Nitric Oxide 66 (2017) 71–77

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Exercise training rescues high fat diet-induced neuronal nitric oxide synthase expression in the hippocampus and cerebral cortex of mice

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ARTICLE INFO

Article history:
Received 28 November 2016
Received in revised form
4 March 2017
Accepted 9 March 2017
Available online 14 March 2017

Abbreviations:
HFD
High fat diet
nNOS
Neuronal nitric oxide synthase
EE
Environmental enrichment

Keywords:
High fat diet
Wheel running exercise
nNOS

ABSTRACT

Consumption of a high fat diet (HFD) and being overweight both induce functional deterioration and atrophy of the hippocampus. These alterations are associated with mental disorders such as depression and anxiety. Exercise combats obesity and enhances brain health. There is substantial evidence that neuronal nitric oxide synthase (nNOS) is a key regulator of affective behavior, and that increased brain nNOS leads to anxiety while environmental enrichment (EE), which reduces brain nNOS, has anxiolytic effects. In this study we investigated the effects of HFD with and without exercise on nNOS protein and gene expression levels in the brains of mice. Twelve weeks of HFD consumption increased body and mesenteric fat weight, as well as nNOS protein levels in the hippocampus and cerebral cortex. Six weeks of exercise training reduced body fat and rescued hippocampal and cortical nNOS expression levels in HFD-fed mice. Cerebellar nNOS expression was unaffected by HFD and exercise. Our results suggest that HFD-induced brain dysfunction may be regulated by hippocampal and/or cortical nNOS, and that exercise may have therapeutic potential for the treatment of HFD-induced depression and anxiety via the nNOS/NO pathway. In conclusion, exercise reverses HFD-induced changes in hippocampal and cortical nNOS protein levels in mice.

1. Introduction

Obesity is becoming increasingly prevalent and is an important contributor to the worldwide burden of disease [1]. Energy imbalance is a major cause of obesity and often leads to type 2 diabetes due to skeletal muscle insulin resistance and glucose intolerance. Although most research on obesity and glucose metabolism has focused on adipose tissue [2] or skeletal muscle [3], recent evidence suggests that obesity also has detrimental effects on the brain. For instance, epidemiological evidence suggests that consuming a Western diet or being overweight are both associated with smaller hippocampal volume [4,5]. An association between consuming a Western diet and mental disorders, such as depression and anxiety, has also been shown [6]. Cordner et al. [7] reported a strong association between exposure to a high fat diet (HFD) and cognitive impairment. Animal studies have shown that HFD-induced obesity reduces brain-derived neurotrophic factor (BDNF) expression [8–10] and neurogenesis [9], and increases inflammation in the hippocampus [10]. These findings may indicate that mental disorders such as depression and anxiety, which are associated with a HFD or Western dietary pattern, may be induced by brain (particularly hippocampal) degeneration.

Nitric oxide (NO) acts as a neuronal messenger in the brain [11]. NO is synthetized from its precursor L-arginine by three isoforms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In the adult brain, nNOS is the major isomer of NOS in the central nervous system [12,13]. In rodents, pharmacological selective inhibition of nNOS increases hippocampal neurogenesis [14], improves spatial memory [15], and inhibits aggression [16]. Previous studies have shown that nNOS in the brain is involved in anxiety-like behaviors [17–20], and that a lack of nNOS or nNOS-selective inhibition reduced anxiety. Recently, we reported an association between anxiety-like behaviors in aged mice and the age-related increase in hippocampal nNOS expression [21]. Thus, considerable evidence suggests that

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http://dx.doi.org/10.1016/j.niox.2017.03.002
1089-8603/

reducing brain nNOS may allow regulation of affective behavior such as depression or anxiety.

Exercise has many health benefits and is commonly recommended to combat obesity and improve glucose homeostasis in skeletal muscle. In the brain, studies in elderly people have shown that exercise increases hippocampal volume and enhances hippocampus-dependent learning and memory [22]. In rodents, exercise has been shown to increase hippocampal BDNF [23,24] and enhance hippocampal neurogenesis [24]. We have previously reported that EE including voluntary running reversed the ageing-induced increase in hippocampal nNOS expression and had anxiolytic effects [21]. Abbott et al. [25] suggested that excessive NO in the brain can act as a neurotoxicant. A histological study demonstrated that starvation-induced suppression of hippocampal nNOS was improved by treadmill exercise [26]. These results indicate that exercise training is important to the maintenance of hippocampal nNOS expression levels and the nNOS/NO pathway.

The effects of exercise on HFD-induced obesity in the brain are poorly understood. Phosphorylation of the hippocampal cAMP response element-binding protein (CREB) is essential for the anxiolytic effects of nNOS [27]. A recent study in rats reported that exercise improved the obesity and reduction in brain function associated with HFD consumption, and also increased the proportion of phosphorylated CREB relative to that in sedentary rats fed a standard diet [28]. However, whether HFD-induced obesity alters hippocampal nNOS expression levels remains unclear. Moreover, although exercise training is an effective strategy for reducing obesity, the effects of exercise on hippocampal nNOS expression in HFD-fed obese mice are unknown.

We hypothesized that the feeding a HFD would increase hippocampal nNOS expression, and that this change could be reversed by exercise training. The purpose of this study was to examine the effects of exercise training in HFD-fed obese mice on nNOS expression levels in the hippocampus.

2. Materials and methods

2.1. Animals

Twenty 4-week-old male C57BL/6J mice (Japan SLC, Shizuoka, Japan) were used in this study. The animals were housed in an accredited animal facility that was maintained at a constant temperature (23.5 ± 0.7 °C), humidity (34.0 ± 5.7%), and light-dark cycle (12 h-12 h). The animals were allowed feed and water *ad libitum*. All experiments were approved by the Animal Care and Use Committee of Fukuoka University.

2.2. High fat diet and exercise training

After 2 weeks of acclimatization the mice were divided into three groups: the SD group (n = 5) was fed a standard diet (CE-7, Clea Japan, Tokyo, Japan; 62% carbohydrate, 14% fat, and 25% protein) for the 12 week experimental period, the HFD group (n = 7) was fed a high fat diet (HFD-32, Clea Japan, Tokyo, Japan; 23% carbohydrate, 57% fat, and 20% protein) for the 12 week experimental period, and the HFD + Ex (n = 8) group was fed HFD-32 for the 12 week experimental period and allowed to voluntarily exercise in a running wheel for the final 6 weeks of the experiment (Fig. 1). Both the HFD and the HFD + Ex groups were reared in a cage with a running wheel, but only the HFD + Ex group was allowed free access to the running wheel. In preliminary testing, we confirmed that the wheel running speed of HFD + Ex mice (20.9 m/min) was equivalent to the lactate threshold (50%–60% of maximum oxygen intake) for the rats, as reported in previous studies [29,30]. Body weight, food intake, and wheel rotation counts were measured every second day. Total energy intake and running distance were calculated based on food intake and wheel rotation count, respectively.

2.3. Tissue preparation

At the end of experimental period the mice were killed by decapitation. The hippocampus, cerebral cortex, cerebellum, and tibialis anterior muscle were rapidly collected and frozen in liquid nitrogen. All specimens were stored at −80 °C for subsequent analysis.

2.4. Citrate synthase activity

Citrate synthase (CS) activity assay was performed to determine the skeletal muscle CS activity using colorimetric assay kits (Biovision Inc., San Francisco, CA), according to the manufacturers' instructions. CS activity from the tibialis anterior muscle served as a marker for mitochondria, and adaptation of animals to the exercise training.

2.5. Western blot

Western blotting was performed to determine nNOS, Akt, and phosphorylated Akt^{Ser473} ($pAkt^{Ser473}$) protein levels in each of the three collected brain loci. Total protein was extracted as previously described [21]. Proteins from each sample (10 μg total protein per lane) were separated by electrophoresis on a 7.5% (w/v) sodium dodecyl sulfate polyacrylamide gel for 35 min at 200 V and transferred to a polyvinylidene fluoride membrane (Millipore, MA, USA) using the semi-dry method. After transfer, the membrane was blocked with 3% (w/v) skim milk at room temperature for 1 h and then incubated overnight with the primary antibodies anti-nNOS (1:500; BD Biosciences, San Jose, CA), anti-Akt (1: 1000; Cell Signaling, Beverly, MA), anti-$pAkt^{Ser473}$ (1: 2000; Cell Signaling) and anti-GAPDH (1:50,000; Acris Antibodies, Herford, Germany) at 4 °C. Next, the membrane was incubated in horse-radish peroxidase-conjugated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA) or horseradish peroxidase-conjugated anti-rabbit IgG antibody (Vector Laboratories) for 1 h at room temperature. Bound antibodies were detected by ECL select Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ) and analyzed using an Amersham Imager 600 (GE Healthcare Life Sciences, Tokyo, Japan). nNOS protein levels were expressed relative to GAPDH levels and normalized to the SD group. The $pAkt^{Ser473}$ levels were evaluated using a value relative to Akt levels.

2.6. Gene expression

Cerebellar and cortical tissues were homogenized using a Polytron homogenizer. Hippocampal gene expression was not analyzed because it took more than 5 min to collect the hippocampus, thus significant RNA degradation had likely taken place. Total RNA was extracted from all samples using the Maxwell® 16 LEV System (Promega, Tokyo, Japan). Real-Time RT-PCR was performed using TaqMan Gene Expression Assay probes to analyze nNOS mRNA levels (*Nos1*-Mm00435175_m1; Applied Biosystems, Foster City, CA) with the Step One Real-Time PCR system (Applied Biosystems). nNOS mRNA levels were normalized to GAPDH mRNA levels (*Gapdh*-Mm99999915_g1; Applied Biosystems) and quantified using the ΔΔCt method.

2.7. Statistics

Data are shown as means ± SE. All statistical analyses were