Role of the Central Melanocortin System in Cachexia

Daniel L. Marks, Nicholas Ling, and Roger D. Cone

Department of Pediatric Endocrinology [D. L. M.], Vollum Institute [R. D. C.], Oregon Health Sciences University, Portland, Oregon 97201, and Neurocrine Biosciences, Inc. [N. L.], San Diego, California

ABSTRACT

Individuals affected with either acute or chronic diseases often show disorders of nutrient balance. In some cases, a devastating state of malnutrition known as cachexia arises, brought about by a synergistic combination of a dramatic decrease in appetite and an increase in metabolism of fat and lean body mass. Stimulation of the hypothalamic melanocortin 4 receptor (MC4-R) produces relative anorexia and increased metabolic rate, even in a relatively starved state. Here we demonstrate that cachexia induced by lipopolysaccharide administration and by tumor growth is ameliorated by central MC4-R blockade. MC4-R knockout mice or mice administered the MC3-R/MC4-R antagonist, agouti-related peptide, resist tumor-induced loss of lean body mass, and maintain normal circadian activity patterns during tumor growth. The final tumor mass is not affected in these animals, providing further support for the potential role of MC4-R antagonism in the treatment of cachexia in disease states.

INTRODUCTION

The severity of cachexia in many illnesses may be the primary determining factor in both quality of life and in eventual mortality (1, 2). Indeed, body mass retention in AIDS patients has a stronger correlation with survival than any other current measure of the disease (3). At this point, most authors suggest that cytokines released during inflammation and malignancy act on the CNS to alter the release and function of a number of key neurotransmitters, thereby altering both appetite and metabolic rate (1, 4–7). Most features of the cachexia observed in prolonged illness can be reproduced by chronic infusion of cytokines (4, 8–12). LPS potently stimulates the release of numerous cytokines and reliably produces anorexia in experimental animals (13–18). Thus, LPS administration provides a useful model for analysis of appetite and metabolism during the early stages of illness-induced cachexia.

Cachexia is commonly observed in patients with cancer, particularly in children and elderly individuals (19). The resulting malnutrition and loss of lean body mass reduces the quality of life for the affected individual and compromises recovery by decreasing tolerance to therapy and increasing postsurgical complications (2, 7). Attempts at drug therapy for cachexia with a variety of agents have met with limited success (20–23). The most widely used agent, megestrol acetate, has shown some promise in reversing weight loss, but this is primarily attributable to increases in fat mass and water retention, primarily via production and release of α-MSH from the POMC precursor. α-MSH binds to central melanocortin receptors (including MC4-R). Central administration of MC4-R agonists can inhibit energy intake, increase energy expenditure (30, 31), and reduce body weight (32, 33). In contrast, disruption of melanocortin signaling with antagonist administration or deletion of the MC4-R (MC4-RKO) leads to an increase in feeding and eventually to obesity (30, 34). POMC neurons in the arcuate nucleus express the leptin receptor and MC4-RKO mice are leptin resistant, leading several investigators to propose that melanocortin neurons mediate the anorexie and metabolic effects of elevated leptin (35, 36). Remarkably, leptin is a member of the IL-6 superfamily of proteins and has many biochemical features of a cytokine molecule (37, 38). However, initial studies of cytokine-induced anorexia in mice with disrupted melanocortin signaling (viable obese yellow, A(13) demonstrated enhanced anorexia in this model, perhaps because of increased release of endogenous corticotropin-releasing factor (39, 40). In a more recent study, Huang et al. demonstrated a reversal of LPS-induced anorexia in rats treated with a central melanocortin antagonist (41). Thus, it remains plausible that central melanocortin receptors may integrate a number of physiological signals that produce the combination of decreased energy intake and increased energy utilization that characterizes illness-induced cachexia. To test the potential role of central melanocortin signaling in the pathology of cachexia, we examined the effects of central melanocortin blockade on activity, feeding, and weight homeostasis in several models of murine cachexia.

MATERIALS AND METHODS

Animals. MC4-RKO mice and their WT controls were derived from the original C57BL/6J×129 colony (34) maintained within the Vollum Institute that had been bred five generations into the C57BL/6J strain. All mice were raised group-housed in a 12-h light/dark cycle. For studies measuring food intake, mice were housed individually, and food intake was estimated by measuring the weight of powdered food remaining in feeding chambers designed to maximize spill capture. Mice were weaned at 21 days and allowed ad libitum access to powdered Laboratory Rodent Diet (Purina), which was weighed and replaced daily. To minimize error attributable to loss of food particles, all bedding was screened before and after the experiment to capture any spilled food. Food remaining in the feeding chamber was also screened to remove any bedding or other debris. For the first LPS injection, male animals aged 6–7 weeks were used. In an identical repeat experiment, female animals aged 5 weeks were used. In the tumor models, male animals, age 4 weeks at the start of the experiment, were used. C57Bl/6J mice (25–33 g, Jackson Laboratory) were housed and fed similarly. All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animal and approved by the Animal Care and Use Committee of the Oregon Health Sciences University.

Cannula Placement. C57Bl/6J mice were anesthetized with halothane and placed in a stereotaxic apparatus (Cartesian Research, Inc.). A sterile guide
cannula with obdurator stylet was stereotaxically implanted for i.c.v. injection with the coordinates of 0.5 mm posterior to the bregma, 1–1.6 mm lateral to the midline, and 2 mm below the bregma. The cannula was then fixed in place using dental cement. The animals were housed separately after surgery at least 1 week for recovery before experiments. The positions of the cannulae were verified at the end of experiments by histological analysis; in animals in which CSF return was not obvious, the position of the cannulae were tested by dye administration before the animals were killed.

**AGRP and LPS Administration.** Each animal was handled daily for a minimum of 5 consecutive days before the initiation of the experiment, simulating the restraint used during the injection of the compounds. ACSF or AGRP diluted in ACSF was infused in a total volume of 2 μl over 30 s in lateral ventricle-cannulated mice. In the LPS experiments, LPS (Escherichia coli 055:B5; Sigma Chemical Co.) was dissolved in normal saline and administered i.p. MC4-RKO mice and littermate controls had basal feeding monitored for 2 days and then during each 12-h period after an i.p. saline injection (1700 h) before injection of 100 μg/kg LPS. In C57Bl/6 WT animals, AGRP (84–132 amino acid fragment; Neuronic Biosciences, Inc., San Diego, CA) was administered at 1500 h, and 50 μg/kg LPS was administered at 1700 h. A second dose of 100 μg/kg was given 60 h after the first dose in the second experiment. No food was available between AGRP administration and LPS administration, and 24-h feeding was measured starting at 1700 h. In the tumor models, AGRP or ACSF was administered at 1400 h with each administration.

**Tumor Models.** LLC cells and EHS sarcoma tumors were maintained either as a primary culture in DMEM with 10% fetal bovine serum or in vivo, respectively, as recommended by the supplier (American Type Culture Collection, Manassas, VA). LLC tumor cells were harvested during exponential growth of the culture, washed in HBSS, and 1 × 10^6 cells were injected s.c. into the upper flank of the mice. EHS sarcoma tissue was dissected from a donor animal, and an approximately 3-mm cube of tissue was implanted s.c. above the rear flank. Sham-operated animals received an implant of a similar amount of donor muscle tissue. In all cases, the time of appearance of a tumor mass was noted in the log, and all animals were found to have a palpable tumor above the rear flank. Sham-operated animals received an implant of a similar amount of donor muscle tissue. In all cases, the time of appearance of a tumor mass was noted in the log, and all animals were found to have a palpable tumor within 4 (LLC) or 8 (EHS) days of the start of the experiment. At the time the animal was killed, tumors were dissected away from surrounding tissue and weighed. Gross examination of all organs did not reveal the presence of any observable metastasis. Trunk blood was collected at the time the animal was killed for measurement of serum leptin with a rat leptin RIA kit (Linco Research, Inc., Manassas, VA).

**Motor Activity and Feeding Activity.** Animals were housed individually in metabolic cages equipped with a running wheel (Mini-Mitter Co., Sunriver, OR). The metabolic cages usage allowed telemetric monitoring of circadian rhythms as assessed via multiple physiological parameters. The wheel revolutions were quantified by recording the magnetic switch closures of a magnet placed on the revolving wheel. For feeding recordings, feeding counts and duration were recorded when the animals interrupted an infrared beam above the feeding chambers.

**Statistical Methods.** Differences between feeding, activity, and water consumption curves in all experiments were analyzed by two-way, repeated measures ANOVA, with time and treatment as the measured variables. Final tumor and body weights were analyzed by Student’s t test when two groups were included, or one-way ANOVA with post hoc analysis when three groups were included. Data sets were analyzed for statistical significance using either the PRISM software package (GraphPad) for ANOVA with repeated measures or EXCEL (Microsoft) using Student’s t test.

**RESULTS**

**AGRP Administration Prevents LPS-induced Cachexia.** Basal feeding was measured every 6 h in two age- and sex-matched groups after simulated i.c.v. injection and i.p. saline injection. Twenty-four h later, AGRP was administered at 1500 h, and LPS was administered i.p. at 1700 h. i.c.v. injection of the 84–132 amino acid fragment of AGRP (2.5 nmol in 2 μl ACSF) prevented the LPS (50 μg/kg)-induced decrease in feeding (Fig. 1A), even in the 24- to 36-h period after LPS treatment. Feeding was measured every 6 h for 24 h and then every 12 h for 48 h more. The difference between feeding curves

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**Fig. 1.** Effect of AGRP administration on LPS-induced cachexia. A, food consumption with LPS and AGRP administration. AGRP (i.c.v.) injection ameliorates LPS anorexia, with effects seen for as long as 24–36 h. B, AGRP prevents LPS anorexia. Cumulative normalized food intake after LPS injection. C, net weight change over 58 h. AGRP prevents LPS-induced weight loss. D, AGRP prevents LPS anorexia. Tolerance to repeated LPS injection is observed in both groups (*, P < 0.001; **, P < 0.0001).
was significant when expressed both as weight-normalized intake ($n = 6, P < 0.001$, Fig. 1B) and as a percentage of basal feeding ($n = 6, P < 0.001$ versus postsaline and sham i.c.v. injection, data not shown). Thereafter, the ACSF-treated group demonstrated a recovery of normal feeding as expected (24-h feeding from 36 to 58 h post-LPS; $91 \pm 3$ g/kg$^{0.75}$ AGRP versus $92 \pm 8$ g/kg$^{0.75}$ ACSF, $P = 0.98$). AGRP also prevented weight loss in this model of illness ($n = 6, P < 0.0001$, Fig. 1C). In a second experiment, a second dose of LPS (100 µg/kg) was given after recovery from the first dose 3 days after the first LPS injection (Fig. 1D). In this experiment, the ACSF-treated animals recovered to 95% of basal feeding on the 3rd day after the first LPS injection, after showing a significant drop in food intake relative to the AGRP group ($n = 7$ AGRP, $n = 6$ ACSF, $P < 0.0001$). Interestingly, the AGRP-treated animals continued to show a relative hyperphagia and consumed 118 ± 6% of basal food intake on that day ($n = 7, P < 0.02$). The second LPS injection did not result in a significant decrease in feeding in either group, demonstrating LPS tolerance in these animals (42).

**MC4-RKO Mice Resist LPS-induced Cachexia and Illness Behavior.** To extend the findings of the previous experiments, MC4-RKO mice were tested for their response to LPS injections. Parameters monitored included food and water intake, lick counts, wheel running activity, and weight gain. Six-week-old male MC4-RKO mice were slightly but not significantly heavier than their wild-type littermates (KO $17.5 \pm 0.8$ g versus WT $15.7 \pm 0.4$ g, $P = 0.07$), and all feeding data were normalized to weight. Basal feeding after i.p. saline was not different between groups ($n = 5, P = 0.8$). LPS administration resulted in a significant decrease in feeding in the WT animals, which was apparent for 36 h after injection (Fig. 2A). This decrease in intake was not seen in MC4-RKO animals ($n = 5, P < 0.01$ versus WT) when measured either as total food intake or as a percentage of basal intake after saline injection (Fig. 2B). Water intake as a percentage of the basal value was also greater in MC4-RKO mice, but the difference was not significant (MC4-RKO 70 ±12% versus WT 46 ±14%, $P > 0.05$). However, total lick counts were significantly different after LPS (MC4-RKO 1.8 ± 0.4 cpm versus WT 0.4 ± 0.09 cpm, $P < 0.05$). Wheel running activity was similar between groups after saline injection but greatly decreased in the WT animals after LPS injection (Fig. 2C). Twenty-four-h total number of turns per min was similar after saline injection (MC4-RKO 2.5 ± 0.6 turns/min versus WT 2.8 ± 0.8 turns/min, $P = 0.8$), whereas after LPS injection, the WT animals showed a decrease in total 24-h turns, whereas MC4-RKO animals maintained normal activity (MC4-RKO 2.4 ± 0.9 turns/min versus WT 0.1 ± 0.05 turns/min, $P < 0.05$). WT animals failed to gain weight during the 60-h experimental period, whereas MC4-RKO mice continued to gain weight (Fig. 2D, $P < 0.05$).

**Effect of AGRP Administration in C57Bl/6J Mice Bearing a Syngenic Sarcoma.** We next examined the effects of AGRP administration in animals with hypophagia and weight loss attributable to the presence of a growing sarcoma. In an initial experiment, daily food intake and weight was followed until the tumor-bearing animals had food intake that was 75–80% of basal for 3 consecutive days. This occurred on day 12 postimplant, on average 4 days after a palpable tumor was present. i.c.v. injection of the 84–132 amino acid fragment of AGRP (2.5 nmol in 2 μl ACSF) caused a return to basal feeding levels in the treated group within 48 h of injection (Fig. 3A). AGRP injected 96 ± 5% on day 14. However, the difference between treatment groups was not significant on that day ($n = 5, P = 0.2$). A second injection on day 14 postimplant sustained this normalization of food intake, whereas the ACSF-treated animals continued to have gradually decreasing intake. This effect lasted for 3 days, with a return to the relatively anorexic state by day 18 postimplant. A third injection of AGRP on this day again raised the food intake of AGRP-treated animals, with both groups killed on day 19 because of the growth of the tumor. Overall ANOVA for feeding in this study was significant
Resistance to Cachexia in MC4-RKO Mice Bearing a Syngenic Adenocarcinoma. To confirm and extend the findings in the sarcoma model, we next tested the response of MC4-RKO mice to the growth of a cachexigenic adenocarcinoma (25, 26). Parameters monitored included food and water intake, lick counts, meal frequency and duration, wheel running activity, weight gain, and tumor mass. WT control mice began to show decreased 24-h feeding at day 3 after tumor implantation, before the presence of a palpable tumor (Fig. 4A). The overall feeding curves were noticeably different from that day onward, with the WT animals consuming 56% of MC4-RKO levels by the final day of the experiment (Fig. 4A; n = 5; P < 0.0001). The change in water lick counts paralleled the change in food consumption (Fig. 4B; P < 0.0001). WT animals showed a prompt decline in wheel running activity to 30 ± 10% of basal activity by day 7 postimplantation, whereas MC4-RKO mice showed a slower decline (day 7, 70 ± 14%, P < 0.05). However, the MC4-KO animals eventually decreased their running activity as well (66 ± 18% of basal on the final day), and the overall activity curves were not different between groups (n = 5, P = 0.09). MC4-RKO animals gained carcass weight, whereas control animals lost weight (Fig. 4C; n = 5; P < 0.05). The final tumor mass was not different between groups (Fig. 4D; P = 0.9). Serum leptin levels also were not different between groups (WT 2.3 ± 1.4 ng/ml versus KO 2.2 ± 1.1 ng/ml, P = 0.98). A repeat trial of this experiment revealed similar results with significant differences observed in carcass weight change (WT −0.3 ± 0.5 g versus KO 1.4 ± 0.4 g, P < 0.05) but not in final tumor mass (WT 1.7 ± 0.3 g versus KO 1.4 ± 0.4 g, P = 0.61). Preliminary carcass analysis indicated that the majority of the difference between groups was attributable to a larger amount of lean body tissue in the MC4-RKO animals.  

DISCUSSION

Under normal circumstances, animals and humans respond to starvation with a complex neuroendocrine response that ultimately leads to an increase in appetite, a relative sparing of lean body mass and burning of fat stores, and an overall decrease in basal metabolic rate (43, 44). In contrast, cachexia refers to a pathological state of malnutrition wherein appetite is diminished concomitant with an increase
Fig. 4. MC4-RKO mice resist cachexia attributable to growth of a syngenic adenocarcinoma. A, food intake in carcinoma-bearing animals. Hypophagia during tumor growth in WT animals is not seen in MC4-RKO animals (ANOVA, \( P < 0.0001 \)). B, total daily water lick counts. Parallel changes in water lick counts are also observed (ANOVA, \( P < 0.0001 \)). C, change in body weight. MC4-RKO animals gained carcass weight, whereas WT animals lost weight (+, \( P < 0.05 \) versus WT), but final tumor mass was not affected (D).

in metabolic rate and a relative wasting of lean body mass (1, 7, 12, 19, 45). This combination is found in a number of disorders including cancer, cystic fibrosis, AIDS, rheumatoid arthritis, and renal failure (1). The severity of cachexia in these illnesses is often the primary determining factor in both quality of life, and in eventual mortality, particularly in the pediatric population (1, 2). Numerous previous studies have demonstrated that cytokines released during inflammation and malignancy act on the CNS to alter the release and function of a number of key neurotransmitters, thereby altering both appetite and metabolic rate (1, 4, 5, 7). However, the neural systems involved in transducing these complex signals remain poorly defined. Previous pharmacological studies have demonstrated an acute and chronic effect of central melanocortin peptides on feeding behavior (30, 36, 47) that parallels the alterations in metabolic rate and a relative wasting of lean body mass (1, 7, 12, 19, 45). This combination is found in a number of disorders including cancer, cystic fibrosis, AIDS, rheumatoid arthritis, and renal failure (1). The severity of cachexia in these illnesses is often the primary determining factor in both quality of life, and in eventual mortality, particularly in the pediatric population (1, 2). Numerous previous studies have demonstrated that cytokines released during inflammation and malignancy act on the CNS to alter the release and function of a number of key neurotransmitters, thereby altering both appetite and metabolic rate (1, 4, 5, 7). However, the neural systems involved in transducing these complex signals remain poorly defined. Previous pharmacological studies have demonstrated an acute and chronic effect of central melanocortin peptides on feeding behavior (30, 36, 47) that parallels the alterations observed during the development of cachexia. The data presented here provide evidence that the hypothalamic MC4-R plays an integrative role in regulating the response to different cachexigenic stimuli and suggest that blockade of this receptor may ameliorate the pathological metabolic state observed in a number of diseases. Furthermore, the specificity of this response is highlighted by the previous demonstration that in anorectic, tumor-bearing animals, hypothalamic content of the potent endogenous orexigen NPY is increased, and animals respond to NPY injections with worsened anorexia (48, 49).

One model that has been particularly useful in studying cachexia has been administration of a purified product found in the cell wall of Gram-negative bacteria, which is known generically as LPS. Early experiments focused on the ability of LPS injections to reliably produce anorexia in experimental animals (13, 14). It is now known that LPS potently stimulates the release of numerous cytokines from immune cells in the periphery and glia within the CNS and that these cytokines are primarily responsible for the observed response (15–18). Previous studies of the impact of central melanocortins in transducing signals from cytokines have had mixed results. In an early study using the A\(^{VY}\) mouse, an enhanced anorexigenic response to peripheral IL1-\(\beta\) was observed (40). Of course, the MC4-R blockade by agouti in this model is reversible. In contrast, a recent study has demonstrated that MC4-RKO mice resist the inhibition of locomotion produced with central IL1-\(\beta\) administration (50). Huang et al. investigated the impact of central administration of \(\alpha\)-MSH or the melanocortin receptor subtype3/subtype4 antagonist SHU-9119 on LPS-induced anorexia and fever in rats (41). In this study, the investigators found a significant potentiation of the suppressive effects of LPS on food intake with administration of \(\alpha\)-MSH and a reversal of LPS-induced anorexia with SHU-9119 administration. These same treatments reduced and increased LPS-induced fever, respectively. Our data are in agreement with these latter findings and demonstrate that both genetic and pharmacological blockade of central MC4-R signaling can prevent the hypophagia, hypodipsia, and decreased locomotor activity seen after induction of a complex and pleiotropic cytokine response. Additionally, we have also demonstrated that in young, rapidly growing mice, the weight loss that accompanies LPS-induced cachexia can be reversed, allowing the animals to continue to follow a normal growth curve.

Our data demonstrate a preservation of normal motor activity in MC4-KO animals, which contrasts with the inability of SHU-9119 to restore normal motor activity in LPS-treated rats (41). Furthermore, we observed very little illness behavior in our AGRP-treated animals after LPS injection. These differences are likely to result from the fact that in our experiments the animals had a blockade of MC4 receptor signaling (with AGRP or in the receptor knock-out mouse) before the injection of LPS and because of the timing of our LPS injections immediately before the onset of the active dark phase. The effect of LPS on motor activity can be detected quite early in the course of the illness, and the systems involved in this suppression may be activated before the onset of melanocortin blockade when LPS is injected before administration of melanocortin antagonists. Additionally, we
have observed that AGRP administration produces a prolonged effect (>36 h in the sarcoma-bearing animals), and it is possible that SHU-9119 does not have a sufficient duration of action (relative to LPS) to reverse the inhibition of nocturnal locomotion when both compounds are injected in the morning.

The role of melanocortin receptors in transducing the prolonged metabolic derangement observed in experimental cancer has not been previously reported. Many different tumor types have been studied, and it is a common finding that tumor-bearing animals die from cachexia and exhaustion of metabolic fuels rather than from metastasis or infection (28, 45, 51, 52). Our observations demonstrate that hypophagia and carcass weight loss induced by sarcoma growth can be both reversed and prevented by administration of the endogenous MC3/MC4 antagonist, AGRP. In this case, the duration of the experiment in the AGRP-treated animals was limited only by ethical concerns because of the size of the tumor rather than by anorexia or lack of physical activity and grooming. When the animals had already become hypophagic because of the growth of the tumor, there was a delay in the response to AGRP injection, with significant induction of feeding seen after the second and third but not the first injection. The presence of this priming effect may be attributable to lasting activation of neurons downstream from MC4 receptors or may simply be attributable to the need for an accumulation of antagonist to overcome a high melanocortin tone. Prevention of tumor-induced hypophagia with early and repeated AGRP injections resulted in a maintenance of normal food intake, and this enhancement of feeding was much greater than the relative hyperphagia observed in the sham-tumor-implanted animals. The inability to completely mimic the feeding observed in the sham-tumor group is likely to reflect, in part, the energy drain imposed by the rapid growth of a metabolically active tissue. However, our observation that the rate of tumor growth was identical between AGRP and vehicle-treated groups argues strongly that tumor growth produces a global metabolic derangement that is primarily mediated by central melanocortin receptor activation. If the tumor simply represented a metabolic sink, increased nutrient intake would be expected to result in increased tumor growth at the expense of nontumor body mass.

Several lines of evidence exist suggesting that the anorexic and metabolic effects of leptin are transduced by hypothalamic melanocortin receptors (53–56). Furthermore, LPS injection is known to increase the level of leptin in the circulation, leading to the suggestion that enhanced leptin feedback may be responsible for illness-induced cachexia (57). Our data argue that the suppression of feeding in our cachexia models is not attributable to enhanced leptin feedback. An increase in plasma leptin was not observed in tumor-bearing animals, and an increase in feeding was observed before any noticeable increase in body weight and without the presence of any grossly detectable increase in body fat. This idea is consistent with previous observations that leptin-deficient ob/ob mice are hypersensitive to the anorexic effects of LPS injections and to the anorexia and weight loss induced by tumor growth (58–60). Thus, we propose that the observed effects of melanocortin blockade in our experiments are attributable to a lepton-independent activation of hypothalamic POMC neurons with a resultant increase in activity at the hypothalamic MC4-R.

In summary, hypothalamic MC4-R activation appears to integrate peripheral signals that lead to anorexia, hypodipsia, and decreased locomotion during illness. Blockade of this signal results in normalization of food intake, activity, and growth without increasing the morbidity or mortality observed. This system is operative in both acute (LPS-induced) and chronic (cancer-induced) illness, which reinforces the idea that the hypothalamic melanocortin system provides the primary inhibitory tone on food intake. Our data suggest that this system may play an integrative role in mediating the cachexia observed in human diseases such as cancer, heart failure, Alzheimer’s disease, and AIDS, thereby providing a common target for therapeutic intervention.

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