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Ursolic acid improves domoic acid-induced cognitive deficits in mice

Dong-mei Wu [a,b,1], Jun Lu [b,*,1], Yan-qiu Zhang [a], Yuan-lin Zheng [b,*], Bin Hu [b], Wei Cheng [a], Zi-feng Zhang [b], Meng-qiu Li [b]

[a] School of Environment and Spatial Informatics, China University of Mining and Technology, Xuzhou 221008, Jiangsu Province, PR China
[b] Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Xuzhou Normal University, Xuzhou 221116, Jiangsu Province, PR China

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ABSTRACT

Our previous findings suggest that mitochondrial dysfunction is the mechanism underlying cognitive deficits induced by domoic acid (DA). Ursolic acid (UA), a natural triterpenoid compound, possesses many important biological functions. Evidence shows that UA can activate PI3K/Akt signaling and suppress Forkhead box protein O1 (FoxO1) activity. FoxO1 is an important regulator of mitochondrial function. Here we investigate whether FoxO1 is involved in the oxidative stress-induced mitochondrial dysfunction in DA-treated mice and whether UA inhibits DA-induced mitochondrial dysfunction and cognitive deficits through regulating the PI3K/Akt and FoxO1 signaling pathways. Our results showed that FoxO1 knockdown reversed the mitochondrial abnormalities and cognitive deficits induced by DA in mice through decreasing HO-1 expression. Mechanistically, FoxO1 activation was associated with oxidative stress-induced JNK activation and decrease of Akt phosphorylation. Moreover, UA attenuated the mitochondrial dysfunction and cognitive deficits through promoting Akt phosphorylation and FoxO1 nuclear exclusion in the hippocampus of DA-treated mice. LY294002, an inhibitor of PI3K/Akt signaling, significantly decreased Akt phosphorylation in the hippocampus of DA/UA mice, which weakened UA actions. These results suggest that UA could be recommended as a possible candidate for the prevention and therapy of cognitive deficits in excitotoxic brain disorders.

Introduction

Domoic acid (DA) is a naturally occurring neurotoxin produced by members of the diatom genus *Pseudo-nitzschia*. Evidence indicates that DA is the structural analogue of kainic acid (KA) and can induce excitotoxicity, ultimately leading to cognitive deficits and brain damage (He et al., 2010; Sawant et al., 2010; Vranyac-Tramoundanas et al., 2008). In 1987, an acute illness was first identified by gastrointestinal symptoms and unusual neurologic abnormalities when over 107 people met the case definition and 4 died after consuming DA-contaminated mussels harvested from cultivation beds on the eastern coast of Prince Edward Island, Canada (Lefebvre and Robertson, 2010). Anterograde memory deficits were the prominent severe neurological symptom in patients. Neuronal damages, particularly in the hippocampus and the amygdaloid nucleus, were found in all four postmortem examinations (Teitelbaum et al., 1990). Our recent studies have demonstrated that the oxidative stress-induced mitochondrial dysfunction is the underlying mechanism of DA-mediated cognitive deficits (Lu et al., 2012). Forkhead box protein O1 (FoxO1), a member of the FoxO family, is involved in a variety of biological processes, including metabolism, cell proliferation and oxidative stress response. FoxO1 transcriptional activity is positively regulated by stress-activated c-Jun N-terminal kinase (JNK) via promoting its import into the nucleus (Guo et al., 2012; van der Horst and Burgering, 2007). Evidence shows that FoxO1 activation mediated by the JNK pathway negatively regulates the electron transport chain (ETC) activity and causes mitochondrial dysfunction (Cheng et al., 2009). On the contrary, activation of PI3K/Akt signaling phosphorylates FoxO1 at three conserved Ser/Thr residues, leading to nuclear exclusion and the subsequent downregulation of target genes expression (Zhao et al., 2004).

Ursolic acid (UA: 3β-hydroxy-urs-12-en-28-oic acid), a natural pentacyclic triterpenoid, has been reported to possess many biological activities, including antioxidant, anti-inflammatory and antitumoral properties. Shih et al. demonstrated that UA protects hippocampal neurons against KA-induced excitotoxicity in rats through free radical scavenging (Shih et al., 2004). But the precise biological mechanisms underlying the neuroprotective effects of UA against KA-induced excitotoxicity are not well understood. Reports from our laboratory also confirmed that UA exerts the neuroprotective effects through the

Abbreviations: AAV, adeno-associated viral; AP, anterior-posterior; APR, ATP production rate; DA, domoic acid; DCF, 2′7′-dichlorofluorescein; DCFH-DA, 2′7′-dichlorodihydrofluorescein diacetate; DV, dorsal–ventral; EGFP, enhanced green fluorescent protein; ETC, electron transport chain; FoxO1, Forkhead box protein O1; HO-1, heme oxygenase 1; i.c.v, intracerebroventricular; IOD, integral optical density; JNK, c-Jun N-terminal kinase; ML, medial–lateral; MWM, Morris water maze; OCT, optimal cutting temperature medium; RCR, respiratory control ratio; ROI, region of interest; ROS, reactive oxygen species; UA, ursolic acid.

* Corresponding authors. Fax: +86 516 83500348.
E-mail addresses: lu-jun75@163.com (J. Lu), ylzheng@xznu.edu.cn, yzheng170@yahoo.com.cn (Y. Zheng).

[1] These authors contributed equally to this work.

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inhibition of oxidative stress and inflammation in different neurotoxic models (Lu et al., 2007, 2010e, 2011a; Wang et al., 2011). Especially, recent evidence has shown that activation of PI3K/Akt signaling and blockage of FoxO1 activity by UA can reduce muscle atrophy and stimulate muscle hypertrophy in mice (Kunkel et al., 2011). However, no studies have been designed to investigate whether FoxO1 activation plays a key role in the oxidative stress-induced mitochondrial dysfunction which leads to cognitive deficits and brain damage in DA-treated mice and whether UA inhibits DA-induced mitochondrial dysfunction and cognitive deficits via the regulation of the PI3K/Akt and FoxO1 signaling pathways. Based on these considerations, we explored the aforementioned issues and further investigated the potential mechanism.

Materials and methods

Animals and administration

Animals and cannulation. Sixteen-month-old male ICR mice were purchased from the Branch of National Breeder Center of Rodents (Shanghai). All of the experimental protocols and euthanasia procedures were approved by the Institutional Animal Care and Use Committee of Xuzhou Normal University. Prior to experiments, the mice had free access to food and water and were kept under controlled conditions at a temperature of 22–24 °C, with a relative humidity of 60% and a 12/12 h light/dark cycle (light from 8:30 a.m. to 8:30 p.m.). After an acclimatization period of one week, mice were randomly divided into 16 groups of 15 animals each. A double guide cannula (Plastics One, Roanoke, VA) was implanted into the dorsal hippocampus of each mouse 10 days before the experiments. Mice were housed individually to prevent them from damaging each other's cannula. All of the surgical procedures were performed under Equithesin anesthesia (3 ml/kg intraperitoneal injection). Animals were placed in a stereotaxic apparatus (ZH-LanXing B/S type, Huaibei, China), and the double guide cannula (26-gauge, stainless steel) was implanted bilaterally into the dorsal hippocampus using coordinates obtained from Paxinos and Watson relative to the bregma (Franklin and Paxinos, 2001): −1.5 mm anterior–posterior (AP), ±1.0 mm medial–lateral (ML), and 2.0 mm dorsal–ventral (DV) from skull. A 28-gauge dummy cannula was inserted to prevent clogging of the guide cannula.

Adeno-associated viral (AAV) infection and FoxO1 knockdown in vivo. AAV vectors encoding short hairpin RNAs (shRNAs) were constructed as previously described (Musatov et al., 2006). Briefly, vectors expressed shRNAs containing FoxO1 (5′-CGCCCCAGGTGGTGGAGAC-3′) or luciferase (5′-CCGCTGGAGAGCAACTGCAT-3′, used as a negative control) target sequences under the control of the human H1 promoter (Kamagate et al., 2008). In addition, the vectors also expressed enhanced green fluorescent protein (EGFP) as a reporter to allow for visualization of transduced neurons. EGFP was also expressed under the control of a hybrid cytomegalovirus/chicken-α-actin promoter to ensure its stable long-term expression. Virus stocks were prepared by packaging the vector plasmids into AAV serotype 2 particles using a helper-free plasmid transfection system in HEK 293 cells. The vectors were purified using heparin affinity chromatography and dialyzed against phosphate buffered saline (PBS). Genomic titers were determined by quantitative PCR (Musatov et al., 2006) and adjusted to 10^{12} particles/ml.

Hippocampal FoxO1 knockdown experiments were performed 3 days prior to drug administration. For knockdown, 2 μl of each vector (2×10^9 packaged genomic particles total) in PBS was injected into the bilateral hippocampus as described above. Each group received one of the following treatments: Groups 1 (vehicle control group) and 2 (DA treatment group) received intra-hippocampal injections of control shRNA, and Groups 3 (FoxO1 knockdown/DA group) and 4 (FoxO1 knockdown group) received intra-hippocampal injections of FoxO1 shRNA AAV particles, respectively. Each vector was injected once every 2 days using a microinjector (KD Scientific Inc., Holliston, MA, USA) at a rate of 1 μl/min.

Drug treatment and DA injection. In total, 30 μg of SP600125 (an inhibitor of SAPK/JNK signaling, Sigma-Aldrich, St. Louis, MO, USA) and 40 μg of LY294002 (an inhibitor of PI3K/Akt signaling, Cell Signaling Technology, Inc., Beverly, MA, USA), were dissolved in 10 μl of a solvent consisting of 99% sterile saline/1% dimethyl sulfoxide, and given to groups 7 (DA/JNK inhibitor cotreatment group), 8 (JNK inhibitor treatment group) and 12 (DA/UA/PI3K inhibitor cotreatment group) for 4 min by means of intracerebroventricular (i.c.v) infusion, respectively, as described above. The other five groups [groups 5 (vehicle control group), 6 (DA treatment group), 9(vehicle control group), 10 (DA treatment group) and 11 (DA/UA cotreatment group)] were infused with an equal volume of the solvent. Drug infusion was performed daily using a microinjector (KD scientific Inc., Holliston, MA, USA) at a rate of 2.5 μl/min for 3 weeks (Wang et al., 2011). Three hours after SP600125 and LY294002 treatment, DA (90%, Merck, Darmstadt, Germany) was diluted to 2 mg/ml in 0.9% saline and then injected intraperitoneally (i.p.) into mice in groups 6, 7, 10, 11, 12, 14 and 15 (DA/Vitamin E cotreatment group) at a dose of 2 mg/kg. In addition, groups 2 and 3 were also i.p. injected with DA. This dosage of DA caused seizures in all of the mice but did not lead to mortality. DA was injected once every day for 3 weeks. Groups 1, 4, 5, 8, 9, 13 (vehicle control groups) and 16 (Vitamin E group) received injections of saline only. At the same time, the mice in groups 11 and 12 received daily UA (Sigma-Aldrich, St. Louis, MO, USA) of 100 mg/kg/day in distilled water containing 0.1% Tween-80 by oral gavage for 3 weeks, and the mice of groups 9 and 10 were given distilled water containing 0.1% Tween-80 orally at the same dose. The mice in groups 15 and 16 received daily Vitamin E (Sigma-Aldrich, St. Louis, MO, USA) of 65 mg/kg/day in peanut oil by oral gavage for 3 week, and the mice of groups 13 and 14 were given an equal volume of the solvent. The drug dosage and period used in this study were based on earlier reports and the results of our pilot study (Clayton et al., 1999; Kunkel et al., 2011; Lu et al., 2007, 2010e, 2011a; Peng and Ramsdell, 1996; Wang et al., 2011). The experimental procedures are shown in Fig. 1. After the behavioral tests (on the 6th week), the mice were sacrificed and brain tissues were collected for immediate use in experiments or stored at −70 °C.

Behavioral tests

All of the behavioral experiments were performed during the 5th week.

Morris water maze (MWM) test. The MWM test was conducted as previously described (Lu et al., 2009). The experimental apparatus consisted of a circular water tank (100 cm diameter, 35 cm height) containing water at a depth of 15.5 cm. The water was temperature-controlled at 23 °C ± 1 °C and was made opaque by the addition of

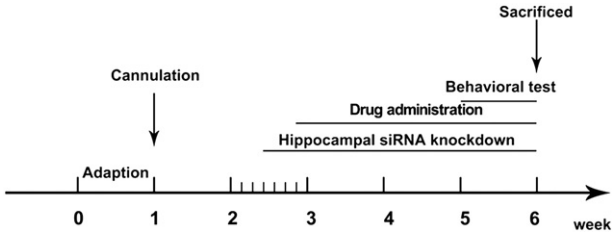

Fig. 1. Timeline of experimental procedures. ICR mice arrived from supplier at 16 months of age. All procedures conformed to the National Institute of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 80-23) and were approved by the Institutional Animal Care and Use Committee of Xuzhou Normal University.