

Leptin signalling in pancreatic islets and clonal insulin-secreting cells

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ABSTRACT

Leptin is a cytokine secreted from adipose tissue at a rate commensurate with the size of the body's fat stores. In addition to its anorectic and thermogenic central actions, leptin is known to act on peripheral tissues, including the pancreatic β -cell where it inhibits insulin secretion and reduces insulin transcript levels. However, the role of leptin signalling through its full-length receptor, OB-Rb, in the β -cell remains unclear. In the present study, we show that leptin activates a signal transducer and activator of transcription (STAT)3 signalling mechanism in pancreatic islets and in a rat model of the pancreatic β -cell, RINm5F. Leptin induced DNA binding to a STAT consensus oligonucleotide and resulted in transcriptional activation from STAT

reporter constructs in a manner consistent with STAT3 activation. Western blot analysis confirmed activation of STAT3 in RINm5F and isolated rat islets. Conditions that mimic increased metabolic activity resulted in attenuation of leptin-mediated STAT DNA binding but had no significant effect on STAT3 tyrosine phosphorylation in RINm5F cells. In addition, leptin activated the mitogen activated protein (MAP) kinase pathway in RINm5F cells. The present study provides a framework for OB-Rb signalling mechanisms in the programming of the β -cell by leptin and suggests that increased metabolic activity may modulate this function.

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INTRODUCTION

Leptin, a hormone secreted from white adipose tissue (Zhang *et al.* 1994) in direct relation to the size of total fat mass (Maffei *et al.* 1995), regulates body weight homeostasis through anorectic and thermogenic mechanisms (Campfield *et al.* 1995, Pelleymounter *et al.* 1995). In addition, it has now been demonstrated in many laboratories that leptin inhibits insulin secretion from mouse, rat and human pancreatic islets (Chen *et al.* 1997, Emilsson *et al.* 1997, Fehmann *et al.* 1997a, Kieffer *et al.* 1997, Kulkarni *et al.* 1997, Pallett *et al.* 1997, Ookooma *et al.* 1998, Poutout *et al.* 1998). Furthermore, leptin reduces insulin transcript levels in rat islets (Kulkarni *et al.* 1997, Pallett *et al.* 1997). The possible importance of leptin in control of β -cell function *in vivo* is underlined by the fact that leptin- and functional leptin receptor-deficient

mice (*ob/ob* and *db/db* respectively) exhibit early onset hyperinsulinaemia, obesity and non-insulin-dependent diabetes mellitus (NIDDM) (Coleman 1978, Zhang *et al.* 1994, Chen *et al.* 1996). Chronic daily leptin administration to *ob/ob* mice leads to a reduction in plasma insulin and glucose levels too rapidly to be accounted for by the changes in body weight (Campfield *et al.* 1995, Halaas *et al.* 1995, Pelleymounter *et al.* 1995). Moreover, it has been demonstrated that a single injection of leptin into *ob/ob* and normal-fed mice produces an acute reduction in plasma insulin levels, indicative of a direct action on the β -cell (Kulkarni *et al.* 1997). These findings have led us and others to postulate that the high levels of leptin that occur in obese individuals may exert an inappropriate inhibitory action on β -cell insulin secretion and contribute to the development of NIDDM (Emilsson *et al.* 1997, Kulkarni *et al.* 1997, Pallett *et al.* 1997).

Several recent studies have addressed the mechanism whereby leptin exerts its inhibitory action on β -cells. Thus there is evidence to suggest that leptin can hyperpolarize the β -cell by directly acting on the ATP-sensitive potassium channels (Harvey *et al.* 1997, Kieffer *et al.* 1997). Leptin also lowers the intracellular free calcium concentration ($[Ca^{2+}]_i$) (Fehmann *et al.* 1997a,b, Kieffer *et al.* 1997) and chronic exposure to leptin results in intra-islet triglyceride depletion, thus rendering the β -cell unresponsive to nutrients (Koyama *et al.* 1997, Shimabukuro *et al.* 1997, Wang *et al.* 1998). The combined action of leptin on cellular membrane potential, $[Ca^{2+}]_i$ and triglyceride stores could explain the observed acute inhibitory effects of leptin on insulin secretion and the longer-term reduction in insulin transcript levels. Although leptin has been shown to act through its full-length receptor, OB-Rb, to activate the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway in a number of systems (Baumann *et al.* 1996, Ghilardi *et al.* 1996), OB-Rb signalling in pancreatic islets has not been defined. In the present study we present evidence that the effects of leptin are mediated by a STAT3 mechanism in primary rat islets and through STAT3 and mitogen activated protein (MAP) kinase in RINm5F β -cells.

MATERIALS AND METHODS

Cell culture

RINm5F cells were a gift from Dr S Islam (Rolf Luft Centre, Karolinska Institute, Stockholm, Sweden). Cells were routinely cultured in 25 cm² flasks in RPMI-1640 medium (Gibco BRL, Paisley, Strathclyde, UK) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 5.6 mM glucose and 10% fetal calf serum at 5% CO₂ in a humidified 37 °C cabinet. Flasks were pre-incubated for a minimum of 24 h in serum-free (0.1% BSA) RPMI-1640 before exposure to recombinant murine leptin (Peprotech, London, UK), forskolin/3-isobutyl-1-methylxanthine (IBMX), acetylcholine or ionomycin (all from Alexis Corp., Nottingham, UK) for the indicated times and concentrations in serum-free RPMI-1640. Serum-free pre-incubation was necessary to reduce basal STAT3 activation and to remove activity of residual leptin present in the routine culture medium, as described previously (Islam *et al.* 1997).

Nuclear extracts

After treatment, the cells were washed in ice-cold PBS, and then in ice-cold PBS with phosphatase

inhibitors (1 mM Na₃VO₄, 5 mM NaF). Cells were lysed in a hypotonic buffer (20 mM Hepes, pH 7.9, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 mM Na₂MoO₄, 1 μ M microcystin LR (Alexis Corp.) with protease inhibitors (Boehringer Mannheim Complete Mini Protease Pellets, Boehringer Mannheim, Mannheim, Germany) and 0.2% IGEPAL detergent (Sigma Chemical Co., Poole, Dorset, UK). Nuclei were collected by brief centrifugation at 14 000 g and the pellet re-suspended in a high-salt buffer (hypotonic buffer with 0.42 M NaCl and 20% glycerol, without IGEPAL). Nuclear proteins were extracted for 30 min at 4 °C by rotation before sedimentation of debris at 14 000 g for 20 min. Nuclear extract supernatants were snap-frozen in liquid nitrogen and stored at -80 °C before we undertook gel-shift assays. A kit was used to determine protein concentration, according to the manufacturer's data sheet (Sigma Diagnostics P5656 Protein Assay).

Isolation of islets and islet nuclear extracts

Pancreatic islets were isolated from male Wistar rats of 200–250 g (Harlan Olac, Bicester, Oxon, UK) by collagenase (Sigma type XI) digestion of pancreata in a physiological saline solution as described previously (Pallett *et al.* 1997). Groups of 200–500 islets were washed in RPMI-1640, 0.1% BSA and incubated for 6 h. Islets were then collected and re-suspended in RPMI-1640, 0.1% BSA with or without leptin. After the incubation, islets were collected by brief centrifugation and put on ice. Five hundred microlitres ice-cold hypotonic buffer (10 mM Hepes pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM NaF, 10 mM Na₂MoO₄, 10 mM β -glycerophosphate, 10 mM Na₃VO₄, 10 mM *p*-nitrophenylphosphate, 1 μ M microcystin LR) and one tablet per 10 ml Complete Mini Protease Inhibitor (Boehringer Mannheim) were added to the islets on ice. After 15 min, 25 μ l 10% IGEPAL were added and the islets vortexed briefly. Nuclei were collected by centrifugation at 14 000 g for 20 s and the pellet re-suspended in 50 μ l high-salt buffer (hypotonic buffer with 0.4 M NaCl and 5% glycerol) and one tablet per 10 ml Complete Mini Protease Inhibitor. Debris was removed by centrifugation at 14 000 g for 10 min and the supernatant extracts taken for gel-shift.

Mobility-shift assay

The gel-shift assay was performed with oligonucleotides from the *c-fos* promoter (a mutated *c-sis* inducible element, m67SIE) top strand 5'-CAT

TTC CCG TAA ATC AT-3' and rat β -casein promoter, top strand 5'-GGA CTT CTT GGA ATT AAG GGA-3'. STAT-binding elements are underlined. These were end-labelled with [γ - 32 P]ATP (Amersham, Little Chalfont, Bucks, UK) and T4 polynucleotide kinase (Gibco). Binding reactions included 1 mM DTT, 1 μ g/ml polydI:polydC (Pharmacia, Uppsala, Sweden), 10% glycerol and 10 mM Tris, pH 7.9. The mixture was incubated at room temperature for 20 min and protein-DNA complexes were separated on 4% native polyacrylamide gels containing 5% glycerol. Gels were dried under vacuum for 40 min and then exposed to Kodak Biomax film (Sigma) at -80°C overnight.

Western blot

Nuclear extracts were obtained as described above. An equal volume of $2\times$ sample-loading buffer (100 mM Tris, pH 6.8, 10% SDS, 20% glycerol, 2% β -mercaptoethanol, 0.2% bromophenol blue) was added to the extracts and the samples boiled for 3 min. Samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Antibodies used were STAT3 N-terminal specific (S21320, Transduction Labs, Lexington, KY, USA), anti-phospho-STAT3 Y705 and anti-phospho-MAP kinase Y204 (9131S, New England Biolabs, Hitchin, Herts, UK).

Constructs and transcriptional reporter assays

Chloramphenicol acetyl transferase (CAT) reporter constructs were used. These were: GAS-CAT, containing four copies of the human Fc γ RI interferon- γ activated sequence, GAS (5'-AGC TTG AGA TGT ATT TCC CAG AAA AGA-3'), IRE-CAT, containing four copies of the human intercellular adhesion molecule-1 (ICAM-1) interferon- γ /interleukin-6 response element, IRE (5'-AGC TTA GTT TCC GGG AAA GCA C-3'), and β -casein-CAT, containing four copies of the β -casein STAT-binding site, β -CAS (5'-AGC TTA GAT T TC TAG GAA TTC AAA TCA-3'). STAT-binding sites were cloned into the thymidine kinase (TK)-CAT vector pBLCAT2 using a method described previously (Caldenhoven *et al.* 1996). For transfection experiments, cells were split 1:3 and 24 h later transfected with 10 μ g supercoiled plasmid DNA by the DEAE-dextran technique. Briefly, DNA precipitates were prepared by mixing the DNA with 1 ml of 0.5 mg/ml DEAE-dextran solution (Pharmacia) in Tris-buffered saline (TBS). Cells were washed twice with TBS, after which the cells were exposed to the DNA precipitate for

30 min at room temperature. After washing the cells twice with TBS, medium was added with 0.1 mM chloroquine, and the cells were incubated for 6 h at 37°C . Cells were then washed twice with PBS, and fresh medium was added. Twenty-four hours later cells were serum starved for 16 h, after which they were stimulated for 12 h with leptin or vehicle and subsequently harvested for CAT assay. Cells were lysed by repeated freeze thawing in 250 mM Tris, pH 7.4, 25 mM EDTA. Fifty microlitres of cellular extract were then incubated in a total volume of 100 μ l containing 250 mM Tris, pH 7.4, 2% glycerol, 0.3 mM butyryl coenzyme A and 0.05 μ Ci [14 C]chloramphenicol for 2 h at 37°C . Reaction products were then extracted using 400 μ l xylene:pristane (1:2) and the percentage of acetylated products determined using liquid scintillation counting.

RESULTS

Leptin induces STAT DNA binding in nuclear extracts from RINm5F and isolated rat islets

Leptin treatment of RINm5F cells caused induction of a single STAT DNA binding complex with the m67SIE oligonucleotide (Fig. 1). This probe contains a mutated STAT-binding site from the human c-fos promoter and has a high affinity for STAT3 and STAT1. Induction was apparent in RINm5F nuclear extracts by 15 min and continued to increase up to the 30 min time point. In experiments using nuclear extracts from isolated rat pancreatic islets, leptin induced a single STAT DNA binding complex using the m67SIE probe that was dose dependent over the concentration range 0.2–20 nM (Fig. 2, left panel) in a 15 min incubation. Time course studies in pancreatic islets showed that induction of this single complex was observed by 2 min, peaked by 15 min and returned to basal levels by 30 min (Fig. 2, right panel).

Leptin induces STAT3 activation in RINm5F and isolated rat islets

Crude cell and nuclear extracts from leptin-treated RINm5F cells were probed with an anti-phospho-Y705 STAT3 antibody. Leptin produced a time-dependent tyrosine phosphorylation and nuclear translocation of two immunoreactive STAT3 species (Fig. 3a). The rapidly induced more slowly migrating band at approximately 90 kDa is consistent with STAT3 α . The faster migrating band is consistent with STAT3 β and was induced by 15 min in crude RINm5F cell extracts. This suggests differential activation of the two isoforms

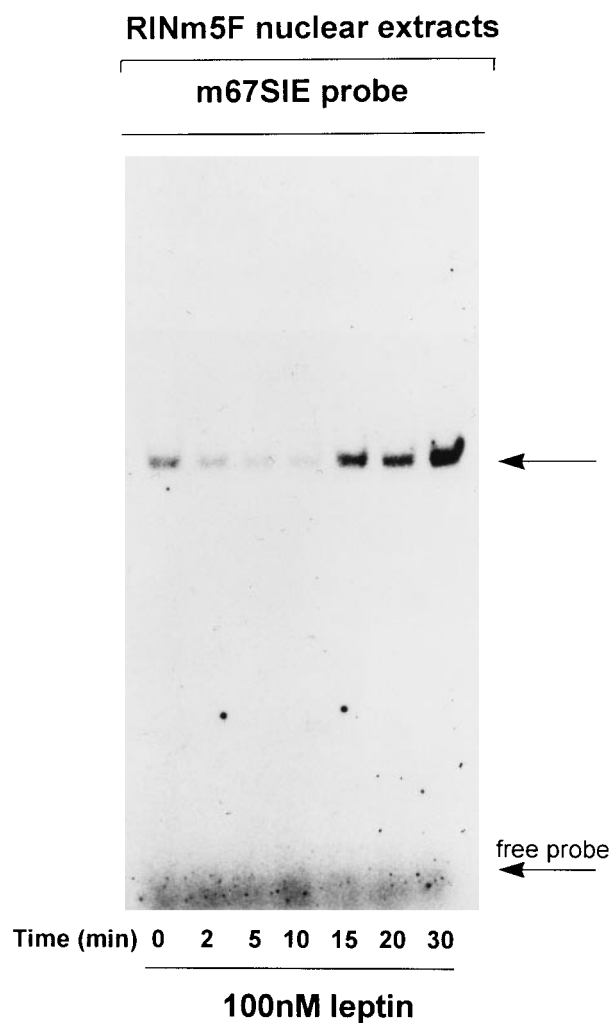


FIGURE 1. Time-dependent induction of STAT DNA binding to the m67SIE element from the c-fos promoter by leptin in RINm5F nuclear extracts. Cells were pre-incubated for 36 h in serum-free medium and then exposed to leptin (100 nM) for the time indicated below the lanes. Nuclear extracts were isolated and run in a DNA binding assay with end-labelled m67SIE oligonucleotide. Protein/DNA complexes were separated on a 5% native PAGE gel. The upper arrow indicates the single complex induced by leptin.

in response to leptin in the cytosol and subsequent nuclear translocation in RINm5F cells. Low levels of STAT3 β could be detected after leptin treatment in nuclear extracts of RINm5F cells by 30 min upon longer exposure of the blots (data not shown). Leptin caused nuclear activation of STAT3 α over the concentration range 0.2–200 nM in RINm5F but STAT3 activation was lost at 2 μ M leptin in RINm5F cells (Fig. 3b). Leptin treatment of

isolated rat pancreatic islets produced an increase in tyrosine phosphorylated STAT3 that peaked by 15 min and dropped to basal levels by 30 min in crude islet extracts (Fig. 4, upper gel and graph). The increase in STAT3 α and STAT3 β tyrosine phosphorylation was significantly greater at 15 min than at the 0 time point as normalized to the level of STAT3 detected with an N-terminal-directed STAT3 antibody (Fig. 4 lower gel). (The increase compared with 100% at 0 time point was: STAT3 α , 650 \pm 110%, $P < 0.02$, $n = 4$; STAT3 β , 487 \pm 126%, $P < 0.05$, $n = 4$.) The reference sample in the lower gel of Fig. 4 is from an A431 cell lysate commercially provided as a positive control for STAT3 detection.

Leptin induces transcriptional activation from STAT consensus elements transfected into RINm5F cells

Leptin increased transcriptional activation from reporter plasmids containing GAS (from the Fc γ RI promoter) and IRE (from the human ICAM-1 promoter) STAT-binding elements (Fig. 5). Leptin-induced transcriptional activity was greater with the IRE (which has a higher affinity for STAT3) compared with the GAS construct (which has a higher affinity for STAT1) consistent with STAT3 activation (Caldenhoven *et al.* 1995). Leptin did not induce transcriptional activation of a reporter plasmid driven by the STAT5 consensus element, β -CAS or the control vector, pBL (Fig. 5).

Leptin-mediated STAT DNA binding is modulated by increases in intracellular cAMP and [Ca²⁺]_i

RINm5F cells were pre-incubated with a number of effectors designed to mimic states of increased metabolic activity for 15 min and then in the presence of added leptin (100 nM) for a further 15 min (Fig. 6). As before, leptin alone (100 nM) induced DNA binding of a single STAT complex within 15 min. Acetylcholine (10 μ M), an endogenous parasympathetic stimulator of insulin secretion, caused a marked reduction in leptin-induced STAT DNA binding to the probe. Pre-incubation of the RINm5F cells with 100 nM phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, had no significant effect on leptin-induced STAT DNA binding, although it increased c-fos transcript levels in RINm5F as assessed by Northern blot (data not shown). Pre-incubation with the calcium ionophore, ionomycin (1 μ g/ml) produced a marked attenuation of leptin-induced STAT DNA binding. Finally, we tested the effects of raising intracellular cAMP by pre-incubating

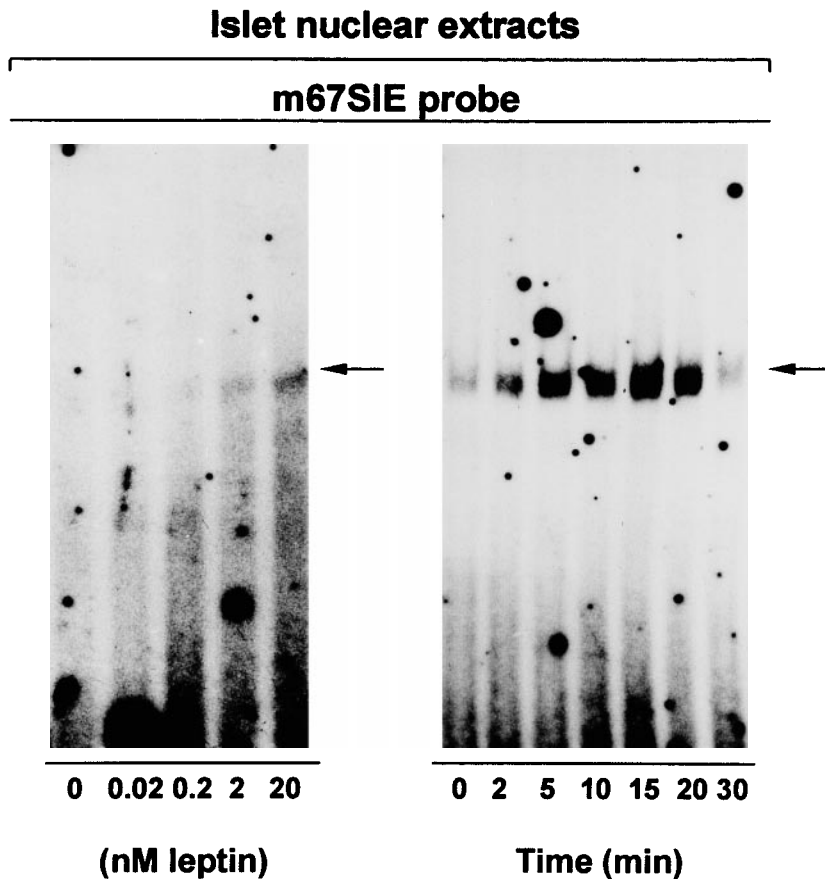


FIGURE 2. Dose- and time-dependent induction of STAT DNA binding to the m67SIE element from the *c-fos* promoter by leptin in rat islet nuclear extracts. Islets were isolated and pre-incubated for 6 h in serum-free RPMI-1640 and then exposed to leptin for 15 min with the leptin concentrations indicated below the lanes (left panel) or to 20 nM leptin for the time indicated below the lane (right panel). Nuclear extracts were then isolated and run in a DNA binding assay with end-labelled m67SIE oligonucleotide. Protein/DNA complexes were separated on a 5% native PAGE gel. The arrow indicates the single complex induced by leptin.

with both the adenylyl cyclase activator, forskolin (10 μ M), and the cAMP phosphodiesterase inhibitor, IBMX (50 μ M). This treatment resulted in a reduction of leptin-induced STAT DNA binding to basal levels; however, this reduction was not as marked as that caused by acetylcholine or ionomycin (Fig. 6).

Increasing cellular metabolic activity does not affect STAT3 tyrosine phosphorylation

RINm5F nuclear extracts, which had been pre-incubated with various effectors and then leptin (100 nM) as described above, were probed with the antibody to phospho-Y705 STAT3 (Fig. 7 upper

gel) and an anti-N-terminal STAT3 antibody (Fig. 7 lower gel). This revealed that leptin induced STAT3 tyrosine phosphorylation; however, there was no significant change in the tyrosine phosphorylation levels of the STAT3 in response to any of the agents tested.

Leptin increases activated MAP kinase-Y204 levels in RINm5F cells

Nuclear extracts of leptin-treated RINm5F cells were probed for active MAP kinase with a phospho-Y204-MAP kinase-specific antibody. Leptin caused an increase in the levels of a 44 and 42 kDa MAP kinase-immunoreactive species within

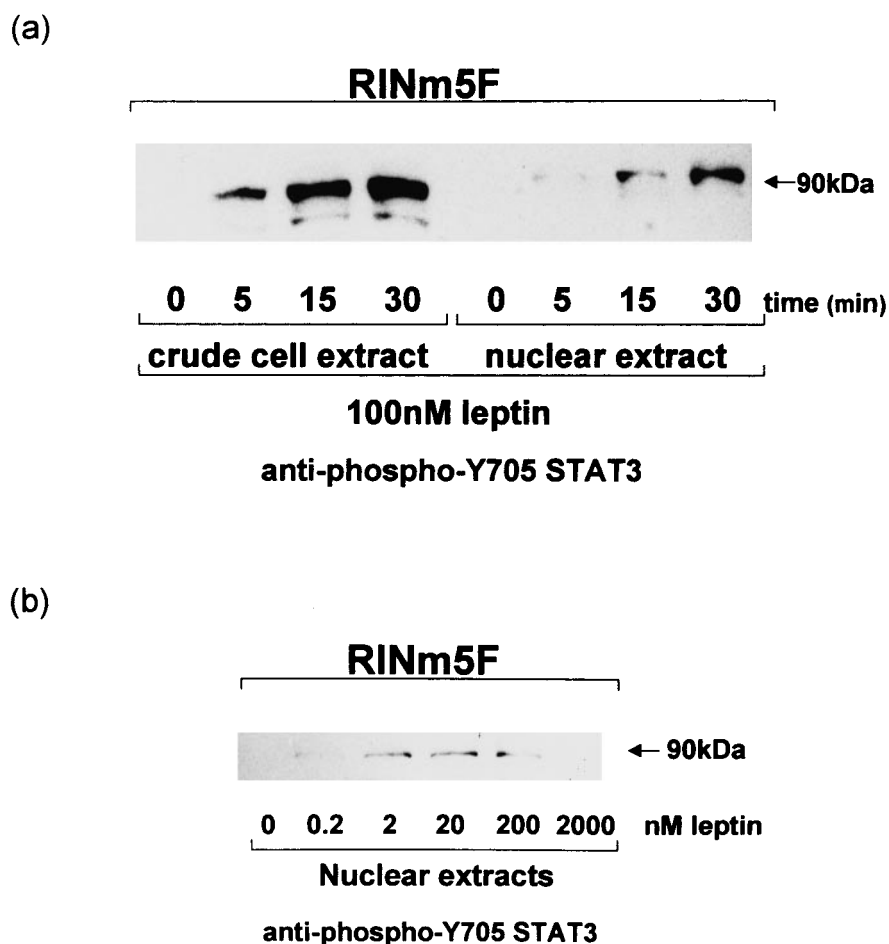


FIGURE 3. Time-dependent induction of tyrosine phosphorylation and nuclear translocation of STAT3 by leptin in RINm5F nuclear extracts. (a) Cells were pre-incubated for 36 h and then exposed to leptin (100 nM) for the time indicated below the lanes. Leptin caused tyrosine phosphorylation of STAT3 α (this band migrated at approximately 90 kDa and is indicated by an arrow) by 5 min and STAT3 β by 15 min in crude cell extracts (left panel) that continued to increase until the 30 min time point. The nuclear translocation of tyrosine phosphorylated STAT3 α was detected in nuclear extracts by 15 min (right panel). (b) RINm5F cells were treated for 15 min with the concentration of leptin indicated below the lane and the nuclear extracts isolated. Tyrosine phosphorylated nuclear STAT3 α was induced over the concentration range 0.2–200 nM leptin.

15 min (Fig. 8a upper gel). The same samples were probed with an antibody that detects ERK1 and ERK2 (anti-panERK) regardless of its activation state (Fig. 8a lower gel). Treatment of isolated rat islets with 20 nM leptin did not lead to an increase in the levels of the active MAP kinase as detected by Western blot (Fig. 8b upper gel). MAP kinase was readily detected in these samples (Fig. 8b lower gel) and tyrosine phosphorylation of STAT3 was detectable in samples prepared in a similar manner for Western analysis (Fig. 4). Treatment of rat islets with concentrations of leptin up to 100 nM did not

result in activation of MAP kinase (data not shown). Nuclear extracts of RINm5F from cells treated with 100 nM leptin for 15 min were used as a positive control in the blots for potential activation of MAP kinase by leptin in pancreatic islets (Fig. 8b).

DISCUSSION

Pancreatic β -cell failure is a hallmark of NIDDM (Pouitout & Robertson 1996). A large proportion of patients with NIDDM are also obese (Groop &

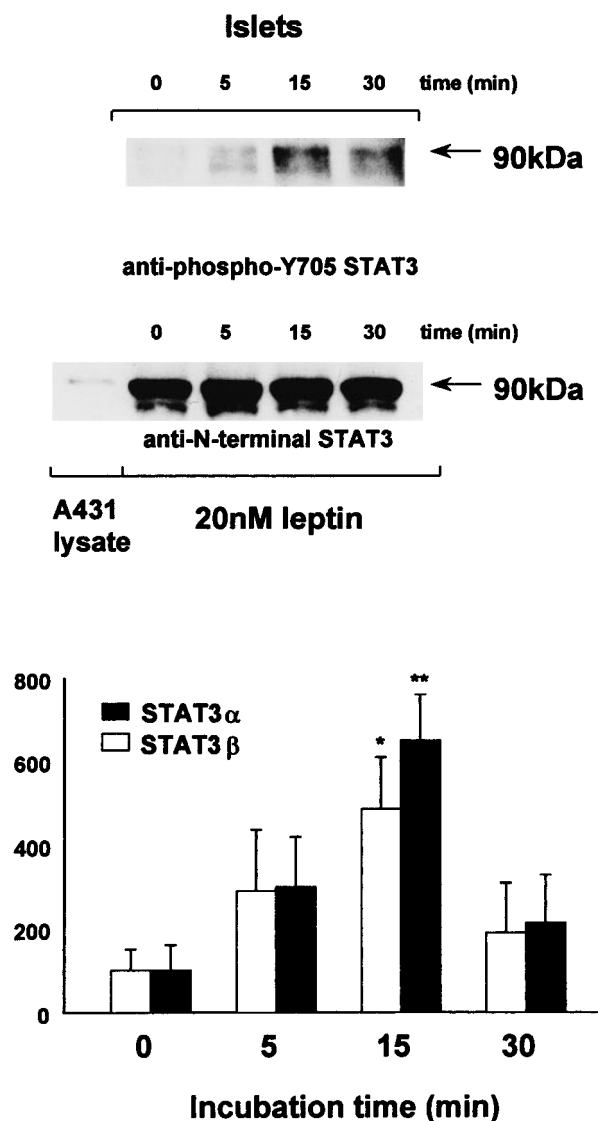


FIGURE 4. Time-dependent induction of STAT3 tyrosine phosphorylation by leptin in isolated rat islets. Rat islets were isolated and pre-incubated for 6 h in serum-free RPMI-1640 and then exposed to leptin (20 nM) for the times indicated above the lanes. Islets were collected by centrifugation, immediately boiled in sample buffer and run on 10% SDS-PAGE gels. STAT3 was detected with an antibody to activated (phospho-Y705) STAT3 (upper gel) and a STAT3 N-terminal-specific antibody (lower gel). The graph shows a quantification of the time-dependent induction of tyrosine phosphorylation of STAT3 α and STAT3 β normalized to the 0 time levels of STAT3 α and STAT3 β respectively. Control samples (lower gel, left) are from A431 cell lysates. Results are expressed as mean \pm S.E.M. and statistical significance determined using Student's unpaired *t*-test (* P <0.05, ** P <0.02 vs 0 time point ($n=4$)).

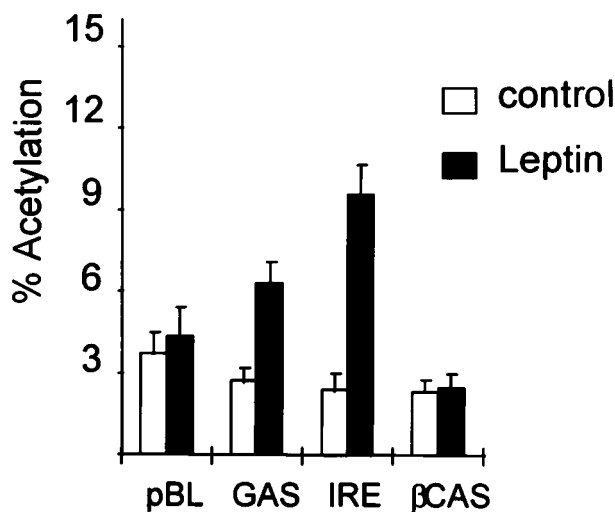


FIGURE 5. Transcriptional activation of reporter constructs driven by STAT element promoters by leptin in RINm5F cells. Cells were transiently transfected with CAT reporter plasmids containing the GAS, IRE or β -CAS STAT responsive promoter elements. Induction of transcription was most marked from the IRE construct and there was no activation of the STAT5-selective construct. Also shown is the control construct (pBL). Results are mean \pm S.E.M. of three experiments.

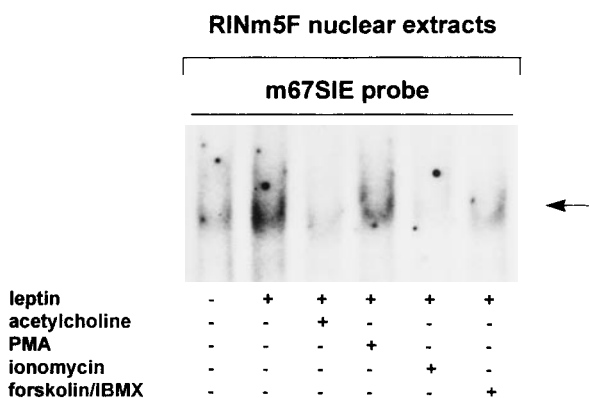
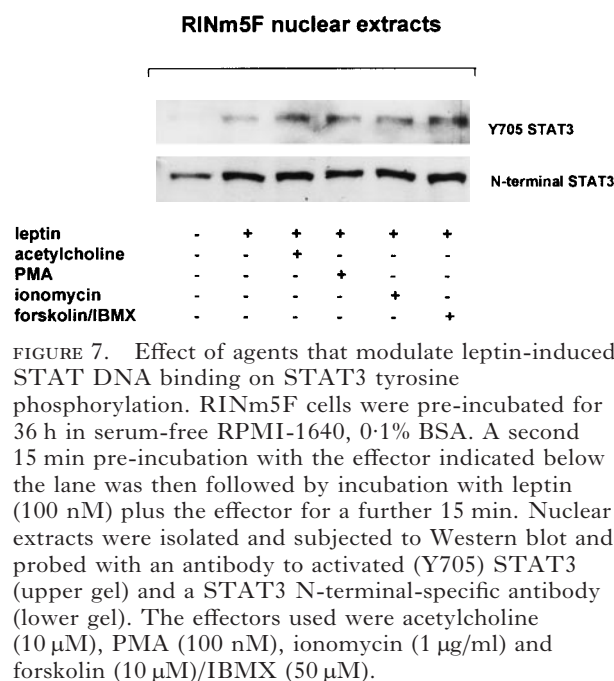


FIGURE 6. Effect of increasing cellular metabolic activity on leptin-induced STAT DNA binding in RINm5F cells. RINm5F cells were pre-incubated for 36 h in serum-free RPMI-1640, 0.1% BSA. A second 15 min pre-incubation with the effector indicated below the lane was then followed by incubation of the cells with leptin (100 nM) plus the effector for a further 15 min. Nuclear extracts were then isolated as described and run on 5% native PAGE gels with the end-labelled m67SIE probe. The effectors used were acetylcholine (10 μ M), PMA (100 nM), ionomycin (1 μ g/ml) and forskolin (10 μ M)/IBMX (50 μ M).



Tuomi 1997) and all are insulin resistant. NIDDM develops when compensatory hyperinsulinaemia fails to overcome the insulin resistance. We have suggested previously that the high levels of leptin found in obesity might be one of the factors that precipitates NIDDM through its inhibitory action on the β-cell (Emilsson *et al.* 1997, Pallett *et al.* 1997). We and others have demonstrated that leptin could cause inhibition of insulin secretion from mouse and rat islets (Chen *et al.* 1997, Emilsson *et al.* 1997, Fehmann *et al.* 1997a, Kieffer *et al.* 1997, Kulkarni *et al.* 1997, Pallett *et al.* 1997, Ookooma *et al.* 1998, Poitout *et al.* 1998) and a reduction in insulin transcript levels in rat islets (Kulkarni *et al.* 1997, Pallett *et al.* 1997). This phenomenon has been shown to involve leptin-mediated activation of the ATP-sensitive potassium channels in islets (Kieffer *et al.* 1997) and insulin-secreting cell lines (Harvey *et al.* 1997) and a reduction in $[Ca^{2+}]_i$ in islets (Fehmann *et al.* 1997a, Kieffer *et al.* 1997) and insulin-secreting cell lines (Fehmann *et al.* 1997a,b). Inhibition of insulin secretion has also been observed *in vivo* in normal-fed mice and with human islets exposed to physiological levels of leptin *in vitro* (Kulkarni *et al.* 1997). Furthermore, chronic leptin exposure leads to islet triglyceride depletion and a loss of nutrient-induced insulin secretion *in vivo* and *in vitro* (Koyama *et al.* 1997, Shimabukuro *et al.* 1997, Wang *et al.* 1998). Taken together, this evidence suggests that leptin may have a physiologi-

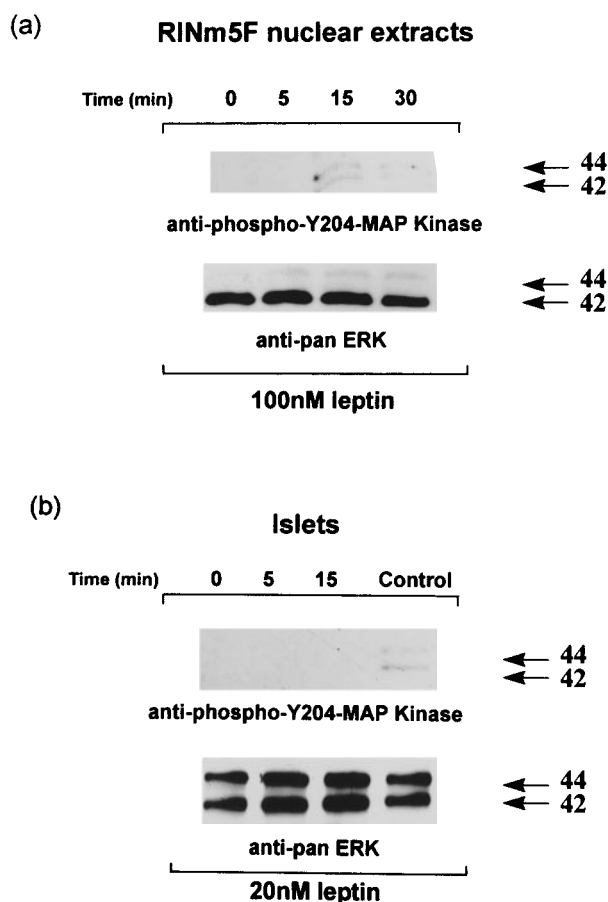


FIGURE 8. Time-dependent induction of MAP kinase p42 and p44 tyrosine phosphorylation by leptin in RINm5F cells but not in rat islets. (a) RINm5F cells were pre-incubated for 36 h in serum-free RPMI-1640, 0.1% BSA and then leptin (100 nM) for the time indicated above the lane. Nuclear extracts were then run on 10% SDS-PAGE gels, transferred to PVDF and probed with an antibody to activated (Y204) MAP kinase (upper gel) and an anti-panERK (lower gel) antibody. Leptin caused an increase in the tyrosine phosphorylated MAP kinases that was detectable by 15 min and dropped by 30 min of incubation. (b) Isolated rat islets were pre-incubated for 6 h and then exposed to leptin (20 nM) for the times indicated above the lane. Crude protein extracts were then probed with the (Y204) MAP kinase (upper gel) and anti-panERK (lower gel) antibodies. The arrows indicate the apparent molecular masses of the bands. Nuclear extracts of RINm5F from cells treated with 100 nM leptin for 15 min were used as positive control in the blots for the potential activation of MAP kinase by leptin in pancreatic islets.

cal influence on the normal glucose-sensing mechanism of the pancreatic β-cell. However, it is also clear that many obese subjects with high circulating

leptin concentrations are not diabetic and animal studies show that exogenously administered leptin generally improves insulin action in obese animals. Thus it seems that the potential diabetogenic activity of leptin is modified by differential efficacy of the leptin signalling pathways in various tissues. The role of leptin signalling through OB-Rb in the long-term control of pancreatic β -cell metabolism and gene expression (β -cell programming) is currently unclear.

The full-length leptin receptor, OB-Rb (Chen *et al.* 1996), is closely related to the type I superfamily of cytokine receptors (Tartaglia *et al.* 1995). OB-Rb is known to homodimerize (White *et al.* 1997) and to activate JAK2 (Ghilardi & Skoda 1997) and STATs 1, 3 and 5B (Baumann *et al.* 1996) or 3, 5 and 6 (Ghilardi *et al.* 1996) in *in vitro* overexpression systems. *In vivo*, the activation pattern appears more restricted. Indeed, despite the availability of STATs 1, 3, 5 and 6, leptin appears to activate only STAT3 in the hypothalamus (Vaisse *et al.* 1996), STAT1 in adipose tissue (Siegrist-Kaiser *et al.* 1997) and STAT5 in the small intestine (Morton *et al.* 1998). We find that leptin activates STAT3 in isolated rat islets where approximately 80% of the cell population are β -cells (Orsi 1986). A STAT3 signalling mechanism in the β -cell is corroborated by activation of STAT3 in nuclear extracts of RINm5 cells, a rat clonal pancreatic β -cell model that we have shown previously responds to leptin and expresses high levels of OB-Rb (Islam *et al.* 1997). Although STAT1 and 5 were detected in both isolated rat islets and RINm5F, we did not detect activation of either molecule in response to leptin treatment by probing with a specific antibody to phospho-Y701 STAT1 or using a STAT5-selective β -casein oligonucleotide (data not shown). We also did not detect transcriptional activation of a reporter construct linked to the β -casein element. It is therefore likely that the leptin signal is transmitted by different STATs depending on the target tissue, time frame and cellular system under study. We also show that leptin can cause induction of transcription from reporter constructs with STAT3-responsive promoter elements in RINm5F. This indicates that STAT3 activation leads directly to transcriptional activation and provides evidence of the first stages of β -cell programming by leptin through STAT3. The time-course for induction of STAT3 α and STAT3 β appears to be more rapid in the primary islet compared with the RINm5F cell. This may be relevant for the kinetics of gene activation in primary β -cells versus clonal β -cells. The delayed activation and nuclear translocation of STAT3 β seen in RINm5F cells may also have important

consequences, since STAT3 α and STAT3 β are reported to have different functional roles in controlling gene expression (Caldenhoven *et al.* 1996, Schaeffer *et al.* 1997). The concentrations of leptin that elicit STAT activation fall within the physiological range (Maffei *et al.* 1995). Thus, induction of STAT DNA binding is detectable in isolated rat islet nuclear extracts with 2 nM leptin and STAT3 activation is observed at concentrations as low as 0.2 nM leptin in RINm5F nuclear extracts. This supports a role for leptin in modulation of β -cell function under normal physiological conditions. An extreme concentration (2 μ M) of leptin led to a loss of nuclear STAT3 activation as assessed by Western blot and may represent saturation of leptin receptor binding as we found previously for the U-shaped dose-dependent inhibition of insulin secretion (Pallett *et al.* 1997).

The insulin synthesis and secretory mechanism of the pancreatic β -cell are controlled by membrane depolarization and calcium influx due to nutrient metabolism (Prentki *et al.* 1997). This process is potentiated by hormone-mediated elevation of intracellular second messengers such as cAMP (Ammälä *et al.* 1993, Holz *et al.* 1993) and D-myo-inositol 1,4,5-trisphosphate (IP3) (Zawalich & Zawalich 1996). We have assessed the impact of increased metabolic activity on leptin-inducible STAT DNA binding activity and STAT3 tyrosine phosphorylation in a clonal insulin-secreting cell line that we had found to respond functionally to leptin (Islam *et al.* 1997). Although RINm5F cells lack the cellular machinery that allows them to respond to glucose (Praz *et al.* 1983), these cells retain many of the features of regulated insulin release and have been used extensively as a model of the pancreatic β -cell. The lack of glucose responsiveness in the RINm5F cells was circumvented by experimental approaches using pharmacological agents that mimic the raised metabolic activity found in native β -cells upon exposure to glucose and hormonal stimuli. Increased $[Ca^{2+}]_i$ is known to be one of the major events that occurs after the exposure of normal β -cells to stimulatory concentrations of nutrients, primarily glucose, and hormonal stimuli (Zawalich & Zawalich 1996, Gromada *et al.* 1997, Prentki *et al.* 1997). Raising $[Ca^{2+}]_i$ with the calcium ionophore ionomycin leads to a marked reduction in leptin-inducible STAT DNA binding to the m67SIE probe. This could be interpreted as evidence that raised metabolic activity attenuates leptin signalling in β -cells and is consistent with a counteraction of the reduction of $[Ca^{2+}]_i$ ascribed to leptin (Fehmann *et al.* 1997a, Kieffer *et al.* 1997, Kulkarni *et al.* 1997). It should be noted, however, that raising intracellular calcium with ionomycin

does not allow discrimination between extracellular and intracellular sources of calcium. We also exposed RINm5F to forskolin and IBMX, compounds that activate adenylyl cyclase and inhibit cAMP phosphodiesterases respectively. These agents raise intracellular cAMP, a second messenger that can potentiate insulin secretion (Ammölö *et al.* 1993, Holz *et al.* 1993, Gromada *et al.* 1997). The RINm5F cell has been reported to secrete insulin in response to increased cAMP through a calcium-independent mechanism (Wollheim *et al.* 1984), whereas intact rat β -cells exhibit calcium-dependent cAMP-inducible insulin secretion (Holz *et al.* 1993). Increasing cAMP resulted in a reduction of leptin-induced STAT DNA binding to basal levels in RINm5F cell nuclear extracts. Since the cAMP response in RINm5F is believed to be uncoupled from increased $[Ca^{2+}]_i$, this could imply a role for cAMP-dependent protein kinase (PKA) in negative modulation of leptin signalling. Agents that raise β -cell metabolic activity by this mechanism, such as the insulinotropic hormone, GLP-1, may counteract leptin signalling by a combined increase in PKA activity and raised $[Ca^{2+}]_i$ in native rat β -cells (Holz *et al.* 1993, Gromada *et al.* 1997). Reversal of leptin-mediated inhibition of insulin secretion by GLP-1 has also been observed (Fehmann *et al.* 1997b). The activation of calcium-dependent protein kinases (PKCs) is another event associated with increased metabolic activity in β -cells. Glucose metabolism (Easom *et al.* 1989) and exposure of the β -cell to stimuli such as the parasympathetic neurotransmitter, acetylcholine (Zawalich & Zawalich 1996), lead to increased activation of PKC, increased $[Ca^{2+}]_i$ and an increase in insulin secretion. We exposed RINm5F cells to both the PKC agonist, PMA, and acetylcholine. Activation of PKC alone did not affect leptin-inducible STAT DNA binding. In contrast, acetylcholine caused a marked reduction in leptin-induced STAT DNA binding that could be due to the rapid increase in intracellular calcium released from internal stores in response to production of the second messenger, IP3, by this neurotransmitter (Zawalich & Zawalich 1996). The lack of effect of direct PKC activation by PMA on STAT DNA binding indicates that a PKC-linked mechanism of raising intracellular calcium, as has been described in RINm5F cells (Yada *et al.* 1989), does not contribute to a reduction in leptin-inducible STAT DNA binding in this cell line under the conditions tested. The present information supports a role for raised intracellular calcium, as opposed to direct action of PKC in the attenuation of leptin-mediated STAT DNA binding activity. This does not, however, rule out modulation of STAT3 activation through a

PKC-mediated change in serine phosphorylation as has been reported by others to occur (Yang *et al.* 1996). The leptin-deficient *ob/ob* mouse is characterized by hyperinsulinaemia due to an exaggerated PKC-linked sensitivity at the β -cell level (Chen & Romsos 1997) that can be 'corrected' by leptin treatment of *ob/ob* islets (Chen *et al.* 1997). Increased parasympathetic stimuli or exposure to other hormones that act through raising intracellular calcium (for example cholecystokinin and vasopressin) are therefore also likely to counteract OB-Rb-mediated signalling in the β -cell. Attenuation of the inhibitory programming of the β -cell by leptin could therefore contribute to the hyperinsulinaemia associated with a raised parasympathetic tone found in some forms of obesity (Bray *et al.* 1990). We did not detect any change in tyrosine phosphorylation of STAT3 in response to any of the effectors tested. This implies that events downstream of STAT activation by JAKs are a target for modulation by these agents.

We have also recently found that leptin can induce the immediate-early gene, *c-fos*, in RINm5F and that this precedes a proliferative response (Islam *et al.* 1997). A similar MAP kinase-dependent effect has subsequently been observed in the MIN-6 β -cell line (Tanabe *et al.* 1997). In the current studies we find a leptin-mediated increase in the levels of active MAP kinase isoforms in RINm5F cells but not in adult primary rat islets. One interpretation of these data is that leptin may have a role in controlling the early development and growth/differentiation of islet β -cells in discrete sub-populations. We proposed previously that high levels of leptin might contribute to the β -cell hyperplasia and hypertrophy associated with obesity by affecting this process in some way (Islam *et al.* 1997). Alternatively, activation of MAP kinase activity in β -cells may be involved in the modulation of STAT signalling. Thus, MAP kinase is known to serine phosphorylate STAT3 leading to a modulation of its DNA-binding activity (Chung *et al.* 1997).

The present study implicates signalling through STAT3 as a likely mechanism whereby regulation of gene expression in the β -cell is modulated by leptin. STAT3 activation has been associated with diverse phenomena such as cell transformation (Karras *et al.* 1997, Turkson *et al.* 1998) and differentiation (Minami *et al.* 1996) and is also the target for leptin action in the hypothalamus *in vivo* (Vaisse *et al.* 1996). The sub-set of target genes under the influence of leptin-inducible STAT3 in β -cells is therefore an important area of future study. Leptin-mediated proliferation of clonal β -cells (Islam *et al.* 1997, Tanabe *et al.* 1997) and

cells of haematopoietic origin (Gainsford *et al.* 1996) associated with MAP kinase activity have also been described. However, since we did not find a similar activation of MAP kinase in isolated rat islets, this could mean that MAP kinase is not a major pathway involved in the regulation of primary β -cell function by leptin. Finally, our observation that raising intracellular calcium and cAMP interferes with leptin signalling suggests that raised metabolic activity may counteract leptin-inducible DNA binding of STAT3 in the β -cell. Since leptin may have a physiological inhibitory role in the regulation of β -cell responses to nutrients and other stimuli, chronic hyperglycaemia, inappropriate hormonal stimuli or insensitivity to leptin action such as that found in obese individuals could contribute to hyperinsulinaemia at early stages in the transition from obesity to NIDDM. Subsequently, perhaps during conditions of β -cell glucose desensitization and glucose toxicity, as is postulated to occur in NIDDM (Poitout & Robertson 1996), the high plasma leptin levels in obese individuals may become diabetogenic.

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