Nutritional Status, Cognition, and Survival

A NEW ROLE FOR LEPTIN AND AMP KINASE*

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Although adequate nutrition is essential for optimal neural activity and survival, mild energy restriction may improve cognition and prolong longevity. Energy status is monitored by the cellular AMP-activated protein kinase (AMPK) system, whereas leptin regulates total energy balance. We investigated the roles of AMPK and leptin in cognition and survival under diet restriction (DR). Hippocampal AMPK activity increases with energy restriction. Modest activation (DR to 60%) induces neurogenesis and improves cognition. However, DR to 40% augmented AMPK activity, reduced cognition and catecholamines, and increased neural apoptosis and mortality. Leptin signaling is preserved only in DR to 60%, countering the effects of AMPK “overactivation” by preventing neuroapoptosis, restoring noradrenergic activity and behavioral performance, and increasing longevity. The balance between leptin and AMPK is crucial in determining neuronal fate, cognitive ability, and survival. Should these findings extend to Man, then controlled activation of AMPK may improve neurodegenerative diseases, and leptin may have a new role in treating stress-associated malnutrition.

Since the Garden of Eden and the trees of knowledge and life, the connection between food, cognition, and survival has been recognized. Today, nutritional status is a well accepted regulator of neural function and longevity.

Studies have shown that mild energy restriction (to 60% of control) improves cognitive function, whereas severe restriction (to 40%) prejudices it (1, 2). Such cognitive dysfunction may manifest as deficits in hippocampal-dependent learning and memory, including spatial information processing (3). The benefits of mild energy restriction have been repeatedly demonstrated in prolonging longevity (4, 5) and in improving neurodegenerative diseases (6, 7). Understanding of the mechanisms for neural damage advanced when apoptosis was shown to be responsible for some of the neural cell loss found in Parkinson and in Alzheimer disease (8, 9). Neuroapoptotic cell death is a multistep process involving several key molecules, modulation of which may inhibit the process. Such neuroprotective agents were also found to overcome poor behavioral performance found in animal models for these diseases (10, 11).

The mammalian forebrain contains populations of cells that divide and differentiate into neurons and glia (12). These neurons are generated continuously from stem cells in specific regions of the adult brain (13). Such constitutive neurogenesis can be modulated by changes in diet. Reduced nutritional status in mice increased the numbers of newly generated cells primarily in the dentate gyrus of the hippocampus (14, 15), which is the principal center for learning and memory (16); suppression of neurogenesis led to impaired learning and memory (17).

Cellular energy status is monitored and controlled by the 5′-AMP-activated protein kinase (AMPK)2 system. AMPK is allosterically activated by 5′-AMP, which accumulates following ATP hydrolysis (18, 19). Recent studies have demonstrated that this activation regulates intracellular signaling pathways involved in cellular survival and apoptotic cell death (20–22). AMPK is considered to act as a “fuel gauge” for cellular metabolism (18), controlling the endogenous energy supply of ATP. It was therefore not surprising when AMPK was also found to regulate feeding behavior (23, 24).

Leptin is an adipokinin hormone that plays a central role in food intake and energy balance, mainly through its hypothalamic receptors (25–27). Studies on leptin function have demonstrated that AMPK is essential for mediating its actions (28). Whereas leptin exerts its catabolic effects in the periphery by stimulating AMPK phosphorylation and activation, its central effects on energy balance act through hypothalamic AMPK dephosphorylation and inhibition (23, 24). Linking systemic energy balance (appetite and autonomic nervous activity) with cellular AMP concentrations makes obvious “biological sense.”

There are also leptin receptors in the hippocampus, suggesting a possible role for leptin signaling in cognition. The involvement of leptin and AMPK in brain function has heretofore not been addressed and is the subject of this report. We have used diet restriction (DR) tools to define the processes underlying the effects of nutrition on cognition and survival. Our results indicate that AMP kinase responds to nutritional status and modulates cognitive ability by affecting the balance between neurogenesis and neuroapoptosis. Leptin reverses the deleterious effects of severe DR, consistent with its role as a “survival” (anti-apoptotic) hormone under such stress conditions.

MATERIALS AND METHODS

Cells and Reagents—PC12 cells were kindly provided by O. Meyuhas, Department of Biochemistry, Faculty of Medicine, Hebrew University-Hadassah Medical School, Jerusalem. These cells were grown either in Dulbecco’s modified Eagle’s medium supplemented with 8% horse serum, 8% fetal bovine serum, glutamine, and gentamicin or with 1% horse serum in the presence of nerve growth factor (50 ng/ml), which causes the cells to differentiate. Murine leptin was provided by A. Gertler. AICAR was obtained from Toronto Research Chemicals.

Mice—The experimental protocol was approved by the institutional committee for the use of animals, number MD-89.52–4. 8–10-week-old C57BL/6 mice were housed in a 12-h light/12-h dark cycle with food and water available ad libitum. The offspring of these animals were housed in clean SPF cages, and the experiments were conducted in accordance with the guidelines of the USA National Institutes of Health for the care and use of laboratory animals. The experimental protocol was approved by the institutional committee for the use of animals, number MD-89.52–4. 8–10-week-old C57BL/6 mice were housed in a 12-h light/12-h dark cycle with food and water available ad libitum. The offspring of these animals were housed in clean SPF cages, and the experiments were conducted in accordance with the guidelines of the USA National Institutes of Health for the care and use of laboratory animals.

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2 The abbreviations used are: AMPK, AMP-activated protein kinase; AL, ad libitum; DR, diet restriction; HPLC, high performance liquid chromatography; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside.
old female Sabra mice (29–32 g) were assigned at random to different groups of 10 mice. The food provided was Purina chow.

Diet Restriction—Control mice received food ad libitum (AL). Diet-restricted mice received a diet of 57 kcal/week/mouse (2.16 g/day/mouse) as 60% of the requirement or 38 kcal/week/mouse (1.44 g/day/mouse) for 40% DR. DR was carried out for 10 days. Behavioral tests were performed using the eight-arm spatial maze during the second week of the experiment. During the behavioral tests, half the animals in each group were treated with AICAR (1–2 mM equivalent to 258–516 mg/kg) or leptin (1 mg/kg) and the other half with saline. The mice were treated with a dose of 1 mg/kg leptin based on previous experiments. We have evaluated the effect of 100–2000 μg/kg of body weight (intraperitoneal) on cognitive function and found that 1000–2000 μg/kg were the optimal dosages, whereas lower concentrations impaired it. This dose is likely to lead to leptin concentrations in the high physiologic range (~100 ng/ml) in the injected animals (characteristic of obese subjects), which declined to lower concentrations after 24 h (29).

AICAR and leptin were dissolved and diluted in saline. All administrations were given intraperitoneally in a volume of 0.1 ml/mouse. Mice were sacrificed by decapitation after the behavioral tests, and the hippocampi were frozen at −70 °C.

RT-PCR Analysis—Total hippocampal RNA was extracted using TriFast reagent according to the manufacturer’s instructions and reverse transcribed. Primers specific for OBRlong were GATGTTCACACCCCCAACGAAACCAGACCATCT, for OBRshort ATCTGGCCTGTTGAGTTTTC and CCAGCTCTCTGGCTTCACC, for BAX CTGAGCTGACTTGGAGC and GACTCCAGGCACCAAAGATG, for BCL-2 GACAGAAGATCATGCCGTCC and GGTCCCAAGAA and CATAGCTGCTGGGACCATCT, for OBRshort GATGTTCCAAACGGGCC and TCACCCACATAGGAGTCCT. All primers were synthesized by Danyel Biotech.

Immunoblot Analysis—Total hippocampal protein was extracted using TriFast reagent. Aliquots of the clarified lysates containing 30 mg of protein were denatured in Laemmli sample buffer (6% SDS, 30% glycerol, 0.02% bromphenol blue, 200 mM Tris-HCl (pH 6.8), and 250 mM mercaptoethanol), at 95 °C for 5 min. The samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Nonspecific binding in a Western blot analysis was prevented by immersing the membranes in blocking buffer (5% nonfat dry milk in Tris-buffered saline-Tween 20 (TBS-T)) for 2 h at room temperature. The membranes were then exposed to the indicated antibodies diluted 1:1000 for 1 h at room temperature. AMPK, phospho-AMPK, and poly-(ADP-ribose) polymerase were obtained from Cell Signaling, BAX, BCL-2, and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The blots were rinsed in TBS-T and then incubated with horse-radish peroxidase-conjugated goat anti-mouse antibodies 1:10,000 for 1 h at room temperature. Antibody-antigen complexes were visualized by detecting enhanced chemiluminescence with x-ray film.

Fluorescence-activated Cell Sorting Analysis—Cells were grown in 60-mm plates and treated with AICAR for 24 h. Apoptosis was evaluated using the annexin V fluorescein isothiocyanate detection kit (Oncogene Research Products, Cambridge MA). Briefly, both attached and floating cells were collected, washed with cold phosphate-buffered saline, resuspended at a density of 5 × 10^6/ml in 0.5 ml of Dulbecco’s modified Eagle’s medium, stained for annexin V, and analyzed by flow cytometry.

Immunohistochemistry—Mice were perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline. Their brains were removed and fixed further in the same solution. 5-mm sections, cut on the same day, were dewaxed and hydrated through graded ethanol, cooked in 25 mM citrate buffer, pH 6.0, in a pressure cooker at 115 °C for 3 min (decolorizing chamber, Biocare Medical), transferred into boiling deionized water, and left to cool down for 20 min. After 5 min of treatment in 3% H2O2, slides were incubated with rabbit polyclonal activated caspase-3 antibody diluted 1:100 in CAS-Block (Zymed Laboratories Inc.) for 3 h at room temperature, washed three times with Optimax (Biogenex), incubated for 30 min with anti-rabbit EnVision (DAKO), and developed with diamino benzidine for 15 min.

Catecholamine Measurements—Catecholamines were measured as described previously (2). The assays for dopamine and norepinephrine were performed by HPLC separation and detection using HPLC-ElectroChemicalDetector. Values are presented as a concentration (ng/mg tissue).

Eight-arm Maze—The animals were placed in an eight-arm maze that is a scaled down version of that developed for rats (30, 31). We used water deprivation, achieved by limiting water consumption overnight and a reward of 50 μl of water presented at the end of each arm. The mice were tested (number of entries) during 5 days until they made entries into all 8 arms or until they completed 24 entries, whichever came first. Hence, the lower the score, the better the cognitive function. Maze performance was calculated per each day for 5 consecutive days. Results were presented as area under the curve utilizing the formula: (number of entries in day 2 + day 3 + day 4 + day 5) – 4 * (day 1) for each mouse, and the scores were averaged for each group. A figure describing the averaged group scores was drawn.

Statistical Analysis—Data are presented as means and S.D. or S.E. Results were evaluated by one-way analysis of variance and two-tailed t test. Post hoc testing was carried out using the Tukey-Kramer multiple comparisons procedure.

RESULTS

Nutritional Depletion Induces Dose-dependent Hippocampal AMPK Activation—To study how nutrition may modulate cognitive function, we measured hippocampal AMPK activity in response to DR. Mice were assigned at random (10 mice/group) to different levels of DR for 5 days and then sacrificed. DR led to elevation of AMPK phosphorylation in a nutrient depletion-dependent manner, reaching a potent AMPK activation (p < 0.01) upon DR to 40% (Fig. 1A). Treatment of the mice with 1–2 mM AICAR (5-amino-imidazole-4-carboxamid ribonucleoside), a specific pharmacological activator of this kinase, could replicate the potent hippocampal AMPK phosphorylation (p < 0.001 for all dosages) achieved by severe DR (Fig. 1B), whereas a wide range of lower AICAR concentrations exhibit more modest AMPK activation (data not shown).

Low Level AMPK Activation Enhances Neurogenesis and Cognition—We questioned the function of AMPK activation in the hippocampus. Because our results showed that nutrient depletion can activate AMPK over a wide range, we used AICAR, which was previously demonstrated to activate hippocampal AMPK in a dose-dependent manner, to mimic these nutritional effects. Mice were treated with bromodeoxyuridine and either saline or 25 mM AICAR (low level stimulation) once daily for 10 days. The number of bromodeoxyuridine-immunoreactive cells in the dentate gyrus measures the generation rate of new cells and was significantly increased from 1000 ± 105 to 1754 ± 139 (p = 0.01) following the treatment (Fig. 2A). Next, we performed behavioral testing using the eight-arm maze to determine whether there was a concomitant improvement in cognition. Mice treated with 25 mM AICAR performed significantly better (p < 0.05) than the controls (Fig. 2B).
High Activation Modulates Catecholamines, Impaired Cognition, and Neuroapoptosis—To study the effect of further augmentation of hippocampal AMPK activation, mice were injected daily with higher doses of AICAR (1–2 mM) for 10 days. Such treatment decreased norepinephrine concentrations progressively ($p < 0.01, 0.001$ for the two dosages, respectively) while elevating those of dopamine ($p < 0.05$) (Fig. 3A). We also determined whether these findings were associated with alterations in hippocampal-dependent spatial memory. Mice given high dose AICAR exhibited a significant deterioration in maze performance compared with the AL group ($p < 0.05$) and in a similar manner to mice under DR to 40% (Fig. 3B). To elucidate the cellular mechanisms involved in these AMPK effects, we measured, by RT-PCR and immunoblotting, the proteins associated with apoptosis. AICAR treatment induced up-regulation of BAX while reducing BCL-2, thereby promoting apoptosis (Fig. 3C).

High AMPK Activation Induces an Intense Apoptotic Cell Death in Vitro—To confirm the cellular effects of AMPK in the hippocampus, we also performed in vitro studies using pc12 cells. This cell line is derived from rat adrenal pheochromocytoma cells and can be differentiated into sympathetic-like neurons, thus providing a suitable model for these studies (32). Treatment of differentiated cells with the same doses of AICAR (from 0.125 to 1.0 mM) induced a dose-dependent reduction in the number of viable cells ($p < 0.01$ for 0.25 mM and $p < 0.001$ for higher dosages), reaching almost total obliteration after 96 h (Fig. 4A). We also evaluated the apoptotic ratio at 48 h by fluorescence-activated cell sorting analysis. Annexin V staining revealed that AICAR treatment induces apoptotic cell death ($p < 0.01$ and $p < 0.05$ for 0.25 and 1 mM, respectively) in a dose-dependent manner (Fig. 4B).

Nutritional Status Differentially Modulates Leptin and Its Receptor Expression—Leptin is a key hormone in the regulation of energy balance and is known to modulate AMPK activity in other systems (27, 28). We next considered how leptin may interact with AMPK in modulating brain function. We analyzed leptin functionality under different degrees of diet restriction. Mice were assigned at random to three groups and fed AL, DR to 60%, or DR to 40% for 10 days. Serum leptin concentrations (enzyme-linked immunosorbent assay) decreased promptly after implementation of both DR regimens and were nearly undetectable after 4 days (Fig. 5A). Analysis of leptin receptor expression by RT-PCR demonstrated an increasing elevation of the short form with the degree of diet restriction. However, although DR to 60% induced significant up-regulation of the long (functional) form of the OB receptor ($p < 0.01$), this was not observed in response to 40% DR (Fig. 5B). Following these results, we studied the effects of leptin administration on hippocampal AMPK phosphorylation. Treatment of 40% diet-restricted mice for 72 h with 1 mg/kg leptin significantly reduced ($p < 0.05$) the AMPK phosphorylation (Fig. 5C).

Leptin Reversed the Effects of Severe DR on Cognition, Catecholamines, and Neuroapoptosis—Next, we investigated the effects of leptin administration (restoration) on neural function in diet-restricted mice. Mice were assigned at random to four groups. The groups were kept under AL or DR to 40% for 10 days and injected daily with either 1 mg/kg leptin or saline for 5 days during the second week. Treatment with leptin reversed the impaired eight-arm maze performance associated with DR to 40% ($p < 0.05$); the control group was not affected by leptin administration (Fig. 6A). Cognitive performance improvement was also observed in the Morris Water Maze (data not shown). We then

**FIGURE 1.** Diet restriction induces neuronal AMPK activation in vivo. AMPK activity was measured by Western blot analysis of AMPK expression and phosphorylation. A, mice under diet restricted to 80, 60, and 40%. B, treatment with 1–2 mM AICAR for 5 days.

**FIGURE 2.** 25 nm AICAR treatment enhances neurogenesis and cognition. A, bromodeoxyuridine+ cells in the granule cell layer and in the subgranular zone of the dentate gyrus were photographed using a fluorescent microscope. B, mice were treated with or without 25 nm AICAR for 10 days and tested in an eight-arm maze. Solid line represents the control group and the broken line the treated group.
evaluated the effects on the concentrations of norepinephrine and dopamine in the hippocampus. Treatment with leptin reversed the reduction of norepinephrine following DR (p < 0.05). Leptin downregulated dopamine concentrations in the AL group (p < 0.05) yet did not induce any significant reduction in the DR group in which dopamine concentrations were already reduced (Fig. 6B). To elucidate the cellular mechanisms involved in leptin effects, we again determined by RT-PCR and immunoblotting the ratio between pro- and anti-apoptotic genes. Although leptin treatment did not significantly change hippocampal Bcl-2 and Bax levels of the control group, it up-regulated Bcl-2 and reduced Bax expression of DR mice. Although this effect was repeated both in RNA and protein levels, BCL-2 accumulation was detected only in the protein level, suggesting the involvement of another regulation mechanism than transcription rate. Analysis of poly(ADP-ribose) polymerase cleavage demonstrated a reduction in the cleaved subunit following leptin treatment, suggesting an increase in the direction of survival (Fig. 6C). We further confirmed the anti-apoptotic effect of leptin by detection of activated caspase-3 expression as pathognomonic of apoptotic cell death. Activated caspase-3 could not be detected in the hippocampi of control animals. Leptin significantly reduced the immunoreactivity of the activated caspase-3 observed following 40% DR (Fig. 6D).

**Leptin Increases Survival under Severe Energy Restriction**—Severe energy restriction leads to deterioration of neural function and eventually death. Because leptin improves cognitive function following DR to 40% and attenuates neural apoptosis, we investigated whether it may also promote mouse survival under these same severe conditions. First, mice were fed AL or DR to 40% for 12 days, and leptin was administrated by daily injection during the second week (5 days). 95% of the mice survived with leptin treatment compared with 65% in the control group (p < 0.05). Next, we repeated our results using mini-osmotic pumps to deliver 0.05 mg/kg/h leptin or saline continuously for 16 days (12 animals in each group). Neither of the saline- or leptin-treated AL groups had any mortality, whereas in the DR groups despite a similar weight loss, leptin-treated mice survived significantly longer (Kaplan Meir log rank, p < 0.05, one-tail). The 50% survival in the treated group increased from 9 to 14 days. (Fig. 7).

**DISCUSSION**

During the course of evolution, survival depended on the ability to conserve body system functionality when food was scarce. To these biological requirements also might be added the ability to think and react appropriately to stress situations. The crucial importance of maintaining cognitive function under such conditions requires development of suitable adaptive mechanisms. We examined how these biological mechanisms were coordinated to promote survival of the organism. We demonstrate here the integration of cellular energy status, nutritional balance, and cognitive function through the concerted cellular and systemic actions of AMPK and leptin, actions directed toward promoting survival.
Nutrient depletion stimulates cellular AMPK activity, which is probably because of the elevation in the AMP/ATP ratio. This activity has a tremendous impact on cell fate. We hypothesized that AMPK may modulate cellular changes in the brain. Investigation of these mechanisms in the hippocampus showed that AMPK is capable of promoting two contradictory cell outcomes, triggering both neurogenesis and neuroapoptosis. The function of AMPK as a neurogenesis promoter is in line with recent work demonstrating its role in neural development (33). It provides a mechanism for neuronal expansion and improved cognitive function during progressive mild energy restriction. Furthermore, these findings may explain the beneficial effects of DR found in neurodegenerative disorders (6, 7) and in the suppression of age-related deficits in learning and memory (6). The mechanism suggested here is that the anti-apoptotic actions of AMPK are limited to conditions of mild energy restriction, where it accelerates catabolism and increases cellular ATP. However, if energy intake is drastically depleted, as in severe diet restriction, cell resources are not sufficient to reverse the AMP/ATP ratio and AMPK activation now leads to apoptosis (34). This model of AMPK function explains the opposite effects of moderate (60%) and severe (40%) DR on cognitive function via regulation of hippocampal cell number.

Leptin concentrations were not different and declined rapidly following 60 or 40% DR. Nevertheless, the long form of leptin receptor was up-regulated in the hippocampus only when energy was restricted to 60%. This result is in line with other work demonstrating the elevation in hypothalamic leptin receptors under energy restriction and reduced leptin activity (35). The leptin receptor belongs to the superfamily of cytokine receptors and activates the JAK-STAT pathway of signal transduction. Obese db/db mice are unable to perform JAK-STAT signal transduction because of the absence of the functional long form receptor (36). When this deficit is reversed by caloric restriction, the receptor capacity is restored (37). Therefore, under energy restriction up to 60%, leptin signaling is preserved by compensatory mechanisms of receptor up-regulation. More severe DR abolishes this response completely. The fact that leptin treatment could inhibit the phosphorylation of AMPK and restore the cellular, neurochemical, and behavioral deficits following 40% DR demonstrates the importance of its signaling in reversing the effects of severe energy restriction. This suggests a role for leptin as a brake to AMPK overactivity in the hippocampus in order to maintain neural function under such conditions.

AMPK regulation of catecholamines implies a novel form of neural modulation. Diet restriction reduces norepinephrine concentrations via AMPK and, as a consequence, contributes to impaired learning and memory. However, restoration of leptin function reduces AMPK activation, increases norepinephrine, and improves cognitive function (38). AMPK-induced dopamine elevation suggests the involvement of dopamine in the apoptotic effects of AICAR, inasmuch as elevation of dopamine levels has been found to cause apoptosis and decreased sur-
Indeed, leptin administration reduces dopamine concentrations, inhibits apoptosis, and induces neural survival. The insight emerging from our work is that AMPK reacts to minimize neural insult. When energy depletion starts to threaten hippocampal function, it enhances neurogenesis. However, when AMPK is unable to reduce the AMP/ATP ratio, as in severe diet restriction, it redirects neuronal fate toward apoptosis. AMPK is thus a regulatory factor in the balance between neuronal growth and death in response to nutritional status and stress. The significance of these results is that a certain level of AMPK activation is beneficial, whereas a higher one is destructive; AMPK is thus a “double-edged sword.” Thus, there may be therapeutic benefits to low dose stimulation of AMPK in the treatment of cognitive disorders.

In addition, the function of leptin as a brake is crucial for the preservation of neuronal activity during fluctuations in energy status throughout life. Indeed, our results detail how leptin treatment prolongs the survival of mice under severe diet restriction by stimulating anti-apoptotic mechanisms. While there is much information about leptin and the effects of “feasting” and positive energy balance, there is far less concerning its function during “fasting.” It appears that following mild caloric restriction, leptin may improve brain function, an obvious beneficial adaptation to the environment. However, under severe diet restriction these effects fail.

The data suggest that, in addition to its many other actions, leptin promotes cognitive function and survival under adverse circumstances. If these results are found to be applicable to Man, then leptin may be used therapeutically in the management of disease states characterized by acute or chronic negative energy balance. These include sepsis and prolonged post-surgical stress and the weight loss associated with severe malnutrition found in developing countries, malabsorption con-
Nutrition Affects Cognition and Survival via Leptin and AMPK

FIGURE 7. Leptin increases survival under severe energy restriction. Evaluation of body weight and survival of 40% DR mice during treatment with either saline or 0.05 mg/kg/h leptin, continuously injected from a subcutaneous 200-μl mini-osmotic pump. Number and weight of living mice were documented every day for 16 days. Solid line represents the control group and the broken line the treated group.

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