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Beta-asarone attenuates ischemia–reperfusion-induced autophagy in rat brains via modulating JNK, p-JNK, Bcl-2 and Beclin 1

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ABSTRACT

Beta-asarone has significant pharmacological effects on the central nervous system. It can attenuate neuronal apoptosis, but its effects on the brain ischemia–reperfusion-induced autophagy have not been reported yet. Our study was a two-stage procedure: evaluation of β-asarone effects on the autophagy at first, and then analysis of the possible mechanism. The middle cerebral artery occlusion (MCAO) model was adopted to make the brain injure and Beclin 1 was used to evaluate the autophagy. We hypothesized that the mechanism might be related to c-Jun N-terminal kinases (JNK), phospho-JNK (p-JNK), Bcl-2 and Beclin 1. To test this hypothesis, we evaluated JNK, p-JNK, Bcl-2 and Beclin 1 levels with flow cytometry. Additionally, we divided the brain into three regions: ischemic region, ischemic penumbra, and normal region, and analyzed them respectively. We found, compared to both groups II (model control) and III (low dose), Beclin 1 levels in groups IV (medium dose) and V (high dose) were significantly decreased. Beclin 1, JNK and p-JNK levels in groups VII (β-asarone) and VIII (JNK inhibitor) were significantly decreased, but Bcl-2 levels were significantly increased. Additionally, Beclin 1, JNK, p-JNK and Bcl-2 levels among the three regions had no significant differences. We conclude that β-asarone can attenuate the autophagy in a dose-dependent manner. The mechanism is likely that β-asarone can decrease JNK and p-JNK levels at first, and then increase Bcl-2 level, finally interfere with the functions of Beclin 1 during the execution of autophagy. Additionally, β-asarone can attenuate autophagy in a widespread manner.

1. Introduction

Autophagy plays important roles in cell survival (Kondo et al., 2005; Kuma et al., 2004; Levine, 2005; Lum et al., 2005). But, unfortunately, autophagy can also kill cells (Andrew, 2008). Autophagic cell death is a distinct form of cell death that differs from other death mechanisms such as apoptosis. Unlike apoptosis, which relies upon the activation of caspases (Luthi and Martin, 2007), autophagic cell death is usually thought of as caspase-independent (Tsujimoto and Shimizu, 2005). Autophagy is low but rapidly upregulated in many processes such as ischemia (Jiang et al., 2010; Luo et al., 2011; Wang et al., 2010, 2011).

Beclin 1 is important in the autophagic machinery (Kihara et al., 2001). Beclin 1 expression promotes autophagy (Liang et al., 1999), and cells with reduced Beclin 1 expression exhibit reduced autophagic activity (Qu et al., 2003; Yue et al., 2003).

Bcl-2 is not only functions as an antiapoptotic protein (Maundrell et al., 1997), but also as an antiautophagy one. It can reduce the pro-autophagic activity of Beclin 1 (Maiuri et al., 2007; Pattingre et al., 2005). The mechanism by which Bcl-2 inhibits autophagy is likely that Bcl-2 interferes with the functions of Beclin 1 (Saeki et al., 2000).

Immunohistochemistry (Dong et al., 2011), western blotting (Russo et al., 2011), and transmission electron microscopy are often to analyze autophagy. Transmission electron microscopy is a gold standard technique for analyzing autophagy (Holt et al., 2011). Immunohistochemistry can be employed to evaluate the autophagy associated proteins (Blatt et al., 2004; Duan et al., 2007; Tal et al., 2005). Flow cytometry is an important quantitative analysis, but Beclin 1 analysis by flow cytometry has been merely reported (Li and Wang, 2010).

β-asarone, a major component of *Acorus tatarinowii* Schott, has significant pharmacological effects on the central nervous system (Cho et al, 2002; Fang et al., 2003, 2008; Zanoli et al., 1998). It can attenuate neuronal apoptosis (Li et al., 2010; Liu et al., 2010), but its effects on autophagy have not been reported yet.

Our study was a two-stage procedure: evaluation of the β-asarone effects on brain ischemia–reperfusion-induced autophagy at first, and then analysis of the possible mechanism.

To evaluate β-asarone effects on the autophagy, we divided the rats randomly into five groups: groups I (sham), II (model control), III (low dose), IV (medium dose), and V (high dose). Beclin 1 levels were used to evaluate the autophagy levels. Transmission electron was to confirm the autophagy. The neuron-specific enolase (NSE) was to evaluate the injure.

Furthermore, we hypothesized that the mechanism might be related to JNK, p-JNK, Bcl-2 and Beclin 1. To test this hypothesis, we

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Table 1
Neurologic deficit scores in groups I (sham), II (model control), III (low dose), IV (medium dose), V (high dose), VI (model control), VII (β-asarone), and VIII (JNK inhibitor).

	Groups							
	I	II	III	IV	V	VI	VII	VIII
Scores	0[b]	2.30 ± 0.48[a]	1.88 ± 0.13[a]	1.20 ± 0.42[a,b]	1.20 ± 0.42[a,b]	2.20 ± 0.42[a]	1.30 ± 0.48[a,b]	1.10 ± 0.32[a,b]

Notes: The scores are given as mean ± S.D. (n = 10). Sig. (2-tailed) P<0.05.
[a] Indicates statistically significant differences *vs* group I (sham) values (P<0.05).
[b] Indicates statistically significant differences *vs* group II (model control) and VI (model control) values (*P<0.05*).

divided the rats randomly into three groups: groups VI (model control), VII (β-asarone), and VIII (JNK inhibitor), and evaluated JNK, p-JNK, Bcl-2 and Beclin 1 with flow cytometry, respectively.

Additionally, we divided the brain into three regions: ischemic region, ischemic penumbra, and normal region, and analyzed them respectively. The MCAO model was adopted to make the brain injure (Longa et al., 1989).

2. Materials and methods

2.1. The preparation of β-asarone

Beta-asarone (cis forms of 2, 4, 5-trimethoxy-1-propenylbenzene) is a strong fat-soluble substance with a small molecular weight (208). The β-asarone used in this study was obtained from *A. tatarinowii* Schott according to the procedure that we have reported (Liu and Fang, 2011). The β-asarone whose purity was up to 99.55% was confirmed by gas chromatography–mass spectrometry, infrared spectrum and nuclear magnetic resonance detection.

2.2. Animals

The study and its experimental protocol were approved monitored by the Ethics Committee of Guangzhou University of Chinese Medicine. One hundred Sprague–Dawley rats (350–400 g) were performed according to the guidelines for the ethical treatment of experiment animals. Local institutional approval for research was obtained before initiation of the study.

2.3. MCAO

To evaluate the β-asarone effect on the autophagy, rates were randomized into groups of 10 animals. The treatment was as follows: group I (sham), 2 ml/kg water intraperitoneally per day for 4 days; group II (model control), 2 ml/kg water intraperitoneally per day for 4 days; group III (low dose), 2 ml/kg of β-asarone (7.5 mg/ml) intraperitoneally per day for 4 days; group IV (medium dose), 2 ml/kg of β-asarone (15 mg/ml) intraperitoneally per day for 4 days; and group V (high dose), 2 ml/kg of β-asarone (30 mg/ml) intraperitoneally per day for 4 days. The dose with significant effects on the autophagy would be used in the study of the possible mechanism.

To analyze the possible mechanism of β-asarone effects on the autophagy, rates were randomized into groups of 10 animals. The treatment was as follows: group VI (model control), 2 ml/kg water intraperitoneally per day for 4 days; group VII (β-asarone), 2 ml/kg of β-asarone (the dose was decided by the first stage study, 15 mg/ml) intraperitoneally per day for 4 days; and group VIII (JNK inhibitor), 2 ml/kg of SP1600125 (sc-200635, Santa Cruz Biotechnology, California, American) (7.5 mg/ml) intraperitoneally per day for 4 days.

Additionally, twenty other rats were also prepared for that some rats might be died or with failure model during the experiment.

At 1 h after the last administration, rats were anesthetized with intraperitoneal injection of 3% chloral hydrate (350 mg/kg). Through a midline incision of the neck, the right common carotid artery, external carotid artery and internal carotid artery were exposed and ligated. A 40-mm length of monofilament nylon suture (Φ0.22–0.24 mm), with its tip rounded by heating near a flame, was inserted from the right common carotid artery to the internal carotid artery through a small incision in the common carotid artery and then advanced to the Circle of Willis to occlude the origin of the right middle cerebral artery (Longa et al., 1989). The sutures remained for 2 h and then removed. Rats in group I (sham) underwent the same surgical procedures except for the MCAO.

The neurologic findings were scored on a five-point scale: a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (falling to the left) a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness (Longa et al., 1989). Scores were recorded when the sutures were removed. The rats died during the experiment and rats with a score of 0 or 4 were excluded for further analysis. The rats were sacrificed after 4 h reperfusion, and the brains were harvested. The brains were divided into three regions: ischemic region, ischemic penumbra, and normal region (Linnik et al., 1993; Shi et al., 1998).

2.4. Flow cytometric evaluation of Beclin 1, Bcl-2, JNK, and p-JNK

2.4.1. Sample preparations

Samples were released by teasing through a steel mesh. Cell suspensions were filtered through sterile nylon filter to remove stroma and then cells were washed twice with PBS. The cells were counted and adjusted to a density of $1\,0\times10^6$ cells/ml. The cells of each sample were divided into four, and then were used to evaluate the Beclin 1, Bcl-2, JNK, and p-JNK, respectively.

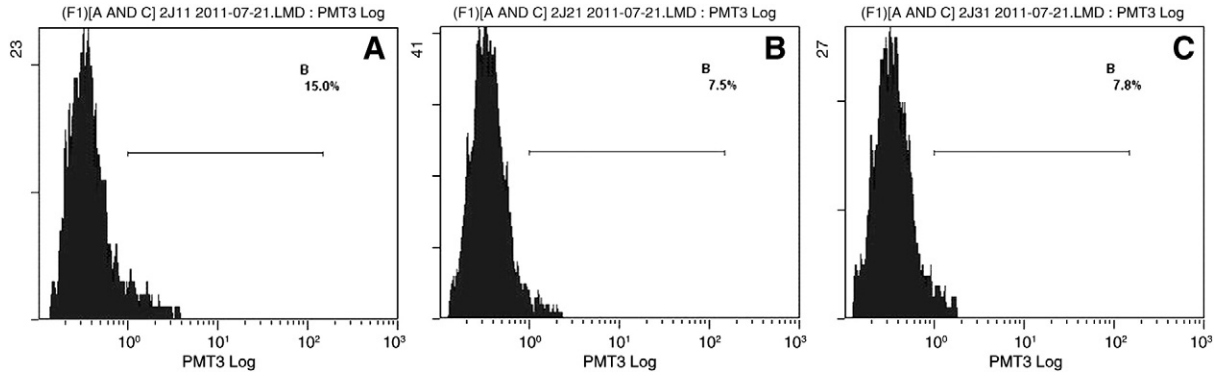

Fig. 1. Flow cytometric evaluation of Beclin 1. A: the representative flow cytometric of Beclin 1 in group II (model control); B: the representative flow cytometric of Beclin 1 in group VII (β-asarone); C: the representative flow cytometric of Beclin 1 in group VIII (JNK inhibitor).