Journal of Stress Physiology & Biochemistry, Vol. 9 No. 4 2013, pp. 101-114 ISSN 1997-0838

ORIGINAL ARTICLE

Role of Leptin in Metabolic Adaptation During Cold Acclimation

Gang-Bin Tang [1], Xiang-Fang Tang [2], Kui Li [2], De-Hua Wang [1]*

[1] *State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang, Beijing 100101, China*
[2] *State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, 2 Yuanmingyuan West Road, Haidian, Beijing 100193, China*

Tel: 8610-64807073 Fax: 8610-64807099
*E-Mail: ***wangdh@ioz.ac.cn***

Received June 11, 2013

Chronic cold exposure stimulates thermogenesis in brown adipose tissue, resulting in fat mobilization and compensatory hyperphagia. Mostly, these physiological events are accompanied by a remarkable reduction in serum leptin levels. However, the physiological roles of hypoleptinemia in cold adaptation are still not fully clear. We hypothesized that leptin is the keystone of the regulatory systems linking energy balance to cold adaptation.

Leptin treatment (5μg/day) decreased food intake, body weight, serum ghrelin levels and hypothalamic melanin-concentrating hormone (MCH) gene expression. Food restriction in the pair-fed group mimicked most of the effects induced by leptin treatment. Central coadministration of ghrelin (1.2 μg/day) partially reversed the effect of leptin on hypothalamic MCH mRNA, but it did not block the reducing effects of leptin on food intake, body weight and serum ghrelin levels. In addition, hypothalamic pro-opiomelanocortin gene expression increased significantly in response to the coadministration of leptin and ghrelin. Collectively, we conclude that the regulatory effects of leptin on energy balance in cold-acclimated rats are dependent on feeding, which may involve the reduction of hypothalamic MCH gene expression. We found no evidence for ghrelin involvement in the regulation of leptin on food intake and body weight during cold acclimation.

Key words: Cold, Leptin, MCH, Metabolism

ORIGINAL ARTICLE

Role of Leptin in Metabolic Adaptation During Cold Acclimation

Gang-Bin Tang [1], Xiang-Fang Tang [2], Kui Li [2], De-Hua Wang [1]*

[1] *State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang, Beijing 100101, China*
[2] *State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, 2 Yuanmingyuan West Road, Haidian, Beijing 100193, China*

Tel: 8610-64807073 Fax: 8610-64807099
*E-Mail: ***wangdh@ioz.ac.cn***

Received June 11, 2013

Chronic cold exposure stimulates thermogenesis in brown adipose tissue, resulting in fat mobilization and compensatory hyperphagia. Mostly, these physiological events are accompanied by a remarkable reduction in serum leptin levels. However, the physiological roles of hypoleptinemia in cold adaptation are still not fully clear. We hypothesized that leptin is the keystone of the regulatory systems linking energy balance to cold adaptation.

Leptin treatment (5μg/day) decreased food intake, body weight, serum ghrelin levels and hypothalamic melanin-concentrating hormone (MCH) gene expression. Food restriction in the pair-fed group mimicked most of the effects induced by leptin treatment. Central coadministration of ghrelin (1.2 μg/day) partially reversed the effect of leptin on hypothalamic MCH mRNA, but it did not block the reducing effects of leptin on food intake, body weight and serum ghrelin levels. In addition, hypothalamic pro-opiomelanocortin gene expression increased significantly in response to the coadministration of leptin and ghrelin. Collectively, we conclude that the regulatory effects of leptin on energy balance in cold-acclimated rats are dependent on feeding, which may involve the reduction of hypothalamic MCH gene expression. We found no evidence for ghrelin involvement in the regulation of leptin on food intake and body weight during cold acclimation.

Key words: Cold, Leptin, MCH, Metabolism

Endothermic animals living in cold environments maintain normothermia by activating a series of physiological events, including increased heat production and compensatory hyperphagia, to ensure survival (Bing *et al*., 1998). Investigation of the neuroendocrine mechanisms underlying thermogenesis and feeding behavior may help in understanding some of the features of cold adaptation.

Leptin (Zhang *et al*., 1994) and ghrelin (Kojima *et al*., 1999) are peptide hormones that take part in the regulation of energy metabolism. Leptin is

mainly secreted by white adipose tissue (WAT) (Masuzaki *et al*., 1995) and acts on the brain to regulate food intake, energy expenditure, and neuroendocrine function (Ahima *et al*., 1996; Halaas *et al*., 1995; Pelleymounter *et al*., 1995). Leptin's central effects are mediated through the long form of the leptin receptor (OB-Rb), which is expressed in the hypothalamic neuropeptide Y/agouti-related protein (NPY/AgRP) and pro-opiomelanocortin/cocaine- and amphetamine-regulated peptide (POMC/CART) neurons (Hakansson *et al*., 1998). It has been demonstrated that leptin regulates these two types of neurons in a reciprocal manner by inhibiting NPY/AgRP neurons while stimulating POMC/CART neurons (Friedman and Halaas, 1998).

Ghrelin, a stomach-derived hormone, has a potent orexigenic effect when administered to rodents and humans (Nakazato *et al*., 2001a). Ghrelin stimulates the activity of neurones expressing NPY and AgRP (Nakazato *et al*., 2001b). On the other hand, ghrelin has an inhibitory effect on POMC neurons (Cowley *et al*., 2003). Thus in the context of energy homeostasis, leptin and ghrelin have opposite effects on hypothalamic neurons.

In this study, we used chronic cold-acclimated rats as models and aimed to determine the effects of chronic intracerebroventricular (ICV) infusion of leptin on energy balance, non-shivering thermogenesis, metabolic hormones and hypothalamic neuropeptides. In addition, we examined the effects of ICV leptin administration on metabolic parameters in food-restricted animals to determine whether the metabolic effects of leptin are mediated by a mechanism separate from its effect on food intake. Lastly, we examined whether chronic ICV administration of ghrelin would block the effects of leptin on energy balance in cold-acclimated rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (8 weeks older) were purchased from Wei-Tong-Li-Hua company and housed at 23 ± 1 °C with a 12L:12D photoperiod (lights on at 08:00). Standard laboratory chow (for rat, Chinese Academy of Medical Sciences) and water were provided ad libitum. All animal procedures were approved by the Animal Care and Use Committee of Institute of Zoology, the Chinese Academy of Sciences.

Peptides

Recombinant leptin was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Recombinant ghrelin was obtained from Phoenix Pharm. Inc. (Mountain View, CA, USA).

Intracerebroventricular (ICV) infusion

Subjects were anesthetized with sodium pentobarbital (60 mg/kg) and stereotaxically implanted with 27-gauge stainless-steel cannulae (Alza Corp., Palo Alto, CA, USA) into the lateral ventricle (stereotaxic coordinates from Bregma: 0.8 mm caudal, 1.6 mm lateral, 3.2 mm ventral). Following surgery, the rats were allowed to recover for 6 days. Angiotensin II drinking tests were performed at the end of the recovery period. Rats that drank ≥5ml of water in 30 min after the ICV injection of 100 ng/10ul angiotensin II (Phoenix Pharm. Inc, Burlingame, CA, USA) were considered to have the cannula correctly placed for ICV microinjection. On the test day, each animal was anesthetized with isoflurane and surgically implanted with an Alzet osmotic minipump 2001 (Alza Corp., Palo Alto, CA, USA) filled with artificial cerebrospinal fluid (aCSF) or peptides under the skin over the dorsal chest. A catheter from the

minipump was connected to the ICV cannula through a subcutaneous tunnel.

Experimental protocols

Rats were cold-acclimated (5 °C) for 15 days and divided randomly into four groups of 6 rats each: The control group received ICV infusions of aCSF for 5 days; the leptin group received ICV infusions of leptin (5 µg/day) for 5 days; the leptin+ ghrelin group received ICV infusions of leptin and ghrelin (leptin: 5 µg/day; ghrelin: 1.2µg /day) for 5 days, and the leptin pair-fed group was allowed to eat only as much chow as consumed by the leptin group for 5 days. The doses of leptin and ghrelin used in this study were determined based on a previous study (Kim *et al*., 2004). Body weight and food intake were measured daily (Between 09:00 and 10:00 am). Body temperature was monitored every 30 s throughout the experiment. The rats were sacrificed by overdose of CO_2 at the end of the experiment, and serum was collected and stored at -80 °C. Body fat and interscapular brown adipose tissue (iBAT) were dissected and weighed. The hypothalamus was immediately dissected as previously described (Bing *et al*., 1998; Tang *et al*., 2009), frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

Metabolic trials

Body weight and food intake

Body weight and food intake were measured at 09.00 h. During each test, rats were weighed and housed individually in metabolic cages, where food and water were provided ad libitum. On next day, the animals were re-weighed and the remained food (not include the spillage from the hoppers).

Core body temperature

Before the cold acclimation, rats were anaesthetized with isoflurane and were implanted with an abdominal transmitter (Mini Mitter, Model G2 E-Mitter, to ± 0.1°C in the temperature range of 33–41°C) according to the guideline (Chi and Wang, 2011). The animals were allowed for 6-day recovery. Implanted transmitters produced temperature-modulated signals, which were sent to a receiver (model ER-4000 Receiver, Mini Mitter Co., Inc.) underneath each rat cage. Core body temperature was recorded telemetrically from the implanted transmitter. Individual cages were placed on the receiver board (Mini Mitter, Model ER-4000). All receivers and the DP-24 DataPort were connected to a computer. Temperature data were collected at 30 s intervals and processed simultaneously by the Vital View data acquisition system (Mini Mitter Co., Inc.). The daily core body temperature was presented as an average.

Non-shivering thermogenesis (NST)

The cold-acclimated rats were transferred from the cold room (5 °C) to 27°C. Two hours' later, NST was measured. The maximum capacity for NST was induced by a subcutaneous injection of noradrenaline (NA) solution with a dosage of 0.4 mg kg^{-1} (Macdonald and Siyamak, 1990). Oxygen consumption and carbon dioxide production were assessed by the LabMaster system (TSE-Systems, Bad Homburg, Germany) at 27 °C (constant-temperature incubator; Yiheng Corp., Shanghai, China). Each animal was measured for 2 h. The flow rate of air was 4000 ml min^{-1}. Volume of oxygen consumed and carbon dioxide produced by each rat were measured for 90 s. In each trial, up to three rats and one obligate reference channel were recorded in parallel, yielding a 3-6 min resolution of metabolic readings. Data collection was automatically controlled by the software. Resting metabolic rate (RMR) was estimated from the lowest stable rate of oxygen consumption over 6-12

min. The value of oxygen consumption was presented as ml $O_2^{-1}g^{-1}h^{-1}$.

iBAT uncoupling protein 1 (UCP1)

Mitochondrial protein concentrations of iBAT were determined by Folin phenol method using bovine serum albumin as standard (Lowry *et al*., 1951). Total iBAT mitochondrial protein (50μg per lane) was separated in a discontinuous SDS-polyacylamide gel (12.5% running gel and 3% stacking gel) and blotted to a nitrocellulose membrane (Hybond-C, Amersham, UK). Unspecific binding sites were saturated with 5% non-fat milk in Tris-buffered saline-Tween for 1 h at room temperature and probed with the indicated antibodies overnight at 4°C. Following incubation with the secondary antibody for 1 h, the bands were visualized by chemiluminescence (Amersham Life Sciences, Little Chalfont, UK). Quantification of the blots was determined with the use of a Quantity One Ver.4.4.0 (BioRad, USA). Primary antibodies used were rabbit anti-UCP1 (1:10,000; ab10983, Abcam, Cambridge, MA, USA) and mouse anti-β-tubulin (1:5000; E7, DSHB, Iowa City, Iowa, USA). The secondary antibodies were goat anti-rabbit IgG (1:5,000; ZSGB-BIO Co., Beijing, China) and goat anti-mouse IgG (1:5,000; ZSGB-BIO Co., Beijing, China).

Hormone assays

Leptin, ghrelin, tri-iodothyronine (T3), thyroxine (T4) and corticosterone concentrations were measured using ELISA kits (Leptin: Linco Research, St. Charles, MO; ghrelin and corticosterone: Ever Systems Biology Laboratory, Inc. Sacramento, CA, USA; T3 and T4 : Jiancheng Corp, Nanjing, China).

Body composition

The visceral organs, including heart, lungs, liver, kidneys, spleen, and gastrointestinal tract (containing contents), were extracted and weighed (±0.01g). Body fat, including subcutaneous and visceral fats, were dissected and weighed (±0.01g).

Quantitative RT-PCR

Four micrograms of total RNA, obtained from the hypothalamus, were reverse- transcribed into first strand cDNA with a reverse transcription kit (Fermentas, Lithuania). Primers set for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and six hypothalamic genes were designed for real-time PCR (Table 1). Real-time PCR was completed using the SYBR Green I qPCR kit (TaKaRa, Japan) in the Mx3005P quantitative PCR system (Stratagene, USA). Real-time RT-PCR was carried out in 25 μL reaction agent comprising 12.5 μL 2 × SYBR® Premix EX Taq™master mix, 1 μL cDNA templates and 0.2μmoL/L primers. Each sample was analyzed in triplicate. The cycle conditions were: initial denaturation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s. At the end of the experiments, melting curve analysis showed that there were no non-specific amplifications. PCR products were further confirmed by DNA sequencing. Standard curves were constructed for each gene via serial dilutions of cDNA (2-fold dilutions). Target genes and GAPDH had similar amplification efficiencies, validating the comparative quantity method. The data derived from the Mx3005P quantitative software were expressed as relative amounts, which were calculated by normalizing the amount of target gene mRNA levels to the amount of GAPDH mRNA levels. No amplification was detected in absence of template or in the no RT control.

Statistical analysis

Data were analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Prior to all

statistical analyses, data were examined for normality of variance using the Kolmogorov-Smirnov test. Repeated measures ANOVA was used to analyze group differences in body weight and body temperature. Differences in food intake were analyzed by repeated measures ANCOVA with body weight as a covariate. Differences in body compositions were assessed by ANCOVA with carcass as a covariate. Differences in hormones, UCP1 content in iBAT, and hypothalamic gene expressions were assessed by one-way ANOVA followed by the Tukey's HSD test for comparisons. Finally, Pearson correlation analysis was performed to determine the correlation between hypothalamic neuropeptides and food intake. Data are expressed as mean ± SEM, $P<0.05$ was considered to be statistically significant.

RESULTS

Food intake and body weight

During the infusion period, animals in all three treatment groups had decreased food intake compared to the control group (group effect, $P < 0.001$; day effect, $P = 0.056$; interaction group × day, $P < 0.001$; Fig. 1A). ICV infusion of leptin induced a decrease in food intake ($P<0.001$). Coadministration of ghrelin did not block the anorectic effect of leptin. Over the 5-day treatment, the cumulative decreases in food intakes of the leptin, leptin+ghrelin and leptin pair-fed group were 40.6 g, 28.1 g and 41.4 g respectively. The cumulative food intake increase in the control group was 16.6 g.

There were significant differences in body weight among the four groups during the 5 days of treatment (Group effect, $P = 0.406$; day effect, $P < 0.001$; interaction group × day, $P<0.001$; Fig.1B). Compared to the control group, 5-day ICV infusion of leptin decreased body weight ($P < 0.001$). The leptin + ghrelin group and the leptin pair-fed group showed similar changes in body weight as the leptin group.

Body compositions

Liver ($P < 0.01$) and alimentary organ mass ($P < 0.01$) decreased significantly after 5-day leptin treatment. Animals in the pair-fed group showed similar changes in liver and alimentary organ mass as the leptin group (Table 2). Coadministration of ghrelin did not reverse the effects of leptin on liver and alimentary organ masses. WAT, heart, spleen, lung, kidneys, testes, seminal vesicles and epididymis masses were not different between the control and treatment groups (Table 2).

NST, iBAT UCP1 and core body temperature

Leptin alone or coadministrated with ghrelin did not affect NST (Fig.2-A) or iBAT UCP1 levels (Fig. 2B). Also, there were no changes of NST (Fig.2-A) or iBAT UCP1 levels (Fig. 2B) in the leptin pair-fed group. Core body temperatures remained constant in all groups throughout the treatment period (Fig. 3).

Hormones

There were no significant changes in serum leptin, T3 and T4 levels among the four groups (Fig.4A, C, D). Leptin treatment resulted in a significant decrease of serum ghrelin levels, but coadministration with ghrelin did not reverse the reducing effect of leptin on serum ghrelin levels. Serum ghrelin levels in the pair-fed group were similar to those in the leptin and leptin + ghrelin groups (Fig. 4B). Leptin alone and coadministrated with ghrelin did not cause significant changes in serum corticosterone levels. The pair-fed treatment caused a significant increase in corticosterone levels compared to the control group (Fig. 4E).

Hypothalamic genes

Gene expressions of hypothalamic NPY (Fig. 5A), AgRP (Fig. 5B), CART (Fig. 5D) and TRH (Fig. 5F) were

not affected by leptin with or without ghrelin treatment. Hypothalamic POMC mRNA (Fig. 5C) was upregulated in the leptin and leptin + ghrelin groups ($P < 0.01$). Leptin administration decreased hypothalamic MCH mRNA (Fig. 5E) ($P < 0.05$), whereas coadministration of ghrelin partially blocked the reducing effect of leptin on MCH mRNA levels. Compared to the leptin group, food restriction resulted in higher gene expression of MCH ($P < 0.05$). There were no differences in NPY, AgRP, CART, TRH or POMC mRNA levels between the leptin group and the pair-fed group (Fig. 5). Correlation analysis showed that hypothalamic MCH mRNA levels were positively correlated with food intake ($r = 0.47$, $P < 0.05$). No significant relationships were found between hypothalamic POMC mRNA levels and food intake.

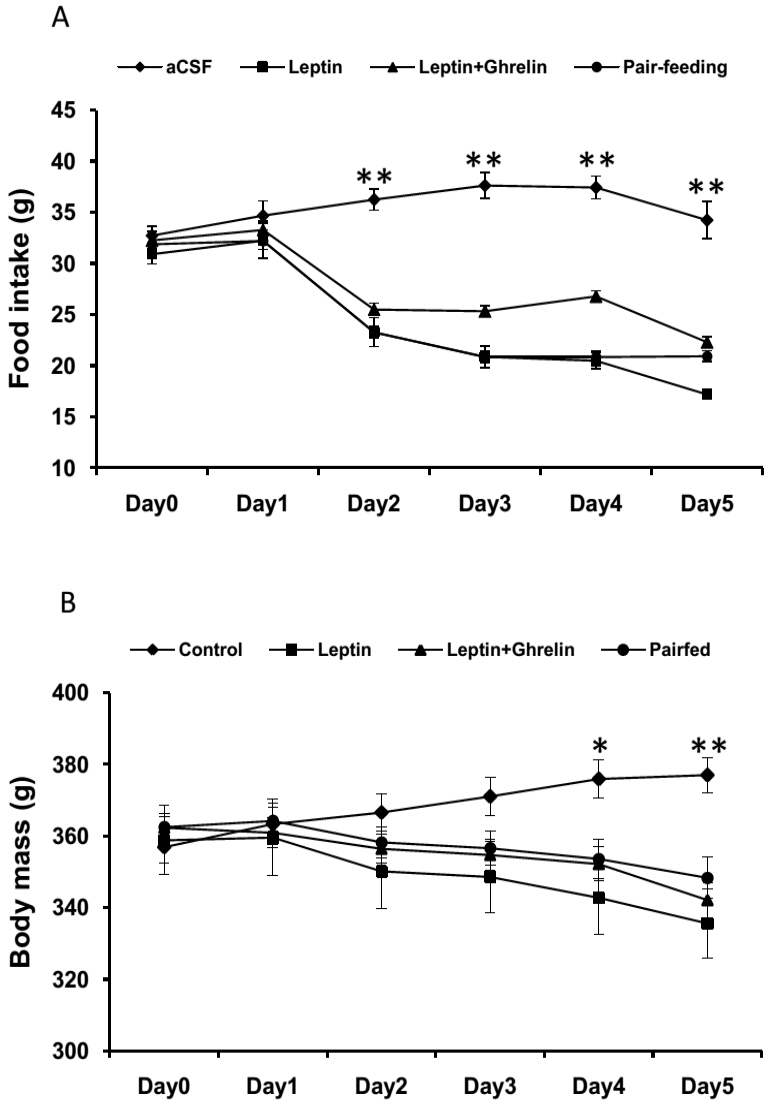

Figure 1. Changes in food intake (A) and body weight (B) in cold-acclimated rats in control, leptin, leptin + ghrelin and pair-fed groups. Cold-acclimated rats were infused with aCSF, leptin, or leptin + ghrelin for 5 days. Rats in the fourth group were pair-fed with the leptin group. * $p < 0.05$; ** $p < 0.01$.

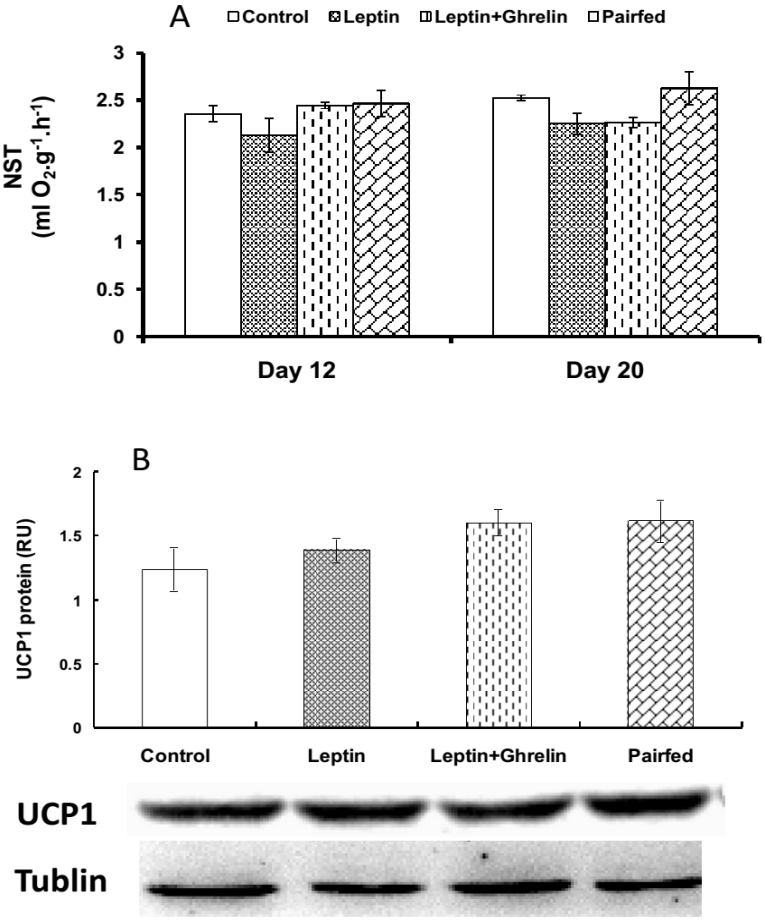

Figure 2. Non-shivering thermogenesis (A) and uncoupling protein 1 (UCP1) (B) in cold-acclimated rats in control, leptin, leptin + ghrelin and pair-fed groups before (4 days prior to the onset of the treatment) and during the treatment (5 days after the onset of the treatment). Cold-acclimated rats were infused with aCSF, leptin, or leptin + ghrelin for 5 days. Rats in the fourth group were pair-fed with the leptin group.

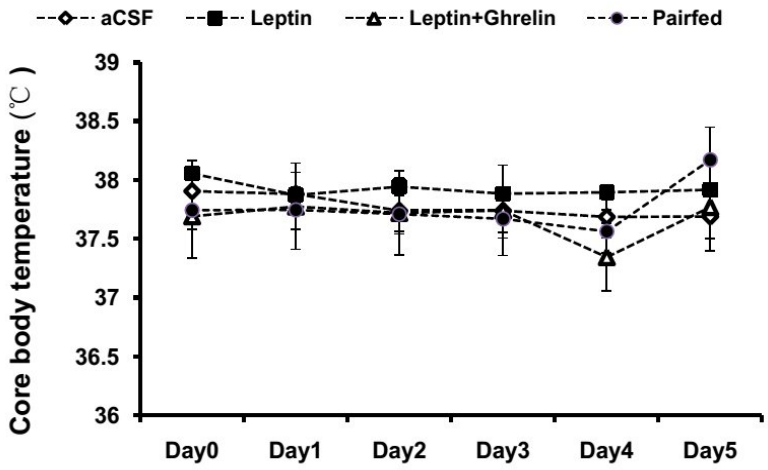

Figure 3. Core body temperature of cold-acclimated rats in control, leptin, leptin + ghrelin and pair-fed groups. Cold-acclimated rats were infused with aCSF, leptin, or leptin + ghrelin for 5 days. Rats in the fourth group were pair-fed with the leptin group.

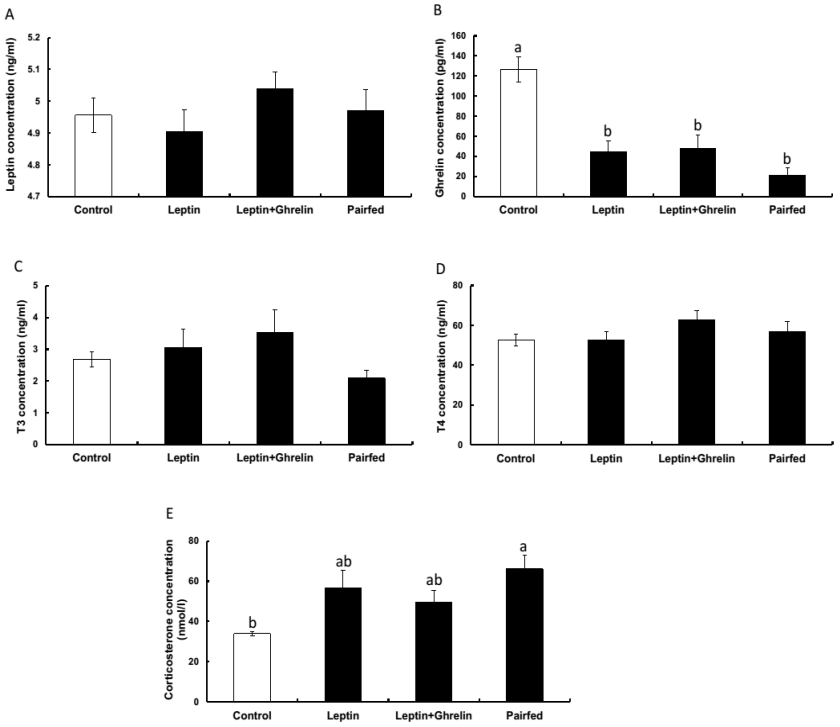

Figure 4. Changes in serum leptin (A), ghrelin (B), T3 (C), T4 (D) and corticosterone (E) concentrations in cold-acclimated rats in control, leptin, leptin + ghrelin and pair-fed groups. Cold-acclimated rats were infused with aCSF, leptin, or leptin + ghrelin for 5 days. Rats in the fourth group were pair-fed with leptin group. Values that do not share a common superscript differ significantly at $P < 0.05$.

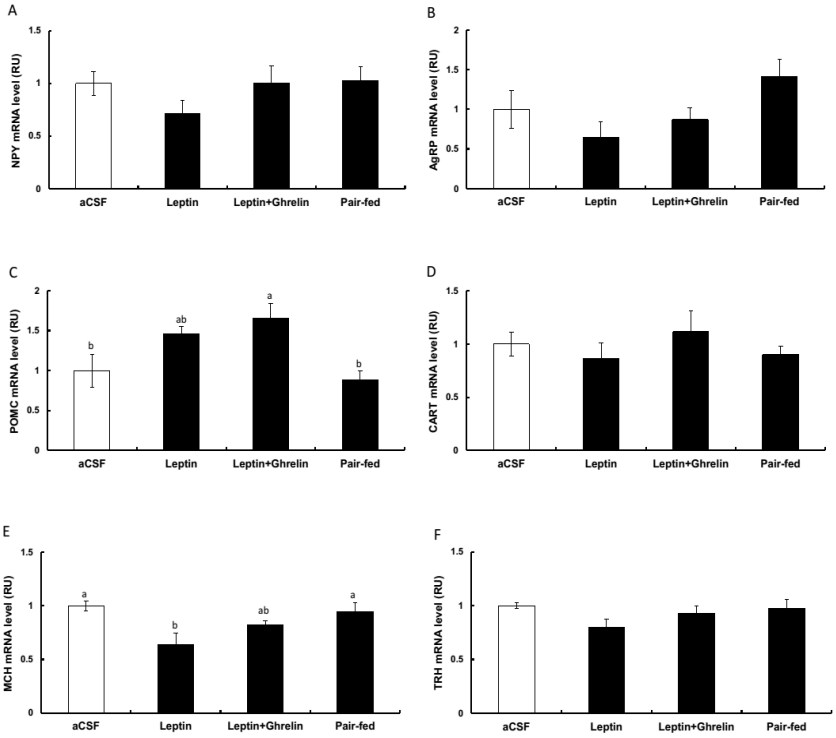

Figure 5. Hypothalamic gene expressions in control, leptin, leptin + ghrelin and pair-fed groups. Cold-acclimated rats were infused with aCSF, leptin, or leptin + ghrelin for 5 days. Rats in the fourth group were pair-fed with the leptin group. Values that do not share a common superscript differ significantly at $P < 0.05$.

Table 1. Gene-specific primers used for Real-time RT-PCR

Primers	Oligonuncleotide sequence (5'-3')	Product size (bp)
NPY (forward)	GCT GGG TCA CTT TCT CAT AG G	116
NPY (reverse)	TCG CTC TGT CCC TGC TCG TGT G	
AgRP (forward)	TCT CTT GCC GTA TCT CTG CCT GGT G	209
AgRP (reverse)	AGA GTT CTC AGG TCT AAG TCT	
POMC (forward)	TGA AGA AGC GGC AGT AGC AC	124
POMC (reverse)	GAC CTC ACC ACG GAA AGC AAC CTG	
CART (forward)	ACT TCC GGG GAT TTT CAG TCA AGG G CTC AAG AGT AAA	160
CART (reverse)	CGC ATT CC	
TRH(forward)	GAA GAA AAA GAG GGA GAG GGT GTC	210
TRH(reverse)	GAA GGG AAC AGG ATA GGG AAT ACA G	
MCH (forward)	CCG CAG AAA GAT CGG TTG TT	200
MCH (reverse)	TGG TCC TTT CAG AGC GAG GTA	
GAPDH (forward)	CGG CAA GTT CAA CGG CAC A	146
GAPDH (reverse)	AGA CGC CAG TAG ACT CCA CGA CA	

P<0.001 =***,p<0.01** ,P<0.05 * and ns= non-significant.

Table 2. Gene-specific primers used for Real-time RT-PCR

Organs	aCSF	Leptin	Leptin+Ghrelin	Pairfed
Carcass (g)	272.29±4.15	255.52±8.12	253.86±6.50	256.39±5.94
Body fat (g)	8.70±0.69	5.49±0.56	8.06±1.12	5.91±0.67
Liver (g)	15.10±0.40[a]	10.71±0.79[b]	12.14±0.59[b]	10.023±0.32[b]
Heart (g)	1.27±0.05	1.19±0.05	1.16±0.04	1.15±0.03
Lung (g)	2.77±0.27	3.20±0.28	2.08±0.25	2.74±0.36
Spleen (g)	0.97±0.07	0.88±0.08	0.95±0.08	0.71±0.05
Kidneys (g)	3.23±0.12	3.04±0.09	2.99±0.123	2.78±0.14
Testes (g)	3.18±0.12	3.19±0.23	3.04±0.14	3.28±0.12
Vesicles (g)	1.28±0.06	1.17±0.10	0.97±0.07	1.07±0.12
Epididymis (g)	1.14±0.03	1.18±0.06	1.02±0.08	1.06±0.04
Alimentary organs (g)	37.93±1.77[a]	25.93±1.46[b]	27.88±1.37[b]	24.63±1.10[b]

Cold-acclimated rats were infused for 5 days with aCSF, leptin, or leptin + ghrelin. Rats in the fourth group were pair-fed with the leptin group. Values that do not share a common superscript differ significantly from each other at P< 0.05.

Alimentary organs include stomach, small intestine, large intestine, and caecum.

DISCUSSION

Body weight gain in cold-exposed rats was suppressed by leptin treatment which is in agreement with our and other previous results (Abelenda *et al*., 2003; Tang *et al*., 2009; Yang *et al*.,

2011). The reducing effect of leptin on body weight may be dependent on feeding, since pair-fed cold-exposed rats showed similar levels of weight loss to the leptin group. In addition, leptin treatment had no effect on non-shivering thermogenesis. Our pilot studies indicated that the effect of leptin on energy expenditure in the cold is mainly mediated by changes in physical activity (unpublished results). These findings lend support to the notion that leptin plays a role in cold adaptation by altering feeding and physical activity, but not thermogenesis. We also showed that central coadministration of ghrelin did not reverse the reducing effects of leptin on food intake and body weight. Another study showed that cold-exposed rats were resistant to the actions of insulin in the hypothalamus, both at the molecular and the functional level (Torsoni *et al*., 2003). At present it is not clear if the effectiveness of ghrelin decreases in cold conditions. This is an interesting point that warrants further investigation.

In this study, serum ghrelin levels decreased in response to leptin administration in the cold. Interestingly, ghrelin levels also decreased in the pair-fed group compared to the control group. As known, food restriction usually increases circulating ghrelin levels in the warm conditions (Gualillo *et al*., 2002). A reasonable explanation for these discrepancies is that high levels of ghrelin levels may suppress thermogenesis, which is advantageous for warm animals to maintain energy balance but may be dangerous for cold-acclimated animals. Therefore, cold-acclimated rats selectively suppressed circulating ghrelin levels to facilitate heat production even though they were hungry and needed to increase their food intake during leptin and food restriction treatment. Additionally, we found coadministration with ghrelin did not reverse the reducing effect of leptin on ghrelin levels, which is also contrary to the findings under warm conditions (Kim *et al*., 2004).

The hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-adrenal (HPA) axes represent major endocrine systems that participate in the regulation of energy balance (Spiegelman and Flier, 2001; Webber and Macdonald, 2000). The neuroendocrine effects of leptin include effects on the HPT and HPA axes (Heiman *et al*., 1999). In this study, neither leptin alone nor coadministrated with ghrelin treatment changed serum T3 and T4 levels. In addition, we also did not detect any effect of leptin, with or without ghrelin treatment, on gene expression of hypothalamic TRH. These findings suggest that leptin and ghrelin may not participate in the modulation of the HPT axis in the cold. Serum T3 and T4, and hypothalamic TRH also remained constant in the pair-fed group, indicating that the HPT axis is not sensitive to changes in feeding status in the cold.

Consistent with the previous studies (Bing *et al*., 1998), our present results showed that leptin treatment had no significant effect on serum corticosterone levels in the cold. Also, serum corticosterone levels remained constant in the leptin and ghrelin coadministration group. We speculate that chronic exposure to cold requires higher thermogenesis and increased food consumption. In this context, cold abolishes the effect of leptin on corticosterone levels, which favors optimization of energy consumption. Compared to control animals, pair-fed animals showed a significant increase in serum corticosterone levels, suggesting a starvation-induced stress response.

In this study, central leptin administration induced a significant decrease in hypothalamic MCH gene expression. As basal and adaptive

thermogenesis were not affected by leptin treatment despite a marked reduction in hypothalamic MCH expression, it seems unlikely that MCH plays a role in thermogenesis. Correlation analysis showed that there was a positive relationship between hypothalamic MCH and food intake, suggesting that MCH may be involved in the regulation of hypoleptinemia-induced hyperphagia in cold-exposed rats. In contrast to our assumptions, a previous study showed that MCH oligonucleotide in cold-exposed rats did not affect food intake (Pereira-da-Silva *et al*., 2003). It should be noted that in that study MCH oligonucleotide also failed to change the food intake in the control group. The duration of stimulus (acute or chronic treatment of MCH) and receptor subtype were assumed to be two key factors influencing the orexigenic effects of MCH (Pereira-da-Silva *et al*., 2003). Therefore, the role of MCH in the regulation of feeding behavior during cold adaptation remains to be determined. We further found that coadminisration of ghrelin partially reversed the effect of leptin on MCH mRNA levels, suggesting that ghrelin may be at least partially involved in the effect of leptin on MCH gene expression.

We showed that leptin treatment had no significant changes on hypothalamic POMC mRNA expression. Simultaneous central administration of leptin and ghrelin resulted in a further increase in POMC mRNA expression. Correlation analysis showed that there was no relationship between POMC mRNA and food intake, making it unlikely that this peptide is involved in the regulation of feeding in long-term cold acclimation. A recent study suggested that POMC neurons might play a role in the short-term cold-induced reduction of spontaneous physical activity (SPA) and may influence cold-induced thermogenesis via enhanced activation of the thyroid axis (De Jonghe *et al*., 2011). In the present study, we found no correlation between POMC mRNA and physical activity (data not shown). In addition, there was no correlation between POMC and NST (data not shown). It seems that POMC neurons may play different roles in short-term and chronic cold exposure. Of note, we only measured mRNA expression of POMC in the hypothalamus, so it is not clear if POMC in other areas of the brain may be involved in the regulation of SPA and thermogenesis. In addition, we did not measure hypothalamic POMC protein levels and neuron activity in the present study, so the role of POMC in the regulation of energy balance during chronic cold acclimation awaits further study.

Hypothalamic NPY and CART mRNA levels remained stable in response to leptin administration. Food restriction induced no changes in hypothalamic NPY, AgRP, POMC, CART and MCH mRNA expressions. In contrast, the expressions of these neuropeptides is sensitive to both leptin (Friedman and Halaas, 1998) and food restriction at room temperature (Sucajtys-Szulc *et al*., 2010). It seems that the regulatory systems of these hypothalamic neuropeptides are different between warm and cold conditions.

In summary, we showed that chronic ICV administration of leptin significantly reduced food intake, body weight gain, serum ghrelin levels and hypothalamic MCH gene expression in cold-acclimated rats, while it had no effect on non-shivering thermogenesis. These catabolic effects of leptin were dependent on feeding. Here, we found no evidence that ghrelin participated in the regulation of leptin on food intake and body weight.

ACKNOWLEDGMENTS

We thank Xiu-Qin Liu for help in taking care of

the animals. This study was supported by National Natural Science Foundation of China Grant (30900140) and open project of State Key Laboratory of Animal Nutrition (2004DA125184O0805).

REFERENCES

Abelenda, M., Ledesma, A., Rial, E., and Puerta, M. (2003). Leptin administration to cold-acclimated rats reduces both food intake and brown adipose tissue thermogenesis. *Jour. Therm. Biol.,* **28**, 525-530.

Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D.Q., Lowell, B., MaratosFlier, E., and Flier, J.S. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature*, **382**, 250-252.

Bing, C., Frankish, H.M., Pickavance, L., Wang, Q., Hopkins, D.F., Stock, M.J., and Williams, G. (1998). Hyperphagia in cold-exposed rats is accompanied by decreased plasma leptin but unchanged hypothalamic NPY. *Am. Jour. Physiol*., **274**, R62-68.

Chi, Q.-S., and Wang, D.-H. (2011). Thermal physiology and energetics in male desert hamsters (Phodopus roborovskii) during cold acclimation. *Jour. Comp. Physiol*. B, **181**, 91-103.

Cowley, M.A., Smith, R.G., Diano, S., Tschop, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., *et al.* (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*, **37**, 649-661.

De Jonghe, B.C., Hayes, M.R., Banno, R., Skibicka, K.P., Zimmer, D.J., Bowen, K.A., Leichner, T.M., Alhadeff, A.L., Kanoski, S.E., Cyr, N.E., *et al.* (2011). Deficiency of PTP1B in POMC neurons leads to alterations in energy balance and homeostatic response to cold exposure. *Am. Jour. Physiol. Endocrinol. Metab*., **300**, E1002-1011.

Friedman, J.M., and Halaas, J.L. (1998). Leptin and the regulation of body weight in mammals. *Nature,* **395**, 763-770.

Gualillo, O., Caminos, J.E., Nogueiras, R., Seoane, L.M., Arvat, E., Ghigo, E., Casanueva, F.F., and Dieguez, C. (2002). Effect of food restriction on ghrelin in normal-cycling female rats and in pregnancy. *Obes. Res*., **10**, 682-687.

Hakansson, M.L., Brown, H., Ghilardi, N., Skoda, R.C., and Meister, B. (1998). Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *Jour. Neurosci*, **18**, 559-572.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science,* **269**, 543-546.

Heiman, M.L., Sloop, K.W., Chen, Y., and Caro, J.F. (1999). Extension of neuroendocrine axes to include leptin. *Jour. Anim. Sci*., **77**, 33-42.

Kim, M.S., Namkoong, C., Kim, H.S., Jang, P.G., Kim Pak, Y.M., Katakami, H., Park, J.Y., and Lee, K.U. (2004). Chronic central administration of ghrelin reverses the effects of leptin. *Int. Jour. Obes. Relat. Metab. Disord.,* **28**, 1264-1271.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, **402**, 656-660.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with

the Folin phenol reagent. *Jour. Biol. Chem*., **193**, 265-275.

Macdonald, I., and Siyamak, A. (1990). Plasma noradrenaline levels and thermogenic responses to injected noradrenaline in the conscious rat. *Exp. Physiol*., **75**, 639-648.

Masuzaki, H., Ogawa, Y., Isse, N., Satoh, N., Okazaki, T., Shigemoto, M., Mori, K., Tamura, N., Hosoda, K., Yoshimasa, Y*., et al.* (1995). Adipocyte-specific expression and regional differences in the adipose tissue. *Diabetes,* **44**, 855-858.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature,* **409**, 194-198.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science,* **269**, 540-543.

Pereira-da-Silva, M., Torsoni, M.A., Nourani, H.V., Augusto, V.D., Souza, C.T., Gasparetti, A.L., Carvalheira, J.B., Ventrucci, G., Marcondes, M.C., Cruz-Neto, A.P*., et al.* (2003). Hypothalamic melanin-concentrating hormone is induced by cold exposure and participates in the control of energy expenditure in rats. *Endocrinology,* **144**, 4831-4840.

Spiegelman, B.M., and Flier, J.S. (2001). Obesity and the regulation of energy balance. *Cell*, **104**, 531-543.

Sucajtys-Szulc, E., Turyn, J., Goyke, E., Korczynska, J., Stelmanska, E., Slominska, E., Smolenski, R.T., Rutkowski, B., and Swierczynski, J. (2010). Differential effect of prolonged food restriction and fasting on hypothalamic malonyl-CoA concentration and expression of orexigenic and anorexigenic neuropeptides genes in rats. *Neuropeptides,* **44**, 17-23.

Tang, G.B., Cui, J.G., and Wang, D.H. (2009). Role of hypoleptinemia during cold adaptation in Brandt's voles (Lasiopodomys brandtii). *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **297**, R1293-1301.

Torsoni, M.A., Carvalheira, J.B., Pereira-Da-Silva, M., de Carvalho-Filho, M.A., Saad, M.J., and Velloso, L.A. (2003). Molecular and functional resistance to insulin in hypothalamus of rats exposed to cold. *Am. Jour. Physiol. Endocrinol. Metab*., **285**, E216-223.

Webber, J., and Macdonald, I.A. (2000). Signalling in body-weight homeostasis: neuroendocrine efferent signals. *Proc. Nutr. Soc*., **59**, 397-404.

Yang, J., Bromage, T.G., Zhao, Q., Xu, B.H., Gao, W.L., Tian, H.F., Tang, H.J., Liu, D.W., and Zhao, X.Q. (2011). Functional evolution of leptin of Ochotona curzoniae in adaptive thermogenesis driven by cold environmental stress. *PLoS. One*., **6**, e19833.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, **372**, 425-432.