Neuroprotective Effects of Endurance Exercise Against High-Fat Diet-Induced Hippocampal Neuroinflammation


*Exercise Biochemistry Laboratory, Korea National Sport University, Seoul, Korea.
†Department of Marine Sports, University of Han-Seo, Seosansi, Korea.
‡Exercise Biochemistry Laboratory, University of West Florida, Pensacola, FL, USA.

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Obesity contributes to systemic inflammation, which is associated with the varied pathogenesis of neurodegenerative diseases. Growing evidence has demonstrated that endurance exercise (EE) mitigates obesity-induced brain inflammation. However, exercise-mediated anti-inflammatory mechanisms remain largely unknown. We investigated how treadmill exercise (TE) reverses obesity-induced brain inflammation, mainly focusing on toll-like receptor-4 (TLR-4)-dependent neuroinflammation in the obese rat brain after 20 weeks of a high-fat diet (HFD). TE in HFD-fed rats resulted in a significant lowering in the homeostasis model assessment of insulin resistance index, the area under the curve for glucose and abdominal visceral fat, and also improved working memory ability in a passive avoidance task relative to sedentary behaviour in HFD-fed rats, with the exception of body weight. More importantly, TE revoked the increase in HFD-induced proinflammatory cytokines (tumour necrosis factor α and interleukin-1β) and cyclooxygenase-2, which is in parallel with a reduction in TLR-4 and its downstream proteins, myeloid differentiation 88 and tumour necrosis factor receptor associated factor 6, and phosphorylation of transforming growth factor β-activated kinase 1, IκBα and nuclear factor-κB. Moreover, TE reduced an indicator of microglia activation, ionised calcium-binding adapter molecule-1, and also decreased glial fibrillary acidic protein, an indicator of gliosis formed by activated astrocytes in the cerebral cortex and the hippocampal dentate gyrus, compared to HFD-fed sedentary rats. Finally, EE up-regulated the expression of anti-apoptotic protein, Bcl-2, and suppressed the expression of pro-apoptotic protein, Bax, in the hippocampus compared to HFD-fed sedentary rats. Taken together, these data suggest that TE may exert neuroprotective effects as a result of mitigating the production of proinflammatory cytokines by inhibiting the TLR4 signalling pathways. The results of the present study suggest that the unique combination of the beneficial effects of TE on the restoration of the blood profile and the anti-inflammatory and anti-apoptotic effects on cognitive function should inspire further investigations into its therapeutic potential for metabolic disorders and neurodegenerative diseases.

Key words: obesity, treadmill exercise, TRL-4 signal transduction, neuroinflammation, memory

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Obesity is emerging as a primary public health concerns in developed countries. Excess body fat is recognised as a major risk factor for various metabolic disorders because it secretes proinflammatory cytokines into the bloodstream and initiates inflammatory signal transduction (1,2). It is well known that obesity is associated with metabolic disorders, such as type-2 diabetes, hypertension and cardiovascular diseases, and recent studies have suggested that it is also closely linked to the disturbance of brain health. For example, recent studies have shown that various neurological disorders, such as apnoea, bipolar disorders and anxiety, are related to obesity (3,4) and, furthermore, neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases, are closely linked to obesity (5).

Recent research has discovered that an adipose tissue-mediated increase in tumour necrosis factor α (TNFα) promotes neuroinflammation and interferes with the action of critical cell survival signalling molecules in the brain such as insulin/insulin-like growth factors.
factor-1 (6). Under normal circumstances, insulin in the brain plays an important role in the development and activation of nerve cells and enhances cognitive abilities (7). However, obesity can interrupt the proper signal transduction of insulin in the brain, which thus impairs cognitive abilities and leads to neurodegenerative diseases in a transgenic model of Alzheimer’ disease (8–10). Moreover, obesity-mediated overproduction of TNFα activates microglia and subsequently promotes the production of other proinflammatory cytokines such as interleukin (IL)-6, and IL-1β, contributing not only to the development, but also the progression of neurodegenerative diseases (11,12). Similarly, recent studies have reported that the infiltration of activated macrophages in the central nervous system (CNS) was approximately 53% greater in an obese mouse compared to a control group (13).

Microglia, differentiated from monocytes in the brain, exist to function as resident immune cells (i.e. macrophages). Interestingly, Koga et al. (14) have reported that microglia and astrocytes in the CNS were both increased in number in obesity. Activation of microglial cells initiates the immune response via the Toll-like receptor-4 (TLR-4) (15,16), whose activation relays the downstream inflammatory signals via an adaptor protein, myeloid differentiation 88 (MyD88) and tumour necrosis factor receptor associated factor 6 (TRAF6). Phosphorylation of transforming growth factor-β-activated kinase-1 (TAK1) activates p38 MAPK and nuclear factor-κB (NF-κB), which promotes the production of proinflammatory cytokines (17–19). Ultimately, an excessive accumulation of body fat leads to the stimulation of neuroinflammation, and hence a disturbance of brain health.

Non-invasive, economical strategies against obesity such as diet and exercise have been suggested to mitigate obesity. In particular, exercise is effective in reducing body fat by increasing the resting metabolic rate (20–22). Moreover, it has been identified as an effective methodology for reversing metabolic diseases by improving insulin sensitivity and restoring ideal blood lipid profiles, including a reduction in low-density lipoprotein and triglycerides in the blood and lipogenesis (23,24).

Although it is clear that obesity contributes to a decline in brain dysfunction via neuroinflammatory response, the molecular mechanisms responsible for this still remain unclear. Moreover, if endurance exercise is protective against obesity, obesity-mediated aberrant proinflammatory responses are largely lacking. Therefore, the present study aimed to investigate whether 8 weeks of treadmill exercise (TE) prevents an unfavourable activation of neuroinflammation in the microglia of brain, mediated by diet-induced obesity.

Materials and methods

Experimental animals

In the present study, 8-week-old male Sprague–Dawley (SD) rats were purchased from Koatech (Pyeongtaek, South Korea) and maintained in an animal room under a 12 : 12 h light/dark cycle at 22 ± 2 °C and 50 ± 5% relative humidity at Korea National Sport University until they were 20 weeks old. At 20 weeks of age, experimental animals (n = 20) were fed with a HFD (60 kcal% fat, #D12492; Research Diets, New Brunswick, NJ, USA) for 20 weeks to induce obesity. The food was purchased from the Central Lab [Seoul, Korea], and the amount of food and water supply were offered ad lib. Experimental animals were further divided into two groups: HF diet control group (HFD-Con, n = 10) and HF diet treadmill exercise group (HFD-TE, n = 10). Normal diet control animals (ND-Con, n = 10) were assigned and fed with a normal diet. The protocols were approved by the Institutional Animal Care and Use Committees of Korea National Sports University (number 09-3-29), Korea.

Treadmill exercise

The exercise protocol in the present study was applied as described previously (25,26) with a minimum revision. Briefly, animals of HFD-TE group were familiarised with treadmill running for 30 min (2 m/min for the first 5 min; 5 m/min for the next 5 min; and 8 m/min for the final 20 min) a day for five consecutive days on a motorised rodent treadmill (D2J-242; Daejong Instrument Industry Co., Seoul, Korea) at 0% grade. On completion of familiarisation, the main exercise (8 m/min for the first 5 min; 11 m/min for the next 5 min; and 14 m/min for the last 20 min at 0% grade) was conducted 5 days a week for 8 weeks.

Passive avoidance task

A passive avoidance task, which is a method of measuring the working memory ability of rodents, was performed 3 days before the conclusion of TE. The apparatus for the passive avoidance task consists of two compartments: the first compartment is a brightly lit white box (18 × 18 × 25 cm) and the second compartment, which is connected to the first one, is a dark box (18 × 18 × 25 cm). The floor of the dark space was constructed with stainless steel to enable the administration of an electric shock. The wall between the first and second compartments was designed to open and close as necessary. The time required for the test animal to enter the second room from the first room was measured, after which an electric shock (0.5 mA) was administered for 2 s. After 5 s, the test animals were then transferred to their cage. The same test was repeated after 72 h, and the time taken to enter the second room from the first (retention latency time) was measured up to a maximum of 300 s.

Body weight and oral glucose tolerance test (OGTT)

Body weight was measured twice a week during the course of the experiment using a laboratory scale, and the mean body weight was used for calculations. OGTT was conducted 12 h after the completion of the 8-week TE. Briefly, under fasting state, 30% glucose solution (1.0 g/kg body weight) was administrated orally, and blood was drawn from the tail vein on five occasions (at 0, 30, 60, 90 and 120 min). Blood glucose was measured using a glucometer (Gluco-Card II; Daichi Kagaku Co., Kyoto, Japan). Using the obtained blood glucose data, the total glucose secretion response area (area under the curve; AUC0–120, mg/dl/min) was analysed via NCSS 2007 (NCSS, Kaysville, UT, USA).

Blood and tissue sample collection

A ketamine/xylazine mixture (2 : 1, 10 mg/kg) was administrated by i.p. injection 24 h after completion of the 8-week TE, and then blood, brain tissue and abdominal fat were collected from six rats to measure protein expression. After separating the hippocampus, the extracted brain tissue was rapidly frozen in liquid nitrogen and stored in a cryogenic freezer (Bio-Freezer; Forma Science, Marietta, OH, USA) at −80 °C until needed for biochemical assays. Serum was obtained from the blood using a centrifugal filter (FLETA-5 centrifuge; Hanil Biomed Inc., Gwangju, Korea) and used for the analysis of glucose and insulin. The abdominal visceral fat (AVF) was washed
with chilled saline solution, and the weight of semi-dried fat tissue was measured after removing surface moisture. Brain tissues for immunohistochemical staining analysis were washed with 50 ml of phosphate-buffered saline (PBS) by perfusion through the left ventricle for 10 min. Subsequently, paraformaldehyde (PFA) fixing solution (4% in 100 ml phosphate buffer) was perfused, and the brain tissues were extracted and immersed in 4% PFA fixing solution for 4 h at 4 °C. The fixed brain tissue was then stored in 30% sucrose solution for 2 days. The anatomical location (from −4.88 to −1.76 mm from the bregma) including the cortex of the fixed whole brain and hippocampus was extracted using Rodent Brain Matrix (RBM-4000C, 1 mm coronal section; ASI Instruments, Inc., Warren, MI, USA) and continuous coronal slices 40 μm in thickness were taken using a freezing microtome (Leica, Nussloch, Germany).

Biochemical analysis

Serum glucose and insulin concentrations were analysed using a glucose hexokinase kit (Bayer, Pittsburgh, PA, USA) and a Mercodia rat insulin enzyme-linked immunosorbent assay kit (Mercodia AB, Uppsala, Sweden), respectively, in accordance with the the manufacturer’s instructions. The homeostasis model assessment-insulin resistance (HOMA-IR) index was calculated according to the formula: HOMA-IR = fasting serum glucose (mmol/l) x fasting serum insulin (μIU/ml)/22.5

Preparation of nuclear fraction

The nuclear fraction was collected using the ProteoExtract Subcellular Proteome Extraction Kit in accordance with the manufacturer’s instructions (Calbiochem; EMD Biosciences, Darmstadt, Germany). In brief, the brain tissue (hippocampus) of each animal was homogenised with 1 ml of extraction buffer I and 5 μl of protease inhibitor cocktail and centrifuged at 10 000 g for 10 min. The obtained pellet were gently agitated with extraction buffer II and 5 μl of protease inhibitor cocktail for 30 min on the rotary shaker and then centrifuged at 6000 g for 10 min. The obtained pellet were gently agitated with 0.5 ml of extraction buffer III, 5 μl of protease inhibitor cocktail and 1.5 μl of benzonase for 10 min on the rotary shaker. The final nuclear fraction was obtained by centrifuged at 7000 g for 10 min.

Western blotting

The brain tissues (hippocampus) were homogenised in RIPA lysis buffer [1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecylsulphate (SDS)]. Protein concentration was determined by the Bradford method. Proteins (30 μg) were separated by SDS-polyacrylamide gel electrophoresis (8% or 12% separating gel), transferred to polyvinylidene fluoride membranes (Millipore, Boston, MA, USA) and blocked with 5% bovine serum albumin. Membranes were incubated with primary antibodies overnight at 4 °C in a dark room, and anti-Iba1 (ab15690) antibodies (dilution 1 : 500; Abcam) at 4 °C. The slices were then blocked with 10% normal donkey serum for 40 min. After blocking, tissue slices were incubated overnight with primary anti-β-Galactosidase (ab10062) and anti-Iba1 (ab15690) antibodies (dilution 1 : 500; Abcam) at 4 °C for 12 h and then washed with 0.01 μ PBS three times, 5 min per wash, followed by incubation at room temperature for 1 h with HRP-conjugated goat anti-mouse antibody (sc-2005; Santa Cruz Biotechnology). The slices were again washed with 0.01 μ PBS three times for 5 min each, and then incubated at room temperature for 30 min with Vectastain-Elite ABC kit (PK-6200; Vector Laboratories, Burlingame, CA, USA). After washing three times with 0.01 μ PBS, slices were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) that was diluted to 0.02% with 0.05 M tris-buffer (pH 7.6) using a DAB peroxidase substrate kit (SK-4100; Vector Laboratories). Hydrogen peroxide (0.02%) was added to bleach for 5 min. The sections were mounted onto gelatin-coated slides, air-dried overnight at room temperature, and mounted using Permount (Fisher Scientific Co., Pittsburgh, PA, USA).

Number of cells in the cerebral cortex and hippocampal dentate gyrus

Four animals from each ND-Con and experimental group (HFD-Con, HFD-TE) were analysed. For measuring cell count, the expression characteristics of the antigen, as specified by the manufacturer of the antibody and previous studies, were confirmed and consulted. For confirmation of ionised calcium-binding adapter molecule-1 (IBA-1) and glial fibrillary acidic protein (GFAP) expression, identical parts of the hippocampal and cortical regions were selected between groups and observed at × 100 and × 200 magnification under a light microscope (DM-2500; Leica Microsystems, Wetzlar, Germany). The mean number of positive cells (subjective/consistent criterion based on a previous study) stained in the immune response to antibodies per unit area (mm2) was quantitatively calculated for each group, and the ratio compared to the normal group was determined.

Statistical analysis

Data were analysed using SPSS, version 18.0 (SPSS, Inc., Chicago, IL, USA). All values are expressed as the mean ± SEM. Statistical significance was determined using one-way ANOVA. When statistical significance was found, a Bonferroni post-hoc test was followed for multiple comparisons. P < 0.05 was considered statistically significant.

Results

The effects of TE on body weight, AUC, HOMA-IR, AVF and AVF/body weight

To determine the effects of a 20-week HFD on the excessive weight gain, body weight was measured at 4-week intervals. The body...
weight of the HFD group was significantly higher than that of the ND-Con group (Fig. 1A). However, despite a decreasing trend in body weight after 8-week TE subsequent to a 20-week HFD compared to HFD group, the difference was not statistically significant. We next measured plasma glucose regulation patterns. Although HFD increased OGTT area under the curve AUC \( F_{2,27} = 26.017, P < 0.001 \), HFD with TE improved glucose tolerance and insulin sensitivity (HOMA-IR, \( F_{2,27} = 22.944, P < 0.001 \)) (Fig. 1B,C). AVF is a key source of low level of chronic inflammation identified as one of the main causes of metabolic disorders. Our data showed that TE prevented HFD-induced gain of AVF \( F_{2,27} = 14.114, P < 0.001 \) (Fig. 1D,E). These results demonstrate that a long-term HFD increased body weight and AVF, decreased blood glucose tolerance capacity, and increased insulin resistance, whereas TE reverses HFD-induced excessive gain of AVF and AVF/body weight, restores the impaired blood glucose disposal capacity, and enhances insulin sensitivity.

The effects of TE on the expression of TLR4-related proteins in the hippocampus of HFD-fed rats

Obesity is closely linked to the chronic inflammation. To investigate whether TE mitigates HFD-induced inflammation, we examined a series of inflammation signalling pathways initiated by TLR4 whose downstream targets are MyD88, TRAF6, TAK1 and \( \mathrm{IkB}\alpha \) in the hippocampus of HFD-fed rats. Our data showed that HFD up-regulated the expression of TLR-4 \( F_{2,15} = 59.310, P < 0.001 \) (Fig. 2A) and downstream signal transduction proteins Myd88 \( F_{2,15} = 18.861, P < 0.001 \) (Fig. 2B) and TRAF6 \( F_{2,15} = 9.567, P < 0.01 \) (Fig. 2C) and promoted phosphorylation levels of TAK1 (phospho-TAK1/t-TAK1 ratio, \( F_{2,15} = 40.373, P < 0.001 \)) (Fig. 2D). \( \mathrm{IkB}\alpha \) functions as a NF-\( \kappa \)B inhibitor under normal conditions. However, upon phosphorylation by TAK1, it becomes a target for ubiquitination and is degraded, thus releasing inhibitory effects on NF-\( \kappa \)B and leading to the activation of NF-\( \kappa \)B. Our data showed that HFD intensified phosphorylation of \( \mathrm{IkB}\alpha \) (p-IkB\( \alpha \)/t-IkB\( \alpha \) ratio, \( F_{2,15} = 19.691, P < 0.001 \)). By contrast, TE reversed HFD-mediated up-regulation of those proteins and phosphorylation.

The effects of TE on the expression of proinflammatory proteins in the hippocampus of HFD-fed rats

Given the remission of HFD-induced inflammatory activation processes via TE, we further examined whether TE prevents the expression of proinflammatory cytokines. NF-\( \kappa \)B is a critical transcription factor tightly linked to the expression of diverse proinflammatory cytokines, whose activity is controlled by phosphorylation. HFD displayed increased levels of phosphorylated NF-\( \kappa \)B (p-NF-\( \kappa \)B/t-NF-\( \kappa \)B ratio, \( F_{2,15} = 35.795, P < 0.001 \)), whereas TE attenuated its elevation (Fig. 3A,B). We next examined

**Fig. 1.** Effects of treadmill exercise (TE) on bodyweight, area under the curve (AUC), homeostasis model assessment-insulin resistance (HOMA-IR) index, abdominal visceral fat (AVF) and AVF/body weight in each group. (a) After 20 weeks of a high-fat diet (HFD), the HFD-control (Con) group showed a significant increase in body weight compared to the normal diet (ND)-Con group and, although there was a tendency for the body weight to decrease in the HFD-TE group compared to the HFD-Con group during 8 weeks of TE, the differences were not significant. \*\( P < 0.001 \) compared to the ND-Con group. (a) The AUC result from the oral glucose tolerance test (OGTT), (c) HOMA-IR obtained through fasting insulin and fasting glucose concentrations, (d) the amount of AVF and (e) AVF/body weight were significantly increased in the HFD-Con group compared to the ND-Con group, and significantly decreased in the HFD-TE group compared to the HFD-Con group.
Fig. 2. The effects of treadmill exercise (TE) on the expression of toll-like receptor-4 (TLR-4)-related proteins in the hippocampus of high-fat diet (HFD)-fed rats. (a) Representative western blots of TLR-4, myeloid differentiation 88 (MyD88), tumour necrosis factor receptor associated factor 6 (TRAF6), β-actin, transforming growth factor β-activated kinase-1 (TAK1) and IκBα proteins. (b–e) Densitometric analysis of the western blot bands normalised to β-actin. (b) TLR-4, MyD88 and TRAF6 proteins showed a significant difference between normal diet (ND)-fed rats and HFD-fed rats. (c) Nuclear factor-κB (NF-κB) proteins showed a significant difference between ND-fed rats and HFD-fed rats. (d–f) Densitometric analysis of the western blot bands normalised to Lamin B (NF-κB). The top demonstrates 30 μg of protein per sample, with an antibody against phospho-TAK1. The bottom demonstrates the total TAK1 protein, which was assessed using antibodies recognising these proteins regardless of their phosphorylated state. IκBα proteins showed a significant difference between ND-fed rats and HFD-fed rats. Nuclear factor-κB (NF-κB) proteins in the hippocampus were significantly reduced in HFD-fed rats after TE. The data shown in the western blot are means from six rat brains. Lamin B and β-actin were probed as an internal control. Bonferroni post-hoc test after ANOVA. Values are presented as the mean ± SEM.

Fig. 3. The effects of treadmill exercise (TE) on the expression of proinflammatory proteins in the hippocampus of high-fat diet (HFD)-fed rats. (a) Representative western blots of nuclear factor-κB (NF-κB), tumour necrosis factor (TNF)-α, interleukin (IL)-1α, cyclooxygenase-2 (COX-2), Lamin B and β-actin proteins. (b–e) Densitometric analysis of the western blot bands normalised to Lamin B (NF-κB) and β-actin (except for NF-κB). (b) NF-κB proteins in the hippocampus were significantly reduced in HFD-fed rats after TE. The data shown in the western blot are means from six rat brains. Lamin B and β-actin were probed as an internal control. Bonferroni post-hoc test after ANOVA. Values are presented as the mean ± SEM.
the protein expression levels of TNF-α, IL-1β and COX-2, a crucial enzyme for inflammation in the hippocampus of HFD-fed rats. Although HFD significantly increased TNF-α, IL-1β and COX-2, TE clearly reverted them to levels of the normal diet group (TNF-α, \(F_{2,15} = 35.795, P < 0.001\); IL-1β, \(F_{2,15} = 29.515, P < 0.001\); COX-2, \(F_{2,15} = 12.698, P < 0.01\)) (Fig. 3A–E).

The effects of TE on the expression of IBA-1 in the cerebral cortex and hippocampal dentate gyrus of HFD-fed rats

IBA1 is a microglia-specific calcium protein involved in phagocytosis upon activation of microglial cells, which ensues in inflammation. We examined whether TE-induced abrogation of proinflammatory cytokine overproduction caused by HFD is linked to levels of IBA-1 expression. Our immunohistochemical data showed that, although IBA-1 was significantly elevated in HFD-fed rats compared to ND and HDF-TE, TE attenuated its overexpression in the cerebral cortex (\(F_{2,9} = 36.043, P < 0.001\)) (Fig. 4A–C, G: note that Fig. 4D–F are magnified images of Fig. 4A–C) and hippocampal dentate gyrus (\(F_{2,9} = 56.809, P < 0.001\)) (Fig. 4H–J, N: note that Fig. 4K–M are magnified images of Fig. 4H–J).

The effects of TE on the expression of GFAP in the cerebral cortex and hippocampal dentate gyrus of HFD-fed rats

GFAP is an important marker of gliosis induced by astrocyte activation and neuroinflammation. We investigated whether TE reduced the expression of GFAP in the cerebral cortex and hippocampal dentate gyrus of HFD-fed rats. Similar to the results for IBA-1, our immunohistochemical data showed that, although GFAP was significantly elevated in HFD-fed rats, TE mitigated its overexpression in the cerebral cortex (\(F_{2,9} = 121.162, P < 0.001\)) (Fig. 5A–C, G: note that Fig. 5D–F are magnified images of Fig. 5A–C) and hippocampal dentate gyrus (\(F_{2,9} = 27.243, P < 0.001\)) (Fig. 5H–J, N: note that Fig. 5K–M are magnified images of Fig. 5H–J).

The effects of TE on the expression of apoptosis-associated proteins in the hippocampus and cognitive function (working memory) of HFD-fed rats

Apoptosis is one of defining hallmarks of neuronal cell death resulting from neuroinflammation, whose initiation and finalisation is controlled by the ratio of pro- and anti-apoptotic Bcl-2 family members.
proteins and caspase-3, respectively. To examine whether TE modulates the expression of the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, as well as caspase-3, we conducted western blot assays and found that, although a 20-week HFD resulted in the activation of caspase-3 (cleaved caspase-3, \( F_{2,15} = 25.805, P < 0.001 \)), TE prevented its elevation in the hippocampus (Fig. 6A,B). Furthermore, TE precluded an increase in Bax (\( F_{2,15} = 12.631, P < 0.01 \)) and a reduction in Bcl-2 (\( F_{2,15} = 31.359, P < 0.001 \)) proteins (Fig. 6A,C,D), with the ratio of Bcl-2/Bax (\( F_{2,15} = 21.682, P < 0.001 \)) remaining higher than that of a HFD group in the hippocampus (Fig. 6E). Finally, we evaluated the working memory of HFD-fed rats. Although a long-term HFD hampered working memory as indicated by a reduced retention latency time, TE improved memory deterioration, resulting from the enhanced retention latency time (\( F_{2,27} = 217.687, P < 0.001 \)) (Fig. 6F).

**Discussion**

The present study examined a potential mechanism concerning how TE protects the brain against HFD-induced neuroinflammation. Several interesting findings have emerged from the present study. First, 8 weeks of TE subsequent to HFD improved insulin resistance, accompanying reduced AVF. Second, in the brain, TE precluded HFD-induced elevation of proinflammatory cytokines caused by activation of TLR-4 signalling pathways. Third, TE prevented HFD-mediated unfavoured activation and damage of microglia (the brain macrophage cells) in both the cerebral cortex and hippocampus. Fourth, TE significantly recovered the memory function of the brain as impaired via a HFD by mitigating apoptosis activation. Taken together, our studies identify TE as a critical repressor of neuroinflammation caused by obesity and demonstrate that restoration of favourable normal metabolic conditions by TE is concurrent with reversal of the diminished plasma glucose regulatory capacity and compromised memory retention ability. A detailed discussion of these findings is provided below.

Obesity was reported to be a primary contributing factor leading to metabolic disorders, such as type 2 diabetes mellitus, cardiovascular diseases and stroke. More specifically, recent studies have suggested that obesity-mediated inflammation and insulin resistance critically contribute to neurodegeneration (6). By contrast, regular endurance exercise exhibits conspicuous protective roles and enhances the memory function of the brain; however, the
The effects of treadmill exercise (TE) on the expression of apoptosis-associated proteins in the hippocampus and cognitive function (working memory) of high-fat diet (HFD)–fed rats. (A) Representative western blots of caspase-3, Bax, Bcl-2 and β-actin proteins. (B–E) Densitometric analysis of the western blot bands normalised to β-actin. (B–E) Caspase-3, Bax and Bcl-2 proteins showed a significant difference between normal diet (ND)-fed rats and HFD-fed rats. Caspase-3 and Bax proteins in the hippocampus were significantly reduced in HFD-fed rats after TE. Bcl-2 proteins in the hippocampus were significantly increased in HFD-fed rats after TE. (C) The Bcl-2/Bax ratio showed a significant difference between ND-fed rats and HFD-fed rats. The Bcl-2/Bax ratio in the hippocampus was significantly different between ND-fed rats and HFD-fed rats. (D) Working memory ability test after performing a passive avoidance task 3 days before the end of the TE. The data shown in the western blot are the means from six rat brains. β-actin was probed as an internal control. A Bonferroni post-hoc test was to determine group differences upon finding significance after ANOVA. Values are presented as the mean ± SEM (passive avoidance task; n = 10 animals).

Fig. 6. The effects of treadmill exercise (TE) on the expression of apoptosis-associated proteins in the hippocampus and cognitive function (working memory) of high-fat diet (HFD)–fed rats. (A) Representative western blots of caspase-3, Bax, Bcl-2 and β-actin proteins. (B–E) Densitometric analysis of the western blot bands normalised to β-actin. (B–E) Caspase-3, Bax and Bcl-2 proteins showed a significant difference between normal diet (ND)-fed rats and HFD-fed rats. Caspase-3 and Bax proteins in the hippocampus were significantly reduced in HFD-fed rats after TE. Bcl-2 proteins in the hippocampus were significantly increased in HFD-fed rats after TE. (C) The Bcl-2/Bax ratio showed a significant difference between ND-fed rats and HFD-fed rats. (D) Working memory ability test after performing a passive avoidance task 3 days before the end of the TE. The data shown in the western blot are the means from six rat brains. β-actin was probed as an internal control. A Bonferroni post-hoc test was to determine group differences upon finding significance after ANOVA. Values are presented as the mean ± SEM (passive avoidance task; n = 10 animals).

Mechanisms responsible for exercise-induced protective effects against obesity-mediated metabolic disorder remain largely unknown. We speculated that TE would induce a negative caloric balance by promoting more caloric expenditure, with fat metabolism enhanced from the adipose tissues, and prevent the accumulation of fat deposits in abdominal adipose tissue and thus reduce the production of proinflammatory cytokines. Our results revealed that 8 weeks of TE prevents the production of proinflammatory cytokines, improves plasma glucose regulation and reduces the mass of AVF and AVF/body weight. These results were consistent with the results reported in other studies (10,27). For example, although obesity-induced AVF accumulation contributes to the induction of the low-grade systemic inflammation, leading to interference with insulin signalling and thus to metabolic disorders (24,28), aerobic exercise reduces AVF and cytokine production (29,30).

In support of this observation, recent studies have reported that reduced AVF correlates with an improvement in insulin responsiveness and sensitivity in the peripheral muscle tissues, which are premier sites for blood glucose storage (31,32).

TE has been suggested to be an economical and practical tool that facilitates anti-inflammatory processes; however, the precise molecular mechanisms by which exercise reduces inflammatory responses in the brain have not been determined. Given that HFD induces the activation of the TLR4 signalling pathway in microglial cells, leading to chronic inflammation and neuronal cell death, the present study focused on the signal transduction of TLR-4. Elevated levels of plasma free fatty acids bind to TLR-4, activate inflammatory responses through the MyD88-dependent and MyD88-independent signal transduction pathways, and result in the production of proinflammatory cytokines (17).

Alternatively, HFD can also increase the production of lipopolysaccharides (LPS), a typical byproduct of bacterial metabolism that is a leading ligand for TLR-4 (33). Additionally, our data reveal that HFD increases expression of TLR-4 protein, and a series of downstream signalling molecules such as MyD88, TAK1 and IκB are increased and activated, resulting in the overproduction of proinflammatory cytokines. By contrast, TE prevents TLR-4 expression and thus forestalls the activation of its downstream target proteins in the hippocampal region of the brain. Consequently, our data suggest that, as a result of the exercise-mediated enhanced metabolic paradigm shift (i.e. reduced body fat via increased utilisation of triglycerides and improved glucose tolerance and insulin sensitivity), low levels of plasma triglycerides and LPS obviate the activation of TLR-4 and the resultant inflammatory responses in the brain tissue.

TLR-dependent signal transduction mediates the activation of a transcription factor, NF-κB, which is critically involved in the expression of various proinflammatory cytokines (34). Under unstressed conditions, NF-κB remains inactive because its endogenous inhibitor, IκB, prevents it from being translocated from the cytosol to the nucleus. However, under stressed conditions, TLR-4 activation phosphorylates IκB, becoming ubiquitinatated and
degraded via an ubiquitin-proteosomal pathway and allowing NF-κB to move into the nucleus for target inflammatory gene expression (35,36). Similarly, our data also confirm that phosphorylation levels of NF-κB from nuclear fraction were increased in the HFD, whereas they were decreased in the HFD-TE group, suggesting that the inactivation of TLR-4 signal transduction through TE resulted in the suppression of NF-κB activation and a reduction in the expression of inflammatory cytokines (TNF-α, IL-1β and COX-2). We provide clear evidence that obesity-induced inflammatory responses can be dampened by engaging in regular endurance exercise.

Generally, activation of astrocytes is known to induce innate and adaptive immunity (37) and the expression of GFAP increases in astrocytes damaged by various causes (38). Moreover, it has been reported that activated microglial cells release proinflammatory cytokines such as IL-1, TNF-α and nitric oxide that contribute to the neurodegenerative process (38,39). We therefore assessed the expression of an astrocyte activation marker, GFAP, and a microglial cell-specific marker, IBA-1, in the cerebral cortex and the dentate to investigate the effects of a HFD on inflammatory responses and to examine whether TE mitigates unnecessary activation and infiltration of glial cells in the brain.

Consistent with other studies (14), our findings show that HFD promotes the expression of GFAP and IBA-1, as shown in immunostained brain tissues that indicate an excessive infiltration of glial cells, also called gliosis, occurring in response to HFD-induced lipotoxicity; however, 8 weeks of TE constrained the HFD-induced activation of GFAP and IBA-1. Because obesity indicative of surplus fat pads around the body is highly associated with a low-grade systemic inflammation, it is considered that TE-mediated reduction in AVF is a key underlying mechanism that ameliorates overexpression of inflammatory cytokines, which otherwise, contributes to developing metabolic disorders and exacerbates metabolic homeostasis.

The hippocampus is an essential part of the brain because it is in charge of memory and cognitive function. Apoptosis has been implicated in the death of brain cells. Especially, many studies have shown that the deterioration of memory and cognitive function seen in various types of neurodegenerative diseases is attributed to a loss of neurons in the hippocampal region as a result of apoptosis (40–42). The active (cleaved) form of caspase-3 is translocated to the nucleus, where it cleaves poly(ADP-ribose) polymerase, an enzyme involved in DNA repair, and thus leads to apoptosis (43). The pro-apoptotic protein Bax translocates to mitochondria and facilitates the release of cytochrome c from the intermembrane space, which in turn activates caspase-3, whereas Bcl-2 protein inhibits apoptosis (44).

Concurrent with the results of the previous studies by Reddy et al. (45,46), the results of the present study also show that HFD promotes neuronal apoptosis in the hippocampus, as indicated by increased active caspase-3 and Bax, reduced Bcl-2 in the hippocampus, and the resultant diminished memory capacity (i.e. shorter retention latency time) in the passive avoidance task. By contrast, TE minimises levels of cleaved caspase-3 and Bax protein but increases the expression of Bcl-2 protein, resulting in improved performance in the passive avoidance task (i.e. longer retention latency time). In addition to the hippocampus, damage to the amygdala has been found to reduce performance in the passive avoidance task, meaning that it can cause impairment of cognitive ability and memory. Therefore, neuroinflammation induced by HFD might affect the amygdala as well as the hippocampus. However, additional studies are needed to confirm this because the present study did not include experiments related to the amygdala.

The present study establishes that TE provides an anti-inflammatory function by inhibiting TLR-4 signal transduction in response to HFD by restoring normal metabolic processes, which forestall glucose intolerance, insulin resistance and neuronal apoptotic cell death. Taken together, the unique combination of the beneficial effects of TE on the restoration of blood profile, including glucose and insulin, and anti-inflammatory and anti-apoptotic effects on the maintenance of the cognitive functions of the brain, should inspire further investigation of its therapeutic potential for metabolic disorders and neurodegenerative diseases.

References

7 Gerozisis K. Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathways. Eur J Pharmacol 2008; 585: 38–49.
10 Maesako M, Uemura K, Kubota M, Kuzuya A, Sasaki K, Hayashida N, Asada-Utsugi M, Watanabe K, Uemura M, Kihara T, Takahashi R, Shishimura S, Kinoshita A. Exercise is more effective than diet control in pre-


24 Marques CM, Motta VF, Torres TS, Aguiar MB, Mandarim-de-Lacerda CA. Beneficial effects of exercise training (treadmill) on insulin resistance and nonalcoholic fatty liver disease in high-fat fed C57BL/6 mice. Braz J Med Biol Res 2010; 43: 467–475.