Resveratrol limits diabetes-associated cognitive decline in rats by preventing oxidative stress and inflammation and modulating hippocampal structural synaptic plasticity

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 21 August 2016
Accepted 22 August 2016
Available online 23 August 2016

Keywords:
Resveratrol
Diabetes
Cognitive function
Structural synaptic plasticity
Oxidative stress
TNF-α

ABSTRACT

Many patients with diabetes are at increased risk of cognitive dysfunction and dementia. Resveratrol, a polyphenol found mainly in grapes and red wine, has antioxidant, anti-inflammatory, and neuroprotective activities. Studies demonstrated that resveratrol could prevent memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. However, whether administration of resveratrol could modulate the structural synaptic plasticity in diabetic rats remains unknown. Therefore, we tested its influence against cognitive dysfunction as well as on hippocampal structural synaptic plasticity in streptozotocin-induced diabetic rats. Our results showed that the cognitive performances in diabetic group were markedly deteriorated, accompanied by noticeable alterations in oxidative as well as inflammation parameters, SYN and GAP-43 expression were reduced in the hippocampus, and cerebral cortex (Baydas et al., 2003; Hasanein and Shahidi, 2010). Elevated blood glucose level stimulates pro-inflammatory cytokines, promotes lipid peroxidation, and further activates the apoptotic pathway which altogether contributes to the pathophysiology of various diabetic complications (Donath et al., 2008). Increased oxidative stress produces serious oxidative damage in the brain under diabetic conditions (Sharma and Singh, 2011; Wang and Jia, 2014). In addition, increased release of inflammatory cytokines and excessive inflammation are observed in diabetics (Kuhad et al., 2009; Wang and Jia, 2014).

1. Introduction

Diabetes mellitus is a common metabolic illness that is accompanied by high blood glucose concentration as a result of the lack of insulin or the presence of insulin resistance in peripheral tissues or both. DM impairs tissues and organs causing serious diseases such as diabetic retinopathy, diabetic nephropathy, and peripheral neuropathy. Besides the most common complications of the peripheral nervous system in diabetic patients, emerging evidences demonstrated that diabetes may also have negative impacts on the central nervous system (Mijnhout et al., 2006; Tuzcu and Baydas, 2006; McCrimmon et al., 2012; Ho et al., 2013; Thomas et al., 2013). Learning and memory deficits also occur in streptozotocin (STZ)-induced diabetic rats (Bayadas et al., 2003; Stranahan et al., 2008; Tiwari et al., 2009; Wang and Jia, 2014), which have been partly associated with the structural and functional deficits in certain brain regions such as the hippocampus and cerebral cortex (Bayadas et al., 2003; Hasanein and Shahidi, 2010).

The multifactorial pathogenesis of learning and memory impairments in diabetes has not been fully elucidated. Several factors such as chronic inflammation, vascular complications, metabolic disturbances, and the release of free radicals are implicated (Hasanein and Shahidi, 2010). Elevated blood glucose level stimulates pro-inflammatory cytokines, promotes lipid peroxidation, and further activates the apoptotic pathway which altogether contributes to the pathophysiology of various diabetic complications (Donath et al., 2008). Increased oxidative stress produces serious oxidative damage in the brain under diabetic conditions (Sharma and Singh, 2011; Wang and Jia, 2014). In addition, increased release of inflammatory cytokines and excessive inflammation are observed in diabetics (Kuhad et al., 2009; Wang and Jia, 2014). Activation of the nuclear transcription factor-κB (NF-κB) signaling pathway was shown to induce cognitive deficits (Kuhad et al., 2009; Wang and Jia, 2014) as well as neuronal apoptosis in diabetics. Previous study (Huang et al., 2009) confirmed that diabetic hyperglycemia indeed worsened seizure severity and status

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epileptics-induced hippocampal neuron damage, and impaired hippocampus-dependent cognition and synaptic plasticity. Indeed, a complex pattern of changes in synaptic plasticity has been observed in hippocampal slices from STZ-diabetic rats (Chabot et al., 1997; Kamal et al., 1999). Synaptic plasticity includes the modifications of the structure and function of synapses, which are integral to learning and memory (Hasan et al., 2011). Cognitive decline associated with the progressive reduction of structural and functional plasticity in the brain regions that play key roles in cognitive functions (Bisaz et al., 2013).

Resveratrol (3,5,4-trihydroxy-trans-stilbene) is a polyphenol found mainly in grapes and red wine with diverse established biological activities, such as antioxidant, anti-inflammatory, cardioprotective and anti-apoptotic effects (Smoliga et al., 2011; Singh et al., 2013). Recently, a number of studies have focused on the neuroprotective and anti-apoptotic effects (Smoliga et al., 2011; Singh et al., 2013). To examine whether resveratrol could attenuate the diabetes-induced cognitive impairments, we tested the learning and memory using the MWM test and the results are shown in Fig. 1. The mean escape latency for the trained rats was decreased over the course of the learning trials in all groups (Fig. 1A). From the third day onwards there was a significant difference in transfer latency between the diabetic and the non-diabetes control rats [F (4,45) = 11.545, (P < 0.01)], while treatment with resveratrol at doses of the 10 mg/kg and 20 mg/kg (P < 0.01) significantly decreased the transfer latency as compared to the diabetic rats. However, there was no significant difference in escape latency between the resveratrol (20 mg/kg) control group and the non-diabetes control group.

In the probe trial of the MWM test, which assesses how well the animals have learned and consolidated the platform location during the four days of training, the animals showed a significant difference. The percentage of time spent in the target quadrant (Fig. 1C) and the number of crossings of the platform area (Fig. 1D) were significantly [F(4,45) = 23.495, (P < 0.01); F(4,45) = 17.844, (P < 0.01)] lower in diabetic group as compared to the non-diabetes control group, reflecting impairment in memory. The percentage of time spent in the target quadrant and the number of crossings of the platform area significantly increased by resveratrol (10 mg/kg, 20 mg/kg) treatment (P < 0.01). Resveratrol (20 mg/kg) control rats exhibited performance similar to that of the control rats in the MWM test, indicating that resveratrol per se had no effect on the learning and memory in the control group (P > 0.05). No significant difference was observed in the swimming speed among the five groups during the 5-day period of the MWM test (P > 0.05; Fig. 1B), indicating that motor deficits in the rats did not contribute to the differences in escape latencies, numbers of crossings, and time spent in the target quadrant.

### 2. Results

#### 2.1. Effects of resveratrol on body weight and the fasting blood glucose levels

As shown in Table 1, eight weeks after streptozotocin injection, diabetic rats exhibit significantly increased (20.97 ± 0.685 mmol/L) fasting blood glucose (FBG) levels as compared to the control rats (4.33 ± 0.158 mmol/L) (P < 0.001). Also, there was a marked decline in the body weights of streptozotocin-treated rats as compared to age matched control rats (P < 0.001). One-way ANOVA revealed that chronic treatment with resveratrol (10, 20 mg/kg) significantly reduced the FBG levels [F(4, 45) = 178.602, P < 0.05] and increased body weights [F(4, 45) = 260.995, P < 0.05] in diabetic rats. Whereas resveratrol per se had no influence on FBG levels and body weight.

#### 2.3. Effects of resveratrol on parameters of oxidative stress in the hippocampus

#### 2.3.1. Effects of resveratrol on diabetes-induced changes in lipid peroxidation

Effects of chronic treatment with resveratrol on lipid peroxidation (LPO) are depicted in Fig. 2A. Malondialdehyde (MDA) levels were significantly increased in the hippocampus [F(4,25) = 45.318, P < 0.001] of diabetic rats as compared to the non-diabetic control animals. However, 8-week resveratrol (10, 20 mg/kg) treatment significantly inhibited the elevation of MDA levels as compared to diabetic rats in the hippocampus (P < 0.05, P < 0.001). However, resveratrol per se did not alter MDA levels in different brain areas of control rats.

#### 2.3.2. Effects of resveratrol on diabetes-induced changes in the antioxidant profile

The reduced glutathione (GSH) levels [F(4,25) = 39.475, P < 0.001, Fig. 2B] and enzyme activities of superoxide dismutase (SOD) [F(4,25) = 29.875, P < 0.001, Fig. 2C] and catalase (CAT) [F(4,25) = 45.473, P < 0.001, Fig. 2D] significantly decreased in the hippocampus of diabetic rats as compared to the non-diabetic control group. This reduction was significantly improved by the treatment with resveratrol in the hippocampus of diabetic rats. However, resveratrol per se did not influence the endogenous antioxidant profile.

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### Table 1 (Results 2.1). Effect of resveratrol on body weight and blood glucose levels (mean ± S.E.M. of 10 observations) in the groups of rats at the onset and the end of the experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Plasma glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Onset of study</td>
<td>End of study</td>
</tr>
<tr>
<td>Control</td>
<td>220.6 ± 4.12</td>
<td>364.6 ± 5.34</td>
</tr>
<tr>
<td>Diabetic</td>
<td>215.1 ± 3.41</td>
<td>175.8 ± 5.82</td>
</tr>
<tr>
<td>Diabetic/RV</td>
<td>215.0 ± 3.62</td>
<td>197.3 ± 6.74</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>219.4 ± 3.92</td>
<td>212.9 ± 4.67</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>210.4 ± 1.64</td>
<td>361.8 ± 5.97</td>
</tr>
</tbody>
</table>

* P < 0.001, compared to the control group

* P < 0.05, compared to the diabetic group.

* P < 0.01, compared to the diabetic group.
2.4. Effects of resveratrol on inflammatory cytokines in the hippocampus

2.4.1. Effects of resveratrol on the levels of TNF-α and IL-1β mRNAs in the hippocampus

The effects of resveratrol on the levels of TNF-α and IL-1β mRNAs in the hippocampus of diabetes were investigated by amplifying the mRNA signals using RT-PCR. Fig. 3 showed that the mRNA levels of TNF-α and IL-1β were increased in the hippocampus of diabetic rats as compared to the non-diabetic control group \(F(4,10) = 37.894, P < 0.001; F(4,10) = 78.243, P < 0.001\). However, resveratrol (10, 20 mg/kg) significantly decreased the mRNA levels of TNF-α \((P < 0.05, P < 0.01)\) and IL-1β \((P < 0.01)\) as compared to diabetic rats in the hippocampus. However, resveratrol per se did not influence the levels of TNF-α and IL-1β mRNAs in the hippocampus.

2.4.2. Effects of resveratrol on the levels of TNF-α and IL-1β inflammatory activity in the hippocampus

The effects of resveratrol on the expression of TNF-α and IL-1β in the hippocampus of diabetes were investigated by ELISA. Fig. 4 showed that the levels of TNF-α and IL-1β were increased in the hippocampus of diabetic rats as compared to the non-diabetic control group \(F(4,25) = 58.348, P < 0.001; F(4,25) = 69.393, P < 0.001\) as compared to the non-diabetic control group. However, resveratrol (10, 20 mg/kg) significantly decreased the levels of TNF-α \((P < 0.001, P < 0.01)\) and IL-1β \((P < 0.001, P < 0.01)\) as compared to diabetic rats in the hippocampus. However, resveratrol per se did not influence the levels of TNF-α and IL-1β in the hippocampus.

2.5. Resveratrol enhances synaptic protein levels in the hippocampus

The levels of SYN and GAP-43 expression were reduced in the hippocampus of diabetic rats versus the non-diabetic control group \(F(4,10) = 6.727, P < 0.01; F(4,10) = 31.591, P < 0.01\) as depicted in Fig. 5. The 8-week resveratrol (10, 20 mg/kg) treatment significantly increased the SYN \((P < 0.05, P < 0.01)\) and GAP-43 \((P < 0.01)\) levels in the hippocampus of diabetic rats. However, resveratrol per se did not influence the levels of SYN and GAP-43 expression in the hippocampus.

3. Discussion

It is becoming increasingly clear that the brain is another site of diabetic end-organ damage and cognitive dysfunction has been listed as one of the many complications of diabetes, along with retinopathy, nephropathy, and cardiovascular disease (Mijnhout et al., 2006; Tuzcu and Baydas, 2006; Ho et al., 2013; Thomas et al., 2013). Lack of satisfactory treatment of the cognitive deficits usually accompanying stress, depression, and associated mental problems present a constant challenge for psychopharmacology research. Traditional hypoglycemic agents have several disadvantages and ill effects. Therefore, employment of safe natural products can be an ideal choice. Resveratrol has a variety of biological effects including anti-inflammatory, antioxidant and neuroprotective activities (Zhu et al., 2008; Fabris et al., 2008; Jing et al., 2013). Although some studies have investigated the neuroprotective actions of resveratrol in diabetes animal models (Kumar et al., 2007; Schmatz et al., 2009; Jing et al., 2013) the effects of
this compound on hippocampal structural synaptic plasticity has not been reported in the literature. The current study demonstrates that structural synaptic plasticity impairment in brain in diabetic condition is one of the reasons for diabetes-associated cognitive decline, and resveratrol significantly enhances the learning ability of the diabetic rats induced by STZ, which is largely due to its anti-oxidant as well as anti-inflammatory activity, and finally facilitate hippocampal structural synaptic plasticity.

The pathogenesis of cognitive impairment caused by diabetes seems to be a multifactorial process (Kodl and Seaquist, 2008). Oxidative stress and inflammation are involved in the pathogenesis of diabetes associated cognitive impairment (Mastrocola et al., 2005; Kuhad and Chopra, 2008; Kuhad et al., 2009). Hyperglycemia results in increased nonenzymatic glycation of intracellular and extracellular proteins, and the eventual formation of advanced glycation end products (AGEs) on these proteins. It is reported that the interaction of AGEs with their receptor elicits the production of reactive oxygen species (ROS), at least in part via the activation of NADPH oxidase (Wautier et al., 2001; Zhang et al., 2011). In both humans and diabetic rats, oxidative stress seems to play a central role in neuronal damage (Arvanitakis et al., 2001; Zhang et al., 2011). Oxidative stress contributes to increased neuronal damage and death through protein oxidation, DNA damage, and peroxidation of membrane lipids (Hawkins and Davies, 2001). In the present study, lipid peroxidation levels were significantly increased in the hippocampus of diabetic rats, whereas reduced GSH, SOD and CAT activities were dramatically reduced in the hippocampus of diabetic rats, which is in agreement with the previous findings (Tuzcu and Baydas, 2006). However, chronic treatment with resveratrol remarkably attenuates all the above alterations of the diabetic condition. ROS can activate nuclear factor-κB (NF-κB) via activation of the mitogen-activated protein kinase signaling pathway (Witte et al., 2010). NF-κB has been shown to modulate gene transcription for the generation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Neumann et al., 1999). Pro-inflammatory cytokines are known to be elevated in several neuropathological states that are associated with learning and memory. In the current study, learning and memory abilities are significantly impaired in STZ-induced diabetic rats, accompanied by the markedly elevated TNF-α and IL-1β levels in the hippocampus, which is mediated by activation of NF-κB signaling. However, chronic administration of resveratrol could remarkably attenuate cognitive deficits caused by diabetes, and could significantly decrease TNF-α and IL-1β levels in the hippocampus of diabetic rats, suggesting that reduction of TNF-α and IL-1β level may contribute to the amelioration of diabetes-associated cognitive decline.

Experimental studies show that the inhibition of long term potentiation (LTP) in the dentate gyrus region of the rat hippocampus by TNF-α, represents a biphasic response, an early phase dependent on p38 mitogen activated protein kinase activation and a later phase, possibly dependent on protein synthesis. LTP inhibition by TNF-α is dependent upon the activation of TNF receptor 1 and mGlu5-receptors along with involvement of ryanodine-sensitive intracellular Ca²⁺ stores (Cumiskey et al., 2007). And it has also been reported that oxidative damage to rat synapses contributes to cognitive deficit (Tuzcu and Baydas, 2006). It is widely recognized that there is relevance between hippocampal synaptic plasticity and memory (Gruart et al., 2006). Synaptic structure is the morphological basis for learning and memory. A close relationship exists between the number of synapses and

Fig. 2. (Results 2.3). Effects of resveratrol on lipid peroxide (A), reduced glutathione (B), superoxide dismutase (C) and catalase (D) levels in the brain. Data are reported as mean ± S.E.M. (n = 6). *P < 0.001 vs. the control group; **P < 0.05, ***P < 0.001 vs. the diabetic group.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)
cognitive decline in patients with diabetic dementia (Zhou et al., 2007); therefore, synaptic loss is considered the best pathological correlate of cognitive impairment (Coleman and Yao, 2003). We focused on synaptic plasticity markers (i.e., SYN and GAP-43) involved in synaptic transmission and long-term potentiation (LTP) in hippocampal pyramidal cells, to further explore the regulatory mechanisms of resveratrol in diabetes animal models. Compelling evidence supports the role of SYN and GAP-43 in stimulating synapse formation and reconstruction (Kwon and Chapman, 2011). SYN is a presynaptic vesicle protein, and it is used as a specific protein marker for the presynaptic terminal, and its level is closely related to the synaptic density. The neuronal growth-associated protein-43 (GAP-43) is a presynaptic membrane phosphoprotein involved in guiding the growth of axons and modulating the formation of new connections (Farina et al., 2004). SYN as well as GAP-43 expression are thought to be correlated with synapse number (Hu et al., 2007), synaptic plasticity in the brain (Ding et al., 2002), and learning and memory abilities (Rune and Frotscher, 2005; Gruart et al., 2006). In our study, SYN as well as GAP-43 expression were lower in the STZ rats than in controls. Resveratrol had no effect on SYN as well as GAP-43 expression in control/RV rats, but it did increase SYN as well as GAP-43 expression in diabetic/RV rats. As synaptic markers are acknowledged to represent changes in synaptic structure (Kwon and Chapman, 2011), these results indicated that the effect of resveratrol on synaptic structure was closely associated with the protein expression of SYN and GAP43. The correlation is closely associated with structural plasticity.
Taken together, the findings of the present investigation suggest that resveratrol exerts its beneficial effects on STZ-induced memory dysfunction may be attributed to its antioxidant and anti-inflammatory activities. And resveratrol enhances synaptic markers (SYN and GAP-43), modulates the fundamental of synaptic structure, and improves cognitive function, which could find clinical use in treating cognitive and neural dysfunction in diabetics. But to elucidate the exact mechanism of its effect, and to examine its potential therapeutic effects further studies are necessary.

4. Experimental procedures

4.1. Animals and housing

Male Sprague-Dawley rats (200–250 g) of 10–12 weeks of age, in the Center of Experimental Animal, Taishan Medical College (Taian, China), where a SPF level laboratory was founded, authorized at by Shandong provincial government. The animals were housed in cages in a temperature and humidity-controlled environment and were maintained on a 12–12 h light-dark cycle. They were allowed free access to food and water. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Taishan Medical College, Taian, China. All efforts were made to minimize animal suffering.

4.2. Induction and assessment of diabetes

A single dose of 60 mg/kg streptozotocin (Sigma Chemical Co., St. Louis, MO, USA) prepared in citrate buffer (pH 4.4, 0.1 M) was injected intraperitoneally to induce diabetes. The age-matched control rats received an equal volume of citrate buffer only. Diabetes was confirmed after 72 h of streptozotocin injection, the fasting blood glucose (FBG) levels were estimated by glucose oxidase peroxidase diagnostic enzyme kit. The rats with FBG level higher than 11.1 mM (200 mg/dl) were considered to be diabetic rats and used for the present study. Body weight and FBG levels were measured before and at the end of the experiment to see the effect of resveratrol on these parameters.

4.3. Treatment schedule

Rats were randomly selected and divided into five groups of 15 animals each, i.e. control, diabetic control, diabetic/RV 10 mg/kg, diabetic/RV 20 mg/kg, and control/RV 20 mg/kg. Resveratrol (Sigma Chemical Co., St. Louis, MO, USA) was suspended in 0.5% w/v sodium carboxymethylcellulose (CMC-Na) solution. The control and diabetic control groups received 0.5% CMC-Na solution only, while the other groups received solution of resveratrol (10 and 20 mg/kg/day; p.o.). The treatment starts from the fifth day of experiment and for 8 weeks once a day and all these solutions were administered in a constant volume of 1 ml/100 g b.w. of rat. After the treatment period, animals were tested for learning and memory task in Morris water maze for five consecutive days. At the end of experiment, all animals were sacrificed under deep anesthesia, blood was collected by femoral vein bleeding and serum was separated. Brains were rapidly removed, and both hippocampi of each were isolated. The samples were stored at –80 °C until utilization.

4.4. Behavioral assessment

4.4.1. Morris water maze test

Animals were tested in a spatial version of Morris water maze test (Morriss et al., 1982; Tuzcu and Baydas, 2006). The apparatus consisted of a circular water tank (150 cm in diameter and 50 cm high), a platform (17 cm in diameter and 31 cm high) invisible to the rats, and a computer program for automatically tracking and
analyzing the path of an animal swimming in a large pool of water. The tank was filled with water maintained at approximately 25 ± 0.5 °C at a height of 32 cm, and the platform was inside and located in middle of a certain quadrant about 22 cm distance from the tank wall. The tank was located in a large room where there were several brightly colored cues external to the maze; these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for 5 consecutive days. The rats received four consecutive daily training trials in the following 5 days, with each trial having a ceiling time of 90 s and a trial interval of approximately 30 s. For each trial, each rat was put into the platform at one of four starting positions, the sequence of which was selected randomly. During test trials, rats were placed into the tank at the same starting point, with their heads facing the wall. The rat had to swim until it climbed onto the platform submerged underneath the water. After climbing onto the platform, the animal remained there for 20 s before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the rat failed to reach the escape platform within the maximally allowed time of 90 s, it was gently placed on the platform and allowed to remain there for the same amount of time (Kuhad and Chopra, 2007, 2008). The time to reach the platform (latency in seconds) was measured.

4.4.2. Memory consolidation test

A probe trial was performed (Tuzcu and Baydas, 2006) wherein a probe of memory consolidation was assessed. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe trial, the hidden platform was removed from the pool, and the rat was placed into the pool as in the training trial. The number of times crossing over the platform site of each rat was also measured and calculated. Further, the percentage of time spent in the former platform quadrant was also taken as a measure of spatial memory retention.

4.5. Measurement of oxidative stress

4.5.1. Determination of MDA level in brain

The content of MDA, an index of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills (Wills, 1966). MDA can be condensed with thiobarbituric acid, which forms a pink product with maximal absorption at 532 nm. Determination of MDA level was performed with a commercial kit (Jiancheng Biotech Co., Nanjing, China). Brain MDA content was expressed as nmol of malondialdehyde per mg protein.

4.5.2. Estimation of GSH level in brain

GSH estimation was done according to the method of Ellman (1959). Briefly, 160 μl of supernatant was added to 2 ml of Ellman’s reagent (5’5’ dithiobis [2-nitrobenzoic acid] 10 mM, NaHCO3 15 mM) and the mixture was incubated at room temperature for 5 min and absorbance was read at 412 nm.

4.5.3. Estimation of SOD activity in brain

Measurements were made as described by Sun et al. (1988). SOD activity was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitro-blue tetrazolium (NTB) to form formazan dye. SOD activity was then measured at 550 nm by the degree of inhibition of this reaction. One unit of enzyme was defined as the amount of enzyme required to inhibit the inhibition rate of 50%. The activity of superoxide dismutase was expressed as units/mg protein.

4.5.4. Estimation of CAT

CAT activity was assayed by the method of Aebi (1984). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml post mitochondrial supernatant (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. The activity of catalase was expressed as units/mg protein.

4.6. TNF-α and IL-1β assay

4.6.1. Reverse transcriptase-PCR (RT-PCR)

For mRNA quantification, total RNA was extracted from hippocampus using the RNAqueous kit (Applied Biosystems, Foster city, CA). The cDNA was synthesized using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster city, CA) according to the manufacturer’s protocol. In brief, 1 μg of total RNA was used for cDNA synthesis. PCR was carried out on cDNA using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) specific for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; assay). GAPDH was used as housekeeping gene in order to normalize IL-1β and TNF-α expression levels. PCR amplification of GAPDH, IL-1β and TNF-α messages was performed using the following primers (Sangon Biotech Co., Ltd.). For GAPDH, forward 5’-TGTGCTCGTCGGATCTGA-3’ and reverse 5’-CTTCTCTCACACCTTTCTGA-3’; for IL-1β, forward 5’-ATGTTCTGTCGGCTGAGCTT-3’ and reverse 5’-TGCCGACATGTCGTTT-3’; for TNF-α, forward 5’-CAAACACTGAGTACAAGCC-3’ and reverse 5’-GAGCTCGTATGCTTAAGT-3’. The samples were first denatured at 94 °C for 3 min, followed by 30 PCR cycles; the temperature profile was 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min. After the last cycle, an additional extension incubation of 5 min at 72 °C was performed. Amplified products were separated by electrophoresis on 1% agarose gel, visualized under UV transilluminator, and then photographed. To verify reproducibility, each brain sample was analyzed in duplicate in two independent experiments for each gene. The values obtained for the target gene expression were normalized to GAPDH and quantitated relative to the expression in control samples. The products were analyzed by densitometry using an image-processing and analysis system (Image) 1.37 software, NIH, USA, and quantities of each product were calculated relative to GAPDH.

4.6.2. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β and TNF-α in the hippocampus were analyzed by ELISA kits (Bios Company, Beijing, China) according to the manufacturer’s instructions. The results are expressed as pg/ml.

4.7. Western blot analysis

The following antibodies were used for analysis: anti-SYN (monoclonal antibody, 1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-GAP-43 (monoclonal antibody, 1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-β-actin (monoclonal antibody, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Hippocampus tissues (30 mg) were homogenized with 0.3 ml of ice-cold buffer containing 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1% Triton X-100, 30 mM Na3P2O7, 50 mM NaF, 5 μM ZnCl2, 100 μM Na2VO4, 1 mM dithiothreitol, 5 mM okadaic acid, 2.5 μg/ml apro tinin, 3.6 μM pepstatin, 0.5 μM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, and 5.3 μM leupeptin. The homogenate was centrifuged at 15,000g for 5 min at 4 °C. The cytosol (supernatant) and nuclear (pellet) fractions were collected. The pellet was re suspended in 100 μl of ice-cold buffer and sonicated for 10 s. The supernatants were collected and centrifuged again with the final supernatants were collected. Protein concentrations in the
supernatants were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Samples were heated at 100 °C for 5 min before gel loading and were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with 5% nonfat dry milk in a washing buffer (Tris-buffered saline containing 0.05% (v/v) Tween-20) for 2 h at 25 °C and subsequently incubated overnight at 4 °C with the primary antibody. Each membrane was washed three times for 15 min followed by incubation with the horseradish peroxidase-linked secondary antibody for 2 h at 37 °C: anti-rabbit antibody or anti-mouse antibody (both obtained from Santa Cruz Biotechnology, CA, USA). After washing the binding was detected by ECL detection reagents. Films were scanned and subsequently analyzed by measuring optical densities of immunostained bands using an image-processing and analysis system (ImageJ 1.37 software, NIH, USA), and the densitometric plots of the results were normalized to the intensity of the β-actin band.

4.8. Statistical analysis

All statistical analyses were performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Values were expressed as mean ± SEM (Standard Error of the Mean). For the Morris water maze test, data were analyzed with a Kruskal-Wallis non-parametric ANOVA test. If the results were significant, the intergroup variation was measured by the Tukey’s post hoc test. Statistical significance was set at P < 0.05. For biochemical assays, differences between groups were analyzed with one-way analysis of variance (ANOVA) followed by the Dunnett’s post hoc test. Results were considered significant if P < 0.05.

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