Calorie Restriction Improves Cognitive Decline via Up-Regulation of Brain-Derived Neurotrophic Factor
Tropomyosin-Related Kinase B in Hippocampus of Obesity-Induced Hypertensive Rats

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SUMMARY

In metabolic syndrome (MetS), previous studies have suggested that cognitive decline is worsened. Among the factors associated with cognition, decreased brain-derived neurotrophic factor (BDNF) in the hippocampus causes cognitive decline. We previously reported that exercise training with calorie restriction yielded protection against cognitive decline via BDNF in the hippocampus of hypertensive rats. The aim of the present study was to determine whether or not calorie restriction results in protection against cognitive decline via BDNF and its receptor tropomyosin-related kinase B (TrkB) in the hippocampus of MetS model rats. We divided dietary-induced obesity-prone and hypertensive rats (OP), as metabolic syndrome model rats, into three groups, fed with a high fat diet (HF), treated with calorie restriction (CR) plus vehicle, and treated with CR and ANA-12 (a TrkB antagonist) (CR+A). After treatment for 28 days, body weight, insulin, fasting blood glucose, adiponectin, systolic blood pressure, and oxidative stress in the hippocampus were significantly lower, and BDNF expression in the hippocampus was significantly higher in CR and CR+A than in HF. Cognitive performance determined by the Morris water maze test was significantly higher in CR than in HF; whereas the benefit was attenuated in CR+A. In conclusion, calorie restriction protects against cognitive decline via up-regulation of BDNF/TrkB through an antioxidant effect in the hippocampus of dietary-induced obesity rats. (Int Heart J 2015; 56: 000-000)

Key words: Metabolic syndrome, Cognition

O ne of the important types of organ damage in metabolic syndrome (MetS) is cognitive decline.1 MetS-associated factors are linked to volume losses in the hippocampus, and MetS negatively impacts cognition by impaired vascular reactivity, neuro-inflammation, oxidative stress, and abnormal brain lipid metabolism.2 Among the factors associated with cognition, brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) are known to be involved in the protective mechanisms against stress and cell death as an antioxidant.3,4 Systemic oxidative stress and/or antioxidant deficiency cause cognitive decline,5 and oxidative stress in the hippocampus in particular impairs cognitive function.6 However, it has not been clarified whether BDNF/TrkB in the hippocampus of metabolic syndrome patients is impaired or not.

Not only pharmacological therapy but also exercise training7-9 and calorie restriction10-12 have been suggested to protect against cognitive decline. The benefits of calorie restriction on metabolic syndrome have been already established via improvement of insulin-resistance.13-16 However, in a previous clinical study, calorie restriction and/or exercise training did not protect against cognitive decline.16 We previously demonstrated that exercise training plus calorie restriction causes synergistic protection against cognitive decline via up-regulation of BDNF in the hippocampus of stroke-prone spontaneously hypertensive rats as hypertensive and vascular dementia model rats.17 We also reported that an angiotensin II type 1 receptor blocker also leads to protection against cognition via BDNF/TrkB in the hippocampus of hypertensive rats.18 In addition, a calcium channel blocker with a statin has the potential to improve cognition via an antioxidant effect in the hippocampus of hypertensive rats.19 It is known that BDNF is negatively correlated to oxidative stress.20 We should focus on the benefit of calorie restriction on cognition in metabolic syndrome via BDNF/TrkB and oxidative stress in the hippocampus.

Considering this background, the aim of the present study was to determine whether calorie restriction has a benefit on cognition via BDNF/TrkB and oxidative stress in the hippocampus of metabolic model rats. To examine our hypothesis, we divided dietary-induced obesity-prone and hypertensive rats...
(OP), as metabolic syndrome model rats, into 3 groups: high fat diet-treated (HF), treated with calorie restriction (CR) plus vehicle, and treated with CR and N-[2-[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzothiophene-2-carboxamide (ANA-12, a TrkB antagonist) (CR+A). As a control, dietary-induced obesity-resistance (OR) rats were subjected to CR. Cognitive function was assessed by the Morris water maze test, which has been widely used as a test of spatial memory and cognition.20

**METHODS**

**Animals:** This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) weighing 350 to 425 g were individually housed in a temperature-controlled room (22° to 23°C) with a 12-hour/12-hour light-dark cycle (lights on at 7:00 AM). The rats were placed on a moderate high-fat diet (32% kcal from fat, Research Diets, New Brunswick, NJ) for 13 weeks. After 5 weeks, rats fed the moderately high-fat diet were classified as OP or OR based on the body weight distribution, as described previously. Briefly, a body weight histogram was constructed to show the distribution of the rats; rats falling within the upper third of the weight distribution were classified as OP (n = 15) and those falling within the lower third were classified as OR (n = 5). We divided OP into 3 groups: HF, CR, and CR+A (n = 5 for each) as described above. Systolic blood pressure and heart rate were measured daily using the tail-cuff method (BP-98A; Softtron, Tokyo). CR, CR+A, and OR groups were given 70% of their mean 24 hour food intake as previously done.20 Food was given daily 2-3 hours before lights off.

**Oral administration of drugs:** The CR group was treated with vehicle, and the CR+A group was treated with ANA-12. The CR+A group was administered ANA-12 (0.5 mg/kg/day, Sigma Aldrich, St. Louis, USA). The vehicle (VEH) group was administered 0.5% methylcellulose. All drugs were dissolved in 0.5% methylcellulose and administered daily by gastric gavage. Aldrich, St. Louis, USA). The vehicle (VEH) group was treated with CR and N-[2-[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzothiophene-2-carboxamide (ANA-12, a TrkB antagonist) (CR+A). As a control, dietary-induced obesity-resistance (OR) rats were subjected to CR. Cognitive function was assessed by the Morris water maze test, which has been widely used as a test of spatial memory and cognition.20

**Western blotting analysis:** To obtain the hippocampus tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with PBS (150 mmol/L NaCl, 3 mmol/L KCl, and 5 mmol/L phosphate; pH 7.4, 4°C). Prior to removal of the brain, an incision was made on the dorsal surface of the head and the bone of the head was opened in a stereotaxic frame. Dye was microinjected into the hippocampus while referring to a rat brain atlas. After these procedures, the brains were removed quickly, and the hippocampal tissue was obtained as 1 mm thick slices by a microtome. The lysate was centrifuged at 6000 rpm for 5 minutes at 4°C with a microcentrifuge. The lysate was collected, and the protein concentration was determined with a BCA protein assay kit (Pierce). An aliquot of 20 μg of protein from each sample was separated on 12% SDS-polyacrylamide gel. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane; Millipore). Membranes were incubated for 2 hours with a rabbit polyclonal antiserum against BDNF (1:1000; Abcam, Cambridge, UK) or α-tubulin (1:1000; Cell Signaling). Membranes were then washed and incubated with a horseradish peroxidase-conjugated horse anti-mouse IgG antibody (1:10,000) for 40 minutes. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (plus Western blotting detection kit; Amersham), and was expressed as the ratio to α-tubulin protein.

**Measurement of oxidative stress in the hippocampus:** The hippocampus tissues obtained as above were homogenized in 1.15% KCl (pH 7.4) and 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5°C for 60 minutes, followed by heating to 100°C for 60 minutes. After cooling, the mixture was extracted with distilled water and n-butanolpyridine (15:1) and centrifuged at 1600 g for 10 minutes. The absorbance of the organic phase was measured at 532 nm. The amount of thiobarbituric acid-reactive substances (TBARS) as an indicator of oxidative stress was determined by absorbance, as described previously.14 Analysis of cognitive function: Spatial learning and memory function of the rats were investigated with the Morris water maze test in a circular pool filled with water at a temperature of 25.0 ± 1°C, which has been widely used as a test of spatial memory and cognition. In the hidden platform test, a transparent platform was submerged 1 cm below the water level. Swimming paths were tracked with a camera fixed on the ceiling of the room and stored in a computer. All the procedures of the Morris water maze were performed for 7 days. A pre-training session was carried out on day 0, in which the animals were given 60 seconds of free swimming without the platform. In the hidden-platform test for 4 days, the rats were given 2 trials (1 session) on day 1 and 4 trials (2 sessions) per day on days 2, 3, and 4. The initial trial interval was about 30 minutes and the inter-session interval was 2 hours. During each trial, the rats were released from 4 pseudo-randomly assigned starting points and allowed to swim for 60 seconds. After mounting the platform, the rats were allowed to remain there for 15 seconds, and were then placed in the home cage until the start of the next trial. If a rat was unable to find the platform within 60 seconds, it was guided to the platform and allowed to rest on the platform for 15 seconds. Probe trials were performed at day 5. In the probe trial, the hidden platform was removed and the rat was released from the right quadrant and allowed to swim freely for 60 seconds. The time spent in the target quadrant, where the platform had been located during training, and the time spent in the other quadrants were measured. In the visible-platform test performed at day 6, the platform was elevated above the water surface and placed in a different position.

**Statistical analysis:** All values are expressed as the mean ± SEM. Comparisons between any two mean values were performed using Bonferroni’s correction for multiple comparisons. ANOVA was used to compare all the parameters in all groups. Differences were considered to be statistically significant at a P value of < 0.05.
RESULTS

Total body weight, visceral fat weight, and metabolic profiles: Body weight, visceral fat weight, fasting blood glucose, blood insulin, serum triglycerides, and serum free fatty acid were significantly lower in CR and CR+A than in HF, and significantly higher in HF than in OR after 4 weeks of treatment (Table I). Plasma adiponectin concentrations were significantly lower in HF than in OR, and significantly higher in CR and CR+A than in HF after 4 weeks of treatment (Table I).

Physiological data: Systolic blood pressure and heart rate were significantly higher in HF than in OR, findings that are similar to those of our previous report (Table II). In CR and CR+A, systolic blood pressure and heart rate were not significantly altered compared to HF (Table II).

Expression of BDNF in the hippocampus: The expression of BDNF in the hippocampus was significantly lower in HF than in OR, and was significantly higher in CR and CR+A than in HF (Figure 1). The up-regulation of BDNF in the hippocampus was similar between CR and CR+A (Figure 1).

TBARS levels in the hippocampus: TBARS levels in the hippocampus were significantly higher in HF than in OR, and were significantly lower in CR and CR+A than in HF (Figure 2). CR and CR+A did not differ in TBARS levels in the hippocampus (Figure 2).

Morris water maze test: In the hidden platform test, escape latency was significantly lower in CR than in HF, and the benefit was attenuated in CR+A (Figure 3A). In the probe test, CR resulted in significantly more time in the target quadrant as compared with HF, and the benefit was attenuated in CR+A (Figure 3B). In the visible platform test, there were no significant differences in escape latency among all of the groups.

Table I. Metabolic Profiles

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<thead>
<tr>
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<th>OR</th>
<th>OP</th>
<th>HF</th>
<th>CR</th>
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<td>Body weight, g</td>
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<td>807 ± 39</td>
<td>668 ± 25</td>
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<td>Visceral fat, g</td>
<td>26 ± 9</td>
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<td>Fasting BG, mg/dL</td>
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<td>87 ± 3</td>
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<td>Fasting BI, ng/mL</td>
<td>0.4 ± 0.1</td>
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<td>Adiponectin, vγ/mL</td>
<td>2.7 ± 0.2</td>
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<td>Serum TG, mg/dL</td>
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<td>90 ± 5</td>
<td>71 ± 8</td>
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<td>Serum FFA, umol/L</td>
<td>351 ± 68</td>
<td>762 ± 79</td>
<td>533 ± 83</td>
<td>572 ± 59</td>
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OR indicates obesity-resistance rats; OP, obesity-prone rats; HF, high fat diet; CR, calorie restriction; CR+A, calorie restriction + ANA-12; BG, blood glucose; BI, blood insulin; TG, triglycerides; and FFA, free fatty acid. *P < 0.05 versus HF in OR or CR or CR+A, +P < 0.05 versus OR in HF, CR or CR+A.

Table II. Physiological Data

<table>
<thead>
<tr>
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<td>Systolic BP, mmHg</td>
<td>118 ± 13</td>
<td>162 ± 19</td>
<td>154 ± 22</td>
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<tr>
<td>Heart rate, bpm</td>
<td>319 ± 11</td>
<td>341 ± 15</td>
<td>335 ± 9</td>
<td>344 ± 13</td>
<td>344 ± 13</td>
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</table>

OR indicates obesity-resistance rats; OP, obesity-prone rats; HF, high fat diet; CR, calorie restriction; CR+A, calorie restriction + ANA-12; BP, blood pressure; and bpm, beats per minute. *P < 0.05 versus OR in HF, CR or CR+A.

DISCUSSION

In the present study, we have demonstrated two major findings. First, calorie restriction has a protective effect on the cognitive decline via up-regulation of BDNF and reduction of oxidative stress in the hippocampus of dietary-induced obesity and hypertensive rats. Second, the benefit of calorie restriction on cognition was attenuated by blockade of BDNF receptors in dietary-induced obese and hypertensive rats. These results suggest that calorie restriction protects against cognitive decline via up-regulation of BDNF/TrkB through an antioxidant effect in the hippocampus of dietary-induced obese rats.

The major finding of the present study was that calorie restriction resulted in protection against cognitive decline in MetS model rats independent of a depressor response. Calorie

Figure 1. Expression of brain-derived neurotrophic factor (BDNF) in the hippocampus in each group. BDNF/α-tubulin expression was expressed relative to that in OR which was assigned a value of 1. *P < 0.05 versus OR, n = 5 for each. BDNF indicates brain-derived neurotrophic factor; OR, obesity-resistance rats; OP, obesity-prone rats; HF, high fat diet; CR, calorie restriction; and CR+A, calorie restriction + ANA-12.

Figure 2. Thiobarbituric acid-reactive substances (TBARS) levels in the hippocampus in each group. *P < 0.05 versus OR, and +P < 0.05 in CR+A and CR versus HF, n = 5 for each. OR indicates obesity-resistance rats; OP, obesity-prone rats; HF, high fat diet; CR, calorie restriction; and CR+A, calorie restriction + ANA-12.
restriction has already been established to have benefits for MetS, especially with respect to the improvement of insulin resistance.\textsuperscript{13,14} Although we could not determine the effects of calorie restriction on blood pressure and sympathetic nerve activity in patients with MetS,\textsuperscript{20} our previous basic research indicated that calorie restriction caused a depressor response with sympathoinhibition in dietary-induced obese and hypertensive rats.\textsuperscript{20} However, the effect of calorie restriction on cognition in MetS is still controversial.\textsuperscript{12,13,19} In hypertensive (not MetS) model rats, we also could not determine the benefit of calorie restriction on cognition.\textsuperscript{20} With respect to the discrepancy between the benefit of calorie restriction on cognition in the hypertensive or MetS model, we should consider that insulin resistance and obesity worsen cognition. Many previous studies have indicated that excessive dietary energy intake and insulin resistance have adverse effects on cognition, and that dietary energy restriction enhances neural plasticity and reduces vulnerability of the brain,\textsuperscript{23} which is consistent with our present results. However, calorie restriction alone would be insufficient to improve cognition and BDNF in the hippocampus of hypertensive rats, as demonstrated in our previous study,\textsuperscript{20} because we used a hypertensive model without insulin resistance. Considering this background, the present results have important clinical implications. Calorie restriction should be undertaken in patients with MetS because it has potential benefits on multiple factors in MetS, such as hypertension, sympathoexcitation, and additive cognitive decline. Interestingly, the present benefits of calorie restriction on cognition in MetS could be obtained independent of a depressor response. We believe that antihypertensive agents with calorie restriction could yield additive protective benefits on cognition in MetS.

We focused on BDNF/TrkB and oxidative stress in the hippocampus of MetS in the present study, and demonstrated that calorie restriction increased BDNF with reduction of oxidative stress in the hippocampus of dietary-induced obese and hypertensive rats. In only hypertensive rats, calorie restriction alone could not cause protection against cognitive decline via up-regulation of BDNF in the hippocampus.\textsuperscript{20} However, oral administration of telmisartan, an angiotensin II type 1 receptor blocker, protected against cognitive decline via BDNF/TrkB in the hippocampus of hypertensive rats.\textsuperscript{21} In that study, the improvement of BDNF/TrkB in the hippocampus was probably due to an antioxidant effect because telmisartan has the potential to reduce oxidative stress in the brain\textsuperscript{15,21,28} and BDNF is negatively correlated to oxidative stress.\textsuperscript{23} We believe that calorie restriction might increase BDNF via reduction of oxidative stress in the hippocampus of dietary-induced obese and hypertensive rats. Moreover, we also determined that blockade of BDNF receptors in the hippocampus attenuated the benefits of calorie restriction on cognition, even though oxidative stress and BDNF expression were not altered. The benefit of calorie restriction on cognition would be mainly due to BDNF/TrkB via reduction of oxidative stress in the hippocampus, and the discrepancy between the benefit of calorie restriction on the cognition of hypertensive or MetS model could be explained by the fact calorie restriction alone did not alter the expression of BDNF in the hippocampus of hypertensive rats.\textsuperscript{20}

We should discuss the mechanisms by which calorie restriction increases BDNF and reduces oxidative stress in the hippocampus of dietary-induced obese and hypertensive rats. In the brain of dietary-induced obese and hypertensive rats, oxidative stress is increased mainly due to activation of the renin-angiotensin system.\textsuperscript{24} Previous studies have suggested that calorie restriction has the potential to inhibit the renin-angiotensin system in several organs.\textsuperscript{29} Although the renin-angiotensin system in the hippocampus was not determined in the present study, we believe that calorie restriction reduced oxidative stress via blockade of the renin-angiotensin system in the hippocampus. With respect to the relationship between BDNF and oxidative stress, it is known that BDNF is negatively correlated to oxidative stress.\textsuperscript{23} Interestingly, we demonstrated that the improvement of cognition by calorie restriction was strongly attenuated by blockade of BDNF in the hippocampus. These results indicate that the benefit of calorie restriction on cognition is associated not only with oxidative stress but also with BDNF in the hippocampus.
There are several limitations in the present study. First, we did not determine the strength and physiological benefits of calorie restriction, such as body maximum O₂ consumption, and did not check the calorie restriction-induced changes in metabolism. Also, we could not clarify the cause-and-effect relationship between calorie restriction and cognitive function. Second, we measured oxidative stress only in the hippocampus, and did not determine whether or not calorie restriction reduced oxidative stress in other sites of the brain. Third, to determine cognitive function, we used the Morris water maze test instead of the shuttle avoidance test so that we could focus on hippocampus function. A spatial working memory task, such as the Morris water maze test, depends on hippocampus function.

Finally, we used ANA-12 given orally to blockade TrkB in the hippocampus. The possibility that ANA-12 may have affected other brain areas and systemic organs cannot be excluded. The use of specific BDNF/TrkB-targeting methods (such as gene transfer methods) locally in the hippocampus would have strengthened our results.

In conclusion, the present results indicate that calorie restriction protects against cognitive decline via up-regulation of BDNF/TrkB through an antioxidant effect in the hippocampus of dietary-induced obese rats, independent of blood pressure. In the treatment of metabolic syndrome, we should consider that calorie restriction with pharmacological therapy might be effective at preventing cognitive decline, and thereby contributing to improving the quality of life of patients with MetS.

**Disclosure**

**Conflict of interest:** The Department of Advanced Therapeutics for Cardiovascular Diseases, Kyushu University Graduate School of Medical Sciences, receives financial support from Astellas Pharma (Tokyo) and Nippon Boehringer Ingelheim (Tokyo). The Department of Advanced Cardiovascular Regulation and Therapeutics, Kyushu University Graduate School of Medical Sciences, receives financial support from Actelion Pharmaceuticals (Tokyo).

**References**


