Title	Possible involvement of uncoupling protein 1 in appetite control by leptin
Author(s)	Okamatsu-Ogura, Yuko; Nio-Kobayashi, Junko; Iwanaga, Toshihiko; Terao, Akira; Kimura, Kazuhiro; Saito, Masayuki
Citation	Experimental Biology and Medicine, 236(11), 1274-1281 https://doi.org/10.1258/ebm.2011.011143
Issue Date	2011-11
Doc URL	http://hdl.handle.net/2115/47824
Rights	Exp Biol Med November 2011 vol. 236 no. 11 1274-1281, doi: 10.1258/ebm.2011.011143. This is the final draft, after peer-review, of a manuscript published in RSM journals: www.rsmjournals.com .
Type	article (author version)
File Information	EBM236-11_1274-1281.pdf

[Instructions for use](#)

Possible involvement of uncoupling protein 1 in appetite control by leptin

Yuko Okamatsu-Ogura¹, Junko Nio-Kobayashi², Toshihiko Iwanaga², Akira Terao¹, Kazuhiro Kimura¹, Masayuki Saito^{1,3}

1 Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

2 Laboratory of Histology and Cytology, Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan

3 Department of Nutrition, Graduate School of Nursing and Nutrition, Tenshi College, Sapporo 065-0013, Japan

Running title:

UCP1 enhances leptin action

Corresponding author:

Yuko Okamatsu-Ogura, Ph.D.

Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Tel: +81-11-706-5205

Fax: +81-11-757-0703

E-mail: keixxx@vetmed.hokudai.ac.jp

Abstract

Leptin reduces body fat by decreasing food intake and increasing energy expenditure. Uncoupling protein (UCP) 1, a key molecule for brown adipose tissue (BAT) thermogenesis, was reported to contribute to the stimulatory effect of leptin on energy expenditure. To clarify whether UCP1 is also involved in the anorexigenic effect of leptin, in this study we examined the effect of leptin on food intake using wild-type (WT) and UCP1-deficient (UCP1-KO) mice. Repeated injection of leptin decreased food intake more markedly in WT mice than in UCP1-KO mice, while a single injection of leptin showed similar effects in the two groups of mice. As chronic leptin stimulation induces UCP1 expression in BAT and ectopically in white adipose tissue (WAT), we mimicked the UCP1 induction by repeated injection of CL316,243 (CL), a highly specific β 3-adrenoceptor agonist, and measured food intake in response to a single injection of leptin. Two-week treatment with CL enhanced the anorexigenic effect of leptin in WT mice, but not in UCP1-KO mice. Three-day treatment with CL in WT mice also enhanced the anorexigenic effect of leptin and leptin-induced phosphorylation of STAT3 in the arcuate nucleus of the hypothalamus, without any notable change in adiposity. These results indicate that UCP1 enhances leptin action at the hypothalamus level, suggesting UCP1 contributes to the control of energy balance not only through the regulation of energy expenditure, but also through appetite control by modulating leptin action.

Key words : uncoupling protein 1, brown adipose tissue, leptin, appetite control

Introduction

Brown adipose tissue (BAT) is a tissue involved in metabolic heat production and has a significant role in cold- and diet-induced thermogenesis ^{1,2}. BAT thermogenesis is principally dependent on the activation of uncoupling protein 1 (UCP1), which uncouples oxidative phosphorylation in mitochondria to dissipate the electrochemical proton gradient as heat. The activity of UCP1 is controlled by the sympathetic nerves to BAT, mainly through the β -adrenergic mechanism. The activation of the sympathetic nerve – β -adrenergic receptor (β -AR) pathway induces lipolysis in BAT to produce fatty acids that activate UCP1 and are used simultaneously as a substrate for thermogenesis. In addition, prolonged activation of this pathway induces hyperplasia of BAT associated with an elevated UCP1 level.

Physiological roles and functional regulation of UCP1 mentioned above were assured by the phenotype of UCP1-deficient (UCP1-KO) mice. UCP1-KO mice are unable to maintain body temperature under cold circumstances ³, and get obese when housed at thermoneutrality ⁴. Brown adipocyte of UCP1-KO mice shows the characteristics of brown adipocyte such as multilocular lipid droplets and higher mitochondrial content compared to white adipocyte, however, accumulates larger lipid droplets reflecting their functional defect ³. In UCP1-KO mice, injection of β 3-AR agonist induces lipolysis from WAT as in wild-type (WT) mice, whereas it fails to show stimulatory effect on BAT thermogenesis, such as increase in oxygen consumption and body temperature observed in WT mice ⁵.

Leptin, a hormone secreted primarily by adipocytes, plays an important role in the regulation of appetite and energy balance ^{6,7}. Leptin inhibits appetite through action on the hypothalamus,

especially the arcuate nucleus (ARC). Leptin secreted from adipose tissue enters the central nervous system, binds to its receptor, and reduces food intake by stimulating anorexigenic peptides, such as pro-opiomelanocortin, and by inhibiting orexigenic peptides, such as neuropeptide Y and agouti gene-related protein. Besides its anorexigenic effect, leptin has been reported to increase energy expenditure. For example, the peripheral or central administration of leptin increases oxygen consumption in rats and mice ^{8,9}. The involvement of BAT in the stimulatory effect of leptin on energy expenditure was established by findings indicating that a single peripheral leptin injection increases sympathetic nerve activity and temperature in BAT ¹⁰⁻¹². Chronic leptin stimulation increases UCP1 expression in BAT, and also induces it ectopically in white adipose tissue (WAT) ^{13,14}. Furthermore, chronic leptin treatment increases oxygen consumption and reduces body fat in WT mice, but not in UCP1-KO mice, compared with pair-fed control mice ^{14,15}, indicating that UCP1 is indispensable for the effect of leptin on energy expenditure. Thus, leptin reduces food intake and increases energy expenditure, thereby reducing body fat.

It is known that feeding conditions also affect the sympathetic nerve-BAT pathway ¹⁶, for example, spontaneous overeating induced by high-fat diets or palatable foods increased norepinephrine turnover rate ¹⁷, GDP-binding to mitochondria, an index of UCP1 activity ¹⁸, and UCP1 expression in BAT ¹⁹, whereas all of these parameters were decreased by fasting ^{17,20}. Such physiological responses to food are referred to as diet-induced thermogenesis, and assumed to be a mechanism to dissipate excess energy as heat. However, it is unknown whether or how leptin is involved in this mechanism. In 1948, Brobeck ²¹ initially proposed a thermostatic hypothesis of food intake that heat or body temperature is involved in appetite control: animals eat to keep warm and stop

eating to stay cool. Subsequently, Himms-Hagen ²² suggested that the activation of BAT leads to an increase in body temperature, which causes the termination of feeding. However, as far as we know, there is no evidence for a BAT-related thermostatic mechanism for appetite control.

In a previous study, we observed a tendency to reduce food intake by chronic hyperleptinemia more in WT mice than in UCP1-KO mice, despite the similar plasma leptin levels ¹⁴. This result suggests the involvement of UCP1 in appetite control. To test this hypothesis, we examined the role of UCP1 in the anorexigenic effect of leptin, particularly focusing on UCP1 ectopically expressed in WAT.

Materials and methods

Animals

UCP1-KO (*ucp1*^{-/-}) mice on a congenic background of C57BL/6J were generated by backcross matings of heterozygous (*ucp1*^{+/-}) mice on a mixed 129/SvPas and C57BL/6J background with C57BL/6J mice for 15 generations, and kindly provided by Dr. L. Kozak (Pennington Biomedical Research Center, Baton Rouge, LA, USA) ³. All WT (*ucp1*^{+/+}) mice were C57BL/6J. Mice were housed in plastic cages placed in an air-conditioned room at 26 °C with a 12-hour light-dark cycle (lights on 07:00-19:00) and given free access to laboratory chow (MF: Oriental Yeast, Tokyo, Japan) and tap water. Both male and female WT and UCP1-KO mice (20-30 weeks old) were used. The experimental procedures and care of animals were approved by the Animal Care and Use Committee of Hokkaido University.

Response to leptin injection

Mice were housed individually and allowed to acclimate for at least 7 days. Then, mice were fasted for 24 hours and injected with recombinant mouse leptin (5 mg/kg, PeproTech, London, UK) or saline intraperitoneally at 19:00 and given food. Food intake for 3 or 12 hours was estimated by measuring the weight of remaining food and spillage in the cage. In another series of experiments, mice were fed *ad libitum* and injected with leptin (1 mg/kg) subcutaneously twice a day at 07:00 and 19:00 for 4 days. Daily food intake was measured. Some mice were killed by cervical dislocation, and fat pads from various regions (interscapular BAT, inguinal and perigonadal WAT) were quickly removed and weighed. Tissue specimens were transferred into liquid nitrogen for Western blot

analysis.

β 3-agonist CL316,243 treatment

WT and UCP1-KO mice were injected with β 3-AR agonist CL316,243 (CL: 0.1 mg/kg, American Cyanamid, Pearl River, NY) or saline subcutaneously once a day at 19:00 for 3 or 14 days. The mice were deprived of food at the time of the last injection. Twenty hours later, a blood sample was taken for plasma leptin assay (Leptin ELISA kit; Morinaga, Yokohama, Japan). After 24-hour fasting, mice were injected intraperitoneally with leptin (5 mg/kg) or saline, and food intake was measured for 3 hours. Then, mice were killed by cervical dislocation, and fat pads from various regions were quickly removed and weighed and transferred into liquid nitrogen for Western blot analysis.

Western blotting

Tissue specimens were homogenized in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 7.4). After centrifugation at 800 g for 10 minutes at 4°C, the obtained supernatant was centrifuged at 100,000 g for 1 hour at 4°C to obtain total membrane protein, and used to determine the content of UCP1 by Western blotting. Briefly, membrane protein of BAT and inguinal WAT was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon; Millipore, Bedford, MA). After blocking the membrane with 5% skimmed milk, it was incubated with polyclonal antibody against UCP1 kindly provided by Drs. Teruo Kawada and Naohito Aoki (Kyoto University, Kyoto, Japan) for 1 hour. The bound antibody was made visible using horseradish-peroxidase-linked

goat anti-rabbit immunoglobulin (Zymed Laboratories, San Francisco, CA) and an enhanced chemiluminescence system (Amersham, Little Chalfont, Bucks, UK).

Immunohistochemistry for phosphoSTAT3 (pSTAT3) following leptin injection

Mice were fasted for 24 hours and injected with leptin (5 mg/kg) or saline intraperitoneally. Thirty minutes after the injection, mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer under anesthesia with sodium pentobarbital. Brains were postfixed, cryoprotected with 30% sucrose, and sectioned coronally at 30 μ m thickness with a freezing microtome. The pSTAT3 was determined by immunohistochemical staining using rabbit anti-pSTAT3 polyclonal antibody (Cell Signaling, MA, USA) and Histfine SAB-PO(R) kit (Nichirei, Tokyo, Japan). pSTAT3 positive cells in the ARC and ventromedial hypothalamus (VMH) were counted using 6 slices per animal.

Data analysis

Values are expressed as mean $\pm$ SE. Statistical analysis was performed using analysis of variance followed by post hoc testing by Tukey-Kramer test unless otherwise noted.

Results

Responses to leptin injection

First, we investigated the acute response to a single injection of leptin in WT and UCP1-KO mice. In WT mice, intraperitoneal injection of leptin reduced food intake to 68% (0~3 hour) and 78% (0~12 hour) of that of saline-injected control mice (Fig. 1A). Almost the same anorexigenic effects of leptin were found in UCP1-KO mice.

We also examined the effect of repeated leptin injections on daily food intake (Fig. 1B). In WT mice, repeated leptin injections reduced food intake to 91% on Day 1, and the reduction tended to increase until Day 3 (79%) and then recover on Day 4 (87%). In UCP1-KO mice, repeated leptin injection reduced food intake to 91% on Day 1, and the effect was sustained at a similar level until Day 4. Two-way ANOVA revealed significant effect of Day ($p<0.05$), the genotype ($p<0.05$), and interaction ($p<0.05$). The effect of leptin on food intake was more apparent in WT mice than in UCP1-KO mice, particularly on Day 2 and Day 3. Thus, the effect of single leptin injection on food intake was not different regardless of the absence or presence of UCP1, but that of repeated leptin injection was greater in WT mice than in UCP1-KO mice. These results suggest that some UCP1-dependent change induced by chronic, but not acute, leptin stimulation is involved in the enhancement of the anorexigenic effect of leptin.

Previously, we and others showed that chronic leptin stimulation for 3-8 days not only increases UCP1 expression in BAT, but also induces ectopic UCP1 expression in WAT^{13,14}. In this study, we confirmed repeated leptin injection for only 2 days induced UCP1 in WAT, without notable effect on UCP1 in BAT (Fig.1C).

Effect of CL316,243 treatment

To investigate if the leptin-induced UCP1 expression is involved in the enhancement of the leptin action, we mimicked the UCP1 induction by injecting the mice with CL, a highly specific β 3-agonist. This treatment was done for 2 weeks before the effect of leptin was examined. As reported previously ⁵, in WT mice, 2-week CL treatment resulted in significant increase of UCP1 expression in BAT and ectopic induction of UCP1 in WAT (Fig.2A). In WT mice, single leptin injection reduced food intake in both the control (-27%) and the CL-treated (-48%) groups, and the effect was greater in the CL-treated group (Fig.2B). In UCP1-KO mice, leptin injection reduced food intake in the control (-26%) and CL-treated (-29%) groups, but there was no difference between the two groups.

The anorexigenic effect of leptin is known to be influenced by adiposity, which is decreased by chronic CL treatment ⁵. To induce UCP1 expression with minimum effect on body fat, next, we examined the effect of short-term CL treatment. In WT mice, 3-day treatment with CL induced UCP1 in WAT without notable effect on UCP1 expression in BAT, body weight, WAT weight, and plasma leptin concentration (Figs. 3A, 3B). A single leptin injection reduced food intake in the control group (-21%), but the reduction was greater in the CL-treated group (-47%) (Fig. 3C). In UCP1-KO, the effect of leptin on food intake was not different between the control (-23%) and CL-treated groups (-26%). These data indicate that the anorexigenic effect of leptin is enhanced by a UCP1-dependent change induced by repeated CL injection.

To confirm the enhanced leptin action after the CL treatment, we also examined the

leptin-induced phosphorylation of STAT3 in the hypothalamus as a marker of leptin signaling. In saline-injected mice, pSTAT3 was not detected in any hypothalamic area in the control and CL-treated groups (Fig. 4A). Thirty minutes after leptin injection, pSTAT3-immunoreactive cells were detected in both ARC and VMH. The number of pSTAT3-positive cells in the ARC was significantly higher in the CL-treated group than the control group, but those in the VMH were almost the same in the two groups (Fig.4B). Furthermore, the pSTAT3 immunoreactivity in the ARC of CL-treated group was increased in intensity as compared with that in control group.

Discussion

The purpose of the present study was to investigate the role of UCP1 in the anorexigenic effect of leptin. The major findings were as follows: (1) repeated leptin injections for 2-3 days decreased food intake more markedly in WT mice than in UCP1-KO mice, although a single injection of leptin showed similar effects in the two groups of mice, (2) a two-day leptin treatment in WT mice induced ectopic UCP1 expression in WAT, (3) a two-week treatment with CL increased UCP1 expression in BAT, induced ectopic UCP1 in WAT, and enhanced the anorexigenic effect of leptin in WT mice, but not in UCP1-KO mice, and (4) a three-day treatment with CL in WT mice also induced ectopic UCP1 in WAT, and enhanced the anorexigenic effect of leptin and leptin-induced phosphorylation of STAT3 in the ARC of hypothalamus.

A difference in the anorexigenic effect of leptin between WT and UCP1-KO mice was found when leptin was injected repeatedly, whereas a single injection had similar effects on the two groups. This was consistent with our previous report showing more reduced food intake by chronic hyperleptinemia in WT mice than in UCP1-KO mice¹⁴. These results indicate that the anorexigenic effect of leptin is modulated by UCP1 and/or some UCP1-dependent changes induced by chronic, but not acute, leptin stimulation. Chronic leptin stimulation was reported to increase UCP1 expression in BAT and induces ectopic UCP1 in WAT^{13,14}. In this study, we found repeated leptin injection showed no notable effect on UCP1 in BAT, but induced ectopic UCP1 in WAT on Day 2 when the different leptin effect in WT and UCP1-KO mice were observed. These results raised the possibility that UCP1 ectopically induced in WAT contributes to the enhancement of leptin action. To test this idea, we injected CL, a highly specific β -3 AR agonist, which is known to increase UCP1 expression

in BAT and to induce ectopic UCP1 in WAT. As predicted, a 2-week treatment with CL enhanced the anorexigenic effect of leptin in WT mice, but not in UCP1-KO mice.

Previously, we reported that chronic treatment with CL reduces body fat in a UCP1-dependent manner ⁵. Since the action of leptin is largely affected by adiposity ^{6,7}, it is possible that the enhancement of leptin action by the chronic CL treatment is secondary to the decreased adiposity. However, a 3-day CL treatment in WT mice increased the anorexigenic effect of leptin without any notable change in body weight, adiposity, UCP1 in BAT, and plasma leptin concentration, indicating that the effect of CL treatment on the leptin action was not a consequence of decreased adiposity. The CL-induced enhancement of leptin action was further confirmed by leptin-induced STAT3 phosphorylation, an important component of leptin signaling, in the ARC of hypothalamus ²³, suggesting an increase in leptin sensitivity at the hypothalamus level.

In this study, we injected CL to increase UCP1 expression in BAT and to induce ectopic UCP1 in WAT. Besides CL, overeating is an alternative stimulant of UCP1 expression. In mice and rats, feeding on high-fat diets has been shown to increase UCP1 expression in BAT, and induces UCP1 expression in WAT. Recently, Feldmann et al. ⁴ reported that when fed with high-fat diets, food intake was larger in UCP1-KO mice than in WT mice, while there was no difference when fed on a normal diet. It is possible that the induction of UCP1 expression by high-fat feeding resulted in the enhancement of leptin action in WT mice, and hence UCP1-KO mice lacking this pathway consumed more food than WT mice.

The administration of CL has been reported to suppress food intake in rats and mice ²⁴⁻²⁶, but the mechanism involved was not clear. It has been shown that the suppressive effect of CL on food

intake is not exhibited in mice lacking $\beta 3$ -AR²⁵, and rescued by the expression of $\beta 3$ -AR in WAT and BAT, but not in BAT alone²⁶. These results indicate the importance of $\beta 3$ -AR in WAT in CL-induced suppression of food intake. Considering that ectopic UCP1 in WAT is induced by the β -adrenergic mechanism²⁷, it is likely that the ectopic expression of UCP1 in WAT is involved in the suppressive effect of CL on food intake, probably through the increased leptin sensitivity. This is consistent with the findings of White et al.²⁸ that CL showed more reduced food intake in S5B/P1 rats than in Osborne-Mendel rats, where UCP1 expression in WAT was induced more in the former than the latter. These results also support the role of ectopic UCP1 in WAT in feeding control through the modulation of leptin action.

The mechanism by which UCP1 in WAT modulates leptin action is not clear. Considering that the expression level of UCP1 induced in WAT is as low as 1~2% of that in BAT, it is doubtful that ectopic UCP1 in WAT contributes significantly to whole-body energy expenditure and thermogenesis. However, ectopic UCP1 in WAT may have a role other than that in thermogenesis, such as acting as a sensor to monitor local changes in WAT. The sensory innervation of WAT is suggested by the existence of substance P and calcitonin gene-related peptide, typical marker peptides of primary sensory neurons²⁹. Retrograde-tracing experiments also revealed that peripheral pseudounipolar dorsal root ganglion cells innervate WAT³⁰. Song et al.³¹ further showed that afferent nerves in WAT project into many areas of brain, including the hypothalamus. Thus, it is possible that the local change in metabolism or temperature induced by the ectopic expression of UCP1 in WAT is transmitted to the central nervous system through the afferent nerves, and enhances leptin sensitivity in the hypothalamus. In support of this, Yamada et al.³² reported that afferent nerve signals from

intra-abdominal fat tissue regulate food intake by modulating hypothalamic leptin sensitivity.

Collectively, it is most likely that UCP1 ectopically induced in WAT modulates the anorexigenic action on leptin. However, it is to be noted that this conclusion does not necessarily rule out the possible involvement of BAT in the UCP1-dependent enhancement of leptin action. Leptin or CL not only chronically increases UCP1 expression both in WAT and BAT, but also acutely activates UCP1 in BAT, leading to the secondary changes such as elevation of body temperature^{5,10-12}. It is possible that such secondary changes may also alter leptin action on the ARC, and it takes 2-3 days to be manifested. Another possibility is that secretion of some humoral factor(s) is modified by the induction or activation of UCP1. Leptin itself can be excluded from the candidates because we found no difference in plasma leptin level between the control and CL-treated mice. Plasma triglyceride (TG) was reported to induce leptin resistance at the blood-brain barrier³³. However, CL decreased plasma TG level to the same extent in WT and UCP1-KO mice (Okamatsu-Ogura et al., unpublished observation), implying that plasma TG is not the causative factor in the enhancement of leptin action induced by CL treatment. Further studies are needed to elucidate the precise mechanism involved.

In conclusion, our results show the possible involvement of UCP1 in the enhancement of leptin action. UCP1 may contribute to the control of energy balance via two pathways, directly through the regulation of energy expenditure by its thermogenic activity and indirectly through appetite control by modulating the anorexigenic effect of leptin.

Author contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; YOO performed the experiments and wrote the manuscript, JNK and IT assisted on histological studies, AT and KK assisted with the design of experiments, MS conceived and designed the study.

ACKNOWLEDGEMENTS

We thank Dr. L. Kozak (Pennington Biomedical Research Center) for kindly providing us with UCP1-KO mice, Drs. Teruo Kawada and Naohito Aoki (Kyoto University) for their kind gifts of the anti-UCP1 antibody, and American Cyanamid Co. for providing CL316,243. This study was supported in part by JSPS Research Fellowships for Young Scientists to Y.O.-O.

REFERENCES

1. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 2004;84(1):277-359
2. Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. *Nature* 2000;404(6778):652-60
3. Enerbäck S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* 1997;387(6628):90-4
4. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* 9 (2009) pp.203-209
5. Inokuma K, Okamatsu-Ogura Y, Omachi A, Matsushita Y, Kimura K, Yamashita H, Saito M. Indispensable role of mitochondrial UCP1 for antiobesity effect of beta3-adrenergic stimulation. *Am J Physiol Endocrinol Metab* 2006;290(5):E1014-21
6. Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;404(6778):661-71

7. Coll AP, Farooqi IS, O'Rahilly S. The hormonal control of food intake. *Cell* 2007;129(2):251-62
8. Mistry AM, Swick AG, Romsos DR. Leptin rapidly lowers food intake and elevates metabolic rates in lean and ob/ob mice. *J Nutr* 1997;127(10):2065-72
9. van Dijk G. The role of leptin in the regulation of energy balance and adiposity. *J Neuroendocrinol* 2001;13(10):913-21
10. Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI. Receptor-mediated regional sympathetic nerve activation by leptin. *J Clin Invest* 1997;100(2):270-8
11. Morrison SF. Activation of 5-HT1A receptors in raphe pallidus inhibits leptin-evoked increases in brown adipose tissue thermogenesis. *Am J Physiol Regul Integr Comp Physiol* 2004;286(5):R832-7
12. Collins S, Kuhn CM, Petro AE, Swick AG, Chrnyk BA, Surwit RS. Role of leptin in fat regulation. *Nature* 1996;380(6576):677
13. Commins SP, Watson PM, Padgett MA, Dudley A, Argyropoulos G, Gettys TW. Induction of uncoupling protein expression in brown and white adipose tissue by leptin. *Endocrinology*

1999;140(1):292-300

14. Okamatsu-Ogura, Y, Uozumi, A, Toda, C, Kimura, K, Yamashita, H, Saito, M. Uncoupling protein 1 contributes to fat-reducing effect of leptin. *Obesity Res Clin Pract* 2007;1:233-241
15. Commins SP, Watson PM, Frampton IC, Gettys TW. Leptin selectively reduces white adipose tissue in mice via a UCP1-dependent mechanism in brown adipose tissue. *Am J Physiol Endocrinol Metab* 2001;280(2):E372-7
16. Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. *Obes Res* 1997;5(6):650-6
17. Young JB, Saville E, Rothwell NJ, Stock MJ, Landsberg L. Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat. *J Clin Invest* 1982;69(5):1061-71
18. Nedergaard J, Raasmaja A, Cannon B. Parallel increases in amount of (3H)GDP binding and thermogenin antigen in brown-adipose-tissue mitochondria of cafeteria-fed rats. *Biochem Biophys Res Commun* 1984;122(3):1328-36
19. Margareto J, Marti A, Martinez JA. Changes in UCP mRNA expression levels in brown adipose tissue and skeletal muscle after feeding a high-energy diet and relationships with leptin, glucose

and PPARgamma. *J Nutr Biochem* 2001;12(3):130-137

20. Rothwell NJ, Stock MJ. Effect of chronic food restriction on energy balance, thermogenic capacity, and brown-adipose-tissue activity in the rat. *Biosci Rep* 1982;2(8):543-9
21. Brobeck JR. Food intake as a mechanism of temperature regulation. *Yale J Biol Med* 1948;20(6):545-52
22. Himms-Hagen J. Role of brown adipose tissue thermogenesis in control of thermoregulatory feeding in rats: a new hypothesis that links thermostatic and glucostatic hypotheses for control of food intake. *Proc Soc Exp Biol Med* 1995 ;208(2):159-69
23. Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, Friedman JM. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat Genet* 1996;14(1):95-7
24. Kumar MV, Moore RL, Scarpace PJ. Beta3-adrenergic regulation of leptin, food intake, and adiposity is impaired with age. *Pflugers Arch* 1999;438(5):681-8
25. Susulic VS, Frederich RC, Lawitts J, Tozzo E, Kahn BB, Harper ME, Himms-Hagen J, Flier JS, Lowell BB. Targeted disruption of the beta 3-adrenergic receptor gene. *J Biol Chem*

1995;270(49):29483-92

26. Grujic D, Susulic VS, Harper ME, Himms-Hagen J, Cunningham BA, Corkey BE, Lowell BB. Beta3-adrenergic receptors on white and brown adipocytes mediate beta3-selective agonist-induced effects on energy expenditure, insulin secretion, and food intake. A study using transgenic and gene knockout mice. *J Biol Chem* 1997;272(28):17686-93
27. Jimenez M, Barbatelli G, Allevi R, Cinti S, Seydoux J, Giacobino JP, Muzzin P, Preitner F. Beta 3-adrenoceptor knockout in C57BL/6J mice depresses the occurrence of brown adipocytes in white fat. *Eur J Biochem* 2003;270(4):699-705
28. White CL, Ishihara Y, Dotson TL, Hughes DA, Bray GA, York DA. Effect of a beta-3 agonist on food intake in two strains of rats that differ in susceptibility to obesity. *Physiol Behav* 2004;82(2-3):489-96
29. Shi H, Song CK, Giordano A, Cinti S, Bartness TJ. Sensory or sympathetic white adipose tissue denervation differentially affects depot growth and cellularity. *Am J Physiol Regul Integr Comp Physiol* 2005;288(4):R1028-37
30. Fishman RB, Dark J. Sensory innervation of white adipose tissue. *Am J Physiol* 1987;253(6 Pt 2):R942-4

31. Song CK, Schwartz GJ, Bartness TJ. Anterograde transneuronal viral tract tracing reveals central sensory circuits from white adipose tissue. *Am J Physiol Regul Integr Comp Physiol* 2009;296(3):R501-11
32. Yamada T, Katagiri H, Ishigaki Y, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Niiijima A, Mano H, Aburatani H, Asano T, Oka Y. Signals from intra-abdominal fat modulate insulin and leptin sensitivity through different mechanisms: neuronal involvement in food-intake regulation. *Cell Metab* 2006;3(3):223-9
33. Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, Nakaoke R, Morley JE. Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes* 2004;53(5):1253-60

Figure legends

Figure 1. Effects of leptin injection on food intake in WT and UCP1-KO mice

(A) WT and UCP1-KO mice were fasted for 24 hours and injected with leptin (5 mg/kg, i.p.) or saline. Food intake for 3 and 12 hours after the injection was measured and expressed relative to the 3-hour food intake of saline-injected controls (1.18 ± 0.07 g in WT and 1.29 ± 0.08 g in UCP1-KO mice). Values are means $\pm$ SE for 4 mice. * $P < 0.05$ vs. saline-injected control of the same genotype. (B) Mice were injected with leptin (1 mg/kg, s.c.) twice a day for 4 days. Daily food intake was measured and expressed relative to that before injection on Day 0 (3.24 ± 0.08 g in WT and 3.03 ± 0.12 g in UCP1-KO mice). Values are means $\pm$ SE for 10 mice. Two-way ANOVA revealed significant effect of Day ($p < 0.05$), the genotype ($p < 0.05$), and interaction ($p < 0.05$). (C) UCP1 expression in BAT and inguinal WAT (I-WAT) of WT mice were analyzed by Western blotting. To detect UCP1, 5 μ g (BAT) or 30 μ g (I-WAT) of membrane protein was used. UCP1 content was expressed as relative to that in BAT of the Day0 group. * $P < 0.05$ Day0 vs. Day2 by Student's t-test.

Figure 2. Effects of 2-week treatment with CL316,243 in WT and UCP1-KO mice

WT and UCP1-KO mice were injected with CL316,243 (CL; 0.1 mg/kg, s.c.) or saline once a day for 2 weeks. (A) UCP1 expression in BAT and inguinal WAT (I-WAT) of WT mice were analyzed by Western blotting. To detect UCP1, 5 μ g (BAT) or 20 μ g (I-WAT) of membrane protein was used. UCP1 content was expressed as relative to that in BAT of the control group. Values are means $\pm$ SE for 6 mice. * $P < 0.05$ control group vs. CL group by Student's t-test. (B) After 2-week treatment

with CL316,243, WT and UCP1-KO mice were fasted for 24 hours and injected with leptin (5 mg/kg, i.p.) or saline. Food intake for 3 hours after the injection was measured. Values are means $\pm$ SE for 6 mice. *P<0.05 vs. saline-injected mice of the same group. †P<0.05 Leptin-injected control group vs. CL group.

Figure 3. Effects of 3-day treatment with CL316,243

WT mice were injected with CL316,243 (CL; 0.1 mg/kg, s.c.) or saline once a day for 3 days. (A) Body weight, adiposity, and plasma leptin concentration were measured. (B) UCP1 expression in BAT and inguinal WAT (I-WAT) were analyzed by Western blotting. To detect UCP1, 5 μ g (BAT) or 30 μ g (I-WAT) of membrane protein was used. UCP1 content was expressed as relative to that in BAT of the control group. Values are means $\pm$ SE for 6 mice. *P<0.05 control group vs. CL group by Student's t-test. (C) After 3-day treatment with CL316,243, WT and UCP1-KO mice were fasted for 24 hours and injected with leptin (5 mg/kg, i.p.) or saline. Food intake for 3 hours was measured. Values are means $\pm$ SE for 6 mice. *P<0.05 vs. saline-injected mice of the same group. †P<0.05 Leptin-injected control group vs. CL group

Figure 4. Effects of 3-day treatment with CL 316,243 on leptin-induced phosphorylation of STAT3 in hypothalamus

WT mice were injected with CL316,243 (0.1 mg/kg, s.c.) or saline once a day for 3 days. Mice were fasted for 24 hours and injected with leptin (5 mg/kg, i.p.) or saline, and sacrificed 30 minutes later. (A) Phospho-STAT3 (pSTAT3) in hypothalamus was detected by immunostaining. (B) The numbers

of pSTAT3-positive cells in the arcuate nucleus (ARC) and the ventromedial hypothalamus (VMH) were counted. Values are means $\pm$ SE for 3 mice. *P<0.05 vs. control group by Student's t-test.

Figure 1

Figure 2

Figure 3

A

B

C

Figure 4