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Leptin activation of Stat3 in the hypothalamus of wild-type and *ob/ob* mice but not *db/db* mice

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Leptin, a hormone secreted by adipocytes, regulates the size of the adipose tissue mass through effects on satiety and energy metabolism[1–5]. Leptin's precise sites of action are not known. The leptin receptor (Ob-R) is found in many tissues in several alternatively spliced forms raising the possibility that leptin exerts effects on many tissues including the hypothalamus[6–8]. Ob-R is a member of the gp130 family of cytokine receptors which are known to stimulate gene transcription via activation of cytosolic STAT proteins[9,10]. In order to identify the sites of leptin action *in vivo*, we assayed for activation of STAT proteins in mice treated with leptin. The STAT proteins bind to phosphotyrosine residues in the cytoplasmic domain of the ligand-activated receptor where they are phosphorylated. The activated STAT proteins dimerize and translocate to the nucleus where they bind DNA and activate transcription. The activation of STAT proteins in response to leptin was assayed in a variety of mouse tissues known to express Ob-R. Leptin injection activated Stat3 but no other STAT protein in the hypothalamus of *ob/ob* and wild-type mice but not *db/db* mice, mutants that lack an isoform of the leptin receptor. Leptin did not induce STAT activation in any of the other tissues tested. Activation of Stat3 by leptin was dose dependent and first observed after 15 minutes and maximal at 30 minutes. Our data indicate the hypothalamus is a direct target of leptin action and that this activation is critically dependent on the gp-130-like leptin receptor isoform missing in C57BLKS/J *db/db* mice[7,8,11]. This is the first *in vivo* demonstration of leptin signal transduction.

STAT activation was assayed in several tissues from C57BL/6J *ob/ob* mice injected with a single dose of leptin. Nuclear extracts were prepared 30 minutes after injection. This time interval has been shown to be sufficient for STAT activation by growth hormone and EGF *in vivo*[12,13]. The tissues tested were all known to express the leptin receptor[6–8]; they included lung and kidney — two sites with especially high levels of *Ob-R* RNA. Activation of STAT complexes was assayed using electrophoretic mobility shift analysis (EMSA). The oligonucleotide probe used, M67-SIE (*sis* inducible element), is a variant of the c-fos SIE and has been shown to bind most activated dimer STAT complexes (including Stat1,3,4,6) with high affinity. The M67-SIE binds Stat5 with low affinity. However, Stat5 binds with high affinity to a rat β-casein promoter element. An oligonucleotide probe containing this element was also used in separate experiments[14].

Leptin treatment specifically induced an M67-SIE DNA-binding activity in the hypothalamus 30 minutes after injection (Fig. 1*a*). Assays performed using nuclear extracts from hypothalami of individual mice revealed a DNA binding activity in leptin treated animals not seen in animals receiving PBS. Nuclear extracts from leptin treated animals failed to shift the β-casein oligonucleotide, suggesting that Stat5 is not activated by leptin (data not shown).

The leptin-induced DNA binding activity co-migrated with SIF-A (*sis* inducible factor; Stat3 homodimer). The protein component of the leptin induced DNA binding activity was identified as Stat3 using antibody mediated supershift analysis (Fig. 1*b*). Hypothalamic nuclear extracts from five leptin treated *ob/ob* animals were pooled, aliquoted and preincubated with each of five different anti-STAT antibodies (Fig. 1*b*). Preincubation with the anti-Stat3 antibody abolished DNA binding while preincubation with anti-Stat1,4,5,6 antibodies had no effect, providing evidence that Stat3 was and the other STAT proteins were not activated by leptin in the hypothalamus. This, along with absence of STAT activation in whole brain (see Fig. 1*c*) shows evidence for hormone-induced STAT activation in a localized region of the brain.

Although *Ob-R* RNA is expressed at a high level in lung and kidney and a lower level in liver and adrenal (data not shown), the SIF-A-like complex was not detectably induced by leptin in these tissues (Fig.1*c*). In these studies, LPS injections were used as a positive control for STAT activation. LPS activates STATs in various tissues in an indirect manner via cytokine release (including IL-6) during the acute phase response[15]. LPS has been reported to induce STAT activation in liver 75 minutes after injection and induces three distinct M67-SIE DNA binding activities: SIF-A (Stat3 homodimer), SIF-B (Stat3:Stat1 heterodimer), and SIF-C (Stat1 homodimer)[12]. Nuclear extracts were prepared 75 minutes after LPS injection of *ob/ob* mice. While LPS induced M67-SIE DNA-binding activities in liver (as described[12]) lung, kidney, adrenal and brain, leptin did not (Fig. 1*c*). As shown, there was some DNA binding activity present in all tissues, but the inter-animal variation for each tissue was similar for both leptin and PBS treated animals indicating leptin did not induce a DNA binding activity in these tissues. The STAT components of the LPS-induced DNA-binding activities were confirmed by supershift analysis to contain both Stat1 and Stat3 proteins (data not shown). Additionally, β-casein DNA-binding activities were not induced by leptin in any of the tissues tested when compared to PBS-injected controls.

The receptor for leptin is known to exist in several different isoforms, only one of which, Ob-Rb, is mutant in C57BLKS/J *db/db* mice[7,8]. The cytoplasmic region of Ob-Rb contains the JAK-binding domains, box 1 and box 2, and a potential consensus site for Stat3 binding. All other Ob-R isoforms defined to date have short cytoplasmic regions that lack these motifs. These short forms could transduce cellular signals after dimerizing with other cell surface receptors as has been reported for IL-6 and CNTF[16,17]. To test if Stat3 activation is dependent on the presence of the Ob-Rb isoform, Stat3 activation was assayed in hypothalamic nuclear extracts of individual *db/db* animals injected with a high dose of leptin (Fig. 2). Nuclear extracts from the hypothalamus of leptin injected *db/db* animals did not show evidence of Stat3

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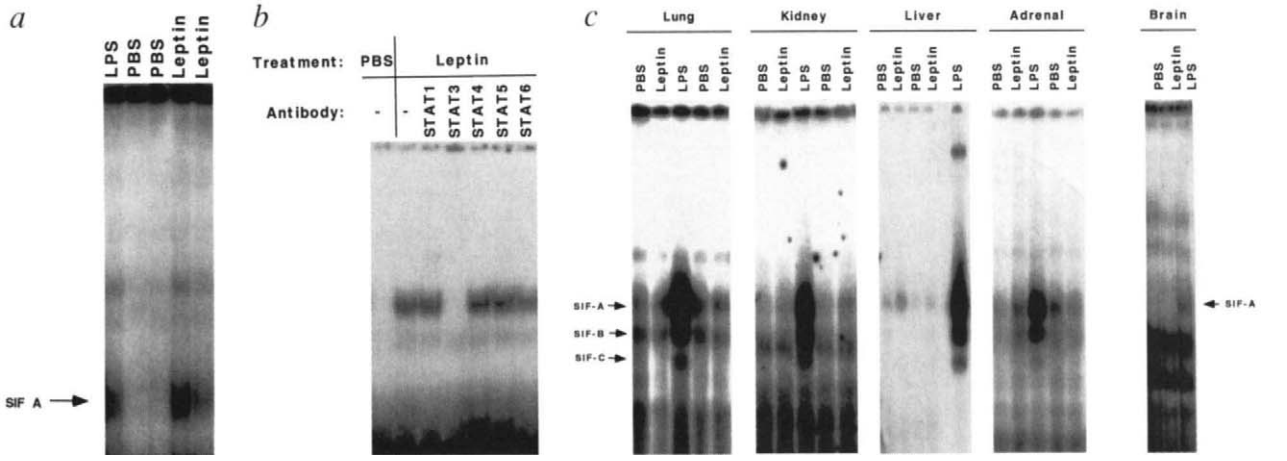

Fig. 1 DNA-binding activities from tissues of leptin *versus* PBS and LPS treated *ob/ob* mice. *a*, Six-week-old female C57BL/6J *ob/ob* mice (Jackson Laboratory) were simultaneously given a single intraperitoneal and intravenous dose of recombinant leptin (10 μg/g) or PBS. Control animals received an intraperitoneal dose of LPS (Lipopolysaccharide, Salmonella Minnesota R595- List Biological Laboratory, 12.5 μg/g in 250 μl). Animals were killed by cervical dislocation 30 min (PBS and leptin treated) or 75 min (LPS treated) after the injection. Nuclear extracts from hypothalami were prepared from individual animals and analysed for STAT activation by EMSA using an M67-SIE probe. Each lane represents the results from an individual animal. *b*, EMSA and supershift of M67-SIE binding activity from hypothalamic nuclear extracts 30 min after leptin or PBS treatment. One 6-week-old female C57BL/6J *ob/ob* animal was PBS treated while five animals were leptin injected with the same dose as in (*a*). Hypothalamic nuclear extracts of the five mice stimulated with leptin for 30 min were pooled, and realiquoted for supershift analysis with individual anti-STAT antibodies. *c*, Leptin and LPS induced STAT activation was also assayed in nuclear extracts from lung, kidney, liver, adrenal and whole brain. Tissues were dissected from the same animals used in (*a*), except brain, for which three different animals were used.

activation. In contrast, Stat3 was activated in the hypothalamus of LPS treated *db/db* animals and in the hypothalamus of leptin treated wild-type littermates of *db/db* (C57BLKS/J *db*/+) (Fig. 2). This indicates that Ob-Rb is required for Stat3 activation by leptin *in vivo*.

The dose response and time course of activation of the leptin induced activation of Stat3 in hypothalamus was assessed in both wild-type and *ob/ob* mice (Fig. 3*a*). In both cases, Stat3 activation in the hypothalamus is dose dependent. Stat3 activation is even observed at a low dose (0.1 μg/g) that does not cause weight loss in *ob/ob* or wild-type mice suggesting this is a physiological and not a pharmacological response to leptin. The sensitivity for Stat3 activation is equivalent in *ob/ob* and wild-type mice despite the increased potency of the weight reducing effects of leptin in *ob/ob* mice[2].

Intravenous administration of a low dose of leptin (0.1 μg/g) to *ob/ob* mice induces Stat3 activation in the hypothalamus after 15 minutes with a maximal response at 30 minutes (Fig. 3*b*). A similar time course for Stat3 activation was seen in wild-type mice (data not shown). By comparison, LPS injection, which is known to activate Stat3 in liver by an indirect mechanism, does not result in any detectable Stat3 activation in the brain after 30 minutes. Stat activation by LPS is readily observed 75 minutes after injection.

The rapid effect of leptin strongly suggests that it directly activates Stat3 in the hypothalamus. This is consistent with the fact that most of the pleitropic effects of leptin are known to be at least partially regulated by the hypothalamus. The lack of a response in *db/db* mice indicates that Ob-Rb, the signalling form of the leptin receptor, is required for this effect. However, activation of Stat3 is not necessarily unique to leptin and specificity of leptin action could involve additional signal transducing molecules.

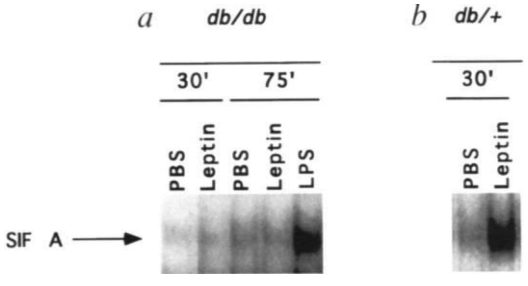

Fig. 2 *db/db* resistance to leptin mediated Stat3 activation in hypothalamus. *a*, Six-week-old female C57BLKS/J *db/db* mice were treated with leptin, PBS, or LPS and killed at the time points indicated. Hypothalamic nuclear extracts were prepared and EMSA was performed as described. *b*, C57BLKS/J *db*/+ lean littermates of *db/db* animals were treated with leptin or PBS and hypothalamic nuclear extracts prepared 30 min later.

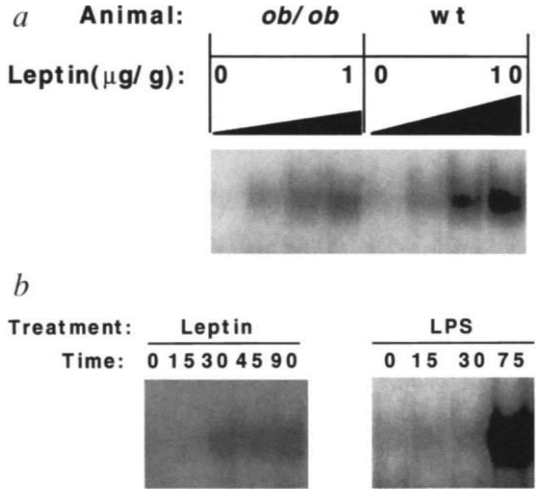

Fig. 3 Dose response and time course of Stat3 activation by leptin in hypothalamus. *a*, Six-week-old female C57BL/6J *ob/ob* mice and their lean C57BL/6J +/? litter mates (wt) were given a single 100 μl IV injection of either PBS or leptin. *ob/ob* animals received doses of 0, 0.01, 0.1 and 1.0 μg/g leptin and lean litter mates (wt) received 0, 0.1, 1.0 and 10.0 μg/g leptin. Hypothalamic nuclear extracts were prepared 30 min later and analysed by EMSA with the M67-SIE probe as described. *b*, Six-week-old female C57BL/6J *ob/ob* mice were given a single IV injection of 0.1 μg/g leptin or 0.25 μg/g LPS and killed at the indicated times. Hypothalamic nuclear extracts were prepared and analysed by EMSA with M67-SIE probe as described.

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The leptin induced activation of Stat3 *in vivo* differs from results *in vitro* showing that leptin activates Stat3, 5 and 6 in COS-7 cells co-transfected with the leptin receptor and the cloned STAT cDNAs[11]. In other cases, STAT activation is less specific *in vitro* than *in vivo*[18,19].

It is not yet clear why STAT activation was not detected in other tissues that express leptin receptor. One possibility is that the truncated isoforms (Ob-Ra, Ob-Rc, Ob-Rd and others) act as dominant negatives and that the ratio of different receptor isoforms favors Stat3 activation only in hypothalamus. This is corroborated by recent data showing that the ratio of Ob-Rb (the signalling form of the receptor) to Ob-Ra (a truncated form) is greatest in the hypothalamus[11]. These data do not exclude the possibilities that a small subpopulation of cells in other tissues respond to leptin or that cell signal transduction in these other tissues does not involve Stat3 activation. However, LPS induction of Stat3 in these tissues is well above background. Given that Stat3 activation in hypothalamus in response to leptin is greater than that seen in response to LPS, it is likely that significant Stat3 activation by Leptin would have been detected in these tissues. It is also possible that there are accessory protein(s) in the hypothalamus required for leptin mediated Stat3 activation.

The activation of Stat3 suggests that leptin modulates gene transcription in the hypothalamus. The identification of leptin inducible genes is likely to further our understanding of the molecular mechanisms by which leptin reduces weight. Further studies are required to elucidate the mechanisms by which leptin exerts its behavioral and metabolic effects.

Methods

Nuclear extract preparation. Tissues were dissected and dounce-homogenized 10 times in 100:1 (v:v) of buffer A (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Hepes pH 7.9, 1 mM DTT, 1 mM $NaVO_4$, 1× complete TM protease inhibitor (Boehringer Mannheim)). After centrifugation at 2,000*g* for 10 min at 4 °C, the pellet was resuspended in 4 volumes of the same buffer, dounced 10 times and centrifuged again at 2,000*g*. The nuclei containing pellet was carefully resuspended in 2 volumes of buffer B (420 mM NaCl, 10 mM KCl, 20 mM Hepes pH 7.9, 20% glycerol, 1 mM DTT, 1 mM $NaVO_4$, 1× complete TM protease inhibitor) and extracted for 30 min at 4 °C on a shaking rotor. After centrifugation at 16,000*g*, the supernatant was diluted 10 fold in buffer C (10 mM KCl, 20 mM Hepes pH 7.9, 20% glycerol, 1 mM DTT, 1 mM $NaVO_4$, 1× complete TM protease inhibitor) and centrifuged for 10 min at 16,000*g*. For brain and hypothalamus, the supernatant was loaded onto a microcon-50 ultrafiltration column (Amicon) and centrifuged for 15 min at 4,000*g*. The protein concentration of the concentrated and desalted sample was detemined by Bradford assay (BIO-RAD). For hypothalami, the inter-sample variation of protein concentration was always less than 50%. Samples were snap-frozen and kept at –80 °C. The procedure was carried out at 4 °C. DTT, $NaVO_4$ and the protease inhibitors were added at the time of the experiment.

Electrophoretic mobility shift assay (EMSA). EMSA was performed as described[20]. Double-stranded oligonucleotide probes were synthesized with 5'–GATC protruding ends for fill-in labelling. M67-SIE probe sequence: 5'–CATTTCCCG-TAAATCAT–3'. 700 ng (hypothalamus, brain) or 2 µg (liver, adrenal, lung, kidney) of nuclear extract protein were incubated at room temperature for 15 min in a volume of 12 µl in the presence of 100 pg of labelled probe (2×10^4–10^5 dpm), 2 µg of poly dI-dC (Pharmacia), 40 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes pH 7.9, 100 µM EGTA, 0.5 mM DTT and 4% Ficoll. Samples were resolved by 4% native PAGE in 0.25× TBE at 4 °C.

Supershift analysis. Antibody mediated supershift analysis was performed by preincubating 4 µl of the nuclear extract containing 700 ng of protein with 1 µl of a 1/30 dilution of anti-STAT antiserum in PBS for 5 min. EMSA was then performed as described above.

Assay for Stat5 activation. Nuclear extracts were prepared from hypothalamus, lung, liver, adrenal and brain after 30 min treatment with leptin or PBS and analysed by EMSA with an oligonucleotide probe containing a Stat5 binding site from the β-casein promoter: 5'–GATTTCTAGGAATTAATC–3' (ref. 14).

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