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Research report

Long-term caloric restriction in mice may prevent age-related learning impairment via suppression of apoptosis

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HIGHLIGHTS

- CR can prevent age-related learning impairments.
- CR increased the expressions of Bcl-2 protein and mRNA and decreased the expression of Bax, Caspase-3 and PARP protein.
- Long-term CR may prevent age-related learning impairments via suppressing apoptosis.

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ABSTRACT

Caloric restriction (CR) is the most reliable intervention to extend lifespan and prevent age-related disorders in various species from yeast to rodents. However, the underlying mechanisms have not yet been clearly defined. Therefore, we aimed to identify the underlying mechanisms of long-term CR on age-related learning impairment in C57/BL mice. Thirty six-week-old male C57/BL mice were randomly divided into three groups: normal control group (NC group, n = 10), high energy group (HE group, n = 10), and CR group (n = 10). After 10 months, the Morris water maze test was performed to monitor learning abilities. Western blotting, immunohistochemistry and real-time polymerase chain reaction were used to monitor changes in protein and mRNA levels associated with apoptosis-related proteins in the hippocampus. The average escape latency was lower in the CR group compared with the NC group, and the average time taken to first cross the platform in the CR group was significantly shorter than the HE group. Both Bcl-2 protein and mRNA expression levels in the CR group were significantly higher than those of the NC group and HE group. The expression of Bax, Caspase-3 and PARP protein in the CR group was significantly lower than the NC group. Our findings demonstrate that long-term CR may prevent age-related learning impairments via suppressing apoptosis in mice.

1. Introduction

Caloric restriction (CR) is defined as limiting calorie intake compared to baseline unrestricted or ad libitum (AL) consumption, with maintained levels of vitamins, minerals, or other essential biomolecules [1]. McCay et al., showed that CR in rodents could markedly extend lifespan [2,3], and many researchers have shown that CR results in a multitude of health benefits in humans [4], including increased insulin sensitivity, and reduced levels of proinflammatory cytokines, reactive oxygen species, and atherosclerotic lipids in the blood [5,6].To date, CR is the only non-genetic intervention that reliably increases life span and health across multiple organisms [7], however, the molecular mechanisms are not well understood.

Alzheimer's disease (AD) is an age-related neurodegenerative disorder with a complex etiology [8], and is characterized by impaired learning, memory, and executive function [9]. Clinical manifestations of AD are closely associated with the formation of senile plaques and neurofibrillary tangles, neuronal loss and cognitive decline [10]. Aging is a complex physiological process caused by accumulation of damage at the molecular, cellular, and organ level [11], and apoptosis plays an essential role. Various factors such as Bcl-2, Bax, caspases, and amyloid beta (Aβ), lead to

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deleterious neurodegenerative disorders like AD [8]. In addition, endothelial apoptosis and de-repression of FOXO3a death axis are in involved in AD pathogenesis [12,13]. Furthermore, another study has shown that the hyperphosphorylated truncated protein tau induces a caspase-3 independent apoptosis-like pathway in an AD cell model [14].

CR is able to delay age-related neurodegenerative diseases and cognitive impairment [15], with adherence to a daily 30% CR regimen in healthy elderly subjects shown to improve performance on memory tests [16]. Our previous study has found that CR can improve learning ability via regulating the PI3K/AKT pathway [17]. Recent research has focused on developing drugs that mimic the health-promoting effects of CR without reducing food intake [18,19]. However, definitive evidence is lacking to support the hypothesis that the neuroprotective effect of long-term CR is mediated via apoptosis. Therefore, in the present study we tested the effect of CR on age-related learning impairment and explored its effect on apoptosis. Our results demonstrated that long-term CR in mice may prevent age-related learning impairments via suppressing apoptosis.

2. Materials and methods

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 one week before experimentation. 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 = 10), high energy group (HE group, n = 10), and CR group (n = 10). Feeding of animals was the same as previously described [17]. The energy ratio of the feed of NC group, HE group and CR group is 1:1.3:0.7. Calorie restriction was a progressive process that was initiated with 10% restriction during the first week followed by 20% and 30% during the second and third weeks, respectively, and maintained at 30% restriction [17]. The total experimental duration was 10 months.

2.2. Behavioral experiments

The Morris water maze was utilized to evaluate learning and memory function. It consisted of a period of 5 days of learning-memory training and a probe trial that was conducted on day 6. The animals were allowed to swim for a maximum of 60 s to find the hidden platform in the probe trial. The average escape latency of the 5 days and the average time of first cross the platform in spatial exploration tests (probe trial) were recorded [17].

2.3. Animal processing

Mice were intraperitoneally anesthetized with 10% (v/v) chloral hydrate at 4 ml/kg, and perfused transcardially with 0.9% (w/v) NaCl followed by 4% (w/v) paraformaldehyde. Brains were quickly removed, processed for paraffin embedding, and coronal sections (4 μm thick) were obtained.

2.4. Immunofluorescence

Immunohistochemistry was performed as previously described [20]. The primary antibodies used were rabbit anti-Bcl-2 (1:1000, Abcam), rabbit anti-Bax (1:200, Beijing Zhongshan Biotechnology Co.), rabbit anti-Caspase-3 (1:200, Beijing Zhongshan Biotechnology Co.) and rabbit anti-PARP (1:1000, Abcam). The number of cells positive for the indicated antibodies were counted using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

2.5. Western blot analysis

Mice hippocampus tissues was prepared and western blots performed as described previously [21]. The following primary antibodies at 4 °C overnight: rabbit anti- Bcl-2 (1:2000, Abcam), rabbit anti-Bax (1:1000, Beijing Zhongshan Biotechnology Co.), rabbit anti-Caspase-3 (1:1000, Beijing Zhongshan Biotechnology Co.) and rabbit anti- PARP (1:2000, Abcam). After rinsing with TBS-T, the membranes were incubated with a goat anti-rabbit horseradish peroxidase- (HRP-) conjugated immunoglobulin (Ig)G (H + L) secondary antibody (1:20000, Beijing TDY Biotech Co. Ltd.) for 40 min at room temperature.

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 Bcl-2 were 5′CCAGCGTGTGTGTGCAAGTGTAAAT3′ and 5′ATGTCAATCCGTAGGAATCCCAACC3′, 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 $2-D\Delta Ct$ method.

2.7. Statistical analysis

Data that were normally distributed were expressed as the mean ± SEM, or otherwise expressed as the median (Range). Data were analyzed using a SPSS 11.5 software (Chicago, IL, USA) and were plotted as the mean ± SD. Groups were compared by one way analysis of variance (ANOVA) or repeated measures ANOVAs with Tukey's post-hoc test.

3. Results

3.1. The effect of long-term CR diet on spatial learning and memory in C57/BL mice

Mouse behavior was analyzed by measuring the average escape latency in place navigation tests (Fig. 1A) and the average time taken to first cross the platform in spatial exploration tests (Fig. 1B) of the different groups after 10 months. The escape latency decreased over the course of the 5 day test period, and there was a significant difference among the three groups ($P < 0.05$). Compared with the HE group and NC group, the average escape latency of the CR group on the fifth day was significantly lower ($P < 0.01$). Compared with the HE group, the average time taken to first cross the platform in the CR group was significantly shorter ($P < 0.01$). However, with respect to the average escape latency and average time taken to first cross the platform, there was no statistical difference between the HE group and NC group.

3.2. Effect of CR on the expression of apoptosis-related proteins in hippocampal neurons of C57/BL mice

Immunohistochemistry revealed that the number of Bcl-2-positive cells in the CA1 area of CR mice hippocampi was higher than the HE group, but there were no obvious differences between the CR group and NC group (Fig. 2A, B1). The number of Caspase-3-positive cells in the CR group was lower than the HE group (Fig. 2A,