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-qi 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

ARTICLE INFO

Article history:
Received 5 March 2013
Revised 20 April 2013
Accepted 25 April 2013
Available online xxxx

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.

© 2013 Published by Elsevier Inc.

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 (Kefelivre 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 antitumor 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...
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. 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′-CGCCCTGGAGACCAACTGAT-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 × 10^12 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 weeks, 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.](http://dx.doi.org/10.1016/j.taap.2013.04.038)
powdered milk. A platform that was 4.5 cm in diameter and 14.5 cm height (1 cm below the water surface) was placed at the midpoint of one quadrant. The pool was located in a test room that contained different prominent visual cues. Each mouse received 4 training periods on each of the 4 consecutive days. The escape latency from the water maze (i.e., time required to find the submerged escape platform) was calculated for each trial. On day 5, the probe test was conducted by removing the platform and allowing each mouse to swim freely for 60 s. The time that the mice spent swimming in the target quadrant (where the platform was located during the hidden platform training) was measured. For the probe trials, the number of times the mouse crossed over the platform site was recorded. All of the data were recorded using a computerized video system.

**Step-through passive avoidance task.** The step-through passive avoidance apparatus consisted of an illuminated chamber (11.5 cm × 9.5 cm × 11 cm lit with a 25 W lamp) attached to a darkened chamber (23.5 cm × 9.5 cm × 11 cm) containing a metal floor that could deliver a mild electric shock (0.3 mA, 50 Hz, 5 s). A guillotine door separated the two compartments. The step-through test was conducted as previously described (Lu et al., 2011b). Briefly, the mice were placed in a dimly lit room, which contained the apparatus, for 0.5 h before training to allow the mice to acclimate to their new environment. Each mouse was then placed into the illuminated chamber, facing away from the door and toward the dark chamber, and allowed to acclimate for 1 min. As soon as the mouse entered the dark chamber, the door was slid back into place, which triggered an electric shock. The mouse was then immediately removed from the chamber and returned to its cage. The latency (time used to change compartments) was recorded.

**Mitochondrial isolation from mouse hippocampus.** Hippocampal mitochondria were isolated as previously described (Lu et al., 2011b, 2011c, 2010b). Mitochondrial fractions were prepared from hippocampal tissue at 4 °C immediately after tissue collection. Fresh (non-frozen) brain tissue was homogenized in 1/3 (w/v) ice-cold homogenization buffer (50 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich, MO, USA), 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM MgCl₂, 0.2 mM 1,4-dithiothreitol, 1 mM EDTA, 1 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM Na₂VO₃ (Sigma-Aldrich, MO, USA), 20 mM sodium pyrophosphate, 20 mM b-Phosphoglucoglycerol, 1 mM p-nitrophenyl phosphate, 1 mM benzamidine, 1 mM PMSF and 5 mg/ml each of leupeptin, apronin and peptatin A). The homogenates were centrifuged at 1000 × g for 10 min at 4 °C. The pellets were discarded, and the supernatants were centrifuged at 17,000 × g for 20 min at 4 °C to separate the cytosolic fraction (supernatant) from the crude mitochondrial fraction (pellet). The protein concentrations were determined as previously described by (Lowry et al., 1951).

**Evaluation of mitochondrial ETC function.** Oxygen consumption assays. Oxygen consumption was measured as previously described (Cheng et al., 2009; Lu et al., 2012). The respiratory control ratio (RCR) was calculated as the ratio of the state 3 respiratory rate to the state 4 respiratory rate.

**Measurement of mitochondrial ATP content, ATP production rate and ETC activity.** Mitochondrial ATP content, ATP production rate (APR) and ETC activity in the mouse hippocampus were measured as previously described (Cheng et al., 2009; Lu et al., 2012) and expressed as μmol ATP (mg protein)⁻¹, μM (s mg protein)⁻¹ and M (min mg protein)⁻¹, respectively.

**Western blot analysis**

The samples (80 μg protein) were separated by denaturing SDS-PAGE and transferred to a PVDF membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) by electrophoretic transfer. The membrane was blocked with 5% non-fat milk and 0.1% Tween-20 in TBS and incubated overnight with one of the following primary antibodies: mouse anti-phopho-SAPK/JNK (Thr183/Tyr185) (1:2000), rabbit anti-phopho-Akt (Ser473) (1:1500), rabbit anti-Akt (1:1000) and rabbit anti-complex II (Sdhα) (1:1000) (Cell Signaling Technology).
repeated measures; the factors used were treatment and training day. The task were analyzed using a two-way analysis of variance (ANOVA) with repeated measures; the factors used were treatment and training day. All of the other data were analyzed with a one-way ANOVA followed by Tukey’s Honestly Significant Difference (HSD) post-hoc test. Data are expressed as the means ± standard deviation (SD). Statistical significance was set at \( p < 0.05 \).

Results

FoxO1 knockdown restores DA-induced hippocampal mitochondrial dysfunction

We constructed an AAV.H1 for the expression of FoxO1-specific shRNAs according to the method previously described by Musatov et al. (2006). A schematic representation of the AAV vector is shown in the Fig. 2A. Hippocampus-specific FoxO1 knockdown mice were prepared by injecting AAV.H1. FoxO1 bilaterally into the hippocampus. FoxO1 knockdown in mouse hippocampus was confirmed by western blot analysis (Figs. 2B–C). Our experimental results also showed that DA treatment significantly promoted the activation of FoxO1 through decreasing its phosphorylation \([p_{-} FoxO1(\text{Ser256})_{\text{Yoplo}}]: F(3, 16) = 31.114, p < 0.01 \text{ vs. control group}\) and stimulating its nuclear translocation \([FoxO1_{\text{nucel}}]: F(3, 16) = 168.487, p < 0.05 \text{ vs. control group}\) in mouse hippocampus. In turn, FoxO1 activation further significantly elevated HO-1 expression \([HO-1]: F(3, 16) = 23.062, p < 0.001 \text{ vs. control group}\) and impaired ETC activity and mitochondrial function \([mt-Nd6]: F(3, 16) = 19.216, p < 0.001; SdhA: F(3, 16) = 12.130, p < 0.001; Uqcrc1: F(3, 16) = 13.129, p < 0.001; mt-Co1: F(3, 16) = 43.335, p < 0.001; Atp5a1: F(3, 16) = 16.516, p < 0.001; APR: F(3, 16) = 21.697, p < 0.001; ETC: F(3, 16) = 11.323, p < 0.001; ATP: F(3, 16) = 11.250, p < 0.001 \text{ vs. control group}\).

Step-through passive avoidance task. The effect of hippocampus-specific FoxO1 knockdown on memory retention in DA-treated mice was investigated using the step-through passive avoidance task (Fig. 3A). None of the tested mice exhibited obvious weight loss. The latencies measured during training sessions did not differ significantly among the four groups \([F(3, 36) = 0.134, n.s.\text{ vs. control group}\). However, the step-through latency in the 24 h retention trial was significantly decreased in DA-treated mice \([F(3, 36) = 36.841, p < 0.001 \text{ vs. control group}\). The analysis also showed that the latency in DA/FoxO1 shRNA-treated mice was significantly lengthened compared with DA-treated mice \((p < 0.001)\). There was no significant difference in the step-through latency among the DA/FoxO1 shRNA, FoxO1 shRNA and control groups \((p > 0.001)\).

MWM test. Statistical analysis of the escape latency in the MWM test was conducted by a two-way ANOVA for repeated measures with day and treatment as the sources of variation \((Figs. 3B–D)\). There was a significant difference in mean latency between training days \([F(3, 144) = 115.815, p < 0.001 \text{ vs. control group}\) and between treatments \([F(3, 144) = 26.904, p < 0.001 \text{ vs. control group}\), but there was no interaction between the day and treatment factors \([F(9, 144) = 1.122, n.s. \text{ vs. control group}\). DA-treated mice had longer escape latencies than control mice \((p < 0.001)\). A comparison between the DA group and the DA/FoxO1 shRNA group showed that the hippocampus-specific FoxO1 knockdown could decrease the escape latency of DA-treated mice \((p < 0.001)\). There were no significant differences in escape latencies among the DA/FoxO1 shRNA and control groups \((p > 0.001)\).

Collection of brain slices

Mice were transcardially perfused with 25 ml of normal saline (0.9 w/v NaCl). The brain tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) or acetone at 4 °C for 4 h, incubated overnight at 4 °C in 100 mM sodium phosphate buffer (pH 7.4) containing 30% sucrose, and embedded in Optimal Cutting Temperature medium (OCT, Leica Microsystems, Nussloch, Germany) for cryosectioning. Coronal sections (12 μm) of cryosectioned tissue were collected on 3-aminopropyl-trimethoxysilane-coated slides (Sigma-Aldrich, MO, USA) and stored at − 70 °C.

Immunofluorescence staining

Cryosectioned sections were cut and mounted as described above and subjected to immunofluorescence staining. Double immunostaining was performed by simultaneously adding 2 primary antibodies, e.g., rabbit anti-FoxO1 (1:250; Cell Signaling Technology, Inc., Beverly, MA, USA) and mouse anti-FOX-1 (1:200; Abcam, Cambridge, UK), followed by overnight incubation at 4 °C. The secondary antibodies (Texas Red-conjugated anti-rabbit 1:80 and FITC-conjugated anti-mouse 1:80; Santa Cruz Biotechnology, CA) were then applied after washing in PBS. The specificity of the staining was assessed by omitting the primary antibody. Sections were counterstained with Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Stained specimens from 20 mice (5 from each group) were captured using a Zeiss Axioskop 40 microscope (40 x objective) (Carl Zeiss, Oberkochen, Germany), and the images were acquired with a CCD camera (CoollSnAP Color, Photometrics, Roper Scientific, Inc.). Staining brightness was quantified using Image Pro-Plus 6.0 software (Media Cybernetics Inc., USA) to assess the fluorescence intensity. Plaque areas were excluded, and OD values in individual cells (three slides for each brain) were used to assess the intensity of immunoreactivity according to the equation \([\frac{\sum \text{IOD}}{\sum \text{Area}}]\), where IOD is the integral optical density in the region of interest (ROI) and Area is the area of the ROI (Lu et al., 2011b). In this study, an ROI represents an individual cell. Σ Area is the total area of all cells in the photograph, and Σ IOD is the sum of integral optical density of all cells in the photograph.

Statistical analysis

All of the statistical analyses were performed using SPSS version 11.5. Group differences in the escape latency during the MWM training task were analyzed using a two-way analysis of variance (ANOVA) with repeated measures; the factors used were treatment and training day.
shRNA, FoxO1 shRNA and control groups (non-significant vs. control group). All of these mice had the same level of performance at the start of the experiment (no significant individual effect was observed in the first four trials on day 1).

On the 5th day, the platform was removed, and the probe test was conducted. Mice in DA/FoxO1 shRNA, FoxO1 shRNA and control groups spent more time swimming in the target quadrant (where the platform had been located) (non-significant vs. control group) than the DA group \(F(3, 36) = 21.375, p < 0.001\) vs. control group].

Similar results were obtained for the mean number of times the animals crossed the platform location. DA-treated mice crossed over the platform less frequently than control mice \(F(3, 36) = 27.675, p < 0.001\) vs. control group]. Hippocampus-specific FoxO1 knockdown significantly increased crossing numbers of DA-treated mice \(p < 0.001\) vs. control group]. No significant difference was found among the DA/FoxO1 shRNA, FoxO1 shRNA and control groups.

**JNK mediates FoxO1 nuclear translocation of DA-treated mice**

As shown in Fig. 4, DA treatment significantly increased ROS and protein carbonyl levels in mouse hippocampus \(F(3, 16) = 35.919, p < 0.001\); protein carbonyl: \(F(3, 16) = 20.188, p < 0.001\) vs. control group], which enhanced the phosphorylation of JNK, decreased the phosphorylation of Akt and promoted FoxO1 activation \(p\text{-JNK (Thr183/Tyr185)}: F(3, 16) = 50.254, p < 0.001; p\text{-Akt (Ser473)}: F(3, 16) = 81.614, p < 0.001; p\text{-FoxO1 (Ser256)}: F(3, 16) = 130.618, p < 0.001; FoxO1nucleocytoplasm: F(3, 16) = 91.222, p < 0.001 vs. control group]. To determine whether oxidative stress is responsible for the JNK/Akt switch, the treatment with vitamin E, a well-known antioxidant was used in the DA-treated mice. Our results showed that vitamin E significantly blocked phosphorylation of JNK and increased the phosphorylation of Akt in the hippocampus of DA-treated mice \(p < 0.001\) vs. DA group) back to near-normal level (non-significant vs. control group). To further evaluate whether the activation of JNK had an effect on the phosphorylation status of Akt and the nuclear translocation of FoxO1 in the hippocampus of DA-treated mice, we blocked SAPK/JNK pathway using an inhibitor of SAPK/JNK signaling, SP600125. Our results showed that i.c.v infusion of SP600125 to DA-treated mice partially blocked JNK activation \(p < 0.01\) vs. control group; \(p < 0.001\) vs. DA group) and inhibited FoxO1 nuclear translocation \(p < 0.001\) vs. control group; \(p < 0.001\) vs. DA group). However,
Fig. 3. Hippocampus-specific FoxO1 knockdown improves cognitive deficits of the DA-treated mice. (A) Step-through passive avoidance task. (B) Mean latency in the Morris water maze (MWM) task hidden platform test. (C) Comparison of the number of crossings over the exact former location of the platform during the MWM task probe test. (D) Comparison of the time spent in the target quadrant (where the platform was located during hidden platform training) during the MWM task probe test. Data are from ten mice per group (A–D). All of the values are expressed as the mean ± SD. ***p < 0.001 compared with control group; ###p < 0.001 compared with DA group.

Fig. 4. JNK activation decreases Akt phosphorylation and mediates FoxO1 activation in the hippocampus of DA-treated mice. (A) Comparison of ROS and protein carbonyl levels. (B, D) Representative immunoblot for p-SAPK/JNK (Thr183/Tyr185), total-SAPK/JNK (t-SAPK/JNK), p-Akt (Ser473), total-Akt, FoxO1 activation in the cytoplasmic and nuclear extracts of hippocampus, β-actin and Histone H3 in all treated groups. (C, E) Relative density analysis of the p-SAPK/JNK, p-Akt, p-FoxO1 and FoxO1 protein bands. The relative densities are expressed as the ratios (p-SAPK/JNK/t-SAPK/JNK; p-Akt/t-Akt; p-FoxO1/β-actin; FoxO1/Histone H3). Data are from three independent experiments with five mice per group. All of the values are expressed as the mean ± SD. **p < 0.01, ***p < 0.001, compared with control group; ###p < 0.001, compared with DA group.

Please cite this article as: Wu, D., et al., Ursolic acid improves domoic acid-induced cognitive deficits in mice, Toxicol. Appl. Pharmacol. (2013), http://dx.doi.org/10.1016/j.taap.2013.04.038
SP600125 treatment had no effect on ROS and protein carbonyl levels in the hippocampus of DA-treated mice ($p < 0.01$ vs. control group; non-significant vs. DA group). In addition, SP600125 treatment alone also had no effect on the levels of ROS and protein carbonyl, the phosphorylation status of JNK and Akt and the activity of FoxO1 in mouse hippocampus (non-significant vs. control group). These results indicate that the activation of Akt and FoxO1 is regulated by SAPK/JNK signaling.

UA restores DA-induced hippocampal mitochondrial dysfunction in mice

As shown in Fig. 5, DA treatment significantly decreased the expression of p-Akt (Ser473) [p-Akt (Ser473): $F(3, 16) = 42.029$, $p < 0.01$ vs. control group], activated FoxO1 through decreasing its phosphorylation [FoxO1nucleus: $F(3, 16) = 41.390$, $p < 0.01$ vs. control group] and stimulating its nuclear translocation [FoxO1nucleus western blot: $F(3, 16) = 109.486$, $p < 0.01$; FoxO1nucleus immunofluorescence: $F(3, 16) = 19.377$, $p < 0.01$ vs. control group] in the hippocampus of mice, which increased HO-1 levels [HO-1 western blot: $F(3, 16) = 65.536$, $p < 0.01$; HO-1nucleus immunofluorescence: $F(3, 16) = 61.426$, $p < 0.01$ vs. control group] and sequentially impaired ETC activity and mitochondrial function [mt-Nd6: $F(3, 16) = 84.065$, $p < 0.01$; Sdhα: $F(3, 16) = 134.139$, $p < 0.01$; Uqcr1c: $F(3, 16) = 100.066$, $p < 0.01$; mt-Co1: $F(3, 16) = 132.16$, $p < 0.01$; Ap3pα1: $F(3, 16) = 60.294$, $p < 0.01$; Rcr: $F(3, 16) = 26.044$, $p < 0.01$; APR: $F(3, 16) = 20.675$, $p < 0.01$; ETC: $F(3, 16) = 18.857$, $p < 0.01$; ATP: $F(3, 16) = 24.140$, $p < 0.01$ vs. control group]. Interestingly, oral administration of UA to DA-treated mice for 3 weeks significantly reversed the abnormal expression of these proteins and abnormal mitochondrial parameter values ($p < 0.01$ vs. DA group; non-significant vs. control group). However, i.c.v infusion of LY294002, an inhibitor of PI3K/Akt signaling, significantly decreased the phosphorylation of Akt ($p < 0.01$ vs. control group; $p < 0.01$ vs. DA group) in the hippocampus of DA/UA mice and then weakened the protective effect of UA [p-Akt (Ser473)gplasma: $F(3, 16) = 0.001$, $p < 0.01$; FoxO1nucleus western blot: $F(3, 16) = 0.001$, $p < 0.01$; HO-1nucleus immunofluorescence: $p < 0.01$, mt-Nd6: $p < 0.01$; Sdhα: $p < 0.01$; Uqcr1c: $p < 0.01$; mt-Co1: $p < 0.01$; Ap3pα1: $p < 0.01$; Rcr: $p < 0.01$; APR: $p < 0.01$; ETC: $p < 0.05$; ATP: $p < 0.01$ vs. control group; p-Akt (Ser473)gplasma: $p < 0.05$; FoxO1nucleus western blot: $p < 0.05$; HO-1nucleus immunofluorescence: $p < 0.05$, mt-Nd6: $p < 0.01$; Sdhα: $p < 0.01$; Uqcr1c: $p < 0.01$; mt-Co1: $p < 0.01$; Ap3pα1: $p < 0.01$; Rcr: $p < 0.05$; APR: $p < 0.05$; ETC: $p < 0.05$; ATP: $p < 0.05$ vs. DA group].

UA improves cognitive deficits of the DA-treated mice

We further investigated the effect of UA on behavioral performance in a step-through passive avoidance task and a Morris water maze task in the DA-treated mice (Fig. 6). Our results showed that oral administration of UA reversed DA-induced cognitive deficits [step-through test: $F(3, 36) = 0.149$, non-significant; $F(3, 36) = 30.334$, $p < 0.01$, vs. DA group; Morris water maze task: $F(3, 36) = 105.667$, $p < 0.01$; $F(3, 36) = 22.518$, $p < 0.01$; $F(9, 144) = 1.341$, non-significant; $F(3, 36) = 25.484$, $p < 0.01$; $F(3, 36) = 25.078$, $p < 0.01$, vs. DA group]. Similarly, i.c.v infusion of LY294002 to mice co-treated with DA and UA partially blocked the protective effect of UA.

Discussion

DA is a naturally occurring shellfish toxin that can induce brain damage in mammals. Many investigations using rats and mice have demonstrated that DA injection causes epilepsy-like syndrome and cognitive deficits (Costa et al., 2010). Our recent findings suggest that hippocampal damage and memory loss induced by DA are involved in excessive ROS-mediated mitochondrial dysfunction (Lu et al., 2012). But the precise biological mechanisms underlying these effects are not well understood. FoxO1 belongs to the Forkhead family of transcription factors and plays an important role in metabolism, cell proliferation and oxidative stress response. Several studies in the last few years indicate that FoxO1 is an important regulator of mitochondrial function (Cheng et al., 2009; Sparks and Dong, 2009). Cheng et al. show that the activation of FoxO1 promotes the upregulation of HO-1 expression and sequentially impairs ETC and mitochondrial function in the insulin resistant liver (Cheng et al., 2009). Remarkably, deletion of hepatic FoxO1 can reverse the mitochondrial abnormalities in the insulin resistant liver. However, it is not clear whether FoxO1 knockdown can reverse the ROS-induced mitochondrial dysfunction in the hippocampus of DA-treated mice.

In the present study, our results demonstrated that the activation of FoxO1 is involved in the neuropathological process of mitochondrial dysfunction in the hippocampus of DA-treated mice (Fig. 2). FoxO1 knockdown reversed the mitochondrial abnormalities and cognitive deficits induced by DA in mice (Figs 2 and 3). Other investigations have also shown that ROS-induced nuclear localization of FoxO1 (FoxO1 activation) has been linked to the activation of JNK pathway (Huang and Tindall, 2007). Our recent studies have confirmed that ROS-activated JNK pathway is involved in DA-induced brain damage (Lu et al., 2012; Wu et al., 2012). In addition, oxidative stress or JNK pathway activation decreases the activity of Akt in HFT cells, leading to the decreased phosphorylation of FoxO1 following nuclear localization (Roy et al., 2010). So, we further investigated whether JNK pathway is involved in FoxO1 activation (Fig. 4). Our results revealed that DA treatment induced excessive ROS production and resulted in activation of the JNK pathway, which agreed with our recent findings (Lu et al., 2012). In turn, the activation of the JNK pathway decreased the phosphorylation of Akt and promoted the nuclear translocation of FoxO1. However, evidence has also shown that oxidative stress-induced phosphorylation of Akt may be dependant largely on the activation of growth factor receptors, which is a protective mechanism for cellular survival in response to oxidative injury (Martindale and Holbrook, 2002). Our results revealed that the long-term persistence of oxidative stress might result in the downregulation of the phosphorylation of Akt in hippocampus of mice with the chronic treatment of DA. In addition, administration of the inhibitor of JNK signaling, SP600125, significantly enhanced Akt phosphorylation and FoxO1 nuclear exclusion in the hippocampus of DA-treated mice, which further confirmed that the activation of JNK pathway is necessary for FoxO1 activation.

UA, a pentacyclic triterpenoid acid, has been reported to possess neuroprotective effects in different animal models (Lu et al., 2007, 2010e, 2012; Shih et al., 2004; Wang et al., 2011; Wilkinson et al., 2011). Additionally, several lines of evidence have also shown that DA can exert its protective activities through enhancing insulin signaling (Jayaprakasam et al., 2006; Kunkel et al., 2011; Lu et al., 2012) and promoting FoxO1 nuclear exclusion (Kunkel et al., 2011). We therefore hypothesize that UA may attenuate DA-induced mitochondrial dysfunction and cognitive deficits in mice via the regulation of these two signaling pathways. Our data did show that UA significantly increased Akt phosphorylation in the hippocampus of DA-treated mice, subsequently downregulated FoxO1-HO-1 signaling, restored mitochondrial function and cognitive deficits (Figs 5 and 6). However, Li et al. found that UA (130 mg/kg, i.p. injection) exerts its neuroprotective effects against acute ischemic injury in mice via the activation of Nr2f/HO-1 pathway (Li et al., 2013). The effect of UA on the HO-1 expression was not consistent with our present finding, which may be due to different pathological mechanisms of mouse models and different dosage and period of UA. LY294002, an inhibitor of PI3K/Akt

Please cite this article as: Wu, D., et al., Ursolic acid improves domoic acid-induced cognitive deficits in mice, Toxicol. Appl. Pharmacol. (2013), http://dx.doi.org/10.1016/j.taap.2013.04.038
Fig. 5. UA restores mitochondrial dysfunction in the hippocampus of DA-treated mice. (A) Representative immunoblot for p-Akt (Ser473), total-Akt, mt-Nd6, Sdhα, Uqcr1, mt-Co1 and Atp5a1, porin, HO-1, FoxO1 activation in the cytoplasmic and nuclear extracts of hippocampus, β-actin and Histone H3 in all treated groups. (B) Relative density analysis of p-Akt, mt-Nd6, Sdhα, Uqcr1, mt-Co1, Atp5a1, porin; HO-1, p-FoxO1 and FoxO1 protein bands. The relative densities are expressed as the ratios (p-Akt/total-Akt; mt-Nd6/porin; Sdhα/porin; Uqcr1/porin; mt-Co1/porin; Atp5a1/porin; HO-1/Histone H3; p-FoxO1/β-actin; FoxO1/Histone H3). (C) RCR. (D) APR. (E) ETC activity. (F) ATP content. (G) Hippocampal CA1 sections from all treatment groups were stained with FITC and Texas Red to visualize FoxO1-positive cells (red) and HO-1-positive cells (green), respectively (scale bar = 25 μm). The nucleus was visualized by DPAI staining. In merged images, the nuclear colocalization of FoxO1 and HO-1 is indicated by the arrows (right). (H) Analysis of the mean OD values of FoxO1-positive cells and HO-1-positive cells in the hippocampal CA1 sections. Data are from three independent experiments with five mice per group (A–H). All of the values are expressed as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control group; #p < 0.05, ##p < 0.01, ###p < 0.001, compared with DA group.
signaling, significantly weakened the protective effect of UA, which indicated that Akt phosphorylation is required for UA to exert its neuroprotective effects (Fig. 5). Our previous report demonstrated that LY294002 injection (40 μg/day, i.c.v.) had no effect on behavioral performance in a step-through passive avoidance task and a Morris water maze task when administered alone (Lu et al., 2010c). These results implied a potential mechanism for the neuroprotective effects of UA in the hippocampus of DA-treated mice.

In conclusion, ROS-induced JNK signaling pathway mediates mitochondrial dysfunction and cognitive deficits by decreasing Akt phosphorylation and promoting FoxO1/HO-1 signaling in the hippocampus of DA-treated mice. Whereas, UA attenuates DA-induced mitochondrial dysfunction and cognitive deficits in mice via the regulation of Akt and FoxO1 pathways. These results indicate that UA could be recommended as a possible candidate for the prevention and therapy of cognitive deficits in excitotoxic brain disorders. A diagram of the neuroprotective effects of UA against DA-induced cognitive deficits is shown in Fig. 7.

Conflict of interest

No conflict of interest is declared.

Acknowledgments

This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the 2010 “Qinglan Project” of the Scientific and Technological Innovation Team Training Program of Jiangsu College and University, the 2012 “Qinglan Project” of the middle-aged academic pioneer of Jiangsu College and University (12QLD008), the National Natural Science Foundation of China (81171012; 30950031; 81271225), the Major Fundamental Research Program of the National Science Foundation of the Jiangsu Higher Education Institutions of China (07KJA36029), grants from the National Science Foundation for Colleges and Universities in Jiangsu Province (09KJB180009), grants from the Key Laboratory of Jiangsu Province, PR China and grants from the Natural Science Foundation of Xuzhou Normal University (08XLR09; 09XLY05; 09XKK02; 10XLA05).

Please cite this article as: Wu, D., et al., Ursolic acid improves domoic acid-induced cognitive deficits in mice, Toxicol. Appl. Pharmacol. (2013), http://dx.doi.org/10.1016/j.taap.2013.04.038
Quercetin activates AMP-activated protein kinase by reducing PP2C expression protecting old mouse brain against high cholesterol-induced neurotoxicity. J. Pathol. 222, 199–212.


