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ORIGINAL ARTICLE

L-3-n-butylphthalide attenuates cognitive deficits in db/db diabetic mice

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Abstract

Numerous epidemiological studies have shown that diabetes mellitus (DM) is associated with dementia and cognition decline. However, there is currently no effective treatment for diabetes-induced cognitive dysfunction. The neuroprotective effect of L-3-n-butylphthalide (L-NBP) has been demonstrated in vascular dementia animal models. The purpose of this study was to determine whether L-NBP can ameliorate cognitive deficits in db/db mice, a model of obesity and type 2 diabetes. The mice were administered with vehicle or L-NBP (120 mg/kg) by gavage daily for 6 weeks. Then, Morris water maze tasks were performed, and hippocampal LTP was recorded in vivo. Next, the synaptic structure of the CA1 hippocampus region was investigated via electron microscopy. Finally, the expression levels of MDA, SOD, 8-OHdG, and NADPH oxidase subunits gp91 and p67, as well as the expression of NF-κB p65, TNF-α, IL-1β and caspase-3 were measured by Western blot, RT-PCR and ELISA. Treatment with L-NBP significantly attenuated the learning and memory deficits in db/db mice. Concomitantly, L-NBP also increased hippocampus synaptic plasticity, characterized by an enhanced in vivo LTP, and suppressed oxidative stress, as indicated by increased SOD activity and decreased MDA, 8-OHdG, and NADPH oxidase subunits p67 and gp91. L-NBP also significantly decreased NF-κB p65, TNF-α, IL-1βand caspase-3 levels in the hippocampus. L-NBP significantly ameliorated cognitive decline in type 2 diabetic mice, and this effect was accompanied by an improvement in hippocampal plasticity and an amelioration of oxidative stress, inflammation and apoptosis cascades. Thus, L-NBP may be a promising therapeutic agent against DM-mediated cognitive dysfunction.

Keywords L-3-n-butylphthalide · Diabetes · Cognitive dysfunction · LTP · Oxidative stress · Inflammation · Apoptosis

Introduction

Numerous clinical and epidemiological studies have demonstrated a close relationship between diabetes mellitus (DM) and dementia (Cooray et al. 2008; Plastino et al. 2010). Patients with type2 diabetes have twice the risk of developing Alzheimer's disease (AD) and other types of dementia as individuals with normal blood glucose levels. There is growing evidence that type2 diabetes is accompanied by poor neurocognitive outcomes including impaired working and semantic memories and visuospatial ability. These findings highlight the importance of diabetes-induced cognitive impairment. Thus, there is an urgent need to determine the pathogenesis of DM-mediated cognitive dysfunction and further explore potential treatments to prevent or ameliorate these cognitive symptoms.

Cognitive decline in diabetes could be caused by various factors (Kodl and Seaquist 2008). Some evidence has demonstrated that DM-mediated cognitive dysfunction strongly correlates with hippocampal synaptic plasticity. Synaptic plasticity includes changes in the efficacy of synaptic transmission and synaptic structure. Structural plasticity includes morphology of dendrite spines as well as the expression of synaptic proteins, for example synaptophysin. Furthermore, long-term potentiation (LTP) of synaptic transmission is deemed to be the basis for the molecular mechanism of learning and

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memory (Lüscher and Malenka 2012). A recent study reported that disturbed hippocampal LTP correlated well with learning disturbance in streptozotocin (STZ)-induced rats (Davari et al. 2013).

Oxidative stress is regarded as a basic mechanism behind the development of diabetes (Aly and Mantawy 2012). An imbalance between oxidants and antioxidants in the brain in the diabetic state eventually contributes to a series of oxidative damage events. A previous investigation revealed that oxidative stress may be associated with learning and memory deficits in various brain regions of rats (Fukui et al. 2001). Thus, application of antioxidants could prevent neural injury in various neurodegenerative conditions (Baydas et al. 2004). In addition, it is widely known that inflammation is involved in the onset of DM and progression of its complications (Kalmijn et al. 2000). Numerous proinflammatory markers and cytokines, such as tumor necrosis factor-(TNF-) α, interleukin-(IL-) 6, and IL-1β, were shown to be increased in DM (King 2008). It was further ascertained that many of the proinflammatory markers were associated with cognitive dysfunction and dementia (Akiyama et al. 2000). Yaffeet al. (Yaffe et al. 2006) reported that cognitive decline was observed in DM patients with unregulated CRP and IL-6 levels but not in patients with normal marker levels.

L-3-n-butylphthalide (L-NBP) was extracted as a pure component from seeds of *Apium graveolens* Linn, Chinese celery. Previous studies indicated that L-NBP showed potent neuroprotective effects in middle cerebral artery occlusion rats by decreasing oxidative damage (Dong and Feng 2002), inhibiting inflammatory responses (Hao-Liang and Yi-Pu 2000) and reducing neuronal apoptosis (Chang 2003). In addition, Xu et al. reported that L-NBP could attenuate spatial learning deficits caused by chronic cerebral ischemia in rats by facilitating LTP (Xu et al. 2012). The positive effects of L-NBP on ischemic cerebrovascular disease have been verified in ischemic patients and animal models, but little is known about the effect of L-NBP on DM-mediated cognitive dysfunction. Therefore, we set out to examine the effects of long-term L-NBP treatment on learning and reference/working memory, inflammation, oxidative stress, and electrophysiological behavior in db/db mice, a classic model of type 2diabetes. The results indicate that L-NBP may be a promising therapeutic agent against diabetic cognitive decline.

Results

L-NBP treatment ameliorated learning and memory impairment in db/db mice

Outcomes were evaluated blindly. After treatment of db/db mice with L-NBP, the time course of non-fasting blood glucose and body weight was tested. There was no difference in blood glucose ($P > 0.05$; Fig. 1a) and body weight ($P > 0.05$; Fig. 1b) between the L-NBP group and db/db group.

In the place navigation trial, the escape latencies among the groups were not significantly different on the first day ($P > 0.05$; Fig. 1c). Interestingly, from the second day to the fifth day, the escape latency in the db/db group was longer than that of the control group ($P < 0.05$; effect of day-by-group interaction, $P > 0.05$; Fig. 1c), and L-NBP treatment significantly decreased the prolonged escape latency in the db/db + L-NBP group ($P < 0.05$; effect of day-by-group interaction, $P > 0.05$). In the spatial probe trial, the number of individuals in the db/db group crossing the platform was markedly reduced compared with that of the control group ($P < 0.05$; Fig. 1d, e). In contrast, the db/db mice treated with L-NBP showed a significantly increased number of target crossings compared to that of the db/db group ($P < 0.05$).

The effects of L-NBP on hippocampal LTP of db/db mice

The results of the hippocampus LTP experiment should be in line with those of the behavioral tests measuring hippocampus-dependent learning and memory. The results indicated that hippocampal LTP was modestly inhibited in the db/db group (130.97 ± 14.08%) after HFS, persisting for more than 60 min, compared with that of the control group (255.90 ± 54.24%, $P < 0.01$; Fig. 2a, b), and markedly increased in the db/db + L-NBP group (176.17 ± 18.96%, $P < 0.01$).

Effect of L-NBP treatment on synaptic ultrastructure

Electron microscopy was used to observe morphological changes of the synapse in the hippocampusCA1 region. In CA1 neurons of the control group, presynaptic and postsynaptic membranes with intact boundaries were clear and synaptic vesicles were clearly visible. Uniform and compact PSD were also observed that mostly had a disked or fenestrated, irregular shape (Fig. 3a). However, in the db/db group, synaptic components were incomplete, presynaptic and postsynaptic membranes were unclear, and the number of synaptic vesicles was significantly decreased. Therefore, typical synaptic structures were not observed, and some synapses seemed to show vacuolar degeneration of neuronal processes (Fig. 3b). In contrast, the synaptic ultrastructure of the db/db + L-NBP group was significantly better than that of the db/db group yet worse than that of the control group. Thus, presynaptic and postsynaptic structures were relatively clear and intact (Fig. 3c).

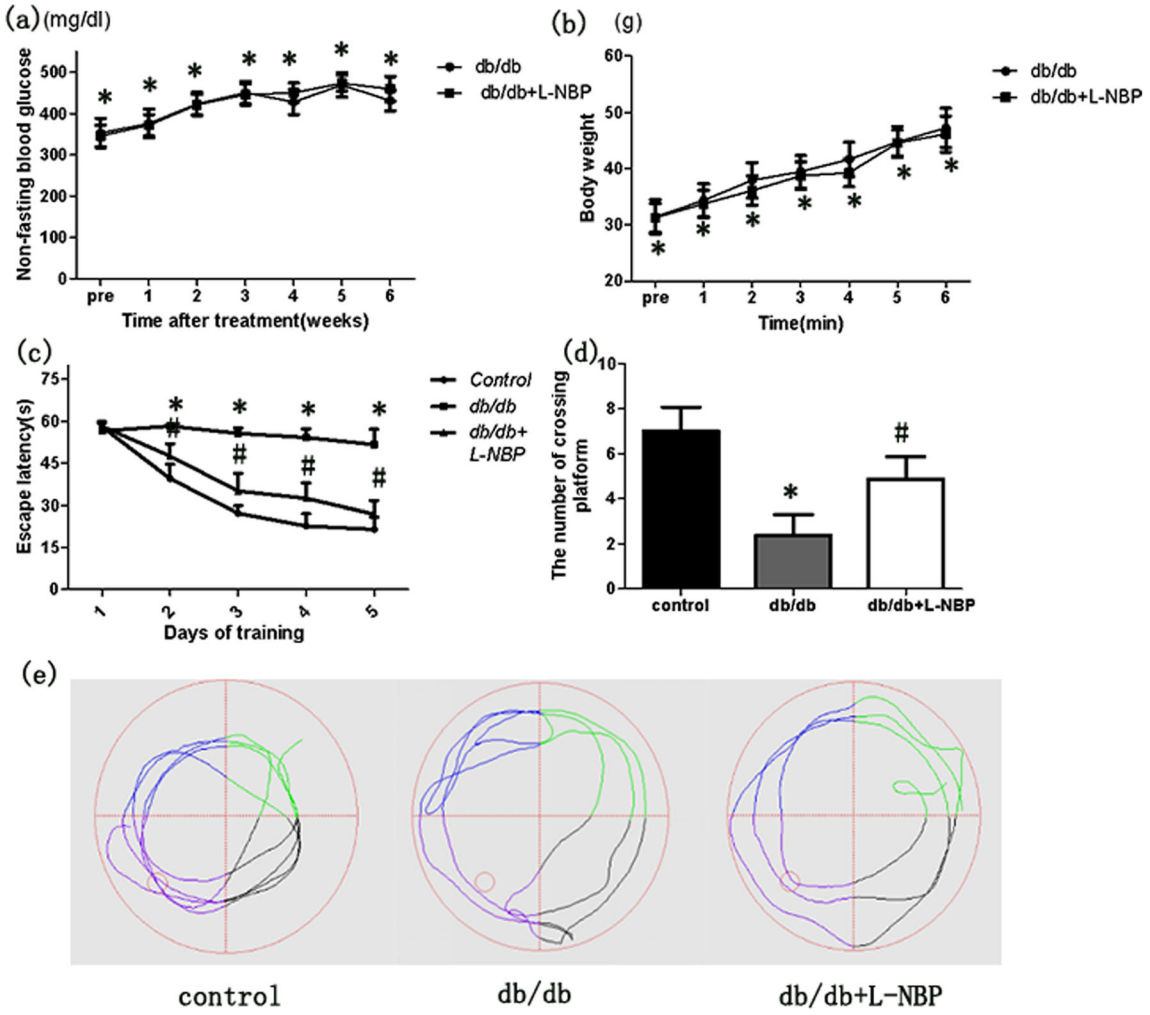

Fig. 1 L-NBP treatment ameliorates learning and memory impairment in db/m and db/db mice. Effects of L-NBP administration on non-fasting blood glucose (**a**) and body weight (**b**) of db/db mice. Values are the mean ± SEM; $n = 10$. $^{*}P > 0.05$ compared with the control group. **c** Mean escape latency on 5 consecutive days in the place navigation phase. **d** The number of target crossings during the spatial probe phase. **e** Representative swim paths during the spatial probe test. Values are the mean ± SEM; $n = 10$. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the db/db group

Effect of L-NBP treatment on oxidative damage factors

The SOD level of the hippocampus in the db/db group decreased compared to that of the control group ($P < 0.01$; Fig. 4a). Additionally, the MDA ($P < 0.01$; Fig. 5b) and 8-OHdG ($P < 0.01$; Fig. 4c) levels of the db/db group were markedly increased compared to those of the control group. However, L-NBP treatment inhibited those changes in SOD, MDA and 8-OHdG levels. In addition, Western blot analysis showed that the protein levels of NADPH oxidase subunits p67 ($P < 0.01$; Fig. 4d, f) and gp91 ($P < 0.01$; Fig. 4e, f) were markedly increased in the db/db group. In contrast, reduced p67 and gp91 protein levels were found in the db/db + L-NBP group compared to those of the db/db group.

Effect of L-NBP treatment on inflammatory cytokines

Both RT-PCR and Western blot analysis showed that the mRNA and protein levels of major inflammatory factors including NF-κB ($P < 0.01$; Fig. 5a–g), TNF-α($P < 0.01$; Fig. 5b–g) and IL-1β ($P < 0.01$; Fig. 5c–g) were notably increased in the hippocampus of the db/db group, in comparison to those of the control group. However, L-NBP treatment significantly suppressed the expression of those inflammatory cytokines in the hippocampus of db/db mice.

Effect of L-NBP treatment on apoptotic factors

Both RT-PCR and Western blot analysis showed that caspase-3 levels were obviously augmented in the hippocampus of the db/db group ($P < 0.01$; Fig. 6a–c), compared with those of the

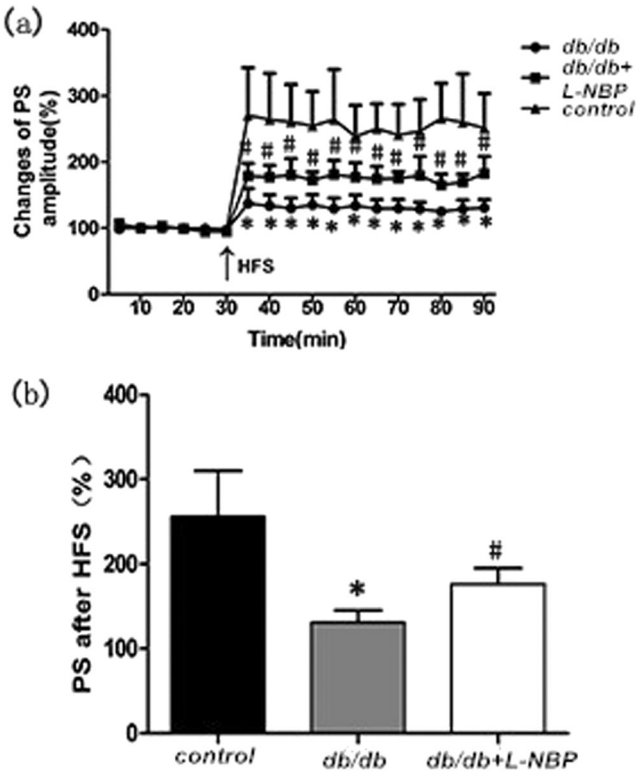

Fig. 2 Effect of L-NBP treatment on hippocampal LTP from the DG in response to stimulation of the PP in db/m and db/db mice. HFS was given at the end of 30 min of baseline recording of PS. **a** The time course of PS after HFS. **b** Summary of PS at 60 min post-HFS. Values are the mean ± SEM; $n = 6$. $^{*}P < 0.01$ compared with the control group; $^{\#}P < 0.01$ compared with the db/db group

control group. Nevertheless, L-NBP statistically diminished the content of caspase-3 in db/db mice.

Discussion

Epidemiological evidence has demonstrated that DM is associated with a high risk of dementia (Cukierman et al. 2005). However, there are currently no effective treatments to prevent or ameliorate cognitive decline.

Previous studies (Stranahan et al. 2008; Li et al. 2002) indicate that db/db mice are regarded as a classical model to study DM-mediated cognitive dysfunction because of their characteristic cognitive impairment. Therefore, in the present research, we investigated the influence of L-NBP on cognitive ability in db/db mice by analyzing behavioral, electrophysiological, and molecular and biochemical aspects of brain functions. The results demonstrate that L-NBP can decrease oxidative stress, attenuate the inflammatory response, suppress neuronal apoptosis, and improve synaptic plasticity and cognitive function. Thus, L-NBP is expected to be a promising and novel agent for treatment of cognitive dysfunction in diabetes.

In behavioral experiments, diabetic mice showed impaired cognitive function (learning and working/reference memory), as manifested in a MWM test. Escape latency of the db/db group was obviously greater from the second day to the fifth day, and the number of passages across the platform on the sixth day was smaller than that of the control group. It has been reported that diabetic rats failed on some behavioral tasks, such as passive avoidance (Grzeda et al. 2007), the radial maze and object learning (Kamal et al. 2000), and studies have further revealed that DM is associated with lower levels of global cognition, semantic memory and working memory (Arvanitakis et al. 2004; Hassing et al. 2004), as observed in our mice. Moreover, as shown by the results of the water maze test, L-NBP significantly prevented the progression of cognitive impairment in diabetic mice. Furthermore, a recent report demonstrated that L-NBP could improve learning and memory impairment in STZ-induced diabetic rats, in partial agreement with our findings (Li et al. 2014).

Learning and memory correlate well with changes in the efficacy of synaptic neurotransmission (Kandel 2001). LTP of synaptic transmission is one of the functional indexes of synaptic plasticity and is also thought to be the primary experimental model for investigating the cellular basis of learning and memory in vertebrates (Bliss and Collingridge 1993). Studies support a progressive deficit of NMDA-dependent LTP in the CA1 field in diabetic rats, gradually reaching a maximum after 12 weeks of diabetes and remaining stable thereafter (Kamal et al. 1999). Kamal et al. (Kamal et al. 2000) demonstrated that the decline in hippocampal LTP in diabetic rats is associated with learning impairments. In this study, the db/db group induced LTP of the PP-dentate granule cell synapses less than the control group did, a result supported by a previous report. L-NBP treatment ameliorated impaired LTP induced by diabetes. Meanwhile, a previous investigation suggested that L-NBP could attenuate the decrease in LTP in the hippocampus induced by chronic cerebral ischemia, a result that partially agrees with our findings (Xu et al. 2012). In the present study, MWM and LTP tests were chosen as markers for the evaluation of the effects of L-NBP on diabetic mice brain learning and memory function. The data suggested that L-NBP attenuated the injury of the hippocampus of diabetic mice. Moreover, the MWM and LTP results are also consistent with the following histological analyses.

Oxidative stress is a basic mechanism behind the development of the diabetic state (Aly and Mantawy 2012) and significantly correlates with diabetic cognitive decline. A large number of experimental and human studies have shown that DM-associated metabolic derangements result in increased neuronal and astrocytic oxidative stress impairments, necrotic brain injury, neuroinflammation and suppression of neuronal cell proliferation in different brain regions (Zhou et al. 2015; Xu et al. 2015). In addition, a previous report revealed that

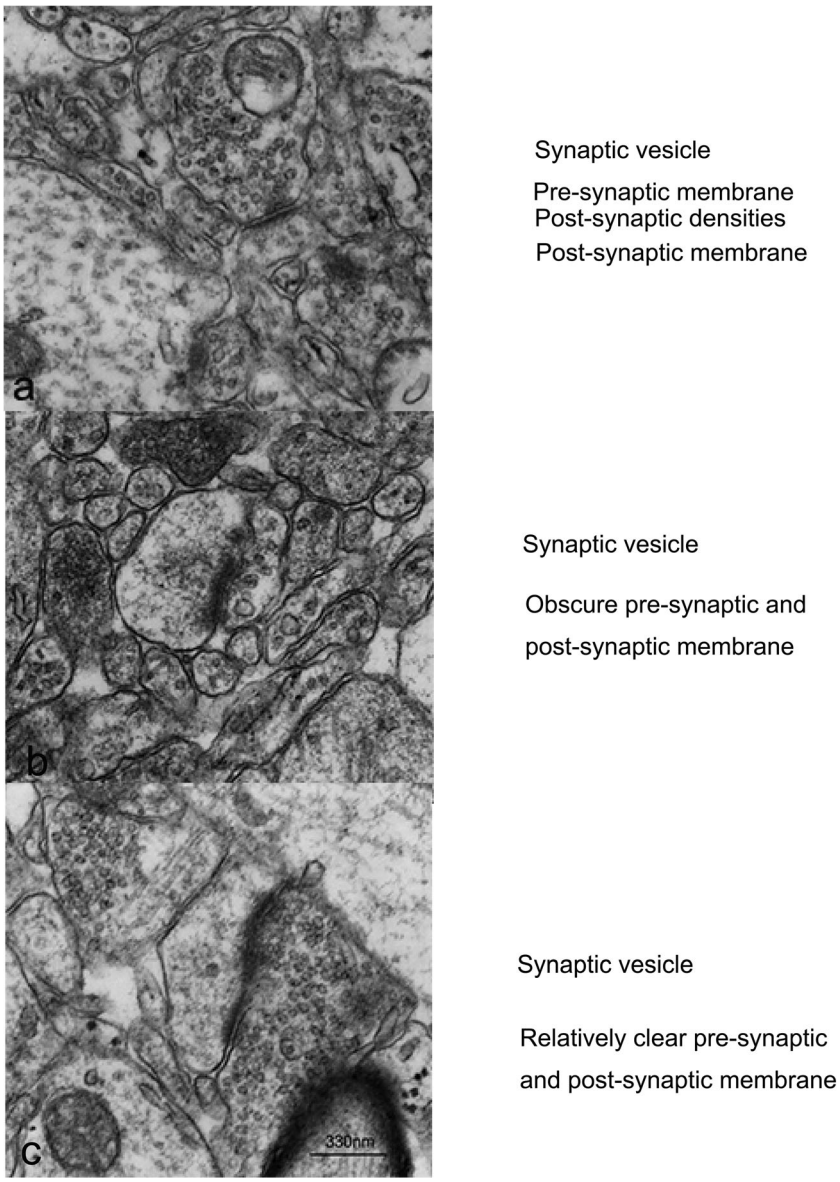

Fig. 3 Effect of L-NBP treatment on synaptic ultrastructure in the CA1 region of the hippocampus. **a** Normal synaptic components (arrows) in the control group. **b** Impaired synapses (arrows) with obscure presynaptic and postsynaptic membranes in the db/db group. **c** Relatively clear and intact presynaptic and postsynaptic structures (arrows) in the db/db + L-NBP group

oxidative damage to rat synapses may contribute to learning and memory deficits in diabetes (Tuzcu and Baydas 2006). Moreover, deregulation in the redox state of the cell leads to oxidative damage of membranes, organelles, and biomolecules such as lipids, proteins, and nucleic acids (Dasuri et al. 2013). In our current work, increased oxidants and decreased antioxidants were observed in diabetic mice, and the phenomenon was partially but significantly reversed after L-NBP treatment. Moreover, we found that L-NBP treatment significantly attenuated cerebral DNA oxidant-induced damage in db/db mice, as shown by the reduction of cerebral 8-OHdG. Meanwhile, Davaria also found an increase in the DNA degradation factor8-OHdG in STZ-injected rats (Davari et al. 2013), consistent with our result. In addition, the reduction of the levels of the hippocampus NADPH oxidase subunits gp91phox and p67 phoxalso agrees with the conclusion that L-NBP treatment significantly attenuated cerebral oxidative stress-induced diabetes.

Inflammation has been implicated in the onset and progression of DM and its complications. Accumulating evidence has indicated that a release of inflammatory markers is associated with diabetes-induced cognitive dysfunction of rats (Liu et al. 2012; Deng et al. 2013). A previous investigation suggested that nuclear factor-κB (NF-κB) is activated through the interaction of advanced glycation end products (AGEs) with the receptor of AGEs (RAGE) on macrophages (Yan et al. 1994). The NF-κB signaling pathway further modulates gene transcription for the generation of pro-inflammatory cytokines suchasIL-1βand TNF-α (Neumann et al. 1999; Kim et al. 2007). These pro-inflammatory cytokines ultimately cause neuronal damage or destruction by directly targeting neurons (Amor et al. 2010). Our current investigations indicated that

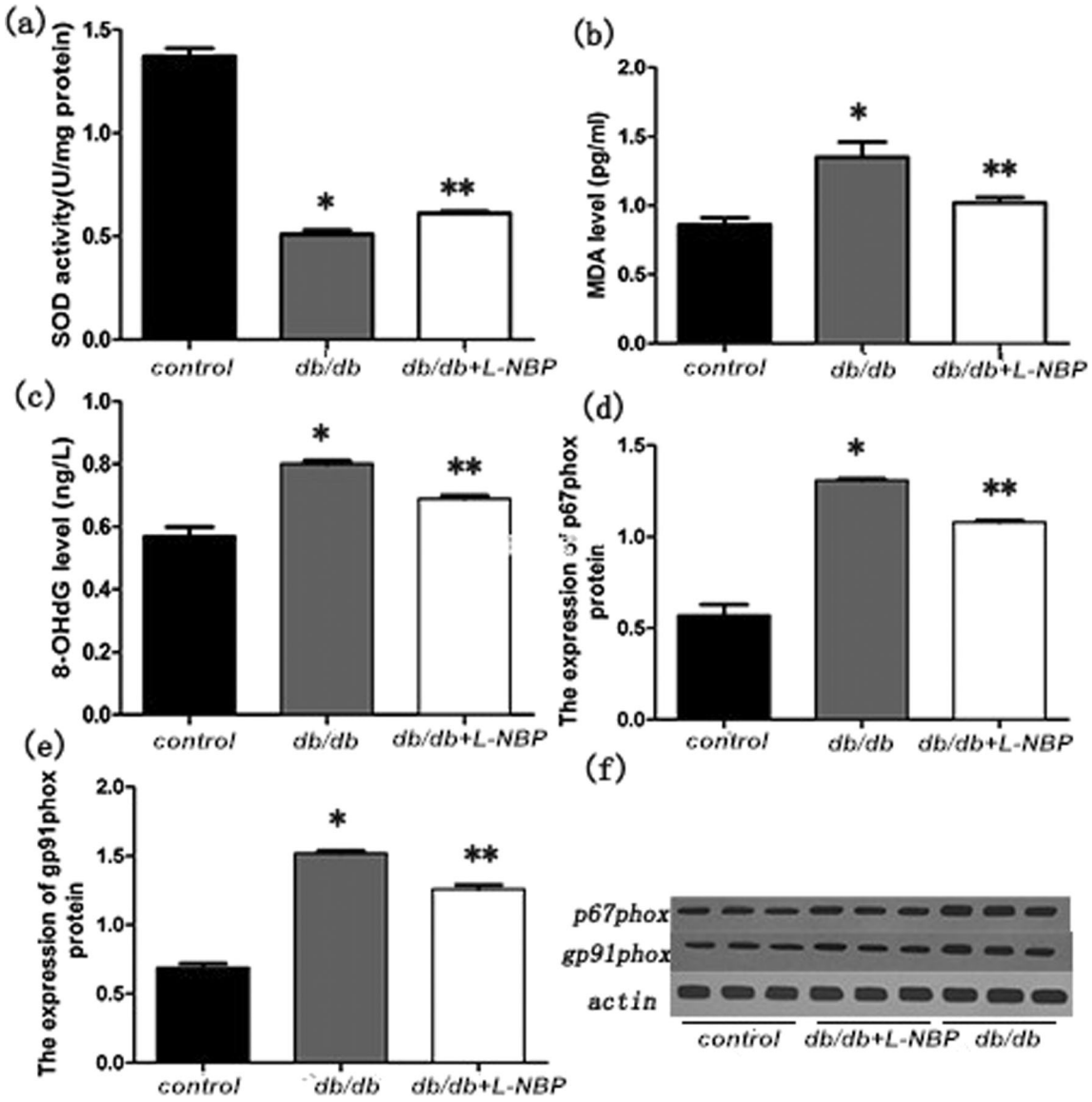

Fig. 4 Effects of L-NBP on oxidative stress in the hippocampus of db/m and db/db mice. SOD (**a**), MDA (**b**) and 8-OHdG (**c**) were detected by ELISA. Values are the mean ± SEM; $n = 6$. **f** Representative immunoblot of p67 and gp91 detected by Western blot; quantification of p67 (**d**) and gp91 (**e**) expressed as percentages is presented. Values are the mean ± SEM; $n = 3$.*$P < 0.01$ compared with the control group; **$P < 0.01$ compared with the db/db group

there was a significant increase in the expression of inflammatory markers but that L-NBP could partly suppress inflammation via inhibition of NF-κB signaling. It was previously reported that L-NBP treatment strongly inhibited the expression of proinflammatory cytokines such as TNF-α and IL-1β in LPS-treated mice, a result that was, in part, in agreement with our current findings (Zhao et al. 2016).

Oxidative stress can cause mitochondrial dysfunction, eventually resulting in a series of caspase-activated apoptosis cascades in neurons (Perry et al. 1998). Thus, we investigated the activity of apoptosis regulatory protein caspase-3 in the brain of diabetic mice, so as to better understand DM-mediated apoptosis mechanisms. In the present study, increased caspase-3 content was observed in diabetic mice. This effect was blocked by L-NBP treatment, implying that L-NBP could inhibit neuronal death in a diabetic mouse model. Consistent with this finding, L-NBP attenuated the expression of caspase-3 and Bax in the hippocampus of cerebral ischemic rats (Yang et al. 2015). In addition, Wang showed that L-NBP treatment also ameliorated serum deprivation-induced apoptosis via inhibition of the mitochondrial pathway in the cardiomyocytes of neonatal rats (Wang et al. 2014).

In conclusion, L-NBP exerts beneficial effects on learning and memory functions by improving synaptic plasticity, reducing oxidative stress, inhibiting the NF-κB signaling pathway and suppressing neuronal apoptosis in diabetic mice. These results provide support for the future use of L-NBP as a potential therapeutic agent for diabetic cognitive decline.

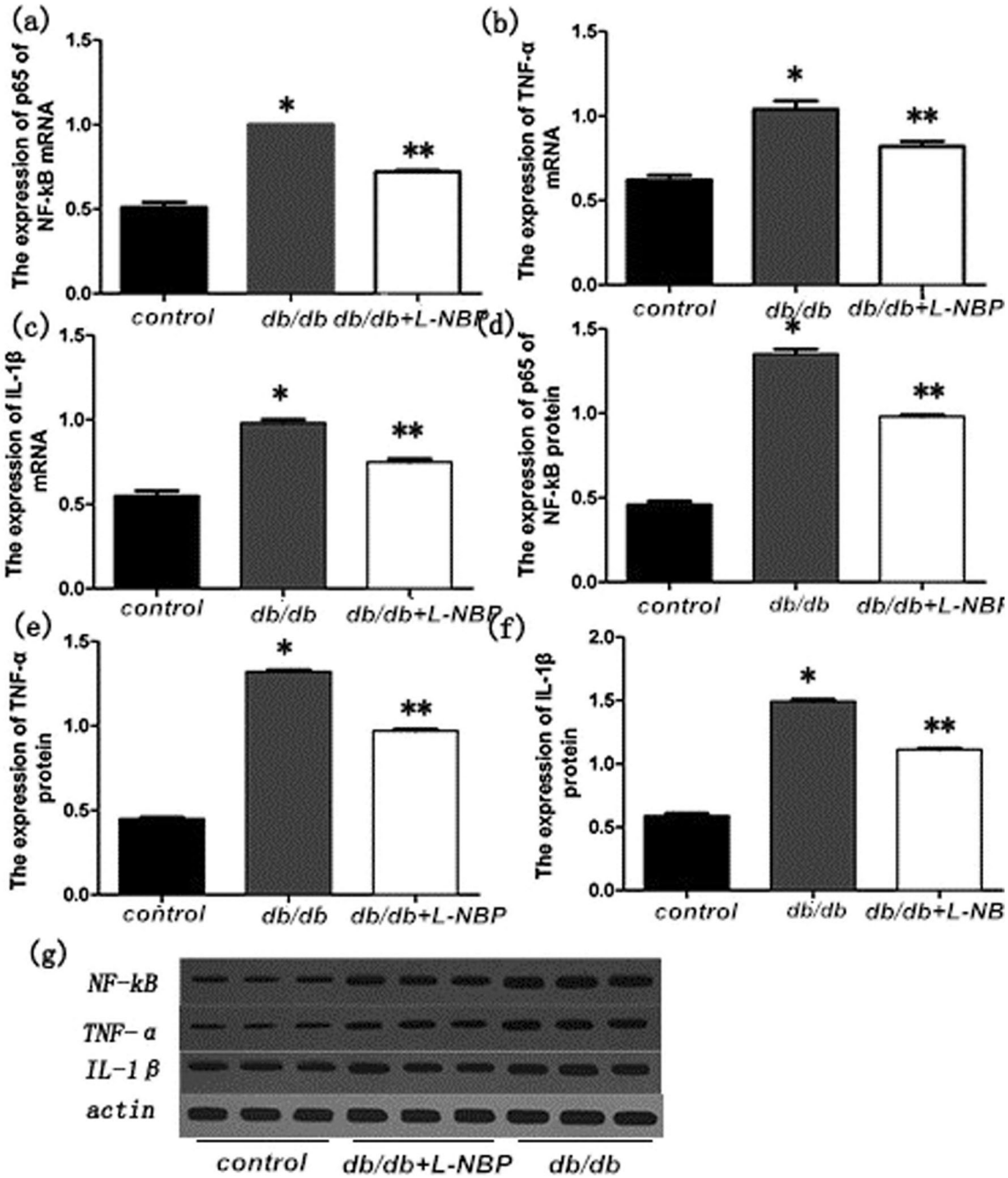

Fig. 5 Effects of L-NBP on inflammatory cytokines in the hippocampus of db/m and db/db mice. Inflammatory cytokines were detected by RT-PCR and Western blot. NF-κB p65 subunit (**a**), TNF-α (**b**) and IL-1β (**c**) mRNA quantification of expression is shown as percentages. **g** Representative immunoblot detected by Western blot; (d-f) protein quantification. Values are the mean ± SEM; $n = 3$. *$P < 0.01$ compared with the control group; **$P < 0.01$ compared with the db/db group

Methods and materials

Chemicals and materials

L-3-n-Butylphthalide (purity>98%) was supplied by CSPC, the Institute of Pharmaceutical Research, Shijiazhuang, China. It was diluted with vegetable oil as previously reported (Peng et al. 2007).

Animals

All procedures were conducted following the Regulations for the Administration of Affairs Concerning Experimental Animals. Male db/db mice (C57BLKS/J-leprdb/leprdb) and male nondiabetic db/m mice (C57BLKS/J-leprdb/+) as controls were obtained from the department of Laboratory Animal Science, Peking University Health Science Center (Beijing,

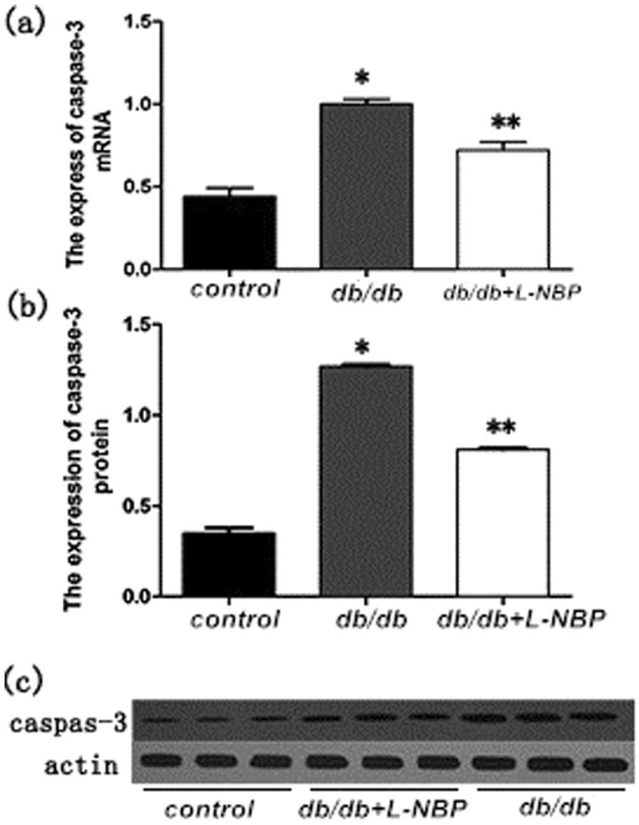

Fig. 6 Effects of L-NBP on apoptosis in the hippocampus of db/m and db/db mice. **a** Representative mRNA quantification of caspase-3 detected by RT-PCR. **c** Representative immunoblot detected by Western blot; **b** protein quantification. Values are the mean ± SEM; $n = 3$.*$P < 0.01$ compared with the control group; **$P < 0.01$ compared with the db/db group

China). The mice were housed in an animal facility with a 12-h light-dark cycle under pathogen-free conditions with access to chow and water ad libitum. The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" and approved by the institute.

Drug administration and experimental design

Seven-week-old db/db mice were randomly divided into 2 groups, with 12 animals in each group: a db/db group and a db/db + L-NBP group. The mice in the db/db + L-NBP group were administered L-NBP at a dose of 120 mg/kg by gavage once a day for 6 weeks, while the mice in the control and db/db groups were dosed with vehicle (vegetable oil). In the course of treatment, body weight and non-fasting blood glucose were measured weekly.

Behavioral testing

Twenty-four hours after the last treatment, all mice were trained and tested in the Morris watermaze (MWM) to monitor their spatial learning and memory behaviors. The apparatus consisted of a large circular water pool (120 cm in diameter and 50 cm in height), divided into 4 equal quadrants (I–IV) by two imaginary perpendicular lines, and a 10-cm-diameter platform submerged 2 cm below the water surface in the center of quadrant III. Milk was dissolved in the water, and the temperature was kept at 22 ± 2 °C. Swimming paths were video-tracked by a digital pick-up camera and analyzed with software.

Mice were allowed 60 s of free swimming and guided to climb onto the hidden platform prior to performing the water maze test. The mice had 4 consecutive training trials at 20-min intervals daily for 5 days. The time required to find the platform (escape latency) was recorded in each trial. If a mouse failed to locate the platform within 60 s, it was placed on it for 10 s, and its escape latency was recorded as 60s.In the spatial probe trial, the hidden platform was removed, and each mouse was subjected to a spatial probe test. The number of times the mouse crossed the original platform location was measured for 60s.

Electrophysiological tests

In vivo electrophysiological tests were carried out after the water maze task. The mice were anesthetized with urethane (1.2 g/kg) by intraperitoneal injection. They were then dissected in a stereotaxic frame, and recording was performed as described previously (An et al. 2011). A bipolar enamel-coated stainless steel electrode was used as the stimulating or recording electrode. The stimulating electrode was inserted 3.8 mm posterior to the bregma and 3.0 mm right of the midline, and the recording electrode was inserted 2.0 mm posterior to the bregma and 1.4 mm right of the midline. The population spikes (PS) were recorded from the DG in response to stimulation of the PP. The amplitude of PS was defined as the average amplitude from the first positive peak to the second negative peak and the amplitude from the negative peak to the succeeding positive peak. In all tests, single 100 μs test stimulation was implemented at intervals of 30s. After the responses stabilized, the stimulus parameters were adjusted to produce PS amplitude that was ~50% of the maximum responses in each test. The PS amplitude needed to be measured every 5 min. In addition, high-frequency stimulation (HFS) induced LTP with 8 pulses at 400 Hz, repeated three times per 10s. All stimulation and recordings were completed by an online oscilloscope-stimulator and data analysis interface system.

Electron microscopy

Mice were sacrificed with sodium pentobarbital (50 mg/kg, i.p.) and then intracardially perfused with 20 mL 0.9%saline, followed by 0.1 M phosphate buffer containing 4% paraformaldehyde and 1.5% glutaraldehyde (Xu and Zhang 2006).

Brains were rapidly removed, and samples were extracted from the hippocampal CA1 pyramidal cell layer. Tissues were dissected into ultrathin sections (50 nm thick) for transmission electron microscopy. Electron micrographs of synapses were obtained at 30,000× magnification using a Hitachi H-7500 TEM (Hitachi, Japan). Adobe Photoshop software was used to modify contrast and brightness of the photomicrographs.

RT-PCR and ELISA analysis

After the LTP tests, the hippocampus tissues were dissected, frozen rapidly on powdered dry ice and stored at −80 °C until processing. RNA was isolated using RNeasy mini-columns (TaKaRa, Japan) with on-column DNase treatment (TaKaRa, Japan) according to the manufacturer's protocol. RNA quantity and quality were determined using A260/A280 readings on a NanoDrop (Thermo Scientific, USA). The RNA obtained was treated with DNase (TaKaRa, Japan) to remove genomic DNA. Reverse transcription (RT) was performed following the manufacturer's protocol using a High Capacity cDNA Reverse Transcription Kit (TaKaRa, Japan). Real-time PCR was performed using a TaqMan Gene Expression assay kit (TaKaRa, Japan) according to the manufacturer's instructions. The following TaqMan probes (TaKaRa, Japan) were used: NF-κB, TNF-α, IL-1β and caspase-3. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. In addition, MDA, SOD and 8-OHdG in the hippocampus tissues were analyzed using commercial kits (Cayman, USA).

Western blot analysis

Proteins were extracted from tissue homogenates for analysis. Tissues were homogenized in 0.1 mL of ice-cold lysis buffer reagent (50 mM Tris base, 150 mM NaCl, 0.1% SDS [pH 8.0]) and centrifuged (16,000×*g*, 30 min at 4 °C). The concentrations of the protein extracts were evaluated using the BCA protein assay. Electrophoresis using a 12% polyacrylamide gel was used to separate proteins, which were then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk at room temperature and incubated overnight at 4°Cwith anti-gp91phox (91 kDa) (Santa Cruz Biotechnology, USA), anti-p67phox (67 kDa) (BD Biosciences, USA), anti-NFκB p65 (65 kDa) (Santa Cruz Biotechnology, USA), anti-IL-1β (30 kDa) (Santa Cruz Biotechnology, USA), anti-TNF-α (17 kDa) (Santa Cruz Biotechnology, USA) or anti-caspase-3 (32 kDa) (Santa Cruz Biotechnology, USA), which were diluted 1:1000 using 5% nonfat dried milk. The membranes were rinsed three times with 0.01% Tween-20 in Tris-buffered saline, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Western blots were prepared using a monoclonal antibody against β-actin (Santa Cruz Biotechnology, USA) at a dilution of1:30,000 as a loading control. Densitometry analysis was performed using ImageJ software (NIH, USA).

Statistical analysis

All data are described as the mean ± SEM. The analyses were performed using SPSS 20.0 software. Escape latencies in place navigation were compared using repeated measure ANOVA. The other data were analyzed by one-way ANOVA followed by an S-N-K analysis for multiple comparisons. Significance levels were set at $P < 0.05$.

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Compliance with ethical standards

Disclosure of conflicts of interest None.

Abbreviations *AD*, Alzheimer's disease; *AGE*, Advanced glycation end-product; *DM*, Diabetes mellitus; *ELISA*, Enzyme-linked immunosorbent assay; *HFS*, High-frequencystimulation; *LTP*, Long-term potentiation; *L-NBP*, L-3-n-Butylphthalide; *MDA*, Malondialdehyde; *PS*, Population spikes; *RT-PCR*, Reverse transcription-polymerase chain reaction; *SOD*, Superoxide dismutase; *8-OHdG*, 8-hydroxydeoxyguanosine

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