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Caloric restriction can improve learning and memory in C57/BL mice probably *via* regulation of the AMPK signaling pathway

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ABSTRACT

Caloric restriction (CR) is effective in slowing aging and delaying aging-related diseases in many species, but the mechanism is complex and not fully elucidated. This study aimed to evaluate the beneficial effects of a caloric restriction diet on learning and memory, and further to elucidate the mechanisms. Thirty-six week-old male C57/BL mice were randomly divided into three groups: normal control (NC group), high-energy (HE group) and CR group. After 44 weeks, the Morris water maze was used to examine learning and memory abilities. Western blotting and immunohistochemistry were used to detect changes in proteins involved in the adenosine monophosphate-activated protein kinase (AMPK) pathway in the mouse hippocampus. Compared with NC group, the swimming distance and escape latency were shorter in the CR group. The protein and mRNA expression of AMPK and glucose transporter type 4 (GLUT4) in the CR group were significantly higher than that in HE group. CR increased serum insulin-like growth factor, adiponectin and vaspin, decreased blood glucose and serum malondialdehyde, and improved insulin sensitivity. Our findings demonstrate that a CR diet may improve hippocampus-dependent spatial learning ability of C57/BL mice, accompanied with an increase in AMPK and GLUT4 expression, which indicates AMPK pathway was associated with the neuroprotective effect of CR in mice.

1. Introduction

Caloric restriction (CR) refers to a plan to reduce energy from the food supply, usually on the basis of ensuring adequate vitamin and mineral levels, minus 30–50% of the energy from normal free feeding (*ad libitum*, AL) (McCay et al., 1935). In 1935, McCay first reported that CR could prolong the lifespan of rats, which was confirmed by many studies on yeast, nematodes, fish, spiders, and mammals (McCay et al., 1935; Mei and Brenner, 2015). A large number of experiments have shown that CR is the most powerful anti-aging method; in addition to genetic operation, it can extend the individual lifespan by as much as 50% (Wiesenborn et al., 2014). CR can postpone the pathological process of aging-related diseases such as cardiovascular disease, cancer, diabetes, ischemic injury and nerve injury, and it has a neuroprotective effect on nervous system degenerative diseases (Gonzalez et al., 2011; Mattson and Wan, 2005; De Lorenzo et al., 2011; Morris et al., 2011; Chouliaras et al., 2012; Kuhla et al., 2013). Recent studies have found that CR can induce hippocampal neurogenesis in adult mice, increase synaptic plasticity in aged mice, thereby regulating the expression of mouse neuromuscular synapses, and weaken the aging-related changes in synuclein in endothelial cells in the brain of aged rats (Mladenovic Djordjevic et al., 2010; Valdez et al., 2010).

Recent studies have found that CR can prolong the aging process, which is related to signaling pathways such as adenosine monophosphate-activated protein kinase (AMPK), silent information regulator 2 related enzyme 1 (SIRT1), insulin/insulin-like growth factor (insulin/IGF-1), peroxisome proliferator activated receptor gamma coactivator 1, and target of rapamycin signaling regulatory pathway; among these, AMPK is the most important signaling pathway (Greer and Brunet, 2009; Fontana et al., 2010). Various signaling networks responsible for anti-aging and anti-senescence have been discussed. It has been suggested that a counter-balancing model involving the crosstalk between SIRT1 and AMPK controls the senescence program in mammalian cells (Wang et al., 2011). The energy balance is usually regulated by SIRT1 and AMPK, and SIRT1 is required for the activity of AMPK (Fontana et al., 2010). CR is the most powerful means of intervention for slowing down aging and delaying aging-related diseases in a wide variety of species, but the mechanism by which CR improves health and prolongs the lifespan of mammals is complex and not yet fully elucidated, although some of the CR effects may be mediated by SIRT1 and AMPK

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(Morris et al., 2011). We previously confirmed a beneficial effect of a CR diet on learning and memory, and found that neuronal phosphoinositide 3-kinase (PI3K)/Akt pathway and SIRT1/mammalian target of rapamycin (mTOR) pathway might be involved (Ma et al., 2014; Ma et al., 2015a; Xu et al., 2015; Dong et al., 2015). Energy metabolism and metabolic regulators play pivotal roles in controlling longevity and cellular senescence, and SIRT1 and AMPK are the two key energy sensor systems (Narala et al., 2008; Chen et al., 2010). However, as the most important pathway for energy balance, does neuronal AMPK pathway have a possible role in this process? There is no evidence from the literature. Thus, we hypothesize that AMPK pathway was associated with the neuroprotective effect of CR in mice in this study.

2. Experimental procedures

2.1. Experimental animals

Thirty-six week-old male C57/BL mice from the Academy of Military Medical Sciences (Beijing, China) were fed *ad libitum* for 1 week before the experiment began. The study has been carried out along the "Principles of laboratory animal care". All animal study protocols were approved by the Institutional Animal Care and Ethics Committee of Xuan Wu Hospital, Capital Medical University in Beijing, China.

Animals were weight-matched and randomly divided into three different groups: normal control group (NC group, n = 12), high-energy group (HE group, n = 12), and CR group (n = 12). Food was purchased from the Experimental Animal Center of the Military Medical Science Academy of the People's Liberation Army. The energy of NC diet, HE diet, and CR diet was 3.484 kcal/g, 4.589 kcal/g and 2.479 kcal/g, respectively, and the NC: HE: CR caloric ratio was 1:1.3:0.7. The NC diet was composed of 19.1% protein, 4% fat and 59% carbohydrate. The CR diet was composed of 19.1% protein, 2.5% fat and 37.2% carbohydrate. The HE diet was composed of 19.1% protein, 21.5% fat and 47.2% carbohydrate. Mice were single housed for the duration of the experiment. A controlled volume of food made available to mice every day. Food consumption data were collected manually daily to ensure each mouse has the consistent food intake. Body weight and blood glucose measurements were taken every week. The total experimental duration was 44 weeks.

2.2. Behavioral experiments

The Morris water maze (MWM) protocol consisted of 5 days of learning-memory training and a probe trial that was conducted on day 6, as described previously (Ma et al., 2014). The swimming distance and escape latency of each mouse spent were recorded by a computerized video system.

2.3. Measurement of serum metabolites

The levels of serum insulin, serum IGF-1, serum adiponectin, serum vaspin and serum malondialdehyde (MDA) were measured using the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions (RapidBio, US). The optical density (OD) value of each sample was measured at 450 nm, and serum insulin, serum IGF-1, serum adiponectin, serum vaspin and serum MDA concentrations were calculated based on a standard curve. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated as (fasted glucose (mM) × fasted insulin (pU/mL) / 22.5).

2.4. Immunofluorescence

Mice were sacrificed after MWM test. Brains were extracted and hippocampal tissues were isolated. The sections were subjected to heat-mediated antigen retrieval treatment after regular deparaffinization and rehydration. Then the sections were blocked with normal goat serum in PBS for 1 h at room temperature, and then incubated with the primary antibody, rabbit anti-AMPK (1:500, Abcam) or rabbit anti-glucose transporter type 4 (GLUT4) (1:500, Abcam), at 4°C overnight. Next day, sections were incubated with goat anti-rabbit Alexa-Fluor 594 (1:200, Beijing Zhongshan Biotechnology Co.) at room temperature for 2 h. Finally, sections were coverslipped using the appropriate anti-fading mounting medium for fluorescence and viewed by fluorescence microscopy. DAPI staining was used as a kind of nuclear marker to avoid false on cellular counting. Three slides were chosen at random from each animal. The number of cells positive in the CA1 region of the hippocampus for the indicated antibodies was counted in three visual fields per slide using Image-Pro Plus 6.0 software. CA1 region was shown in the blank frame in Fig. S1.

2.5. Western blot analysis

The hippocampi of 6 mice from each group were collected in a 1.5-ml centrifuge tube, washed twice with cold Tris-buffered saline-Tween, and homogenized in lysis buffer with an electric homogenizer on ice for 2 min. The crude hippocampal homogenates were incubated on ice for 30 min and then centrifuged for 30 min at 15,000 rpm at 4 °C. The supernatant was collected and the protein concentration was measured by the bicinchoninic acid assay. Protein samples boiled in Laemmeli buffer for 5 min were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking, the membrane was incubated with the indicated antibodies overnight at 4 °C: rabbit anti-AMPKα (1:5000, Cell Signaling, Beverley, MA, USA) and rabbit anti-p-AMPKα (1:2000, Cell Signaling, Beverley, MA, USA). Next, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:20,000, Beijing TDY Biotech Co. Ltd) for 2 h at room temperature. GAPDH served as a loading control.

2.6. Real-time PCR

Total RNA was extracted using the RNA Extraction Kit (CWBIO. Co. Ltd). Total RNA was then reverse-transcribed using an ExScript RT reagent kit (CWBIO. Co. Ltd). Real-time PCR was then performed using an ABI 7500 (Applied Biosystems) with UltraSYBR Mixture (CWBIO. Co. Ltd). The forward and reverse primers for AMPK were 5′ AGCCAAAT CAGGGACTGCTACT 3′ and 5′ AGGGAGGTGACAGATGAGGTAAG 3′, respectively. The forward and reverse primers for GLUT4 were 5′ CCC CATTCCCTGGTTCATT 3′ and 5′ GACCCATAGCATCCGCAAC 3′, respectively. The efficiency of the PCR was determined using a series of dilutions of a standard vascular sample. The specificity of the product was assessed by melting curve analysis. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analysis

Data were analyzed using the SPSS 11.5 software (Chicago, IL, USA) and plotted as mean ± SD. Comparisons among the groups of animals were made with one-way analysis of variance (ANOVA), except the comparison among the three groups for average swimming distance of MWM, which was analyzed by a two-way repeated measures ANOVA followed by a *post hoc* Tukey's test was applied. Results were considered to be significantly different at $P < 0.05$.

3. Results

3.1. CR diet improves spatial learning and memory

Mouse behavior was analyzed by measuring the average swimming distance, escape latency and escape trajectory of the spatial probe test after 44 weeks (Fig. 1). There was a significant difference among the