plated into the wells of triplicate 24 well plates in 300µl of medium. Differentiation of human preadipocytes was induced by adding medium supplemented with 200µM indomethacin, 3-Isobutyl-1-methylxanthine, 0.1µM dexamethasone, 1µM insulin, 1% L-Glutamine, 10% foetal bovine serum and GA-1000 (Lonza). After 10days, when oil red O staining showed lipid droplet formation,²⁰ the medium was replaced with medium containing 0, 10 or 100ng/ml insulin and the cells were incubated for a further 0, 4, 8, 24, 48, 72 or 120hours prior to removing both the conditioned medium and harvesting the cells to form lysates. Leptin was estimated in the medium using ELISA and in the lysates by western blotting.

ELISA

Leptin in cell culture medium was measured using the Human Leptin Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions. Absorbance was measured at 570nm and the range of sensitivity of the kit was 1.56-100pg/ml/10⁴ cells.

Western blotting

To prepare lysates cell monolayers were washed with phosphate buffered saline (PBS) and harvested by trypsinisation. Cells were counted, centrifuged at 250 x g for 10minutes at 4°C and re-suspended in lysis buffer (25mM Tris pH 7.5, 300mM sucrose, 10mM monoethyglycerol, 1 mM EDTA, 1%(v/v) Igepal, 0.002% protease inhibitor cocktail, 0.01% phosphatase inhibitor II (Sigma)). Lysates were stored at -80°C. Proteins were resolved using 10% polyacrylamide gel electrophoresis (PAGE). The Bradford method²¹ was used to determine protein concentration in cell lysates and each lane of the gel was loaded with 100g of sample protein taken from treated normal or type 2 diabetic human adipocytes, or control cells. 10µl of Kaleidoscope pre-stained molecular weight markers (Sigma) was used to facilitate protein size determination. Electrophoresis was

conducted in 25mM Tris, 19mM glycine buffer for 1hour at 40mA.⁶ Proteins were then electroblotted onto nitrocellulose membranes in ice cold transfer buffer (25mM Tris, 80mM glycine, 20% (v/v) methanol) using a Miniprotean blotting system (Bio-Rad) at 100v⁶ for 2hours.

Membranes were then blocked by immersion in 5% dried milk in 25mM Tris, 150mM NaCl (TBS) for 1hour. Anti-leptin antibody (antiOB, R&D Systems) was then added and membranes were incubated overnight at 4°C, washed (10minutes x6) with 0.1% Tween in TBS followed by washes (10minutes x 3) with TBS. Membranes were then incubated with alkaline phosphatase (AP) conjugated secondary antibodies (Bio-Rad) in 3% dried milk in TBS for 2hours at room temperature with gentle agitation. The membrane was washed again as described above and visualized by incubating (10minutes) with Immuno-Star AP Chemiluminescence enhancer (50µl) and substrate solution (2.5ml, Bio-Rad) evenly coating the nitrocellulose blot. Blots were stripped using glycine buffer (200mM glycine pH 2.2, 0.1% sodium dodecyl sulphate, 1% Tween 20) prior to treatment with anti-β-actin antibody (Sigma). Results were analyzed by densitometry and normalised using a Chemi Doc (v. 4.2.1) imaging system²² with β-actin as reference.

Data and statistical analysis

All experiments were repeated at least once on different occasions and data are presented from typical experiments involving five replicates. Data sets were subjected to Shapiro-Wilk normality tests and expressed as mean±s.d. Subsequent comparisons were made using two tailed Student's *t*-test with Bonferroni correction. Values of P<0.05 were considered significant. Graph Pad Prism software (v. 5.0b) was used.

Results

Increased leptin production in response to insulin was not observed at time points earlier than 72hours. Data are therefore presented for 72 and 120hours treatments only.

Effect of insulin on leptin secretion from adipocytes from normal human donors

ELISA showed that insulin treatment of normal human adipocytes for 72hours caused an increased leptin secretion of 5.68±0.82pg/ml/10⁴ cells (P<0.001) at a physiological concentration (10ng/ml) and of 16.21±2.72pg/ml/10⁴ cells (P<0.001) at a supraphysiological concentration (100ng/ml, Figure 1). Insulin treatment of these cells for 120hours increased leptin secretion approximately a further twofold at both physiological (12.76±1.04pg/ml/10⁴ cells, P<0.001) and supraphysiological (26.94±0.95pg/ml/10⁴ cells, P<0.001) concentrations (Figure 1).

Effect of insulin on leptin secretion from adipocytes from human type-2 diabetic donors

ELISA showed that, unlike normal adipocytes, insulin treatment for 72hours of type 2 diabetic human adipocytes at a physiological concentration (10ng/ml) demonstrated no observable stimulation of leptin secretion. However, a supraphysiological insulin concentration (100ng/ml) for 72hours did increase significantly leptin secretion to 3.10±0.36pg/ml/10⁴ cells (P<0.039). Incubation periods of 120hours increased leptin secretion at least twelvefold at both physiological (25.92±1.74pg/ml/10⁴ cells, P<0.001) and supraphysiological (38.56±2.48pg/ml/10⁴ cells, P<0.001) insulin concentrations (Figure 2).

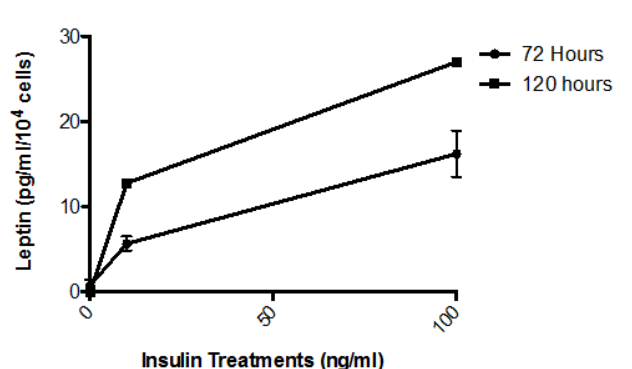


Figure 1 Effect of insulin on leptin secretion by normal human adipocytes.

Cells were treated with media supplemented with 0ng/ml, 10ng/ml or 100ng/ml insulin for 72 or 120hours. Leptin concentrations in the media were then determined by ELISA. Treatment at both insulin concentrations and times resulted in significant ($P<0.001$, $n=5$) increases in leptin secretion from basal levels. The insulin effect is seen to be both concentration dependent and time dependent. Error bars are omitted when their range would be smaller than the symbol used to indicate mean value.

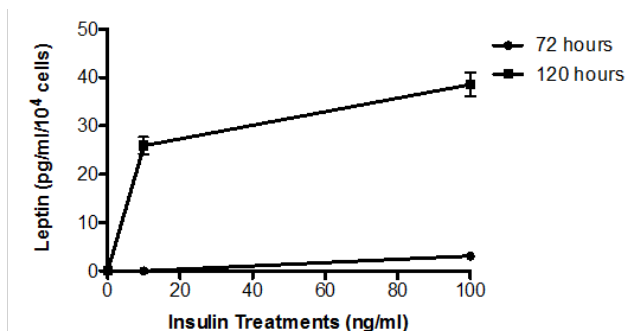


Figure 2 Effect of insulin on leptin secretion by type 2 diabetic human adipocytes.

Cells were treated with media supplemented with 0ng/ml, 10ng/ml or 100ng/ml insulin for 72 or 120hours. Leptin concentrations in the media were then determined by ELISA. Insulin treatment at 10ng/ml resulted in a significant ($P<0.039$, $n=5$) increase in leptin secretion from basal levels only after 120hours. Insulin treatment at 100ng/ml for both times resulted in significant ($P<0.001$, $n=5$) increases in leptin secretion from basal levels. The insulin effect is seen to be both concentration dependent and time dependent with the time dependency particularly marked ($P<0.001$, $n=5$ at both insulin concentrations). Error bars are omitted when their range would be smaller than the symbol used to indicate mean value.

Comparison of insulin effects on adipocytes from normal and type-2 diabetic samples

Leptin concentrations following 120hours of insulin treatment were examined in both culture media ($n=5$) and cell lysates ($n=3$) using ELISA and western blotting respectively. ELISA data (previously shown as Figure 1 & 2) showed that type 2 diabetic human adipocytes secreted significantly more leptin than normal human adipocytes following treatment with physiological (10ng/ml, $P<0.003$) and supraphysiological (100ng/ml, $P<0.013$) concentrations of insulin for 120hours. ELISA data for 120hour treatments were supported by western blot results showing increased leptin band intensity at this time point following treatment of both normal human and type 2 diabetic human adipocytes with insulin at physiological (10ng/ml, $P<0.001$) and supraphysiological (100ng/ml, $P<0.001$) concentrations (Figure 3).

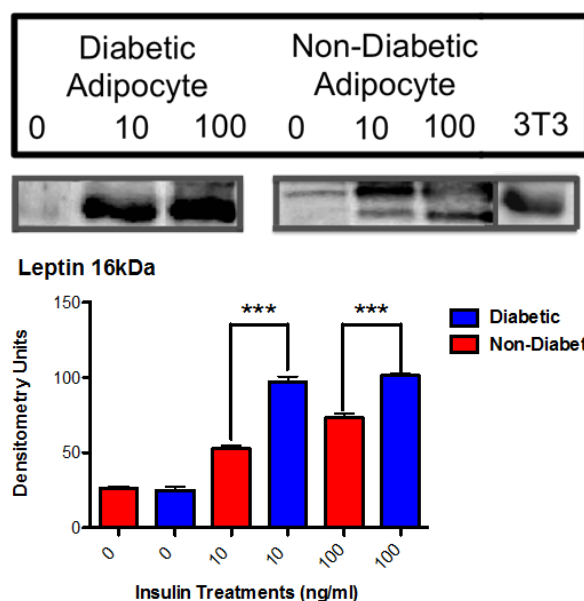


Figure 3 Effect of insulin on leptin concentrations in normal and type 2 diabetic human adipocyte cell lysates.

Cells were treated with media supplemented with 0ng/ml, 10ng/ml or 100ng/ml insulin for 120hours. Leptin production (16kDa MW) was then determined by densitometry of immunostained western blots of cell lysates. At both insulin concentrations type 2 diabetic adipocytes showed greater amounts of leptin compared to their non-diabetic counterparts ($P<0.003$, $n=5$, 10ng/ml; $P<0.001$, $n=5$, 100ng/ml).

Discussion

Starvation has been shown to decrease both plasma insulin and leptin concentrations in non-diabetic rodents.²³ Conversely, obesity in humans is often associated with hyperinsulinaemia and hyperleptinaemia^{12,24} with serum leptin concentrations correlated with BMI.¹² Since obesity is associated with the development of metabolic conditions such as type 2 diabetes,^{7,25} relationships between insulin and leptin secretion have become of great interest to researchers. In particular, leptin may be a therapeutic target to circumvent both obesity and diabetes. Cammisotto & Bukowiecki²⁶ found that insulin stimulated leptin expression and secretion in adipocytes *in vitro* and these results have been replicated *in vivo*.^{25,26}

While studies have shown that leptin secretion in the absence of diabetes is positively associated to insulin,^{12,27,28} others have produced conflicting results with Mac Dougald et al.⁹ and Sliecker et al.²⁹ both reporting no or very modest leptin rises following insulin treatment. To attempt to resolve these conflicting findings and to further extend the data to include adipocytes from type 2 diabetics, the present study investigated the effect of both physiological (10ng/ml) and supraphysiological (100ng/ml) concentrations of insulin on leptin secretion by adipocytes from normal human and type 2 diabetic human donors. The insulin concentrations chosen reflect typical values observed in the serum of non-diabetic and diabetic humans, and also those used in previous investigations of the role of leptin.^{12,27}

Our data showed leptin to be produced and secreted in an insulin dose dependent manner in both cell types. However, increasing incubation times with insulin 1.7-fold from 72 to 120hours resulted in an approximately 2-fold increase in the amount of leptin secretion by normal human adipocytes (Figure 1), but a more than 12-fold

increase for type 2 diabetic human adipocytes (Figure 2). A possible interpretation of these findings is a combination of

- i. The onset of secretion of leptin in the case of the diabetic adipocytes was delayed compared to their normal counterparts and
- ii. When stimulated, the rate of secretion of leptin by diabetic adipocytes was greater than their normal counterparts. The eventual amount of leptin secreted by type 2 diabetic human adipocytes at a supraphysiological insulin concentration was significantly higher than that secreted by normal adipocytes ($P < 0.013$).

These findings were obtained from determinations of leptin concentrations in cell culture supernatants using ELISA. They were mirrored by determinations of leptin in cell lysates using antibody staining of western blots (Figure 3). Overall, our data may provide some explanations for the previously described conflicting results concerning the reduced sensitivity of type 2 diabetic adipocytes to insulin; a phenomenon termed insulin resistance.^{30,31} If adipocytes of type 2 diabetics, compared to their normal counterparts, have

- i. a delayed response to insulin and
- ii. a wider range of eventual responsiveness to changes in insulin concentration, the extent of insulin treatment that they receive will have major effect on their observed cellular responses.

In this context Kolaczynski et al.¹² found that short-term hyperinsulinemia (74 to $>10,000\mu\text{U/ml}$; equivalent to approximately 3 to $>300\text{ng/ml}$) had no effect on circulating leptin concentration or levels of leptin *in vitro*. Similar results were also obtained by Haffner et al.,²⁷ but MacDougald et al.,⁹ found that insulin treatment of rats with induced diabetes increased leptin mRNA in adipose tissue to 60% of that of their non-diabetic counterparts within 4 hours. We propose support for the theory of Haffner et al.,²⁷ of a control mechanism for the regulation of leptin secretion, which is in place for the fasting or low circulatory insulin state. This typically leads to insulin sensitivity in adipocytes of non-diabetics.⁹ However, this mechanism seems to be delayed in our diabetic human adipocytes and results in hypersensitivity following prolonged exposure to insulin. The work of Kurlawalla-Martinez et al.,³² supports such a control mechanism and identified phosphatase and tensin homolog (PTEN) as a potential negative regulator of insulin sensitivity within the PI3K/Akt pathway because loss of PTEN in mutant mice models led to improved insulin sensitivity. Intracellular calcium may also be an important factor because it is upregulated by insulin,³³ which causes robust Akt phosphorylation leading to increased leptin secretion. It is possible that prolonged exposure of adipocytes to insulin may increase leptin secretion by a combination of such mechanisms. Furthermore, it has been suggested that a delayed effect of insulin treatment on leptin secretion in adipocytes is compatible with the effect being indirect¹² and additional physiological factors may be involved *in vivo* with strong candidates being hormonal³⁴ and inflammatory^{35,36} influences, and a role for the hypothalamus.²³ Should the delayed effect of insulin treatment on leptin secretion in adipocytes from type 2 diabetic donors be proven in subsequent studies, the phenomenon may be analogous to the biphasic response of insulin secretion to glucose³⁷ where impairment of early and late phase insulin secretion leads to different clinical outcomes.³⁸

A final point is that the contrasting findings regarding the effect of insulin on leptin secretion may reflect the different situations in which data has been collected. For example, the *ob/ob* mouse is perhaps the most commonly used model for investigating the metabolic effects of obesity including diabetes. These mice have a defect in the gene that

produces leptin and the administration of leptin corrects the deficiency in these animals. However, this contrasts with the observation that obese humans typically have high levels of leptin in their serum.¹² Furthermore, the few obese humans who do have leptin deficiency do not typically develop diabetes.³⁹

Conclusion

In conclusion our results suggest that insulin stimulates leptin secretion by both type 2 diabetic and non-diabetic adipocytes in a dose dependent manner. Secretion is also increased following extended insulin incubation. For normal human adipocytes there was a two-fold rise in leptin secretion between 72-120hours. For type 2 diabetic human adipocytes, however, shorter-term insulin incubation produced limited leptin secretion and significant increases (>12 -fold) occurred between 72-120hours. The data from diabetic human adipocytes contrast greatly with those obtained from normal human adipocytes and suggest that the cells from diabetic donors may have a delayed response to insulin and also a wider range of eventual responsiveness to changes in insulin concentration. We propose further examination of induced leptin secretion and subsequent PI3-Akt pathway activation following prolonged insulin incubation within type 2 diabetic human adipocytes and, in particular, the role of intercellular calcium in mediating these effects.

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Conflict of interest

Author declares that there is no conflict of interest.

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