

Biphasic regulation of extracellular-signal-regulated protein kinase by leptin in macrophages: role in regulating STAT3 Ser⁷²⁷ phosphorylation and DNA binding

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Activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) requires dimerization that is induced by phosphorylation of Tyr⁷⁰⁵, but its activity can be further modulated by phosphorylation at Ser⁷²⁷ in a manner that is dependent on cell context and the stimulus used. The role of STAT3 Ser⁷²⁷ phosphorylation in leptin signalling is currently not known. While cells transfected with the signalling-competent long form of the leptin receptor (ObRb) have been used to study leptin signalling, these are likely to be of limited use in studying STAT3 Ser⁷²⁷ phosphorylation due to the importance of cell background in determining the nature of the response. However, we have recently found that J774.2 macrophages endogenously express high levels of ObRb, and using these cells we find that leptin stimulates STAT3 phosphorylation on both Tyr⁷⁰⁵ and Ser⁷²⁷. The phosphorylation of Ser⁷²⁷ was not affected by rapamycin or the protein kinase C inhibitor H7 [1-(5-isoquinolylsulphonyl)-2-methylpiperazine dihydrochloride]. While the MEK-1 [mitogen-activated protein kinase (MAP kinase)/extra-

cellular-signal-related kinase (ERK) kinase-1] inhibitor PD98059 [(2-amino-3'-methoxyphenyl)oxanaphthalen-4-one] had no effect on leptin-stimulated phosphorylation of STAT3 Tyr⁷⁰⁵, it greatly attenuated leptin's effects on STAT3 Ser⁷²⁷ phosphorylation. Further, Ob's effect on the DNA binding activity of STAT3 was also greatly reduced at all time points by PD98059. Leptin-induced ERK activation in J774.2 cells shows a biphasic pattern, with an initial reduction in ERK phosphorylation for up to 10 min following leptin stimulation, while at later time points phosphorylation of ERK was increased above basal levels. The increase in ERK activity corresponded with an increase in both phosphorylation of Ser⁷²⁷ and STAT3 DNA binding activity. These data provide the first evidence that ERK-mediated phosphorylation of Ser⁷²⁷ is required for full stimulation of STAT3 by leptin.

Key words: leptin receptor signalling, serine kinase, serine phosphorylation, signal transducer and activator of transcription.

INTRODUCTION

Leptin (product of the *ob* gene) is a 16 kDa adipocyte-derived protein, and mouse models that lack functional forms of leptin (e.g. *ob/ob* mice) are characterized by obesity due to hyperphagia [1]. Leptin has an important role in controlling appetite, and there has been a great deal of interest in understanding the molecular basis of its actions. The cell-surface leptin receptor (ObR) shows a high degree of sequence similarity to members of the cytokine receptor superfamily and has signalling capabilities similar to interleukin-6 (IL-6)-type cytokine receptors [2]. These receptors do not possess kinase activity themselves, but are commonly associated with other signalling molecules, such as the tyrosine kinase Janus kinase-2 (JAK-2), which are activated following binding of ligand to the receptor. The ObR gene is widely expressed, and a number of splice variants exist. Most cell types express truncated forms that have cytoplasmic domains of only 30–40 amino acids, which is thought to be too short to mediate downstream signalling [3–5]. The full-length ObR (ObRb) has a 302-amino-acid intracellular domain which confers full signalling capabilities to the receptor. Full-length ObRb was thought to be predominately expressed in the hypothalamus [4]; however, we have also recently detected high levels of ObRb expression in macrophages and demonstrated that Ob activates cognate signalling pathways in these cells [6]. This, combined

with the fact that leptin has profound effects on macrophage phagocytic function, cytokine production [7,8] and lipid metabolism [6], indicates that macrophages are a direct target for leptin. As cell lines from the hypothalamic ObRb expressing neurons are not available, macrophage cell lines represent the best cell model for studying leptin receptor signalling in a natural cellular context.

A major consequence of leptin binding to ObRb is activation of JAK-2, which in turn phosphorylates at least three tyrosine residues in the tail of the receptor [9]. These sites, when phosphorylated, allow SH2-domain-mediated recruitment and subsequent tyrosine phosphorylation of the SH2-domain-containing tyrosine phosphatase SHP-2 and the transcription factor STAT3 (signal transducer and activator of transcription-3) [10]. Tyrosine phosphorylation of SHP-2 plays a key role in leptin-mediated activation of extracellular-signal-regulated protein kinases (ERKs) [9,11]. Tyrosine phosphorylation of STAT3 allows dimerization mediated by its SH2 domains, which in turn allows nuclear translocation and the transactivation of STAT3-responsive genes [12]. While the role of the tyrosine phosphorylation is clear, STAT3 can also be phosphorylated at a serine residue (Ser⁷²⁷). There is conflicting evidence about the identity of the kinases responsible for this serine phosphorylation and also its functional consequence. Some reports attribute the phosphorylation to the ERKs [13], while other reports indicate

Abbreviations used: ObRb, long form of the leptin receptor (ObR); ERK, extracellular-signal-regulated protein kinase; H7, 1-(5-isoquinolylsulphonyl)-2-methylpiperazine dihydrochloride; PD98059, (2-amino-3'-methoxyphenyl)oxanaphthalen-4-one; IL, interleukin; STAT, signal transducer and activator of transcription; JAK, Janus kinase; SHP, SH2-domain-containing tyrosine phosphatase; CNTF, ciliary neurotrophic factor; MAP, mitogen-activated protein kinase; MEK-1, MAP kinase/ERK kinase-1; mTOR, mammalian target of rapamycin.

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the ERKs are not involved [13–15]. For example, the p70 S6 kinase pathway is implicated in the case of ciliary neurotrophic factor (CNTF)-induced STAT3 serine phosphorylation [14], and a pathway that is inhibited by the protein kinase C inhibitor H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride] is implicated in IL-6-induced STAT3 serine phosphorylation [15,16]. Some reports provide evidence that the phosphorylation of Ser⁷²⁷ is important in allowing maximal activation of STAT3 [16–19], while other reports indicate it is not [15,20]. Whilst leptin-stimulated STAT3 tyrosine phosphorylation has been demonstrated, no previous studies have established STAT3 as a leptin-mediated serine kinase target. Indeed the only previous report with relevance to this comes from studies using heterologous expression of erythropoietin–leptin receptor chimaeras in Chinese-hamster ovary cells [9]. These data suggested that the ERK pathway was not required for full activation of STAT3 activity by ObRb. Here we demonstrate that, in macrophages, endogenously expressing high levels of ObRb, leptin induces STAT3 Ser⁷²⁷ phosphorylation in an ERK-dependent manner and that this phosphorylation is required for maximal activation of STAT3 DNA binding.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies for STAT3, phospho-STAT3, phospho-ERK and total ERK were obtained from New England Biolabs (Beverly, MA, U.S.A.). Anti-(p70 S6 kinase) antibodies were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). (2-Amino-3'-methoxyphenyl)oxanaphthalen-4-one (PD98059) and H7 were obtained from Calbiochem. Rapamycin was purchased from Sigma. Leptin was obtained from Biogenesis Ltd., Poole, Dorset, U.K.

Cell culture

The murine macrophage cell line J774.2 were grown in RPMI medium (2 g of glucose/l), supplemented with 10% (v/v) foetal-bovine serum and 1% antibiotic/antimycotic. J774.2 macrophages were serum-starved in RPMI media without foetal-bovine serum containing 5 mg/ml BSA (fatty-acid-free). After incubation for 1 h, media was replaced with fresh RPMI/BSA containing either inhibitors or DMSO and incubated for a further 1 h before stimulation with 2 nM murine recombinant leptin.

Cell lysis and immunoblotting

Following stimulation, cells were washed once with ice-cold PBS and lysed in buffer containing 1% Triton X-100, 10 mM Tris base, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml pepstatin and 200 kallikrein-inhibitory units of aprotinin. Lysates were then centrifuged for 10 min at 24000 *g* at 4 °C. The supernatants were combined with the appropriate amount of 4×SDS sample buffer containing 20% (v/v) glycerol, 4% (w/v) SDS, 200 mM dithiothreitol, 0.2 M Tris buffer, pH 6.8. Samples were subjected to SDS/PAGE, then transferred on to PVDF membrane and immunoblotted with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (ECL[®]) in accordance with the manufacturer's instructions (Amersham) using a luminescent image analyser (Fujifilm LAS1000).

DNA binding assay

Affinity purification of DNA-bound STAT3 was performed essentially as described previously [20]. Whole-cell extracts were prepared by lysis of approx. 20×10^6 cells/ml in RIPA buffer containing 0.1 M Tris, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.5% deoxycholate. Cell lysates were incubated with 1 µg of double-stranded, 5'-biotinylated oligonucleotide for 1 h at 4 °C. The oligonucleotide sequence used was derived from the high-affinity STAT-binding site of the *c-fos* gene (GTGCACATTTCCCGTAA-ATC). To isolate the DNA-binding proteins the oligonucleotide was coupled to 35 µl of a 50% streptavidin/agarose suspension for 1 h at 4 °C. Complexes were washed three times in lysis buffer before separation by SDS/PAGE and Western blotting with anti-STAT3 antibody.

RESULTS

Phosphorylation of STAT3

Stimulation of J774.2 macrophages with low-nanomolar doses of leptin significantly increased phosphorylation of STAT3 at both Ser⁷²⁷ and Tyr⁷⁰⁵, with the total amount of STAT3 in the detergent-extractable fraction remaining unchanged (Figure 1). Both the ERK kinases and p70 S6 kinase have previously been linked with phosphorylation of STAT3 Ser⁷²⁷, so we investigated the activation of these kinases by leptin in J774.2 macrophages using phosphospecific antibodies that recognize activated forms of the kinase. We find that p70 S6 kinase is activated by leptin, as indicated by increased levels of Ser³⁸⁹ phosphorylation (Figure 2). ERK1 and ERK2 showed significant levels of activity in the basal state, as evidenced by phosphorylation of Thr²⁰² and Tyr²⁰⁴. However, we consistently saw a reduction in the level of phosphorylation after 5 min of leptin stimulation, followed by increased phosphorylation, compared with basal, at 15 min and longer stimulations with leptin (Figure 2).

Specific inhibitors were used to determine which pathways were involved in regulating phosphorylation of STAT3 Ser⁷²⁷. Rapamycin was used to inhibit mammalian target of rapamycin (mTOR) and thus the activation of p70 S6 kinase [21], while PD98059, an inhibitor of MEK1-induced phosphorylation of ERKs, was used to block the ERK pathway. With the addition

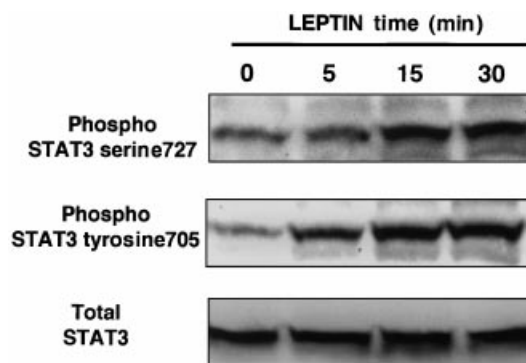


Figure 1 Leptin stimulates phosphorylation of STAT3 on serine and tyrosine

J774.2 macrophages were stimulated with 2 nM murine leptin for the indicated time. Cells were then lysed and lysates were analysed by SDS/PAGE and Western blotting using phospho-specific antibodies for STAT3 Tyr⁷⁰⁵ and Ser⁷²⁷ and an antibody that recognized total STAT3.

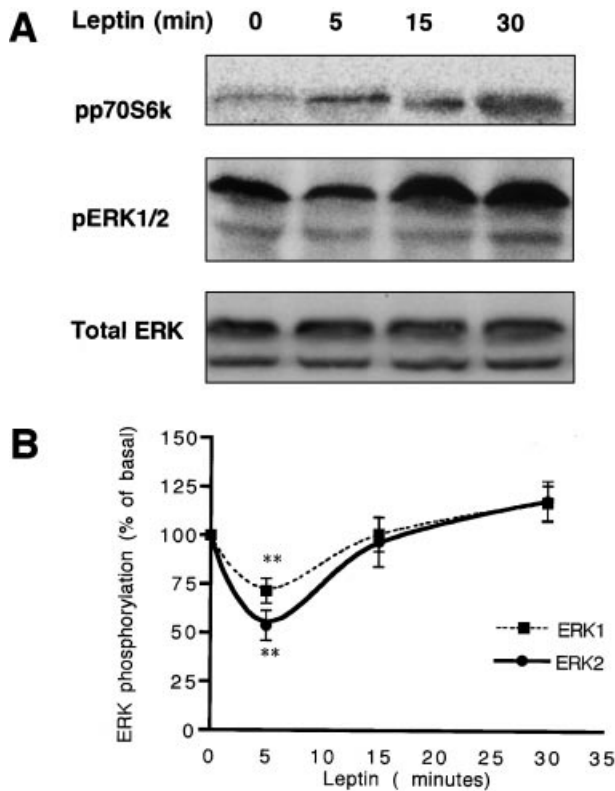


Figure 2 Leptin activates p70 S6 kinase and ERK

J774.2 macrophages were stimulated with 2 nM murine leptin for the indicated time. Cells were then lysed, and lysates were analysed by SDS/PAGE and Western blotting using phospho-specific antibodies that recognize either Ser³⁸⁹ of p70 S6 kinase or Thr²⁰²/Tyr²⁰⁴ of ERK or total ERK. Results show (A) a typical Western blot and (B) quantification, using a phosphoimager, of combined results from five determinations (performed in duplicate) of leptin's effects on ERK phosphorylation (■, pERK1 (phosphorylated ERK1); ●, pERK2) (means ± S.E.M.). ** Indicates points significantly different from basal ($P < 0.01$).

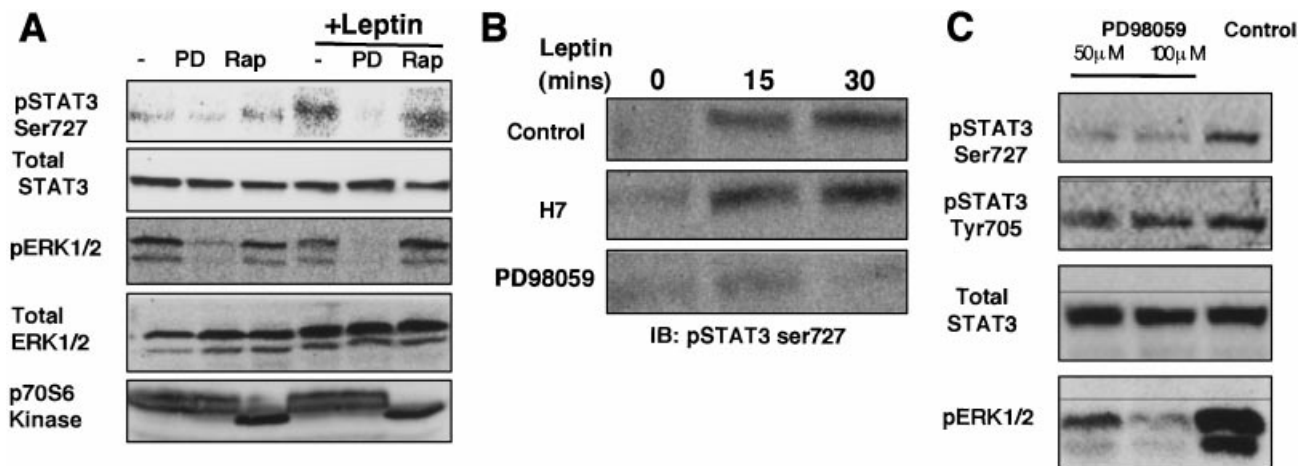


Figure 3 Effect of kinase inhibitors on leptin stimulation of STAT3 phosphorylation

(A) Serum-starved J774.2 macrophages were preincubated with inhibitors for 1 h and then stimulated with 2 nM murine leptin for 15 min. Cells were then lysed and lysates were analysed by SDS/PAGE and Western blotting as indicated using phospho-specific antibodies for STAT3 Tyr⁷⁰⁵ and Ser⁷²⁷, ERK Thr²⁰²/Tyr²⁰⁴ and with antibodies that recognized total STAT3, total ERK and total p70 S6 kinase. (B) Effect of H7 (100 μM) and PD98059 (50 μM) on leptin-stimulated STAT3 Ser⁷²⁷ phosphorylation. (C) Effect of indicated concentration of PD98059 on leptin-stimulated phosphorylation of STAT3 and ERK.

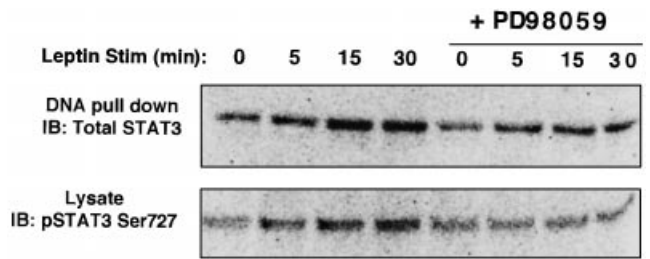


Figure 4 Effect of PD98059 on leptin stimulation of DNA-binding activity of STAT3

Serum-starved J774.2 macrophages were stimulated with 2 nM leptin for the indicated time in the presence or absence of 50 μM PD98059. Cell lysates were incubated with biotinylated oligonucleotides and bound proteins were precipitated as described in the Materials and methods section. The amount of STAT3 bound was determined by Western blotting (top panel). The amount of STAT3 phosphorylated on Ser⁷²⁷ in the original lysates was determined by Western blotting (bottom panel). Similar results were obtained in three independent experiments.

of rapamycin, the slower-migrating phosphorylated forms of the downstream mTOR effector p70 S6 kinase can no longer be seen, indicating that activation by stimulation of p70 S6 kinase is blocked (Figure 3). Using PD98059, leptin-induced phosphorylation of Thr²⁰²/Tyr²⁰⁴ was almost completely blocked, indicating that there was almost total inhibition of ERK activation (Figure 3). Preincubation of J774.2 macrophages with rapamycin or H7 did not affect leptin-induced STAT3 Ser⁷²⁷ phosphorylation (Figure 3). Incubation with PD98059 had no effect on leptin-induced STAT3 Tyr⁷⁰⁵ phosphorylation, whereas phosphorylation of Ser⁷²⁷ was greatly reduced (Figure 3).

STAT3 activity was estimated using a DNA-binding assay in which biotinylated oligonucleotides consisting of the STAT3-inducible element of the *c-fos* gene were used to affinity-purify STAT3 bound to the DNA. The data in Figure 4 show that leptin induces an increase in DNA binding after 15 min stimulation of

J774.2 macrophages. The same samples were Western-blotted for serine-phosphorylated STAT3, and it can be seen that the leptin-stimulated increase in serine phosphorylation corresponds with the increase in DNA binding. Treatment of the cells with PD98059 blocked the leptin-mediated STAT3 Ser⁷²⁷ phosphorylation and, while it greatly reduces STAT3 DNA binding activity at all time points, there is still a clear stimulation above basal levels that parallels the stimulation in the absence of the inhibitor.

DISCUSSION

The long form of the leptin receptor is expressed at high levels in macrophages, and an increasing amount of evidence indicates both an immunoregulatory role for leptin [22–24] as well as an effect on lipid metabolism in these cells [6]. Previously, we have demonstrated STAT3 tyrosine phosphorylation in response to leptin in macrophages [6]; however, it has not been established whether STAT3 is a serine kinase target in leptin-stimulated macrophages and whether this might be involved in regulating STAT3 activity.

With the use of phosphospecific antibodies we have shown that STAT3 tyrosine and serine phosphorylation are both increased after 15 min of leptin stimulation. The finding that PD98059 inhibits leptin-mediated STAT3 serine phosphorylation suggests that ERK is involved in regulating this effect. In support of this we observed increased ERK1/2 phosphorylation at time points corresponding to increased STAT3 Ser⁷²⁷ phosphorylation. ERK-dependent regulation of Ser⁷²⁷ phosphorylation is also observed in response to growth factors such as epidermal growth factor and platelet-derived growth factor [15,25]. However, neither IL-6 nor CNTF requires ERK for phosphorylation of Ser⁷²⁷. IL-6 uses an H7-sensitive pathway [16,26] and CNTF employs a pathway that requires mTOR activity [14]. Inhibition of H7-sensitive kinases and mTOR activity, however, did not affect leptin-induced STAT serine phosphorylation. The basis for these differences is not clear, but it is likely that these reflect cell specificity and differences in the types of pathways activated by different ligands.

Previous findings show that the tyrosine and serine phosphorylation of STAT3 can be interdependent or independently regulated [15,20,26]. When we blocked STAT3 serine phosphorylation with PD98059, we found that leptin-stimulated tyrosine phosphorylation was unaffected, suggesting that the tyrosine phosphorylation in response to leptin is independent of the serine phosphorylation. It is not clear if leptin-mediated serine phosphorylation could occur in the absence of tyrosine phosphorylation, although Chung et al. [15] have previously shown that activating Raf and its downstream MAP kinase pathway could induce Ser⁷²⁷ phosphorylation without tyrosine phosphorylation. This would suggest that tyrosine phosphorylation is not always a prerequisite for ERK-dependent STAT3 serine phosphorylation.

Our results show that leptin increased DNA binding of STAT3 in the same timeframe that it increased STAT3 serine phosphorylation, and inhibition of ERK reduced this DNA binding dramatically, as well as the STAT3 serine phosphorylation. From this we can conclude that leptin can induce tyrosine phosphorylation, dimer formation and STAT3 DNA binding in the absence of STAT3 Ser⁷²⁷ phosphorylation, but that leptin-stimulated modification of this serine residue increases DNA binding activity to a maximal level. It is clear from the available evidence that different signalling pathways can contribute to STAT3 serine phosphorylation and that the serine phosphorylation of STAT3 is of varying importance in STAT3 activation, with these effects being dependent on the cell type and the

stimulus used. Therefore previous findings for other cytokine signalling systems cannot be extrapolated to predict the situation with leptin stimulation in cells endogenously expressing ObRb, such as macrophages. For example, despite ObRb exhibiting similar signalling and functional specificity to IL-6 type cytokine receptors, there are also clear differences in signalling from that in STAT3. The YXXQ motif of the gp130 receptor molecule is required for the activation of the H7-sensitive pathway and STAT3 serine phosphorylation in response to IL-6 [16], whereas in leptin signalling it is presumably the conserved YXXL motif, as this is the motif in ObRb that is crucial for the activation of the ERK. Even in the case of leptin signalling it is clear that cellular context can greatly influence the contribution of different pathways to a particular response. For example, Banks et al. [9] found that activation of ERK was not required for full STAT3 activation induced by activation of leptin receptors transfected into Chinese-hamster ovary cells [9]. It must be hypothesized that the differences in the levels of expression of signalling molecules in different cell types are responsible for these differences.

The rapid and transient reduction in ERK phosphorylation at 5 min is intriguing and is similar to the effect of leptin on the ERK pathway observed in human blood mononuclear cells [27]. This could play an important role in the temporal control of the leptin-induced signal, as it has been reported that the MEK/ERK pathway is involved in the inhibition of JAK-1 and JAK-2 activation as well as STAT activation [17]. In serum-starved macrophages we saw relatively low levels of phosphorylated STAT3, despite significant levels of activated ERK. It is possible therefore that this basal ERK activation is acting to inhibit JAK-STAT signalling. Typically we see ObRb and JAK-2 phosphorylation start to increase after 5 min of leptin stimulation [6], the same time that we see the reduction in ERK activation. Therefore it is possible leptin first acts to reduce basal ERK activation to allow JAK/STAT signalling to be initiated, then increases ERK activation to maximally increase STAT3 signalling. Over time the increased ERK activity contributes to increased STAT3 activity and so acts to shut off leptin signalling through mechanisms such as induction of suppressor of cytokine signalling 3 ('SOCS3') gene expression [28].

In conclusion, our results establish a clear role for the ERK kinase cascade and Ser⁷²⁷ phosphorylation in the regulation of STAT3 activity by leptin in macrophages, and these effects are likely to be important in leptin's effects on immune function and lipid metabolism in this cell type. This provides the first description of the role of these pathways in regulating STAT3 activity in a physiologically relevant target tissue for leptin and suggests that such mechanisms may also be important in the hypothalamic neurons, which are the major leptin target tissue. However, this is by no means certain given the previous findings that cell context plays a crucial role in determining the contribution that STAT3 Ser⁷²⁷ plays in regulating its activity and will need to be confirmed in subsequent studies.

This work was funded by the British Heart Foundation.

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