Effect of Leptin on Hypothalamic Gene Expression in Calorie-Restricted Rats

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Diminished leptin signaling to the arcuate nucleus of hypothalamus (ARH) may induce calorie restriction (CR)-specific neuroendocrine and metabolic adaptation, which is potentially relevant to the effect of CR. The present study investigated whether restoration of leptin signaling to the ARH could reverse CR-induced alterations in neuropeptide gene expression in rats. Male F344 rats, fed ad libitum or a 30% CR diet from 6 weeks of age, received leptin or vehicle intracerebroventriculally for 14 days via osmotic mini-pumps implanted in the subcutis at 34 weeks of age. The messenger RNA levels were quantified by realtime reverse transcription–polymerase chain reaction using total RNA extracted from microdissected tissues containing the ARH. The results indicated that leptin administration reversed the upregulated expression of neuropeptide Y and agouti-related protein genes in CR rats, suggesting the possibility of a role for the leptin–ARH pathway in the effect of CR.

CALORIE restriction (CR) is a nutritional intervention that reduces morbidity and mortality beyond reproductive age in a wide range of laboratory organisms (1,2). Hence, this effect has been designated “anti-aging.” An evolutinal theory of aging and CR assumes that the effect of CR occurs via neuroendocrine and metabolic adaptation in response to reduced calorie intake (3,4). Organisms suspend growth and reproduction under conditions of food shortage, and simultaneously enhance the stress response machinery leading to maximization of survival. The evolutinal viewpoint also predicts the presence of signals that elicit neuroendocrine and metabolic adaptation. Although the identification of these types of signals may lead to novel interventions that can mimic the effect of CR (5), our knowledge is currently incomplete.

Recent studies on the regulation of food intake and energy expenditure have elucidated nutrient-sensing pathways that provide information regarding the energy intake and storage status from peripheral organs to the central nervous system (6). Among these pathways, leptin is a well-known adipose tissue–derived hormone that participates in the regulation of appetite and energy expenditure via interactions with hypothalamic neuronal circuitries (6). Our previous reviews stressed a role for leptin and its signaling pathways in the arcuate nucleus of hypothalamus (ARH) for the adaptive response to CR (7,8). We hypothesized that reduced leptin signaling to the ARH could evoke the neuroendocrine and metabolic adaptation induced by CR. In the ARH, two neuronal populations antagonistically regulate neuroendocrine and energy expenditure. Neuropeptide Y (NPY)-expressing neurons, most of which also coexpress agouti-related protein (AGRP), are activated by fasting (9,10) and an increase in appetite and food intake when food is available. Activation of NPY and/or AGRP neurons also increases parasympathetic activity, while reducing sympathetic activity and energy expenditure (6). In contrast, proopiomelanocortin (POMC)-expressing neurons, many of which also coexpress cocaine- and amphetamine-regulated transcript (CART) (6,11), are activated when food is sufficient. Activated POMC and/or CART neurons exhibit opposing effects on appetite, food intake, autonomic activity, and energy expenditure to activated NPY and/or AGRP neurons (6,12,13).

Accumulated evidence has indicated that the leptin–ARH pathway also regulates the endocrine system. Increased NPY and/or AGRP signaling reduces the activity of neurons that express gonadotropin-releasing hormone (GnRH), growth hormone–releasing hormone (GHRH), and thyroid-releasing hormone (TRH), while it stimulates neurons that express corticotropin-releasing hormone (CRH) (14–19). Reduced POMC and/or CART signaling diminishes the activity of TRH neurons, but releases CRH neurons from suppression by α-melanocyte-stimulating hormone (α-MSH), a cleavage product of POMC (6,13,19–21). These changes are expected to activate the hypothalamus–pituitary–adrenal axis but attenuate reproductive, growth, and thermogenic functions. Most of these endocrine changes are observed in CR rodents (22–26). The above evidence suggests that leptin acts as a signal that elicits neuroendocrine and metabolic alterations relevant to the anti-aging effect of CR through modulating the activity of ARH neurons. Our previous studies confirmed that CR decreased the plasma leptin concentration in male F344 rats (27) and upregulated the expression level of NPY messenger RNA (mRNA) in the ARH relative to that of POMC mRNA during a feeding cycle (28).
To gain further insights into a possible role for leptin or leptin-responsive ARH neurons in the effect of CR, we conducted the present study to investigate whether restoration of attenuated leptin signaling to the ARH could reverse CR-induced alterations in ARH neuropeptide gene expression. Although many studies have provided data on the leptin–hypothalamic pathway under fasting conditions, there is little available information regarding the effect of long-term CR on this pathway (7).

**METHODS**

**Experimental Animals**

The present study was performed in accord with the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University. Male 4-week-old specific pathogen-free Fischer 344 rats (F344/DuCrj) were purchased from Charles River Laboratory Japan (Yokohama), transferred to a barrier facility (temperature: 22–25°C; 12-h light/dark cycle), and maintained separately under specific pathogen-free conditions. F344 rats were fed ad libitum (AL) with Charles River Formula-1 diet (Oriental Yeast Co. Ltd., Tsukuba, Japan) until 6 weeks of age. The amount of food consumed by the AL group was measured at 1-week intervals until 12 weeks of age, at 2-week intervals until 24 weeks of age, and at 4-week intervals thereafter. In the present study, 30% CR was started at 6 weeks of age with modified every-other-day feeding. Every other day, 30 minutes before the lights were turned off, the CR group received 140% of the mean daily food intake in the AL group. The amount of food provided to the CR group was adjusted every week until 12 weeks of age, every 2 weeks until 24 weeks of age, and fixed thereafter, because the mean food intake in the AL group stayed constant after 20 weeks of age.

This modified every-other-day feeding yielded a 2-day cycle in the pattern of food intake in the CR groups but successfully restricted the food intake by 30% of that of each AL group as a whole. The four consecutive 12-hour light/dark periods after feeding were designated Dark 1, Light 1, Dark 2, and Light 2. We subdivided each CR group into CR1 and CR2 groups, which represented CR rats killed within the first 24 hours (Dark 1 and Light 1) after feeding and during the subsequent 24 hours (Dark 2 and Light 2), respectively. Most of the meals provided to the CR rats were ingested within the CR1 phase. The gene expression levels of NPY and POMC during the 2-day feeding cycle have already been published (28).

**Effect of Leptin Administration on Gene Expression in the ARH in AL and CR F344 Rats**

At 34 weeks of age, rats were administered leptin or vehicle intracerebroventricuclarly for 14 days. In Light 2, rats were anesthetized by intraperitoneal injection of pentobarbital (37.5 mg/kg body weight) and placed in a stereotaxic apparatus. A 28-gauge stainless-steel guide cannula (brain infusion kit 2; ALZA Corporation, Mountain View, CA) was implanted into the lateral ventricle using the following coordinates: 1.0 mm posterior to the bregma, 1.4 mm lateral to the midline, and 5.0 mm ventral to the surface of the skull. The guide cannula was secured to the skull with two stainless-steel screws and dental cement. Each rat had an osmotic pump (Alzet 2002; ALZA Corporation; pumping rate: 0.5 µL/h) implanted subcutaneously; this pump was connected to the guide cannula by a polyethylene tube (PE60). Both groups of rats were administered recombinant leptin (10 ng/h; R & D Systems, Minneapolis, MN) or vehicle (0.01 M phosphate-buffered saline [PBS], pH 7.2) via the osmotic pump for 14 days. The body weights and food intake were monitored daily after the operation (day 0). All rats were killed by decapitation on day 14 (Light 2), because our previous and complementary studies indicated that differences in the gene expressions between the AL and CR groups were greater in the CR2 phase than in the CR1 phase (28) (Supplemental Figure 1). Trunk blood was collected into two tubes, one containing EDTA-2Na and aprotinin for plasma preparation and the other containing a coagulating agent for serum preparation; the plasma and serum were isolated by centrifugation and stored at −80°C for hormone assays. The brain was removed from each rat, weighed, and dissected into slices according to the technique of Palkovits and Brownstein (29) with some modifications. Briefly, after decapitation, the whole rat brain was immediately removed and placed in a dissection device (rodent brain matrix; ASI Instruments Inc., Warren, MI) on crushed ice. An approximately 1.7-mm-thick slice containing the arcuate nuclei was excised using razor blades, frozen in powdered dry ice, and maintained at −80°C until microdissection. Under a dissecting microscope, hypothalamic tissue containing both the arcuate nuclei and the median eminence was punched out from the frozen slice using a dissecting needle with an internal diameter of 800 µm. After the hypothalamic tissue was punched out, the remaining tissue was cut into thin sections to validate the dissection level. We confirmed that each slice was excised between the levels of P1800 and P3900 µm according to Palkovits and Brownstein (29). The white adipose tissue (WAT) weight represented the combined wet weights of perirenal adipose and epididymal adipose tissue; these tissues were immediately dissected out of each rat and weighed after decapitation.

**Extraction of Total RNA and Reverse Transcription Reaction**

Total RNA was extracted from each of the punched-out tissue samples using ISOGEN (Nippon Gene Inc., Toyama, Japan) and the Pellet Paint Co-precipitant (Novagen, Madison, WI). The total RNA concentration was determined by using spectrophotometry (optical density [OD] at 260 nm). Prior to the reverse transcription (RT) reaction, any contaminating residual genomic DNA was eliminated by digestion with DNase I (Promega, Madison, WI). The RT reaction was performed using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer’s protocol. Briefly, total RNA (0.9 µg) was mixed with 5.0 µM random hexamers, RNase inhibitor at 0.4 U/µL, 0.5 mM each dNTP, 5.5 mM MgCl2, Multiscribe reverse transcriptase (Applied Biosystems, Tokyo, Japan) at 1.25 U/µL, and 1 × TaqMan RT buffer in a total volume of
60 μL. The RT reaction was carried out as follows: one cycle of 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, and 4°C for 5 minutes. The complementary DNA (cDNA) samples obtained were stored at −30°C until polymerase chain reaction (PCR) amplification was performed. The gene expression levels in the ARH were evaluated using real-time PCR.

**Real-Time PCR**

The primers and probes used were designed based on the cDNA sequences (GenBank) using the primer design software Primer Express (Perkin Elmer Applied Biosystems, Foster City, CA). The synthesis of these probes was performed by Perkin Elmer Applied Biosystems. The primer and probe sequences were as follows: NPY sense: 5'-GGGCTG-GATCTTTGCCATA-3'; NPY antisense: 5'-GGGCTG-GATCTTTGCCATA-3'; NPY TaqMan probe sequence: 5'-5-carboxyfluorescein (FAM)-CGCTCTGCGACACTA-CATCAAATCTCATCAC-N,N,N',N'-tetramethyl-6-carboxyarodamine (TAMRA)-3'; AGRP sense: 5'-GCAGAGG-GTGCTAGATCCACAGAA-3'; AGRP antisense: 5'-GCAGAGG-GTGCTAGATCCACAGAA-3'; AGRP TaqMan probe sequence: 5'-FAM-GCATGCTGCGGCAGCCTGAC TAMRA-3'; POMC sense: 5'-ACTGTAGACGAA-TCTCCGACATC-3'; POMC TaqMan probe sequence: 5'-FAM-TGCTCAGGCTCCTACTCCACCCATG-3'; CART sense: 5'-GACCTGTTAATTTTGGGACATG-3'; CART antisense: 5'-AATTGGCAGCATGCTCCATTTT-3'; CART TaqMan probe sequence: 5'-FAM-CATTGACCCGCCCATC-3'. The gene expression levels of neuropeptide Y and proopiomelanocortin in the ARH were also presented in the previous article (28).

**Supplemental Figure 1. Messenger RNA (mRNA) expression levels of agouti-related protein (AGRP) (A) and cocaine- and amphetamine-regulated transcript (CART) (B) in the arcuate nucleus of hypothalamus (ARH) in calorie-restricted (CR) rats.**Circle represents mean ± standard deviation (n = 3–5). *p < .05 versus the ad libitum-fed (AL) group at each time point by one-factor analysis of variance followed by Tukey–Kramer post hoc test for multiple comparisons. Materials and methods are described in detail in a previous article (28). The primer sequences for AGRP and CART were as follows: AGRP sense: 5'-GCAGGAGCA-GAGCATGACATG-3'; AGRP antisense: 5'-GCAGGAGCA-GAGCATGACATG-3'; CART sense: 5'-CCAGAGATGATGGGCTCT-3'; CART antisense: 5'-CATTTGAGCCGCCACAT-3'. The gene expression levels of neuropeptide Y and proopiomelanocortin in the ARH during the feeding cycle were also presented in the previous article (28).

**Glucose, Insulin, Leptin, and Desacyl-Ghrelin Levels**

Serum glucose was measured by the glucose dehydrogenase method (Kantokagaku, Tokyo, Japan) in accordance with the manufacturer’s protocol. Plasma insulin was measured using a rat insulin enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Plasma leptin was measured using a rat leptin enzyme-linked immunosorbent assay (ELISA) kit (Amersham...
RESULTS means 6 (SAS Institute, Cary, NC). The results are presented as statistical analyses were performed using StatView software. 0.05 was considered to be statistically significant. All statistical analyses were performed using StatView software (SAS Institute, Cary, NC). The results are presented as means ± standard deviation.

Pharmacia Biotech. Plasma desacyl-ghrelin was measured using a Desacyl-Ghrelin ELISA kit (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan).

Statistical Analysis

Two-way analysis of variance (ANOVA) with repeated-measures was used to determine the main effects of leptin administration (LEPTIN) and time course (TIME) for food intake and body weight. Two-way ANOVA was used to determine the effects of CR (DIET) and leptin administration (LEPTIN) for plasma parameters, adipose tissues, and gene expression levels. A, Two-way analysis of variance (ANOVA) for beta-actin: DIET, F (1, 20) = 0.004, p = .95, not significant; LEPTIN, F (1, 20) = 0.004, p = .83, not significant; DIET × LEPTIN, F (1, 20) = .394, p = .54, not significant. B, Two-way ANOVA for cyclophilin: DIET, F (1, 20) = 1.405, p = .25, not significant; LEPTIN, F (1, 20) = 0.00008, p = .99, not significant; DIET × LEPTIN, F (1, 20) = 0.128, p = .74, not significant. CR = calorie-restricted.

RESULTS

Food Intake and Body Weight in F344 Rats

The amount of food consumed in the AL-PBS group recovered by day 3 after the operation and remained constant thereafter (Figure 1). The amount of food intake in the AL-Leptin group on day 3 was approximately 30% lower than that in the AL-PBS group and remained constant at the lower level until the end of the experiment (LEPTIN, p < .0001). In the CR-PBS group, approximately 80%–90% of a meal allotted for the 2-day feeding period was ingested within the first day, and the remaining food was consumed on the second day. The CR-Leptin group showed a food intake pattern similar to that of the CR-PBS group. However, the amount of food ingested on the first day of the 2-day feeding period was significantly lower in the CR-Leptin group (LEPTIN, p = .0098), and consequently, the amount of food consumed on the second day was slightly greater in the CR-Leptin group than in the CR-PBS group.

Body weight was 30% lower in the CR group than in the AL group at day 0 (Table 1, DIET, p < .0001). Body weight in the AL-PBS group was slightly reduced after the operation, and then remained constant from day 3 to day 13 (Figure 2). The AL-Leptin group lost a significant amount of body weight (p < .0001 by one-way ANOVA with Tukey-Kramer post hoc test for multiple comparisons). Body weight in the CR group fluctuated in parallel with the 2-day food intake pattern. The body weight in the CR-Leptin group did not differ from that in the CR-PBS group, although the range of fluctuation appeared to be smaller in the CR-Leptin than in the CR-PBS group.

WAT weight, normalized by body weight, was decreased in the CR group (Table 1, DIET, p = .0008). Leptin administration induced similar decreases in WAT weight in both diet groups (LEPTIN, p = .0038; DIET × LEPTIN, not significant [NS]). Interscapular brown adipose tissue (BAT) weight did not differ between the AL and CR groups. Leptin administration significantly decreased BAT weight in both diet groups (LEPTIN, p < .0001; DIET × LEPTIN, NS).

Glucose, Insulin, Leptin, and Desacyl-Ghrelin Concentrations in F344 Rats

Serum glucose concentration was decreased in the CR group, and the level was unaffected by leptin treatment
(Table 1; DIET, p = .0006; LEPTIN, NS). Plasma insulin concentration was reduced in the CR group, and the level was further decreased in both Leptin groups (Table 1; DIET, p = .0035; LEPTIN, p = .004; DIET × LEPTIN, NS). The plasma leptin concentration was significantly decreased in the CR group (Table 1; DIET, p < .0001). The plasma leptin level was also reduced in both Leptin groups, and the reduction rate was greater in the AL-Leptin group (LEPTIN, p < .0001; DIET × LEPTIN, p < .0001). The desacyl-ghrelin concentration was greater in the CR group (Table 1; DIET, p = .0016). The level was not significantly affected by leptin treatment.

**Gene Expression Levels in the ARH**

The NPY mRNA expression level was significantly increased in the CR group (Figure 3A; DIET, p = .0011). The NPY mRNA level was decreased by leptin administration in the CR group, but not in the AL group (LEPTIN, p = .0524; DIET × LEPTIN, p = .0087).

### Table 1. Body Weight, Adipose Tissue Weight, Serum Glucose, Plasma Insulin, Plasma Leptin, and Plasma Desacyl-Ghrelin After Leptin Administration in AL and CR Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AL</th>
<th>CR</th>
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<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>Leptin</td>
</tr>
<tr>
<td>BW (g) at Day 0</td>
<td>368.8 ± 17.4</td>
<td>354.8 ± 13.9</td>
</tr>
<tr>
<td>BW (g) at Day 13</td>
<td>350.3 ± 15.4</td>
<td>312.4 ± 12.3</td>
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<tr>
<td>WAT (g/100 g BW)</td>
<td>4.15 ± 0.172</td>
<td>2.83 ± 0.575</td>
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<td>BAT (g/100 g BW)</td>
<td>0.117 ± 0.026</td>
<td>0.072 ± 0.016</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>122.1 ± 5.5</td>
<td>128.7 ± 8.1</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>19.39 ± 5.51</td>
<td>12.25 ± 4.80</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>14.10 ± 1.49</td>
<td>2.25 ± 1.85</td>
</tr>
<tr>
<td>Desacyl-ghrelin (fmol/mL)</td>
<td>323.49 ± 85.32</td>
<td>413.77 ± 128.47</td>
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</table>

**Notes:** Data represent the means ± standard deviation of 4–8 rats.

Two-way analysis of variance (ANOVA) for BW at day 0: DIET, F (1, 24) = 400.34, p < .0001; LEPTIN, F (1, 24) = 2.014, p = .169, NS; DIET × LEPTIN, F (1, 24) = 1.441, p = .242, NS.

Two-way ANOVA for BW at day 13: DIET, F (1, 24) = 276.726, p < .0001; LEPTIN, F (1, 24) = 19.231, p = .0002; DIET × LEPTIN, F (1, 24) = 7.946, p = .0095.

Two-way ANOVA for WAT: DIET, F (1, 22) = 15.021, p = .0008; LEPTIN, F (1, 22) = 10.455, p = .0038; DIET × LEPTIN, F (1, 22) = 0.719, p = .406, NS.

Two-way ANOVA for BAT: DIET, F (1, 24) = 0.339, p = .566, NS; LEPTIN, F (1, 24) = 23.795, p < .0001; DIET × LEPTIN, F (1, 24) = 0.148, p = .704, NS.

Two-way ANOVA for glucose: DIET, F (1, 23) = 15.584, p = .0006; LEPTIN, F (1, 23) = 0.001, p = .972, NS; DIET × LEPTIN, F (1, 23) = 4.522, p = .0444.

Two-way ANOVA for insulin: DIET, F (1, 17) = 11.493, p = .0035; LEPTIN, F (1, 17) = 11.033, p = .004; DIET × LEPTIN, F (1, 17) = 0.381, p = .545, NS.

Two-way ANOVA for leptin: DIET, F (1, 23) = 53.689, p < .0001; LEPTIN, F (1, 23) = 119.272, p < .0001; DIET × LEPTIN, F (1, 23) = 37.632, p < .0001.

Two-way ANOVA for desacyl-ghrelin: DIET, F (1, 24) = 12.594, p = .0016; LEPTIN, F (1, 24) = 1.549, p = .225, NS; DIET × LEPTIN, F (1, 24) = 0.042, p = .84, NS. The values of desacyl-ghrelin were converted to log values and then analyzed.

WAT = white adipose tissue; BAT = brown adipose tissue; BW = body weight; NS = not significant; AL = ad libitum-fed; CR = calorie-restricted; PBS = phosphate-buffered saline.
The AGRP mRNA expression level was also increased in the CR group (Figure 3B; DIET, $p = .0026$), and the level showed reductions after leptin administration in both diet groups (LEPTIN, $p = .0077$), particularly in the CR group although statistically insignificant (LEPTIN $\times$ DIET, $p = .073$).

The POMC mRNA expression level did not change significantly in any of the groups (Figure 3C). The CART mRNA expression level was slightly reduced in the CR group (Figure 3D; DIET, $p = .0006$) and slightly upregulated by leptin in both diet groups (LEPTIN, $p = .0364$; DIET $\times$ LEPTIN, NS).

**DISCUSSION**

In the present study, intracerebroventricular (ICV) leptin administration reversed the CR-induced alterations in the NPY, AGRP, and CART mRNA expression levels in the ARH of F344 rats, whereas neither CR nor ICV leptin had a significant effect on the POMC mRNA expression level. The food intake pattern over the 2-day feeding cycle in the Leptin-CR group suggests that CR rats lost their appetite after ICV leptin administration, which is consistent with the alterations in the neuropeptide gene expression levels. These findings support the hypothesis that leptin signaling to the ARH is involved in the CR-induced neuroendocrine alterations.

The importance of CR-induced NPY neuron activation in the ARH should be stressed for the following reasons. Overexpression of the NPY gene in rats was found to extend their life span (30). Activation of NPY receptors by receptor-specific agonists protected hippocampal neurons from excitotoxic insults (31), and a neuroprotective effect of CR was reported in similar experiments (32). Activation of NPY neurons suppressed GHRH neurons in the ARH (16). Dwarf mice with a mutation of the GHRH-receptor gene lived longer if they were fed a low-fat diet (33). Finally, suppression of the somatotropic axis was linked to longer life spans in several rodent models (34,35). Therefore, activation of NPY neurons could partially contribute, directly or indirectly through suppression of the somatotropic axis, to CR-mediated extension of the life span.

AGRP is an endogenous antagonist of the melanocortin 3 or 4 receptor (MC3/4-R) that binds to $\alpha$-MSH, a cleavage product of POMC (36). TRH and CRH neurons were reported to bear MC4-R (37,38). Binding of $\alpha$-MSH to MC4-R on TRH neurons causes their activation (21), whereas binding of $\alpha$-MSH to MC4-R on CRH neurons suppresses their synthesis or secretion of CRH (6,13). Because the activation of MC4-R depends on the net effect of these opposing $\alpha$-MSH and AGRP effects, and the present study demonstrated an augmented AGRP mRNA expression level due to CR, the antagonistic tone by AGRP could be predominant rather than the attenuated stimulatory tone by POMC and thus $\alpha$-MSH in the hypothalamus of CR rats (Supplemental Figure 1). Therefore, activation of NPY and/or AGRP neurons could be more important for understanding the effect of CR.

In the present study, the CART mRNA expression level, which was slightly reduced by CR, was upregulated by ICV leptin administration. Many neurons in the ARH were reported to coexpress POMC and CART (6,11). However, the present findings suggest that leptin signaling and CR differentially regulate the gene expressions of POMC and CART in individual neurons. A merit of the present finding that CR suppresses CART mRNA expression could be a relationship with the regulation of GnRH neurons. Previous studies have demonstrated that CART activates GnRH neurons (8,39,40). Therefore, we can speculate that diminished leptin signaling due to CR reduces the stimulatory signal from CART neurons for the activation of GnRH neurons. Because NPY was reported to inhibit the activation of GnRH neurons (8,41–43), a reduction in leptin signaling is thought to suppress reproductive function by augmenting the inhibitory tone from NPY neurons and reducing the stimulatory tone from CART neurons.

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**Figure 2.** Effect of intracerebroventricular leptin on body weight in F344 rats fed ad libitum (AL) (A) or a calorie-restricted diet (CR) (B). Values are the means ± standard deviations of 6–8 rats. A, Two-way analysis of variance (ANOVA) with repeated measures for AL rats: LEPTIN, $F (1, 144) = 30.306, p < .0001$; TIME, $F (12, 144) = 40.091, p < .0001$; LEPTIN $\times$ TIME, $F (12, 144) = 19.846, p < .0001$. B, Two-way ANOVA with repeated measures for CR rats: LEPTIN, $F (1, 144) = 0.061, p = .81$, not significant; TIME, $F (12, 144) = 42.83, p < .0001$; LEPTIN $\times$ TIME, $F (12, 144) = 4.496, p < .0001$. PBS = phosphate-buffered saline.
A greater body fat content increases plasma leptin concentration, leading to leptin resistance (6). Responsiveness of ARH neurons might be, therefore, reduced in the AL group, and this leptin resistance might have confounded the experimental outcomes. In the present study, the rate of reduction of body weight after leptin treatment was greater in the AL than in the CR group. The reduced rate of WAT weight after leptin administration did not differ between the AL and CR groups. The plasma leptin level was more greatly reduced in the AL group after leptin administration. Because the ICV administration of leptin can affect the body fat content and the plasma leptin through activation of the sympathetic nervous system into the brown adipose tissue that plays a major role in energy expenditure (44), the present data indicated that the ARH neurons in the AL group reasonably responded to ICV leptin, if compared to that in the CR group.

The present study stressed a role for the leptin–ARH pathway in the CR-induced neuroendocrine alterations. However, the pathway may be dispensable for the effect of CR. In ob/ob mice and Zucker fa/fa rats whose leptin signaling is spontaneously disrupted, CR extended their life spans (45,46). The arcuate nucleus neurons are regulated by multiple hormones (47). For example, ghrelin, a peptide that is secreted mostly by gastric endocrine cells (48), is also known to regulate NPY and AGRP neurons in the ARH (49). Fasting increases the plasma concentration of ghrelin (50,51) and activates NPY and/or AGRP neurons (9,10). In the present study, we have provided data for only the plasma level of desacyl-ghrelin, a major fraction of plasma ghrelin but the inactive form in the circulation (48), and thus, further analyses are still required. However, the plasma desacyl-ghrelin level was significantly higher in the CR group as expected, and thus we can speculate that CR exhibits its effect in ob/ob mice and Zucker fa/fa rats via activation of the ghrelin–ARH pathway. Accumulated evidence has also indicated that insulin displays effects similar to those of

![Figure 3](https://academic.oup.com/biomedgerontology/article-abstract/61/9/890/595975)
leptin in the ARH regarding appetite and energy expenditure (6,52–54). Indeed, the plasma insulin level is known to be reduced by CR (55,56) as observed in the present study. A potential role for insulin is also supported by a study in nematodes, suggesting that reduced insulin-like signaling in the central nervous system is sufficient for life-span extension (57). Holzenberger and colleagues (58) also mentioned in their review (but to our knowledge the data have not been published yet) that brain-specific insulin-like growth factor (IGF)-1 receptor knockout mice had an increased life span. Reduced insulin or IGF-1 signaling in the brain may elicit neuroendocrine alterations similar to those induced by the diminished leptin signaling. Changes by CR in hypothalamic neurons’ activities should be further analyzed in long-lived rodent models with diminished IGF-1 or insulin signaling.

The present study used a modified every-other-day feeding regimen for CR. This regimen yielded a 2-day cycle in the food intake of CR rats, i.e., a cycle that consisted of fed and fasted phases. In the present study, we evaluated the effect of leptin at one time-point in the fasted phase. An analysis of the respiratory quotient in CR rats with a daily feeding regimen has also indicated the presence of a fed-fasted phase in a daily feeding cycle; i.e., fat was used as an energy source when carbohydrate reserves were depleted, i.e., the fasted phase, in the 24-hour feeding cycle (59). Previous studies that demonstrated an elevation of the NPY gene in the hypothalamus also examined gene expression in a fasted phase in CR rodents (24,26). Therefore, the present results from every-other-day feeding could be also applied to CR animals with daily feeding regimens.

Summary

The present results suggest a role for the leptin–ARH pathway in the effect of CR in intact animals. At present, however, it is reasonable to conclude that CR controls the hypothalamic nuclei via multiple signaling pathways, and that disruption of a single pathway may not debilitate the effect of CR. It may therefore be important to identify the putative central pathway(s) that mediates the effect of CR and at which the nutrient-sensing pathways converge.

Acknowledgments

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