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Original article

Leonurine ameliorates cognitive dysfunction via antagonizing excitotoxic glutamate insults and inhibiting autophagy

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ABSTRACT

Background: Chronic cerebral hypoperfusion is related with cognitive deficits in different types of dementia.
Purpose: In this study, we aimed to investigate the effect and potential mechanisms of leonurine on chronic cerebral hypoperfusion both *in vitro* and *in vivo*.
Study Design: Chronic cerebral hypoperfusion was duplicated by oxygen-glucose deprivation (OGD) *in vitro* and by ligation of bilateral common carotid arteries (2-VO) *in vivo*.
Methods: In *in vitro* study, there were control group, OGD group, OGD + 100 μM leonurin group, and OGD + 10 μM donepezil group. The spontaneous excitatory postsynaptic current amplitude and frequency were recorded. In *in vivo* study, the chronic cerebral hypoperfusion model was induced by ligated bilateral common carotid arteries. Rats were randomly divided into Sham group, 2-VO group, 2-VO + 60 mg/kg/day leonurine group, and 2-VO + 4 mg/kg/day donepezil group. After three weeks, the Morris water maze and Long-term depression recording were observed. Then N-methyl-*D*-aspartate receptor-associated proteins and autophagy-associated proteins were detected by Western blot assay.
Results: In *in vitro* experiment, results showed that leonurine could obviously attenuate the spontaneous excitatory postsynaptic current amplitude and frequency on pyramidal neurons. In *in vivo* experiment, leonurine significantly decreased levels of glutamate and hydrogen peroxide, improved both the cognitive flexibility and the spatial learning and memory abilities. Moreover, leonurine obviously enhanced long-term depression, elevated the ratio of N-methyl-*D*-aspartate receptor 2A/2B, and decreased the expression of postsynaptic density protein-95. Interestingly, the ratio of LC3II/LC3I and beclin-1 expression were markedly down-regulated by leonurine.
Conclusion: These findings suggest that leonurine ameliorates cognitive dysfunction at least partly via antagonizing excitotoxic glutamate insults and inhibiting autophagy. Furthermore, it might become a potential drug candidate of chronic cerebral hyperfusion in future.

Introduction

Chronic cerebral hypoperfusion (CCH) has been identified to be related with the cognitive deficits in different types of dementia, such as Alzheimer's disease, vascular dementia and frontotemporal dementia (Osawa et al., 2004; Shu et al., 2013). CCH leads to the inadequate blood supply in different regions of brain, which is accompanied with the deficiency of oxygen and nutrients. As known, the hippocampus formation is the most sensitive brain area to the cerebral ischemia and plays a key role in spatial learning and memory (Li et al., 2011). Accumulating evidences demonstrate that there is a causal relationship between CCH and dementia (Osawa et al., 2004).

Brain ischemia and hypoxia could lead to the depolarization of neurons, and then a large sum of glutamate will release into the synaptic cleft to excessively agitate the N-methyl-*D*-aspartate receptors (NMDARs or NRs), which will cause lots of Ca^{2+} flux into neurons and result in cell death by this excitotoxic glutamate insults. Autophagy is necessary for cell degrading cellular "garbage" and for maintaining cell homeostasis by lysosome-mediated

Abbreviations: CCH, Chronic cerebral hypoperfusion; OGD, oxygen–glucose deprivation; aCSF, artificial cerebral spinal fluid; 2-VO, bilateral common carotid arteries; NMDARs, N-methyl-*D*-aspartate receptors; LTD, long-term depression; MWM, Morris water maze; IT, initial training; IPT, initial probe trials; RT, reversal training; RPT, reversal probe trials.

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catabolic machinery. It plays an important role not only at physiological status, such as cell survival, development and differentiation, but also at pathological condition, such as Alzheimer's disease, Parkinson's disease and brain ischemia disease (Caberlotto and Nguyen, 2014). Interestingly, studies demonstrated that brain hypoxia and ischemia obviously enhanced the neuronal autophagy, and the inhibition of the autophagy was of benefit to hippocampus (Koike et al., 2008). On the contrary, there were also some other results demonstrated that autophagy played neuroprotective effects on brain ischemia (Balduini et al., 2001; Carloni et al., 2008). Therefore, the role of autophagy in brain ischemia and hypoxia still needs to be further investigated. Then, how about the role of autophagy in CCH and what the underlying mechanism is still not completely understand.

Leonurus cardiaca is an herbaceous perennial plant in the mint family and has a long history in traditional medicine to treat a variety of diseases in China, Japan, Korea and European countries. Leonurine (LEO), an active alkaloid extracts from *Leonurus cardiaca*, has recently been demonstrated to be effective on the treatment of cardiovascular disease and nervous system diseases (Liu et al., 2013; Qi et al., 2010; Wojtyniak et al., 2013). Studies have demonstrated that LEO has neuroprotective effects on middle cerebral artery occlusion rats via anti-oxidantion and anti-apoptosis (Qi et al., 2010). However, the effect and underlying mechanism of LEO on CCH are still poorly understood. In this study, the CCH model was duplicated by oxygen-glucose deprivation (OGD) *in vitro* and by ligation of bilateral common carotid arteries (2-VO) *in vivo*. We aimed to investigate whether or not LEO could alleviate the cognitive impairment of CCH.

Materials and methods

The brain slices were removed from 3–4 week Wistar rats and there were control group (CON) incubated in oxygenated artificial cerebrospinal fluid (aCSF); OGD group incubated with aCSF containing 95% N_2 and 5% CO_2 (OGD); OGD group treated with aCSF containing 95% N_2 and 5% CO_2 and different concentrations of LEO (OGD + 25 μM LEO, OGD + 50 μM LEO and OGD + 100 μM LEO groups). The cell viability of brain slices was determined by PI staining, and the patch-clamp recording was performed in CON, OGD and OGD + 100 μM LEO, and OGD + 10 μM donepezil hydrochloride (OGD + DON) groups. Adult male Wistar rats (250–280 g) were randomly divided into four groups, *i.e.* sham operation group with vehicle treatment (Sham); 2-VO group with vehicle treatment (2-VO); 2-VO group treated with 60 mg/kg/day of LEO (2-VO + LEO); and 2-VO group treated with 4 mg/kg/day of DON (2-VO + DON). The CCH model was induced by 2-VO surgery. After three weeks, spatial learning and memory performances of rats were evaluated by Morris water maze (MWM). Then the long-term depression (LTD) recording was performed immediately after MWM test. In order to investigate the neuroprotective mechanism of LEO, the levels of glutamate and H_2O_2 of hippocampus were measured. Moreover, levels of NR2A, NR2B, PSD-95 (1:2000, abcam, UK), LC3I/II (1:1000, Medical & biological laboratories Co. LTD., JPN), beclin-1 (1:2000, Cell Signaling Technology, USA) and β-actin (1:4000, abcam, UK) were tested by Western blot assay. All data were analyzed by SPSS 16.0 software. Escape latencies and swimming speeds in MWM experiment were analyzed by two-way repeated ANOVA followed by the Bonferroni multiple group comparison. Other data were analyzed by one-way ANOVA followed by a post Turkey test. Data were presented as means ± S.E.M and defined differences at $p < 0.05$ as statistically significant. All pictures were processed with Photoshop software (sources and detailed methods in Supporting Text).

Results

LEO attenuated the excitotoxic insults induced by OGD

As shown in Fig. 1A and B, OGD significantly increased brain cell death. However, LEO alleviated the OGD-induced damage in a dose-dependent manner ($p < 0.05$). In the OGD + 100 μM LEO group, LEO could significantly decrease the brain cell death to 130.36 ± 9.06% (fold of CON group), so we selected the 100 μM LEO for next experiments. OGD induced the increase of the amplitude and expressed a markedly right shift in the cumulative probability curve of spontaneous excitatory postsynaptic current (sEPSC) amplitude ($p < 0.01$, Fig. 1C, D, F, G, I and J). However, LEO and DON obviously attenuated the amplitude of pyramidal neurons ($p < 0.05$), and presented a markedly left shift in the cumulative probability curve of sEPSC amplitude (Fig. 1C, D, F, G, I and J). As to the sEPSCs frequency, OGD produced obviously higher time-dependent frequency of pyramidal neurons, which was also ameliorated by LEO and DON ($p < 0.01$, Fig. 1C, E, F, H, I and K).

LEO alleviated the impaired spatial learning and memory in 2-VO model rats

Rats were subjected to MWM test to investigate the spatial learning and memory ability. The average escape latency was obviously decreased in all four groups during initial training (IT) without affecting swimming speeds (Fig. 2A and B). However, the escape latency was much longer in 2-VO group from day 2 to day 4 ($p < 0.05$, Fig. 2A). Interestingly, rats in 2-VO + LEO group and 2-VO + DON group located the platform much more faster on day 3 and day 4 ($p < 0.05$, Fig. 2A). In initial probe trials (IPT), the platform crossings (1.1 ± 0.31) and the quadrant dwell time (29.22 ± 2.44%) were obviously decreased in 2-VO group compared with that of Sham group (2.5 ± 0.54; 50.44 ± 3.06%, $p < 0.01$, Fig. 2C and D). Although there was no significant difference in platform crossings between 2-VO group and 2-VO + LEO group or 2-VO + DON group, LEO and DON remarkably improved the quadrant dwell time compared with that of 2-VO group ($p < 0.05$, Fig. 2C and D).

The average escape latency was markedly longer in 2-VO group than that of Sham group on both day 6 and day 7 during reversal training (RT) ($p < 0.05$, Fig. 2A). Rats of 2-VO + LEO group and 2-VO + DON group spent less time to find the platform than that of 2-VO group without affecting swimming speeds ($p < 0.05$, Fig. 2A and B). There was no significant difference in platform crossings between 2-VO group and 2-VO + LEO group or 2-VO + DON group ($p > 0.05$, Fig. 2E). However, the quadrant dwell time in reversal probe trials (RPT) were obviously increased in 2-VO + LEO group and 2-VO + DON group compared with those in 2-VO group ($p < 0.05$, Fig. 2F).

In addition, we analyzed the temporal distribution of the quadrants. Rats of 2-VO group spent much more time in zone 1 ($p < 0.01$, Fig. 3A) on day 7. In contrast, they spent less time in zone 4 ($p < 0.05$, Fig. 3D). There was no significant difference between 2-VO group and 2-VO + LEO group or 2-VO + DON group in the time spent in both zone 2 and zone 3 ($p > 0.05$, Fig. 3B and C). However, rats administrated with LEO or DON spent less time in zone 1 and more time in zone 4 on day 7 ($p < 0.05$, Fig. 3A and D). Moreover, the swim traces of all rats in RT stage were showed in Fig. 3E. In Sham group, 2-VO + LEO group and 2-VO + DON group, the trajectories of reversal learning stage were shorten, whereas the trajectory was significantly longer in 2-VO group (Fig. 3E). Also, rats of 2-VO group showed much more swimming trajectories in zone 1. In contrast, rats treated with LEO or DON showed much more swimming trajectories in zone 4 rather than other zones (Fig. 3E).