1-3-n-butylphthalide improves cognitive deficits in rats with chronic cerebral ischemia

Jing Xu a, Yiyi Wang a,b, Ning Li a, Lanju Xu c, Hanyu Yang c, Zhuo Yang a, * 

a College of Medicine, Nankai University, 94, Weijin Rd, Tianjin 300071, China
b Tianjin Xiqing Hospital, Tianjin 300380, China
c CSPC, The Institute of Pharmaceutical Research, Shijiazhuang 050051, China

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A B S T R A C T

3-n-Butylphthalide (NBP) has been shown to have protective effects against ischemic stroke. In the present study, we investigated effects of 1-3-n-butylphthalide (1-NBP) on the learning and memory impairment induced by chronic cerebral ischemia in rats. Male Wistar rats were administered 20 mg/kg 1-NBP by gavage daily for 30 days after the bilateral common carotid artery clamping (two-vessel occlusion, 2-VO). Results showed that daily treatments of 20 mg/kg 1-NBP significantly attenuated spatial learning deficits in Morris water maze (MWM) task. Results of long-term potentiation (LTP) indicated that treatment with 20 mg/kg 1-NBP attenuated the inhibition of LTP in rat model of 2-VO. Moreover, 1-NBP reduced glial fibrillary acidic protein (GFAP)-positive astrocytes induced by chronic cerebral ischemia. The present findings demonstrate the protective effect of 1-NBP on chronic cerebral ischemia-induced hippocampus injury, which supports using 1-NBP for therapy of cerebral ischemia in the future.

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1. Introduction

Senile dementia, a progressive aging-related disease, has become an important medical and social problem due to the increase in the number of elderly. Vascular dementia (VaD), as the second most common form of dementia in the elderly (Giacobini, 2004), has gained much attention in recent years. Repeated focal infarct is the most common reason of human VaD. With pathological changes in cardiovascular system, many old people suffer from repeated focal infarct and chronic cerebral ischemia, which may lead to dementia. VaD is characterized by a progressive cognitive and behavioral deterioration induced by loss of blood supply in various areas of the brain (Kalaria et al., 2004). Reduction in cerebral blood flow that arising in chronic cerebral ischemia can lead to selective neuronal injuries in vulnerable regions of the brain, especially the hippocampus (McBean and Kelly, 1998; Pulsinelli and Brierley, 1979). Spatial learning and memory are dependent on the integrity of the hippocampus. The hippocampal formation is centrally involved in the initial phase of memory retention processes. Thus this injury is accompanied by a progressive cognitive decline (Alagona et al., 2004). At present, no specific drug exists to prevent, delay, or cure VaD. Therefore, more and more studies have focused on finding new drugs to improve cognitive deficits caused by VaD.

Previous studies showed that 3-n-butylphthalide (NBP) had the ability to decrease the area of cerebral infarct in focal cerebral ischemic rats (Liu and Feng, 1995). It can also improve energy metabolism in mice with complete brain ischemia (Feng et al., 1995). NBP is a chiral compound, which contains both L- and d-isomers. Peng et al found that 1-3-n-butylphthalide (1-NBP) attenuated learning and memory deficits induced by chronic cerebral hypoperfusion in rats (Peng et al., 2007). Moreover, 1-NBP showed potent neuroprotective effects by decreasing oxidative damage (Dong and Feng, 2002), reducing neuronal apoptosis (Chang and Wang, 2003) and inhibiting inflammatory responses (Xu and Feng, 2000) in middle cerebral artery occlusion rat models. The positive effects of NBP and 1-NBP on cerebral ischemia and cerebral infarct have been verified in ischemic patients and animal models, however little is known about the effect of 1-NBP on chronic cerebral ischemia, especially the electrophysiological behavior. Therefore, we set out to examine the functions of 1-NBP as well as to investigate whether 1-NBP has the ability to protect rat brains from chronic cerebral ischemia.

To test the hypothesis, a chronic cerebral ischemia rat model-the bilateral common carotid artery clamping (two-vessel occlusion, 2-VO), which can reduce the blood flow in the brain to one-third of its normal value was set up (Farkas et al., 2007). As the hypoperfusion also affects the hippocampus (Todd et al., 1984), it...
may exert effects on various neuronal properties, including the neuronal cell viability or its electrophysiological behavior. The best-studied cellular model for hippocampal learning and memory is the detection of long-term potentiation (LTP), which is a long lasting increase in the synaptic transmission efficiency induced by the high frequency stimulation (Bliss and Lomo, 1973). The Morris water maze (MWM) test is a memory test based on the capacity of animals to recover themselves by reaching a hidden goal platform in a pool of water (Morris et al., 1982). Animals with a damaged hippocampus display spatial navigation impairments and perform poorly in the MWM test (Morris et al., 1982).

In the present study, we investigated the effects of l-NBP on improving cognitive deficits in rat model induced by chronic cerebral ischemia. In brief, MWM test, LTP in hippocampus and brain histology analyses were used to evaluate the effects of l-NBP on rat brain function after permanent bilateral occlusion of the carotid arteries, which may provide an interesting view of the potential application of l-NBP for VaD therapy in the future.

2. Materials and methods

2.1. Chemicals and materials

L-NBP was provided by CSPC, the Institute of Pharmaceutical Research, Shijiazhuang, China. It was diluted with vegetable oil (Peng et al., 2007).

2.2. Animals

Male Wistar rats (270–300 g) were subjected to surgery. Animals were group-housed with free access to water and food in an established animal house having a 12 h light: 12 h darkness cycle and a thermo regulated environment. The animal care and experimental protocol were approved by the Ethical Commission at Nankai University, China.

2.3. Surgery

Rats were randomly divided into three groups: sham group, vehicle group and l-NBP group. Rats were anesthetized using 4% chloral-hydrate (intraperitoneal). The common carotid artery was isolated and double ligated with 5/0 silk suture in rats of vehicle group and l-NBP group. As sham-operated controls, rats of another group received the same operation without ligation.

2.4. Drug administration and experimental design

After 2-VO surgery, rats were randomly divided into two groups, both of which consisted of 10 animals with identical mean body weights. The daily administration of l-NBP (20 mg/kg) or vehicle (vegetable oil) by gavage started from the surgery day, lasted for 30 days. Then spatial learning and memory were assessed in all rats. The experimental design is shown in Fig. 1.

2.5. MWM task

The day after the last time of drug treatments, all rats were trained and tested in MWM (RB-100A type, Beijing, China) to monitor their spatial learning and memory. The water maze consists of a large circular pool (150 cm in diameter, 60 cm in height, filled to a depth of 45 cm with water at 23 °C ± 1 °C). The water maze was divided into 4 equal quadrants (I–IV) by two imaginary perpendicular lines crossing in the center of the tank. There was a 10-cm diameter platform submerged 2 cm below the water surface in the center of quadrant III. The water was made opaque using non-toxic black ink. Each rat received two trials every day and the test lasted for 5 days. The escape latency (swimming time to locate the hidden platform) was used to determine the platform day by day by the spatial probe test with the platform removed. The evaluator conducting the MWM was blinded to the treatment groups.

2.6. Electrophysiological recordings

The day after finishing MWM test, rats were given LTP test. Rats were anesthetized with 30% urethane (0.4 ml/kg), and then were placed in a stereotaxic frame (Narishige, Japan). Small holes were drilled in the skull for inserting stimulating and recording electrodes (Advent Co., UK). The tip of the recording electrode was positioned in the stratum radiatum of area CA1 (3.5 mm posterior to bregma and 2.5 mm lateral to the midline). The stimulating electrode was inserted into the CA3 region (4.2 mm posterior to bregma and 3.5 mm to the midline). The test stimuli were delivered to the CA3 region every 30 s at an intensity that evoked a response of 50% of its maximum. After every 20 s for 20 min stable baseline recording, a high frequency stimulation (HFS) consisted of 10 trains of 10 stimuli at 100 Hz with 2 s intertrain interval was given in three groups. The field excitatory postsynaptic potential (fEPSP) was then recorded at 40 kHz (Scope Software, Powlab, ADInstruments, Australia) every 20 s for 60 min. The fEPSP slope (20–80% level of the falling phase) was used to measure synaptic efficacy.

2.7. Histology

After the electrophysiology experiment, rats were deeply anesthetized and were perfused through the left cardiac ventricle with phosphate buffered sodium (PBS, pH 7.2) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brain of each rat was removed and fixed by immersion in the same solution. Fixed tissues were embedded in paraffin. Brains were serially sectioned at 5 micron thick. Sections for hematoxylineosin (HE)-staining were placed onto uncoated slides. Sections intended for use in immunohistochemistry assays were placed onto coated slides (ZS9Q-Bio, China). Sections were routinely HE stained for histomorphological assessment. The experimenter evaluating the histology was blinded to the treatment group of the rats.

2.8. Immunohistochemical procedures for glial fibrillary acidic protein (GFAP)

Sections for immunoreactivity assays were placed onto coated slides (Zhongsan Goldenbridge Biotechnology, China). The labeled dextran polymer (LDP) immunohistochemistry was used to detect expressions of GFAP in CA1 subfield. The deparaffinized sections were boiled in citrate buffer in microwave oven for antigen retrieval. After that, peroxidase activity was inactivated by incubation with 3% H2O2 solution for 30 min at room temperature. Then the sections were incubated with rabbit polyclonal anti-GFAP antibody (diluted 1:100 ZS9Q-Bio, China) in a moist chamber at 4°C overnight. Negative controls were conducted by exchange of primary antibody for PBS. Sections were then incubated with En Vision-Systems polymer-conjugated secondary antibody PV-9000 (GIB, USA), and finally incubated with diaminobenzidin (DAB) at room temperature for 3 min. The slides were washed twice in PBS between steps. Sections were observed and photographed using an OLYMPUS BX5100 microscope with a CCD camera (JVC, Japan). The experimenter evaluating the histology was blinded to the treatment group of the rats.

2.9. Statistics

All data were presented as mean ± SEM. Escape latencies in place navigation were compared using repeated measure ANOVA. Data for spatial probe and LTP recording were compared using one-way ANOVA. Significance levels were established at a level of P < 0.05. The analyses were performed using SPSS 16.0 software.

3. Results

3.1. MWM performance

In order to determine whether the rats’ cognitive function was affected after the oral administration of l-NBP, the rats’ abilities of spatial learning acquisition and memory retention were tested by using MWM test (Morris et al., 1982). In the MWM, animals from all groups became more efficient at locating the platform on successive trials. The escape latencies were progressively shorter in all groups in a day-dependent manner. Two-way repeated measures ANOVA confirmed statistical difference of day (F = 139.34, P < 0.001) and group (F = 7.598, P < 0.01). There was no significant difference in day × group interaction (F = 2.418, P = 0.065). On day 1, group comparisons revealed that animals in the sham group displayed a significantly lower latency in finding the platform when compared with that of vehicle group (sham: 26.25 ± 1.58 s; vehicle: 44.27 ± 5.05 s, P < 0.01), and similarly on day 3 (sham: 7.52 ± 0.94 s; vehicle: 14.50 ± 2.26 s, P < 0.05), day 4 (sham: 5.84 ± 0.94 s; vehicle:
14.31 ± 2.52 s, P < 0.01) and day 5 (sham: 5.29 ± 0.71 s; vehicle: 10.02 ± 1.56 s, P < 0.05) (Fig. 2A). These results indicated that the experimentally induced chronic cerebral ischemia resulted in a significant impairment of memory acquisition, and confirmed the utility of this model in the investigation of l-NBP effects (de la Torre and Aliev, 2005). Compared with vehicle group, when treated with 20 mg/kg l-NBP, the escape latency was markedly decreased at day 4 (8.19 ± 1.60 s, P < 0.05) and also at day 5 (5.86 ± 1.07 s, P < 0.05) (Fig. 2A).

In spatial probe test, it could be seen that there was a marked effect of l-NBP treatment. The quadrant dwell times were 51.13 ± 3.44%, 36.03 ± 2.14% and 50.93 ± 6.56% in sham group, vehicle group and l-NBP group (F = 4.48, P < 0.05). Statistical results revealed that the quadrant dwell time was decreased significantly in vehicle group compared with that of sham group (Fig. 2B, P < 0.05), which was attenuated by 20 mg/kg l-NBP (P < 0.05).

3.2. Recording of LTP

The LTP from Schaffer collaterals to CA1 was recorded, and the fEPSP slope was measured. The fEPSP slope were 143.26 ± 10.40%, 112.68 ± 9.82% and 139.27 ± 14.34% in sham group, vehicle group and l-NBP group (F = 107.61, P < 0.01). In vehicle group, the normalized slope of the fEPSP was significantly reduced compared with that in sham group (Fig. 3B vehicle: 112.68 ± 9.82%; sham: 143.26 ± 10.40%, P < 0.01). It was found that 20 mg/kg l-NBP attenuated this change compared with that in vehicle group (20 mg/kg l-NBP: 139.27 ± 14.34%; vehicle: 112.68 ± 9.82%, P < 0.01).

3.3. HE staining

The typical neuropathological changes were observed in the hippocampus on visual inspection including neuronal cell loss, nuclei shrinkage, cerebral edema and dark staining of neurons. Administration of 20 mg/kg l-NBP attenuated these pathological changes (Fig. 4).

3.4. Immunohistochemistry of GFAP

As shown in Fig. 5, GFAP-positive astrocytes were rare in the hippocampus of rats in sham group. However, in vehicle group, activated astrocytes were markedly increased in the hippocampus on visual inspection. Administration of 20 mg/kg l-NBP reduced the number of GFAP-positive astrocytes in the hippocampus of rats (Fig. 5).

**Fig. 2.** Rats’ performance in MWM test. (A) Mean escape latency calculated for each day in sham, vehicle and 20 mg/kg l-NBP groups in place navigation phase. (B) Mean percentage of time in target quadrant in spatial probe phase. Data are expressed as mean ± SEM. n = 10, *P < 0.05 compared with the sham group; **P < 0.01 compared with the sham group; *P < 0.05 compared with the vehicle group.

**Fig. 3.** Effects of l-NBP on fEPSP of LTP. (A) Changes in fEPSPs slopes after high frequency stimulation (HFS; 10 × 100 Hz for 0.1 s each, 2 s interval); (B) Time course changes in fEPSP slope in three groups. n = 10, **P < 0.01 compared with the sham group; ***P < 0.01 compared with the vehicle group.
4. Discussion

Cerebral ischemia is one of the major leading causes of morbidity and mortality worldwide. The rate of cerebral perfusion and the morphological integrity of the circulatory network of the brain play an important role in the maintenance of proper neuronal function and related memory capacity (Farkas et al., 2002). It is known that 2-VO reduces the blood flow in the brain to one-third of its normal value in rats (Farkas et al., 2007), in which the hippocampus damage is most severe (McBean and Kelly, 1998; Pulsinelli and Brierley, 1979). Spatial learning and memory are dependent on the integrity of the hippocampus, so the injury may lead to a progressive cognitive decline (Alagona et al., 2004). Nowadays more and more studies have focused on finding new drugs to attenuate cerebral ischemia. dl-NBP was synthesized and received approval by the State Food and Drug Administration of China for clinical use in the treatment of stroke in 2002. dl-NBP is a chiral compound, and contains L- and D-isomers. Previous studies showed that L-NBP significantly improved microcirculation in pial arterioles (Xu and Feng, 1999), reduced the area of cerebral infarct, and inhibited platelet aggregation (Peng et al., 2004). There were a large number of studies focused on the protective effects of L-NBP, but to the best of our knowledge, little is known about the effects of L-NBP on chronic cerebral ischemia, especially the electrophysiological behavior. The 2-VO model is one of the global models of cerebral ischemia (Dirnagl et al., 1993; Yao et al., 2009). In this whole brain ischemia, the hippocampal damage was more severe (McBean and Kelly, 1998; Pulsinelli and Brierley, 1979). The purpose of the present study was to investigate whether L-NBP had the ability to protect rats’ brains from chronic cerebral ischemia.

Spatial learning and memory are often used as an index to evaluate the cognitive function in animal models, and the growing evidence reflects the continuing importance of hippocampus for spatial learning and memory (Clark et al., 2007). MWM is a well-validated method for evaluating learning and memory in rodents.

**Fig. 4.** HE staining showed effects of L-NBP on morphologic changes in rats’ hippocampus induced by 2-VO: sham group (A, 100×; B, 400×); vehicle group (C, 100×; D, 400×); 20 mg/kg L-NBP group (E, 100×; F, 400×).
The acquisition of spatial learning and memory can be obtained from the latency in the acquisition phase. The persistence of the spatial memory can be directly reflected through the time spent in the target quadrant in the retention phase. The MWM results showed that the performance of rats was worse in vehicle group than that in sham group either in acquisition phase or in retention phase. These results indicated that the experimentally induced chronic cerebral ischemia resulted in a significant impairment of memory acquisition, and confirmed the utility of this model in the investigation of l-NBP effects (de la Torre and Aliev, 2005). The treatment of 20 mg/kg l-NBP improved the escape latency and the quadrant dwell time compared with those of vehicle group (Fig. 2, \( P < 0.05 \)), which suggested that l-NBP attenuated learning and memory impairment caused by 2-VO.

Learning and memory are associated with changes in the efficacy of synaptic neurotransmission (Kandel, 2001). LTP of synaptic transmission is one of the functional indexes of synaptic plasticity, which is a widely accepted model for learning and memory at the cellular level (Malenka and Nicoll, 1999). Yao et al have reported that chronic cerebral ischemia caused by 2-VO significantly reduced the normalized slope of the fEPSP (Yao et al., 2009). The present results showed that the LTP was inhibited in vehicle group compared with that in sham group (Fig. 3, \( P < 0.01 \)), which were in agreement with the previous results. l-NBP attenuated LTP decreasing in the hippocampus induced by chronic cerebral ischemia compared with that of vehicle group (Fig. 3, \( P < 0.01 \)). Although at later time points, the normalized fEPSP slope appeared bigger in 20 mg/kg l-NBP group than that in sham group, we used the normalized fEPSP slope of the whole 60 min after HFS and found that there was no difference between sham group and 20 mg/kg l-NBP group (Fig. 3B). In the present study, MWM and LTP tests have been chosen as markers for the evaluation of the l-NBP on rat brain function after 2-VO. Our data suggested that l-NBP attenuated the injury of hippocampus after chronic cerebral ischemia. The MWM and LTP results are also supported by the histological and immunohistochemical analyses.
The previous studies found that reduction in cerebral blood flow and the concomitant abnormalities of energy metabolism that arise in chronic cerebral ischemia can lead to selective neuronal injuries in vulnerable regions of the brain, particularly in the hippocampus (Suh et al., 1996). Our results of HE staining agreed with this study. HE staining showed neuronal cell loss, nuclei shrinkage, cerebral edema and dark staining of neurons in hippocampus of vehicle group. While administration of 20 mg/kg l-NBP attenuated the 2-VO induced neuronal damage (Fig. 4).

Recent evidence showed that astrocytes play an active role in partnership with neurons in protecting the CNS against various kinds of insults, including cerebral ischemia and hypoxia (Louw et al., 1998; Vernadakis, 1996), or neurological disorders (Tacconi, 1998). GFAP is the major cytoskeletal protein of astrocytes in the brain (Eng et al., 2000). Numerous studies have shown that expression of GFAP is increased in various types of brain injury including trauma, demyelination and brain ischemia (Eng and Ghirnikar, 1994; Li et al., 1995; Petito and Halaby, 1993; Petito et al., 1990; Tanaka et al., 1992). Our result of GFAP agreed with these studies. There was an up-regulated GFAP expression in vehicle group compared with that in sham group. GFAP was used as these studies. There was an up-regulated GFAP expression in vehicle group compared with that in sham group. GFAP was used as

In conclusion, our present work showed that l-NBP attenuated learning and memory injury induced by chronic cerebral ischemia, which supported the information to use l-NBP for therapy of cerebral ischemia in the future.

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