Leptin signaling is required for leucine deprivation-enhanced energy expenditure

Running title: A role of leptin signaling in leucine deprivation

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CAPSULE

Background: Leucine deprivation decreases fat mass via enhancing energy expenditure; the involvement of leptin signaling is unknown.

Results: Leucine deprivation promoted leptin signaling and the increased energy expenditure was blocked in leptin-signaling disrupted mice.

Conclusion: Leptin signaling is required for leucine deprivation-increased energy expenditure.

Significance: Our studies reveal a physiological mechanism linking leptin signaling with leucine deprivation-enhanced energy expenditure.

ABSTRACT

Leptin signaling in the hypothalamus is crucial in energy homeostasis. We have previously shown that dietary deprivation of the essential amino acid leucine in mice stimulates fat loss by increasing energy expenditure. The involvement of leptin signaling in this regulation, however, has not been reported. Here, we show that leucine deprivation promotes leptin signaling in mice maintained on an otherwise normal diet and restores leptin responses in mice maintained on a high-fat diet, a regimen known to induce leptin resistance. In addition, we found that leucine deprivation-stimulated energy expenditure and fat loss was largely blocked in db/db mice homozygous for a mutation in leptin receptor and a knock-in mice line Y3F with abrogation of leptin receptor tyrosine$^{1138}$-mediated Signal Transducer and Activator Transcripts (STAT)3 signaling. Overall, our studies describe a novel link between hypothalamic leptin signaling and stimulation of energy expenditure under leucine deprivation.

INTRODUCTION

Obesity is a complex, chronic disease and perturbed leptin signaling in the hypothalamus, the major area in the brain regulating energy homeostasis, is known to contribute to its development (1). Leptin, a peptide hormone secreted from white adipose tissue (WAT), works through the leptin receptor (Ob-R), a class 1 cytokine receptor (2). In mice, at least five forms of the receptor have been reported to be produced from the Ob-R gene, including 4 short isoforms (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Re) and a long isoform (Ob-Rb) (3). Although Ob-Rb is detectable in many tissues, it is only highly expressed in the hypothalamus (4), specifically in the arcuate nucleus of hypothalamus (ARC), ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH), with lower levels in the paraventricular nucleus of hypothalamus (PVN) and the lateral hypothalamic area (LHA) (4-6). Binding of leptin to Ob-R stimulates the phosphorylation of receptor Tyr$^{1138}$, which activates the intracellular Janus Kinase (JAK)2 /Signal Transducer and Activator of Transcription (STAT)3 pathway to reduce food intake and increase energy expenditure (7,8). Leptin resistance, which blocks the anorexic and weight-reducing effects of leptin (9), and reduces phosphorylation of STAT3 in the ARC (10), has been observed in obese mice (11) and human (12) models.

Leptin signaling is transcriptionally regulated (13,14) and significantly affected by nutritional status. Both High-Fat Diets (HFD) and high-fructose diets have been shown to cause leptin resistance (15-19). In addition, dietary protein content also influences leptin signaling (20,21). Recent studies have demonstrated that increased serum levels of amino acids are also closely related to human obesity (22), which is usually associated with leptin resistance (23). These results indicate a possible role of essential amino acids in leptin signaling.

Our lab has shown previously that dietary deprivation of leucine stimulates fat loss largely via increasing energy expenditure (24). Given
the importance of leptin signaling in the regulation of energy homeostasis, we speculated that hypothalamic leptin signaling is required for the stimulation of energy expenditure and fat loss during leucine deprivation. The aim of our current study is to investigate this possible link. In this study, we show that leptin signaling is directly involved in the regulation of energy expenditure and fat loss during leucine deprivation, and present evidence that the signaling mediated by leptin receptor Tyr1138-mediated STAT3 pathway.

**EXPERIMENT PROCEDURES**

*Animals and diets.* Male C57BL/6J mice and leptin receptor-deficient (db/db) mice were obtained from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). Y3F mice, with abrogated hypothalamic activation of STAT3 by leptin, were described as previously (25). Both db/db and Y3F mice are in the C57BL/6J background. Mice were maintained on a 12-h light/dark cycle at 25 °C. Control (nutritionally complete amino acid), (-) leu (leucine-deficient), HF (high-fat diet, containing 60% of calories as fat) and HF without leucine diets were obtained from Research Diets, Inc. (New Brunswick, NJ). Eight-to-ten-week-old WT, db/db and Y3F mice were randomly divided into control and (-) leu diet groups, with free access to diets for 7 days. Four-week-old mice received HF Diet (HFD) for 16 weeks to generate the leptin-resistant mice model (26). At the end of experiments, animals were killed by CO2 inhalation. Tissues were isolated, snap frozen, and stored at -80 °C for future analysis. These experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences (INS), Shanghai Institutes for Biological Sciences (SIBS), University of Chinese Academy of Sciences (UCAS).

**Indirect calorimetry.** Mice were maintained in a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments, Columbus, OH) for 20 h to allow them to adapt to this environment and volume of O2 consumption and CO2 production were continuously recorded during the next 24 h according to the instructions of the manufacturer.

**Rectal temperature measurement.** Rectal temperatures of mice were measured at 3 p.m. (basal metabolic state) using a rectal probe attached to a digital thermometer (Physitemp Inc., NJ, USA).

**Leptin sensitivity assay in vivo.** Leptin was administered after mice were maintained on a control or (-) leu diet for 7 days. For control and (-) leu treated mice, mice were intraperitoneally (IP) injected with either PBS or leptin (2 or 3 mg/kg) (27,28). For HFD-fed mice, mice were IP injected with either PBS or leptin (5 mg/kg) (29).

Before each study day, mice were fasted for 24 h and leptin was administered at 9:00 A.M. Food intake and body weight was measured at 1 and 4 h post-injection of leptin.

**Real-time quantitative reverse transcription-PCR (RT-PCR).** RT-PCR was performed using RNA isolated from the hypothalamus of mice after IP injected with 2 mg/kg leptin or PBS for 45 min, as described previously (30). The sequences of primers used in this study are available upon request.

**Western blot analysis.** After fasting for 24 h, mice were subjected to IP injection with vehicle (PBS) or leptin (at 3 or 5 mg/kg) for 45 min before hypothalamus were removed for analysis. Western blot analysis was performed as previously described (24). Primary antibodies phospho-STAT3, anti-total-STAT3 (Cell Signaling Technology, Beverly, MA), and anti-actin antibody (Sigma-Aldrich) were...
incubated overnight at 4°C and specific proteins were visualized by ECL (Amersham Biosciences).

**Immunohistochemistry (IHC) staining.** IHC staining was performed as described previously (16). Briefly, brain coronal sections of 25 μm were cut using a frozen microtome (Leica Microsystems, Germany), incubated with primary antibody anti-phospho-STAT3 (pTyr705) (Cell Signaling Technology, Beverly, MA), and pictures were taken by using an Olympus BX61 microscope (Olympus, Japan). Sections ranging from Bregma -1.34 mm to -2.06 mm, which contain the ARC, VMH, and DMH nuclei, were chosen for quantitative measurement of STAT3 phosphorylation levels, with the third ventricle used as the landmark. The intensity of pSTAT3-positive signals was evaluated for each nucleus using the Image-Pro Plus software program (Media Cybernetics, Inc.).

**Statistical analysis.** All data are expressed as means ± SEM, with the numbers of mice included in each group in each experiment indicated. Significant differences were assessed by two-tailed Student t-test or one-way ANOVA followed by the Student-Newman-Keuls (SNK) test. p < 0.05 was considered statistically significant.

**RESULTS**

**Leucine deprivation increases leptin signaling in mice**

To investigate the effects of leucine deprivation on leptin signaling, C57BL/6J wild-type (WT) mice were maintained on a leucine-deficient diet or control diet for 7 days, as described in our previous studies (24,30,31). Leucine deprivation significantly decreased serum leptin levels compared with mice fed a control diet (Figure 1A). The effect of leucine deprivation on leptin signaling was examined by measuring food intake and body weight following intraperitoneal injection of leptin (3 mg/kg) (28). Although food intake was inhibited to a similar extent in both leucine-deprived and control mice 1 hour after leptin injection, it was significantly decreased in leucine-deprived mice, but not in the control group, 4 hours following leptin injection (Figure 1B). In contrast, body weight was not significantly changed in either group following leptin injection (Figure 1C), which is possibly caused by the continuous water drinking during treatment.

We next examined hypothalamic expression of several key neuropeptides that regulate energy balance (27). These included orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP), anorexigenic pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART), which have previously been shown to be inhibited or stimulated, following IP administration of leptin (2 mg/kg) for 45 min (27). Leptin significantly inhibited hypothalamic expression of Npy and Agrp in leucine-deprived mice, but not in control mice (Figure 1D). We did not see the reported changes in neuropeptide expression following leptin injection in control mice, however, possible due to the lower dose of leptin used in our study, which varies among published experiments (27,28). In addition, leptin increased Cart expression, but had no effect on Pome expression in the hypothalamus of mice maintained on either control or leucine-deficient diets (Figure 1D). Consistent with a much stronger effect of leptin in leucine-deprived mice, leptin produced a much more pronounced phosphorylation of STAT3 (the “gold standard” marker of cellular leptin action (10)) in the hypothalamus of these mice, especially in the ARC area, compared with control mice as shown by immunohistochemistry (IHC) staining (Figure 1E-1G).

**Leucine deprivation restores leptin signaling**
in mice under conditions of leptin resistance induced by maintenance on a HFD

To test if leucine deprivation also stimulates leptin signaling under conditions of leptin resistance, we examined its effect in mice maintained on a HFD for 16 weeks, a regimen known to induce leptin resistance (26). These mice were subsequently maintained on either a HFD or a leucine-deficient HFD for 7 days before PBS or leptin injection. HFD increased serum leptin levels compared with control group, and this increase was significantly reversed by leucine-deficient HFD (Figure 2A). Resistance to leptin in HFD-fed mice was demonstrated by the loss of leptin-induced anorexia, corresponding neuropeptide changes in the hypothalamus and STAT3 phosphorylation in the ARC compared with mice maintained on a control diet (Figure 2B, 2D-2F). In contrast, leucine-deficient HFD largely restored leptin’s ability to reverse the above markers of leptin resistance (Figures 2B, 2D-2F). Body weight was not obviously decreased in control or HFD-fed mice, but was significantly reduced in mice maintained on a leucine-deficient HFD, following leptin injection for 1 h and 4 h (Figure 2C).

Leptin signaling is required for the enhancement of energy expenditure under leucine deprivation

We have previously shown that leucine deprivation stimulates fat loss largely by increasing energy expenditure (24). To investigate a possible role of leptin signaling in the stimulation of energy expenditure and fat loss during leucine deprivation, we fed WT mice and mice homozygous for a mutated leptin receptor (db/db) a control or leucine-deficient diet for 7 days.

Consistent with our previous results (24), leucine deprivation significantly decreased body weight and fat mass in WT mice, but both of these effects were largely blocked in db/db mice (Figure 3A and 3B). The absence of leucine deprivation-induced fat loss in db/db mice could be the result of an increase in food intake, a decrease in energy expenditure, or both. A similar reduction in food intake, however, was observed in both WT and db/db mice maintained on a leucine-deficient diet (Figure 3C). We therefore measured energy expenditure by indirect calorimetry, rectal temperature and physical activity. The total energy expenditure (24 h O2 consumption, normalized to lean body mass) was markedly increased and the respiratory exchange ratio (RER, VCO2/VO2) was low during both dark- and light-phases in WT mice maintained on a leucine-deficient diet compared with WT mice maintained on a control diet (Figure 3D and 3E). By contrast, the effects of leucine deprivation on energy expenditure and RER were absent in db/db mice (Figure 3D and 3E). Consistent with changes in energy expenditure, the increase in body temperature observed in WT mice following leucine deprivation was significantly blocked in db/db mice (Figure 3F). We did not see significant differences in physical activity between WT and db/db mice following leucine deprivation, however, basal activity in db/db mice was decreased compared with WT mice (Figure 3G).

Increased energy expenditure in leucine-deprived mice has previously been shown to correlate with an increase in uncoupling protein (UCP)1 expression in brown adipose tissue (BAT) and phosphorylation of protein kinase A (PKA) and the rate-limiting lipase hormone sensitive lipase (HSL) in white adipose tissue (WAT) (24). Here, we found that increases in UCP1 expression in BAT, as well as phosphorylation of HSL and PKA substrate, were blocked in db/db mice following leucine deprivation, and that basal levels of these mRNAs and proteins were lower in db/db mice (Figure 3H-3J).
Ob-Rb Tyr^1138-mediated STAT3 signaling is required for increased energy expenditure during leucine deprivation

One of the major pathways mediating the effects of leptin depends on STAT3, as evidenced by the observation that neuron-specific deletion of Stat3 recapitulates the obese phenotype of db/db mice (32). To explore the possible role of hypothalamic STAT3 signaling during leucine deprivation, we used knock-in Y3F mice, in which hypothalamic activation of STAT3 by leptin is prevented by a tyrosine-to-phenylalanine substitution at Tyr^1138 of Ob-Rb(25). Y3F and WT control mice were maintained on a leucine-deficient or control diet for 7 days prior to examination of related metabolic parameters. Phenotypically, the response to leucine deficiency in Y3F mice closely matched that of db/db mice (Figure 4A-4J). Although UCP1 expression in BAT was slightly induced in Y3F mice following leucine deprivation, UCP1 levels were much lower compared with those in leucine-deprived WT mice (Figure 4H and 4I).

DISCUSSION

In addition to transcriptional and/or translational regulation of genes related to leptin signaling (13,14), nutritional status may also influence leptin signaling. It is well known that HFD causes leptin resistance (15-17). Chronic fructose-diets have also been shown to be associated with increased plasma leptin levels and induce leptin resistance prior to the onset of obesity (17-19). Moreover, fructose-free but high-fat diets can reverse high-fructose/high-fat diet induced leptin resistance in rats, suggesting that fructose in diet is the bioactive ingredient that causes leptin resistance (33).

Several lines of evidence suggest a relationship between essential amino acids and leptin secretion and signaling. For example, leucine activates leptin expression in rat adipose cells (34) and increases satiety by stimulating of leptin secretion in rats (35). It has also been reported that leucine promotes leptin receptor expression in mouse C2C12 myotubes (36). In addition, dietary supplementation of arginine or histidine has been reported to suppress serum leptin levels (37,38). A direct effect of essential amino acids on leptin signaling, however, has not previously been reported.

In our current study, we showed for the first time that dietary deficiency of leucine has a significant effect on leptin signaling, as demonstrated by decreased food intake, body weight, adiposity, change of neuropeptide, phosphorylation of STAT3 in ARC area following leptin stimulation in mice maintained on a leucine-deficient diet. In contrast to our observation that leucine deprivation improves leptin signaling in mice, it has been reported that high-protein diet decreases caloric intake in human, possibly mediated via increased leptin sensitivity (20), and low-protein diet increases food intake and serum leptin levels in rats, possibly reflecting a state of leptin resistance (21). These results provide important information for understanding of nutritional regulation of leptin signaling. On the other hand, the effects of individual amino acid should not be equated with high- or low-levels of proteins and deserve independent investigation.

Leptin resistance has been identified as one of the major contribution factors in obesity, based on observations in db/db mice, HFD-fed mice and fructose-fed mice (15-17,19,39). Various strategies have been proposed to promote leptin signaling, including over-expression of Src-homology-2 (SH2)-B (13), inhibition of protein-tyrosine phosphatase (PTP)1B expression (14), treatment with drugs such as metformin (40), or feeding a fish-rich diet (41). Here, we showed that dietary leucine deprivation can also efficiently reverse the decreased leptin signaling in a leptin-resistant mice model. These results further indicate a role for dietary amino acid content in leptin
sensitivity and suggest that manipulation of dietary amino acids may be an effective way to improve leptin signaling and thereby decrease body weight. Furthermore, due to the importance of amino acids in leptin signaling, we speculate that the attenuated leptin signaling in obese human patients might be caused by the increased serum levels of amino acids such as leucine.

Our previous work has shown that leucine deprivation stimulates fat loss largely via increasing energy expenditure (24). It is known that leptin increases energy expenditure and promotes fat metabolism in ob/ob mice, which are deficient in leptin secretion (42). Furthermore, it has been shown that central injection of leptin efficiently augments BAT UCP1 and prevents weight gain in HFD-fed rats (43). In our study, a role for leptin signaling in the regulation of energy expenditure during leucine deprivation was confirmed by the observation of leucine deprivation-mediated decreases in fat mass and increases in energy expenditure were blocked in db/db mice. Our observation that leptin levels are decreased in leucine-deprived mice compared with control group, confirm that enhanced leptin signaling is responsible for the stimulation of energy expenditure during leucine deprivation. Lower leptin levels in leucine-deprived mice could result from decreased fat mass, or from a direct effect of leucine deprivation on leptin secretion. Consistent with the latter possibility, it has been reported that leucine stimulates leptin secretion (34,35).

Leptin functions by activating STAT3-dependent and independent intracellular signaling pathways (16,25). Binding of leptin to its membrane receptor Ob-Rb stimulates receptor Tyr1138, Tyr985 and Tyr1077 phosphorylation (44). Phosphorylated Tyr1138 recruits STAT3 and activates the JAK2/STAT3 pathway, which modulates energy homeostasis (7,8,45). By using Y3F mice with abrogated Tyr1138-mediated STAT3 signaling (25), we provided evidence that hypothalamic STAT3 contributes to leptin-dependent regulation of energy expenditure during leucine deprivation. Consistent with our results, STAT3 signaling has been shown to be critical for leptin regulation of UCP1 expression in BAT (8).

One of the downstream targets in mediating effects of hypothalamic STAT3 may be Ribosomal Protein S6 Kinase 1 (S6K1), the downstream target of the Mammalian Target of Rapamycin (mTOR) kinase (46). Our previous study has shown that S6K1 activity is decreased in the hypothalamus and acts as a major regulator of increased thermogenesis and fat loss during leucine deprivation (31). By contrast, S6K1 activity is not decreased in the hypothalamus of leucine-deprived db/db and Y3F mice compared with control groups (our unpublished data). In addition to that Tyr1138-mediated STAT3-dependent pathway, intracellular signaling pathways including extracellular signal-regulated kinase (ERK), suppressor of cytokine signaling (SOCS)3 and STAT5, which are regulated by phosphorylation of Ob-Rb at other tyrosine sites, have been identified as important regulators of energy homeostasis (47-49). The possible role of these effectors in the leucine and leptin-mediated regulation of energy homeostasis need to be investigated in the future.

The mechanism by which leucine deprivation regulates leptin signaling, however, is not understood yet. Previous studies have indicated SH2B1, SOCS3 and PTP1B as important regulators (13,14,50), for leptin signaling. We did not, however, observe any changes in the hypothalamic expression of Sh2b, Socs3 and Ptp1b by leucine deprivation (Data not shown). Recent study have demonstrated that Rho-kinase (ROCK)1, which activity is influenced by nutritional status (51,52), increases phosphorylation of JAK2 and downstream activation of STAT3 to regulate leptin action (45).
A possible role for ROCK1 in connecting leucine deprivation with leptin pathway will be studied in the future.

Taken together, our results show that leucine deprivation promotes leptin signaling in mice maintained on an otherwise normal diet and restores the responses to leptin under leptin-resistant condition in HFD-fed mice. In addition, we show that leptin signaling is directly involved in the stimulation of energy expenditure and fat loss under leucine deprivation and that this effect is likely to be mediated by STAT3-dependent pathway (Figure 4K). These results describe a novel link between hypothalamic leptin/STAT3 signaling and stimulation of energy expenditure under leucine deprivation, and also provide a new perspective for understanding the nutritional control of leptin signaling and the role of leptin signaling in energy homeostasis under deprivation of an essential amino acid. Future studies, however, will be required to elucidate mechanisms underlying leucine deprivation control of improved leptin signaling in the hypothalamus and identify specific STAT3 expressing neurons response for leucine deprivation-increased energy expenditure.

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REFERENCES

1. Sahu, A. (2004) Minireview: A hypothalamic role in energy balance with special emphasis on leptin. Endocrinology 145, 2613-2620
2. Tartaglia, L. A. (1997) The leptin receptor. J Biol Chem 272, 6093-6096
3. Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lee, J. I., and Friedman, J. M. (1996) Abnormal splicing of the leptin receptor in diabetic mice. Nature 379, 632-635
4. Fei, H., Okano, H. J., Li, C., Lee, G. H., Zhao, C., Darnell, R., and Friedman, J. M. (1997) Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. P Natl Acad Sci USA 94, 7001-7005
5. Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., and Trayhurn, P. (1996) Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. Fehs Lett 387, 113-116
6. Elmquist, J. K., Bjorbaek, C., Ahima, R. S., Flier, J. S., and Saper, C. B. (1998) Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neural 395, 535-547
7. Bates, S. H., Stearns, W. H., Dundon, T. A., Schubert, M., Tso, A. W. K., Wang, Y. P., Banks, A. S., Lavery, H. J., Haq, A. K., Maratos-Flier, E., Neel, B. G., Schwartz, M. W., and Myers, M. G. (2003) STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature 421, 856-859
8. Bates, S. H., Dundon, T. A., Seifert, M., Carlson, M., Maratos-Flier, E., and Myers, M. G. (2004) LRb-STAT3 signaling is required for the neuroendocrine regulation of energy expenditure by leptin. Diabetes 53, 3067-3073
9. Dardeno, T. A., Chou, S. H., Moon, H. S., Chamberland, J. P., Fiorenza, C. G., and Mantzoros, C. S. (2010) Leptin in human physiology and therapeutics. *Front Neuroendocrin* 31, 377-393

10. Myers, M. G., Leibel, R. L., Seeley, R. J., and Schwartz, M. W. (2010) Obesity and leptin resistance: distinguishing cause from effect. *Trends Endocrin Met* 21, 643-651

11. Tam, J., Cinar, R., Liu, J., Godlewski, G., Wesley, D., Jourdan, T., Szanda, G., Mukhopadhyay, B., Cheleder, L., Liow, J. S., Innis, R. B., Cheng, K. J., Rice, K. C., Deschamps, J. R., Chorvat, R. J., McElroy, J. F., and Kunos, G. (2012) Peripheral Cannabinoid-1 Receptor Inverse Agonism Reduces Obesity by Reversing Leptin Resistance. *Cell Metab* 16, 167-179

12. Kelesidis, T., Kelesidis, I., Chou, S., and Mantzoros, C. S. (2010) Narrative review: the role of leptin in human physiology: emerging clinical applications. *Ann Intern Med* 152, 93-100

13. Li, Z. Q., Zhou, Y. J., Carter-Su, C., Myers, M. G., and Rui, L. (2007) SH2B1 enhances leptin signaling by both janus kinase 2 Tyr(813) phosphorylation-dependent and -independent mechanisms. *Mol Endocrinol* 21, 2270-2281

14. Zabolotny, J. M., Bence-Hanulec, K. K., Stricker-Krongrad, A., Haj, F., Wang, Y. P., Minokoshi, Y., Kim, Y. B., Elmquist, J. K., Tartaglia, L. A., Kahn, B. B., and Neel, B. G. (2002) PTP1B regulates leptin signal transduction in vivo. *Dev Cell* 2, 489-495

15. Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H., and Cai, D. (2008) Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. *Cell* 135, 61-73

16. You, J., Yu, Y., Jiang, L., Li, W. X., Yu, X. X., Gonzalez, L., Yang, G. Q., Ke, Z. J., Li, W. J., Li, C., and Liu, Y. (2010) Signaling through Tyr(985) of Leptin Receptor as an Age/Diet-Dependent Switch in the Regulation of Energy Balance. *Mol Cell Biol* 30, 1650-1659

17. Huang, B. W., Chiang, M. T., Yao, H. T., and Chiang, W. (2004) The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats. *Diabetes Obes Metab* 6, 120-126

18. Lee, Y. C., Ko, Y. H., Hsu, Y. P., and Ho, L. T. (2006) Plasma leptin response to oral glucose tolerance and fasting/re-feeding tests in rats with fructose-induced metabolic derangements. *Life Sci* 78, 1155-1162

19. Shapiro, A., Mu, W., Roncal, C., Cheng, K. Y., Johnson, R. J., and Scarpace, P. J. (2008) Fructose-induced leptin resistance exacerbates weight gain in response to subsequent high-fat feeding. *Am J Physiol-Reg I* 295, R1370-R1375

20. Weigle, D. S., Breen, P. A., Matthys, C. C., Callahan, H. S., Meeuws, K. E., Burden, V. R., and Purnell, J. Q. (2005) A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. *Am J Clin Nutr* 82, 41-48

21. Du, F. Y., Higginbotham, D. A., and White, B. D. (2000) Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. *J Nutr* 130, 514-521

22. Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., Haqq, A. M., Shah, S. H., Arlottoto, M., Slentz, C. A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B. R., Yancy, W. S., Jr., Eisenson, H., Musante, G, Surwit, R. S., Millington, D. S., Butler, M. D., and Svetkey, L. P. (2009) A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 9, 311-326

23. Fukuda, M., Williams, K. W., Gautron, L., and Elmquist, J. K. (2011) Induction of leptin resistance by activation of cAMP-Epac signaling. *Cell Metab* 13, 331-339

24. Cheng, Y., Meng, Q. S., Wang, C. X., Li, H. K., Huang, Z. Y., Chen, S. H., Xiao, F., and Guo, F. F.
(2010) Leucine Deprivation Decreases Fat Mass by Stimulation of Lipolysis in White Adipose Tissue and Upregulation of Uncoupling Protein 1 (UCP1) in Brown Adipose Tissue. *Diabetes* **59**, 17-25
25. Jiang, L., You, J., Yu, X. X., Gonzalez, L., Yu, Y., Wang, Q., Yang, G. Q., Li, W. J., Li, C., and Liu, Y. (2008) Tyrosine-dependent and -independent actions of leptin receptor in control of energy balance and glucose homeostasis. *P Natl Acad Sci USA* **105**, 18619-18624
26. Munzberg, H., Flier, J. S., and Bjorbaek, C. (2004) Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. *Endocrinology* **145**, 4880-4889
27. Enriori, P. J., Evans, A. E., Sinnayah, P., Jobst, E. E., Tonelli-Lemos, L., Billes, S. K., Glavas, M. M., Grayson, B. E., Perello, M., Nillni, E. A., Grove, K. L., and Cowley, M. A. (2007) Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. *Cell Metab* **5**, 181-194
28. Bewick, G. A., Kent, A., Campbell, D., Patterson, M., Ghatei, M. A., Bloom, S. R., and Gardiner, J. V. (2009) Mice With Hyperghrelinemia Are Hyperphagic and Glucose Intolerant and Have Reduced Leptin Sensitivity. *Diabetes* **58**, 840-846
29. Cristino, L., Busetto, G., Imperatore, R., Ferrandino, I., Palomba, L., Silvestri, C., Petrosino, S., Orlando, P., Bentivoglio, M., Mackie, K., and Di Marzo, V. (2013) Obesity-driven synaptic remodeling affects endocannabinoid control of orexinergic neurons. *P Natl Acad Sci USA* **110**, E2229-E2238
30. Cheng, Y., Zhang, Q., Meng, Q. S., Xia, T. T., Huang, Z. Y., Wang, C. X., Liu, B., Chen, S. H., Xiao, F., Du, Y., and Guo, F. F. (2011) Leucine Deprivation Stimulates Fat Loss via Increasing CRH Expression in the Hypothalamus and Activating The Sympathetic Nervous System. *Mol Endocrinol* **25**, 1624-1635
31. Xia, T., Cheng, Y., Zhang, Q., Xiao, F., Liu, B., Chen, S., and Guo, F. (2012) S6K1 in the central nervous system regulates energy expenditure via MC4R/CRH pathways in response to deprivation of an essential amino acid. *Diabetes* **61**, 2461-2471
32. Gao, Q., Wolfgang, M. J., Neschen, S., Morino, K., Horvath, T. L., Shulman, G. I., and Fu, X. Y. (2004) Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. *P Natl Acad Sci USA* **101**, 4661-4666
33. Shapiro, A., Tumer, N., Gao, Y. X., Cheng, K. Y., and Scarpace, P. J. (2011) Prevention and reversal of diet-induced leptin resistance with a sugar-free diet despite high fat content. *Brit J Nutr* **106**, 390-397
34. Roh, C., Han, J. R., Tzatsos, A., and Kandror, K. V. (2003) Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes. *Am J Physiol-Endoc M* **284**, E322-E330
35. Lynch, C. J., Gern, B., Lloyd, C., Hutson, S. M., Eicher, R., and Vary, T. C. (2006) Leucine in food mediates some of the postprandial rise in plasma leptin concentrations. *Am J Physiol-Endoc M* **291**, E621-E630
36. Mao, X. B., Zeng, X. F., Wang, J. J., and Qiao, S. Y. (2011) Leucine promotes leptin receptor expression in mouse C2C12 myotubes through the mTOR pathway. *Mol Biol Rep* **38**, 3201-3206
37. Fu, W. J. J., Haynes, T. E., Kohli, R., Hu, J. B., Shi, W. J., Spencer, T. E., Carroll, R. J., Meininger, C. J., and Wu, G. Y. (2005) Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. *J Nutr* **135**, 714-721
38. Kasaoka, S., Tsuboyama-Kasaoka, N., Kawahara, Y., Inoue, S., Tsuji, M., Ezaki, O., Kato, H., Tsuchiya, T., Okuda, H., and Nakajima, S. (2004) Histidine supplementation suppresses food intake and fat accumulation in rats. *Nutrition* **20**, 991-996
39. Lin, H. Y., Xu, Q., Yeh, S., Wang, R. S., Sparks, J. D., and Chang, C. (2005) Insulin and leptin resistance with hyperleptinemia in mice lacking androgen receptor. *Diabetes* **54**, 1717-1725
40. Kim, Y. W., Kim, J. Y., Park, Y. H., Park, S. Y., Won, K. C., Choi, K. H., Huh, J. Y., and Moon, K. H. (2006) Metformin restores leptin sensitivity in high-fat-fed obese rats with leptin resistance. *Diabetes*
55. Winnicki, M., Somers, V. K., Accurso, V., Phillips, B. G., Puato, M., Palatini, P., and Paulletto, P. (2002) Fish-rich diet, leptin, and body mass. *Circulation* **106**, 289-291

41. Hwa, J. J., Fawzi, A. B., Graziano, M. P., Ghiaudia, L., Williams, P., VanHeek, M., Davis, H., Rudinski, M., Sybertz, E., and Strader, C. D. (1997) Leptin increases energy expenditure and selectively promotes fat metabolism in ob/ob mice. *Am J Physiol-Reg I* **272**, R1204-R1209

42. Dube, M. G., Beretta, E., Dhillon, H., Ueno, N., Kalra, P. S., and Kalra, S. P. (2002) Central leptin gene therapy blocks high-fat diet-induced weight gain, hyperleptinemia, and hyperinsulinemia - Increase in serum ghrelin levels. *Diabetes* **51**, 1729-1736

43. Kloeck, C., Haq, A. K., Dunn, S. L., Lavery, H. J., Banks, A. S., and Myers, M. G. (2002) Regulation of Jak kinases by intracellular leptin receptor sequences. *J Biol Chem* **277**, 41547-41555

44. Huang, H., Kong, D., Byun, K. H., Ye, C., Koda, S., Lee, D. H., Oh, B. C., Lee, S. W., Lee, B., Zabolotny, J. M., Kim, M. S., Bjorbaek, C., Lowell, B. B., and Kim, Y. B. (2012) Rho-kinase regulates energy balance by targeting hypothalamic leptin receptor signaling. *Nat Neurosci* **15**, 1391-1398

45. Dube, M. G., Beretta, E., Dhillon, H., Ueno, N., Kalra, P. S., and Kalra, S. P. (2002) Central leptin gene therapy blocks high-fat diet-induced weight gain, hyperleptinemia, and hyperinsulinemia - Increase in serum ghrelin levels. *Diabetes* **51**, 1729-1736

46. Kloek, C., Haq, A. K., Dunn, S. L., Lavery, H. J., Banks, A. S., and Myers, M. G. (2002) Regulation of Jak kinases by intracellular leptin receptor sequences. *J Biol Chem* **277**, 41547-41555

47. Banks, A. S., Davis, S. M., Bates, S. H., and Myers, M. G. (2000) Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem* **275**, 14563-14572

48. Bjorbaek, C., Lavery, H. J., Bates, S. H., Olson, R. K., Davis, S. M., Flier, J. S., and Myers, M. G. (2000) SOCS3 mediates feedback inhibition of the leptin receptor via Tyr(985). *J Biol Chem* **275**, 40649-40657

49. Gong, Y., Ishida-Takahashi, R., Villanueva, E. C., Finger, D. C., Muenzberg, H., and Myers, M. G. (2007) The long form of the leptin receptor regulates STAT5 and ribosomal protein S6 via alternate mechanisms. *J Biol Chem* **282**, 31019-31027

50. Mori, H., Hanada, R., Hanada, T., Aki, D., Mashima, R., Nishinakamura, H., Torisu, T., Chien, K. R., Yasukawa, H., and Yoshimura, A. (2004) Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat Med* **10**, 739-743

51. Huang, H., Lee, S. H., Ye, C., Lima, I. S., Oh, B. C., Lowell, B. B., Zabolotny, J. M., and Kim, Y. B. (2013) ROCK1 in AgRP neurons regulates energy expenditure and locomotor activity in male mice. *Endocrinology* **154**, 3660-3670

52. Chuang, S. M., Juan, Y. S., Long, C. Y., Huang, C. H., Levin, R. M., and Liu, K. M. (2011) The effect of L-arginine on bladder dysfunction following ovariectomy in a rabbit model. *International urogynecology journal* **22**, 1381-1388

**FIGURE LEGENDS**

**Figure 1. Leucine deprivation increases leptin signaling.** Mice were fed a control (con) or leucine-deficient [(-) leu] diet for 7 days, followed by measuring serum leptin levels in A; fasted for 24 h prior to intraperitoneally (IP) injecting PBS (- leptin) or 3 mg/kg leptin (+ leptin), followed by measuring food intake and body weight 1 h and 4 h post-injection in B and C, or analyzing STAT3 phosphorylation 45 min post-injection in E and F; or fasted for 24 h prior to IP injecting PBS (- leptin) or 2 mg/kg leptin (+ leptin), followed by analyzing levels of mRNA 45 min post-injection in D. Data are mean ± SEM (n = 5-6 for each group in Figures A-E; n = 3 or 4 in Figure F and G). Statistical significance was determined by the Student *t*-test: *p* < 0.05 [for the effect of (-) leu versus control diet] for panel A and E, or by ANOVA followed by the Student-Newman-Keuls (SNK) test: *p* < 0.05.
[for the effect of any group versus PBS treated-control mice], \#p < 0.05 [for the effect of leptin-treated (-) leu group versus PBS-treated (-) leu group], \&p < 0.05 [for the effect of leptin-treated (-) leu group versus leptin-treated control group] for B-D and G. (A) Serum leptin levels; (B) Food intake; (C) Body weight change; (D) Hypothalamic neuropeptide changes; (E) Hypothalamic STAT3 proteins (upper, western blot; lower, quantitative measurements of p-STAT3 protein relative to total STAT3); (F) Immunohistochemistry staining for p-STAT3 in hypothalamus. 3V, third ventricle; ARC, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus. Images shown are representative of several animals for each group. Scale bar, 500 \(\mu m\) and 200 \(\mu m\); (G) Quantitation of the intensities of positive p-STAT3 signals within the ARC, VMH, and DMH regions as marked in panel F. Relative signal intensities are quantified using Image-Pro Plus software from sections at Bregma -1.34 mm to -2.06 mm for each indicated treatment group.

Figure 2. Leucine deprivation restores leptin signaling in mice under conditions of leptin resistance induced by high-fat diet (HFD). Mice were fed a control or HFD for 16 weeks, followed by feeding control diet (con), HFD (HF) or a HFD without leucine [(-) leu HF] for 7 days. These mice were measured serum leptin levels in A; or fasted for 24 h prior to intraperitoneally (IP) injecting PBS (- leptin) or 5 mg/kg leptin (+ leptin), followed by measuring food intake and body weight 1 h and 4 h post-injection in B and C, or analyze levels of mRNA and STAT3 phosphorylation 45 min post-injection in D and E. Data are mean ± SEM (n = 5-7 for each group in Figures A-D; n = 3 or 4 in Figure E and F). Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls (SNK) test: *p < 0.05 [for the effect of HFD diet versus control diet], \#p < 0.05 [for the effect of leucine-deprived HFD versus HFD] for panel A, and *p < 0.05 [for the effect of leptin-treated group versus PBS-treated group under the same diet], \#p < 0.05 [for the effect of PBS-treated HFD versus PBS-treated control diet], \&p < 0.05 [for the effect of PBS-treated (-) leu HFD versus PBS-treated HFD] for panel B-F. (A) Serum leptin levels; (B) Food intake; (C) Body weight change; (D) Hypothalamic neuropeptide changes; (E) Immunohistochemistry staining for p-STAT3 in hypothalamus. 3V, third ventricle; Arc, arcuate nucleus. VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus. Images shown are representative of several animals for each group. Scale bar, 500 \(\mu m\) and 200 \(\mu m\). (F) Quantitation of the intensities of positive p-STAT3 signals within the ARC, VMH, and DMH regions as marked in panel E. Relative signal intensities are quantified using Image-Pro Plus software from sections at Bregma -1.34 mm to -2.06 mm for each indicated treatment group.

Figure 3. Leptin signaling is required for leucine deprivation-increased energy expenditure. Wild-type (WT) and db/db mice with leptin receptor mutation (DB) were fed a control (con) or leucine-deficient [(-) leu] diet for 7 days. Energy expenditure was measured by indirect calorimetry. Data are mean ± SEM (n = 5-8 for each group). Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls (SNK) test: *p < 0.05 [for the effect of any group versus WT mice with control diet], \#p < 0.05 [for the effect of DB mice with (-) leu diet versus DB mice with control diet], \&p < 0.05 [for the effect of DB mice with (-) leu diet versus WT mice with (-) leu diet]. (A) Body weight change; (B) Adipose tissue mass in proportion to body weight; (C) Daily food intake; (D) 24 h oxygen consumption (VO2); (E) Respiratory exchange ratio (RER); (F) Rectal temperature; (G) Physical activity; (H) Ucp1 mRNA expression in BAT; (I) UCP1 protein in BAT (upper, western blot; lower, quantitative measurements of UCP1 protein relative to actin); (J) p-HSL and p-PKA
substrate protein in WAT (upper, western blot; lower, quantitative measurements of p-HSL and p-PKA substrate protein relative to total HSL and actin, respectively).

**Figure 4. Hypothalamic Ob-Rb Tyr^{1138}-mediated STAT3 is required for leucine deprivation-increased energy expenditure.** Wild-type (WT) and knock-in mice line with abrogated leptin receptor tyr^{1138}-mediated STAT3 signaling (Y3F) were fed a control (con) or leucine-deficient [(-) leu] diet for 7 days. Energy expenditure was measured by indirect calorimetry. Data are mean ± SEM (n = 5-6 for each group). Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls (SNK) test: *p < 0.05 [for the effect of any group versus WT mice with control diet], #p < 0.05 [for the effect of Y3F mice with (-) leu diet versus Y3F mice with control diet], &p < 0.05 [for the effect of Y3F mice with (-) leu diet versus WT mice with (-) leu diet]. (A) Body weight change; (B) Adipose tissue mass in proportion to body weight; (C) Daily food intake; (D) 24 h oxygen consumption (VO \textsubscript{2}); (E) Respiratory exchange ratio (RER); (F) Rectal temperature; (G) Physical activity; (H) Ucp1 mRNA expression in BAT; (I) UCP1 protein in BAT (upper, western blot; lower, quantitative measurements of UCP1 protein relative to actin); (J) p-HSL and p-PKA substrate protein in WAT (upper, western blot; lower, quantitative measurements of p-HSL and p-PKA substrate protein relative to total HSL and actin, respectively); (K) working model.
Figure 1

A. Serum Leptin (ng/ml)

B. Food Intake (g)

C. Body Weight Change (%)

D. Relative mRNA (%)

NPY, AgRP, POMC, CART

E. Leptin

p-STAT3, t-STAT3

- leptin, + leptin, (-) leu, con + lepton, (-) leu + lepton
Figure-1

F

Leptin

con

(-) leu

G

p-STAT3 (relative intensity)

ARC VMH DMH

con (-) leu con (-) leu con (-) leu

* 

& 

# 

10

5

0
Figure-2

A

Serum Leptin (ng/ml)

con  HF  (-) leu HF

0  5  10  15  20

Body Weight Change (%)

con HF con HF

1 h 4 h

con HF (-) leu HF (-) leu HF

B

Food Intake (kcal)

con HF (-) leu HF con HF

1 h 4 h

con HF (-) leu HF

16  10  5  0

C

Body Weight Change (%)

con HF (-) leu HF

1 h 4 h

con HF (-) leu HF

D

Relative mRNA (%)

NPY AgRP POMC CART

con HF (-) leu HF con HF (-) leu HF con HF (-) leu HF con HF (-) leu HF

*  #

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Figure-3

A. Body Weight Change (%)

B. Abdominal Fat Mass /Body Weight (%)

C. Food Intake (g/day)

D. VO2 (ml/kg/hr)

E. RER

F. Rectal Temperature (°C)

G. Locomotor Activity (counts)

- con
- (-) leu

Table:

| Condition | Light | Dark | 24 h |
|-----------|-------|------|------|
| Body Weight (wt) | 10 | 5 | 0 |
| Rectal Temperature (°C) | 37 | 36.5 | 35.5 |
| Vo2 (ml/kg/hr) | 5000 | 4000 | 3000 |
| Food Intake (g/day) | 5 | 2.5 | 1 |
| Abdominal Fat Mass /Body Weight (%) | 10 | 5 | 0 |
| Locomotor Activity (counts) | 2000 | 1000 | 0 |
Figure 3

H. BAT

Relative UCP1 mRNA (%)

|   | WT | DB |
|---|----|----|
| con | 300 | 0
| (-) leu | * | *

I. BAT

UCP1

actin

|   | WT | DB |
|---|----|----|
| con | 300 | 0
| (-) leu | * | *

J. WAT

p-HSL

t-HSL

p-PKA substrate

actin

|   | WT | DB |
|---|----|----|
| con | 300 | 0
| (-) leu | * | *

Arbitrary Units

WT DB WT DB WT DB
Figure 4

A. Body Weight Change (%)

B. Abdominal Fat Mass / Body Weight (%)

C. Food Intake (g/day)

D. VO2 (ml/kg/hr)

E. RER

F. Rectal Temperature (°C)

G. Locomotor Activity (counts)

WT Y3F

light dark 24 h

con (-) leu

* * * * *

* * * * *
**Figure 4**

**H**

**BAT**

Relative UCP1 mRNA (%)  

- **WT**  
- **Y3F**

**I**

**BAT**

|          | WT con | WT (-) leu | Y3F con | Y3F (-) leu |
|----------|--------|------------|---------|-------------|
| UCP1     | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| actin    | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |

**J**

**WAT**

|          | WT con | WT (-) leu | Y3F con | Y3F (-) leu |
|----------|--------|------------|---------|-------------|
| p-HSL    | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| t-HSL    | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| p-PKA substrate | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
| actin    | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |

**K**

- **Hypothalamus**
- **Ob-Rb**
- **pY1138**
- **STAT3**

**Leptin**  

**BAT Energy Expenditure**  

**WAT Lipolysis**  

**Fat Loss**

**(-) leu**

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Leptin signaling is required for leucine deprivation-enhanced energy expenditure
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