

Possible involvement of uncoupling protein 1 in appetite control by leptin

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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 signal transducer and activator of transcription 3 (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.

Keywords: uncoupling protein 1, brown adipose tissue, leptin, appetite control

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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 adipocytes of UCP1-KO mice show the characteristics of brown adipocytes, such as multilocular lipid droplets, and higher mitochondrial content compared with white adipocytes; however, they accumulate larger lipid droplets reflecting their functional defect.³ In UCP1-KO mice, injection of β 3-AR agonist induces lipolysis from white adipose tissue (WAT) as in wild-type (WT) mice, whereas it fails to show stimulatory effects 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 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 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 as to whether or how leptin is involved in this mechanism. In 1948, Brobeck²¹ 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-Hagen²² 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 (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 seven days. Then, mice were fasted for 24 h 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 h 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 four 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, USA) or saline subcutaneously once a day at 19:00 for 3 or 14 d. 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-h fasting, mice were injected intraperitoneally with leptin (5 mg/kg) or saline, and food intake was measured for three 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 mmol/L Tris and 1 mmol/L EDTA, pH 7.4). After centrifugation at 800g for 10 min at 4°C, the obtained supernatant was centrifuged at 100,000g for one 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Immobilon; Millipore, Bedford, MA, USA). 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 one hour. The bound antibody was made visible using horseradish-peroxidase-linked goat anti-rabbit immunoglobulin (Zymed Laboratories, San Francisco, CA, USA) and an enhanced chemiluminescence system (Amersham, Little Chalfont, Buckinghamshire, UK).

Immunohistochemistry for pSTAT3 following leptin injection

Mice were fasted for 24 h 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 mol/L 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 phospho signal transducer and activator of transcription 3 (pSTAT3), was determined by immunohistochemical staining using rabbit anti-pSTAT3 polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) and Histfine SAB-PO[®] kit (Nichirei, Tokyo, Japan). pSTAT3-positive cells in the ARC and ventromedial hypothalamus (VMH) were counted using six 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 the 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 h) and 78% (0–12 h) of that of saline-injected control mice (Figure 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 (Figure 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 analysis of variance revealed significant effects 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 d not only increases UCP1 expression in BAT but also induces ectopic UCP1 expression in WAT.^{13,14} In this study, we confirmed that repeated leptin injection for only two days induced UCP1 in WAT, without a notable effect on UCP1 in BAT (Figure 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 two weeks before the effect of leptin was examined. As reported previously,⁵ in WT mice, two-week CL treatment resulted in a significant increase of UCP1 expression in BAT and ectopic induction of UCP1 in WAT (Figure 2a). In WT mice, a 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 (Figure 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, three-day treatment with CL induced UCP1 in WAT without a notable effect on UCP1 expression in BAT, body weight, WAT weight and plasma leptin concentration (Figures 3a and b). A single leptin injection reduced food intake in the control group (–21%), but the reduction was greater in the CL-treated group (–47%) (Figure 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 (Figure 4a). Thirty minutes after leptin injection, pSTAT3-immunoreactive cells were detected in both the 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 (Figure 4b). Furthermore, the pSTAT3 immunoreactivity in the ARC of the CL-treated group was increased in intensity as compared with that in the 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 d 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 the hypothalamus.

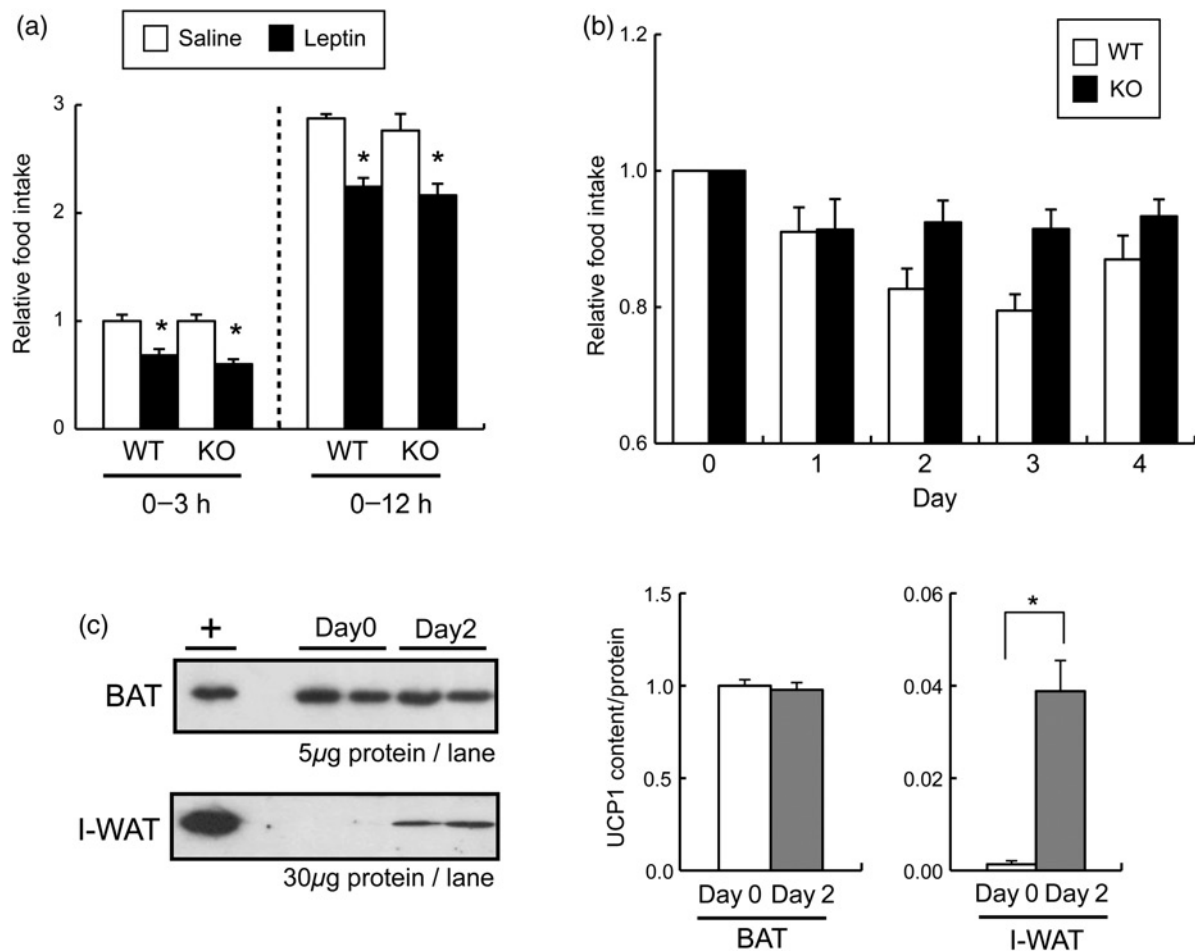


Figure 1 Effects of leptin injection on food intake in WT and UCP1-KO mice. (a) WT and UCP1-KO mice were fasted for 24 h and injected with leptin (5 mg/kg, intraperitoneally) or saline. Food intake for 3 and 12 h after the injection was measured and expressed relative to the three-hour food intake of 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 four mice. * $P < 0.05$ versus saline-injected control of the same genotype. (b) Mice were injected with leptin (1 mg/kg, subcutaneously) twice a day for four 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 analysis of variance revealed significant effects 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 µg (BAT) or 30 µg (I-WAT) of membrane protein was used. UCP1 content was expressed as relative to that in BAT of the Day 0 group. * $P < 0.05$, Day 0 versus Day 2 by Student's *t*-test. WT, wild-type; UCP1, uncoupling protein 1; UCP1-KO, UCP1-deficient; BAT, brown adipose tissue; WAT, white adipose tissue

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 mice.¹⁴ 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 that repeated leptin injections showed no notable effect on UCP1 in BAT, but induced ectopic UCP1 in WAT on Day 2 when the different leptin effects 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 $\beta 3$ AR agonist, which is known to increase UCP1 expression

in BAT and to induce ectopic UCP1 in WAT. As predicted, a two-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 three-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.

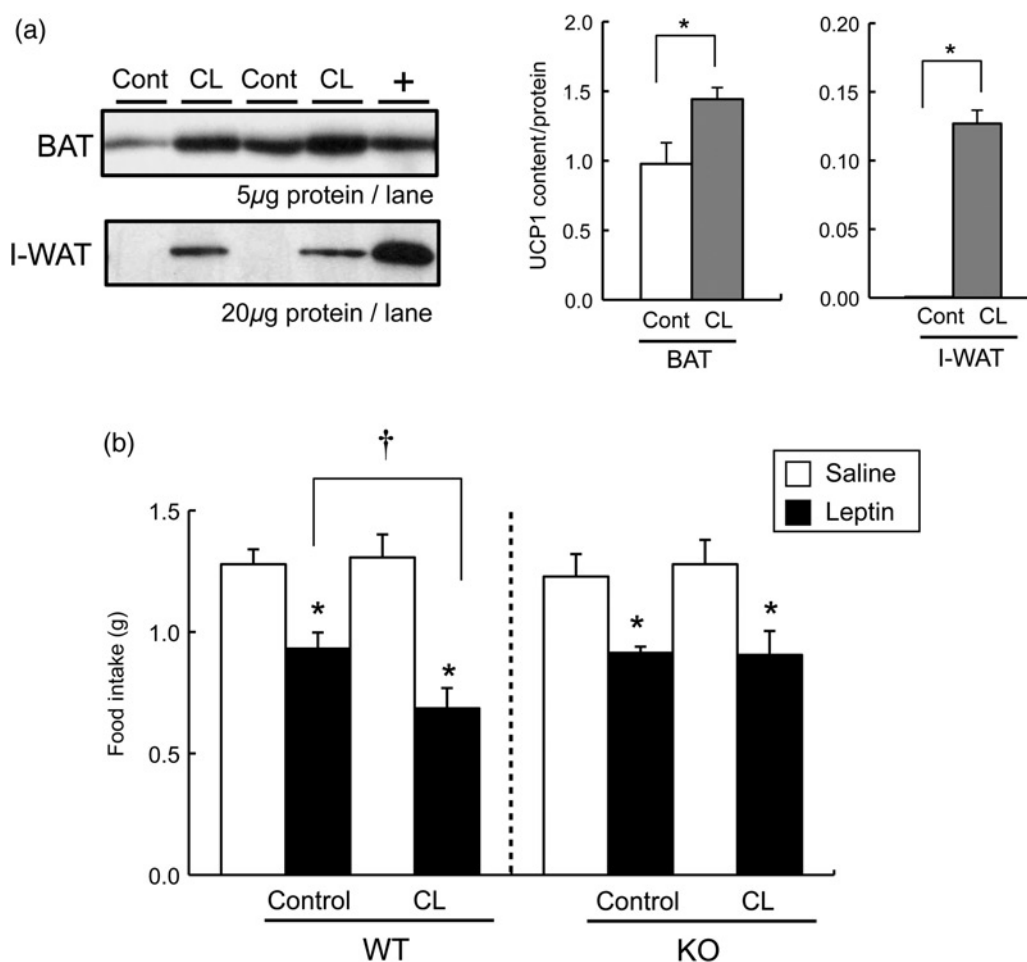


Figure 2 Effects of two-week treatment with CL316,243 in WT and UCP1-KO mice. WT and UCP1-KO mice were injected with CL316,243 (CL; 0.1 mg/kg, subcutaneously) or saline once a day for two weeks. (a) UCP1 expression in BAT and inguinal WAT (I-WAT) of WT mice were analyzed by Western blotting. To detect UCP1, 5 µg (BAT) or 20 µg (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 six mice. * $P < 0.05$, control group versus CL group by Student's *t*-test. (b) After two-week treatment with CL316,243, WT and UCP1-KO mice were fasted for 24 h and injected with leptin (5 mg/kg, intraperitoneally) or saline. Food intake for three hours after the injection was measured. Values are means $\pm$ SE for six mice. * $P < 0.05$, versus saline-injected mice of the same group. † $P < 0.05$, leptin-injected control group versus CL group. WT, wild-type; UCP1, uncoupling protein 1; UCP1-KO, UCP1-deficient; BAT, brown adipose tissue; WAT, white adipose tissue

In this study, we injected CL to increase UCP1 expression in BAT and to induce ectopic UCP1 in WAT. Besides CL, over-eating 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 induce 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 β 3-AR,²⁵ and rescued by the expression of β 3-AR in WAT and BAT, but not in BAT alone.²⁶ These results indicate the importance of β 3-AR in WAT in CL-induced suppression of food intake. Considering that ectopic UCP1 in

WAT is induced by the β -adrenergic mechanism,²⁷ 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 neurons.²⁹ Retrograde-tracing experiments

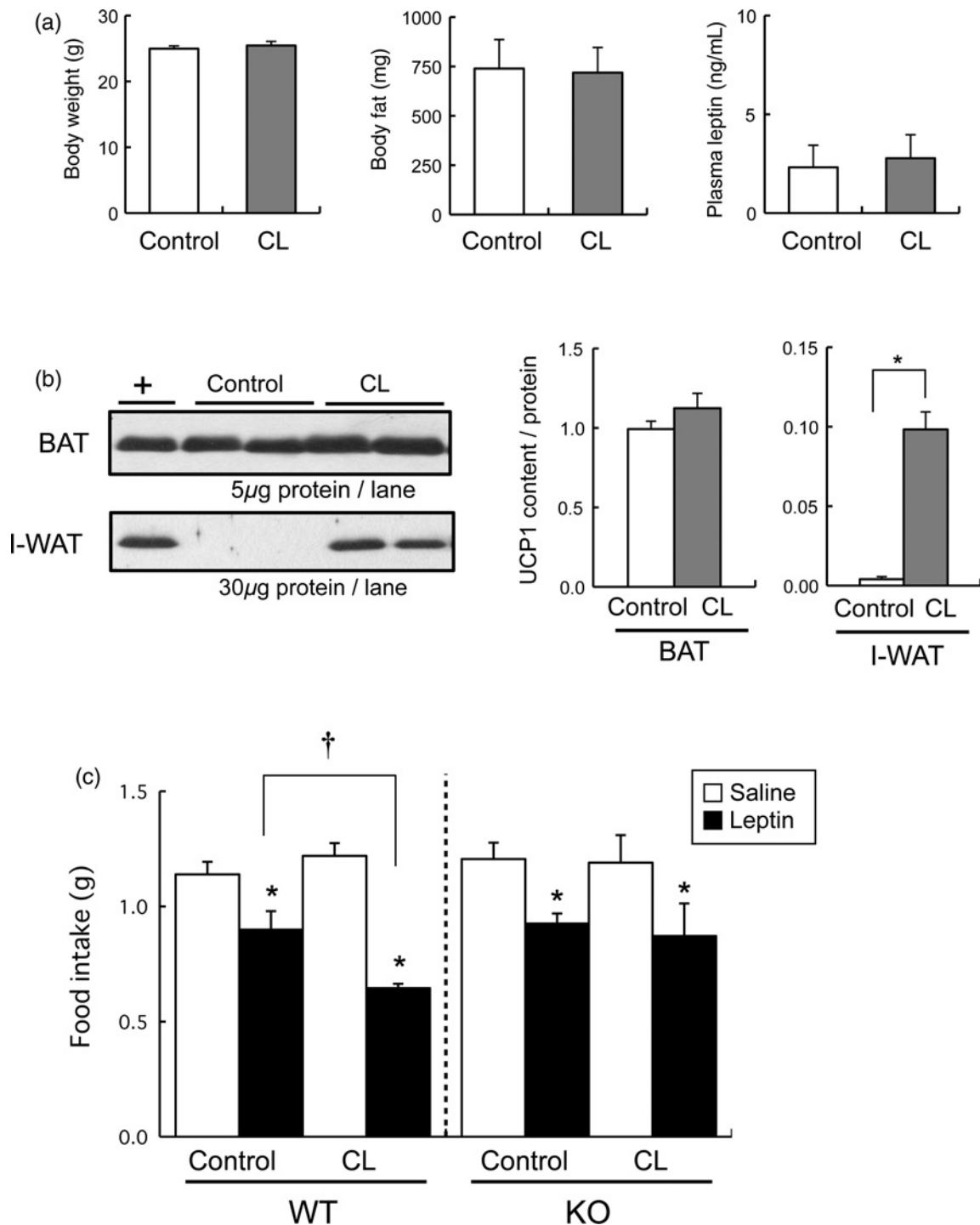


Figure 3 Effects of three-day treatment with CL316,243. WT mice were injected with CL316,243 (CL; 0.1 mg/kg, subcutaneously) or saline once a day for three days. (a) Body weight, adiposity and plasma leptin concentration were measured. (b) UCP1 expression in BAT and inguinal WAT (I-WAT) were analyzed 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 six mice. * P < 0.05, control group versus CL group by Student's t -test. (c) After three-day treatment with CL316,243, WT and UCP1-KO mice were fasted for 24 h and injected with leptin (5 mg/kg, intraperitoneally) or saline. Food intake for three hours was measured. Values are means $\pm$ SE for six mice. * P < 0.05 versus saline-injected mice of the same group. † P < 0.05 leptin-injected control group versus CL group. WT, wild-type; UCP1, uncoupling protein 1; UCP1-KO, UCP1 deficient; BAT, brown adipose tissue; WAT, white adipose tissue

also revealed that peripheral pseudounipolar dorsal root ganglion cells innervate WAT.³⁰ Song *et al.*³¹ further showed that afferent nerves in WAT project into many areas of the 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

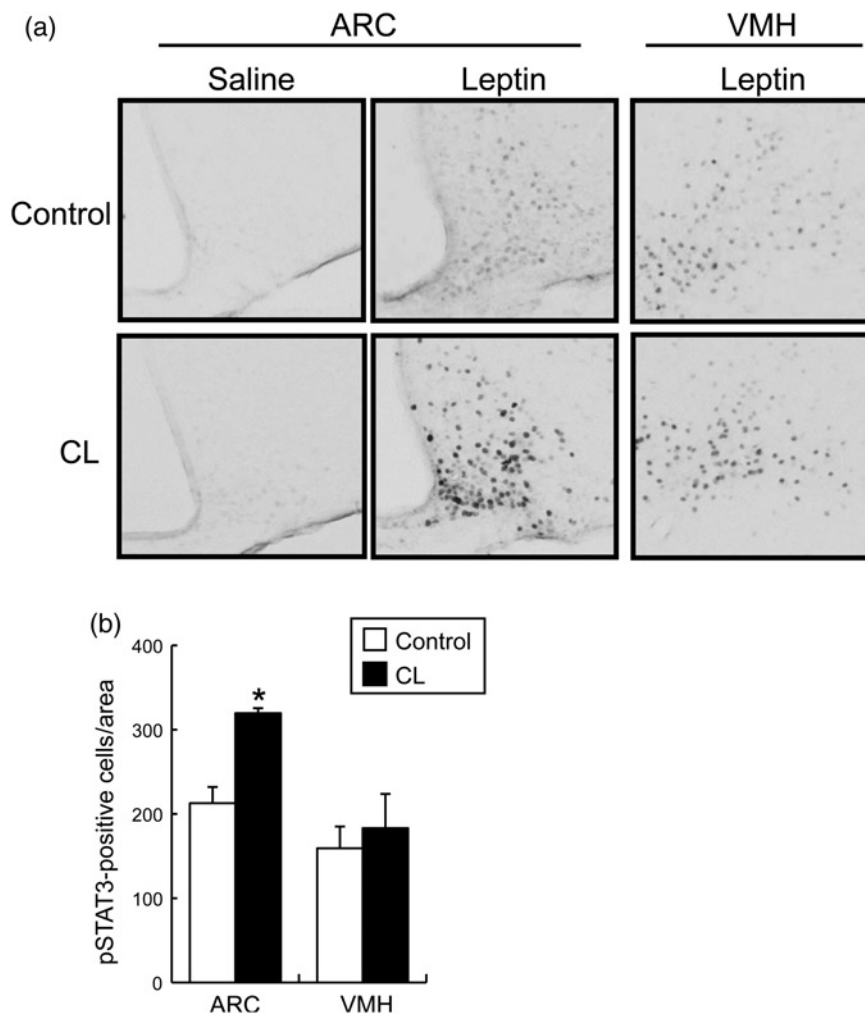


Figure 4 Effects of three-day treatment with CL 316,243 on leptin-induced phosphorylation of STAT3 in hypothalamus. WT mice were injected with CL316,243 (0.1 mg/kg, subcutaneously) or saline once a day for three days. Mice were fasted for 24 h and injected with leptin (5 mg/kg, intraperitoneally) or saline, and were killed 30 min later. (a) 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 three mice. * $P < 0.05$, versus control group by Student's *t*-test. STAT3, signal transducer and activator of transcription 3

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 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 d 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 barrier.³³ 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. YO-O performed the experiments and wrote the manuscript; JN-K and TI assisted on histological studies; AT and KK assisted with the design of experiments; and MS conceived and designed the study.

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