Evidence shows that administration of d-galactose (d-gal) induces reactive oxygen species (ROS) production and inflammatory response resulting in neurodegenerative changes. Ursolic acid (UA), a triterpenoid compound, has been reported to possess antioxidant and anti-inflammatory properties. Our previous studies have demonstrated that UA could protect mouse brain against d-gal-induced oxidative damage. In the present study, we examined the protective effect of UA against d-gal-induced inflammatory response in the prefrontal cortex and explored the potential mechanism of its action. Our results showed that UA administration significantly improved behavioral performance of d-gal-treated mice in step-through test and Morris water maze task. One of the potential mechanisms of this action was decreased advanced glycation end products (AGEs), ROS, and protein carbonyl levels in the prefrontal cortex of d-gal-treated mice. Furthermore, the results also showed that UA significantly reduced the number of activated microglia cells and astrocytes, decreased the expression of CD11b and glial fibrillary acidic protein, downregulated the expression of iNOS and COX-2, and decreased interleukin (IL)-1β, IL-6, and tumor necrosis factor-α levels in the prefrontal cortex of d-gal-treated mice. Moreover, UA significantly decreased AGEs induced the expression of receptor for advanced glycation end products and inhibited NF-κB p65 nuclear translocation in the prefrontal cortex of d-gal-treated mice. The aforementioned effects of UA could attenuate brain inflammatory response.

Keywords: AGEs, d-galactose, inflammatory response, RAGE, ursolic acid

Introduction

Ursolic acid (UA: 3β-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid. Evidence shows that it has many biological activities such as antioxidant, anti-inflammatory, trypanocidal, antirheumatic, antiviral, and antitumoral properties (Huang et al. 1994; Harmand et al. 2003; Saedinia et al. 2004; Liu 2005; Ren et al. 2005). Previous reports from our laboratory also confirmed neuroprotective effects of it against oxidative damage induced by d-galactose (d-gal; Lu et al. 2007).

d-gal is a reducing sugar and can be metabolized at normal concentration. However, at high levels, it induces the production of reactive oxygen species (ROS) and advanced glycation end products (AGEs; Lu et al. 2007, 2009). Recent findings show that ROS and AGEs induced by continuous injection of d-gal in rodent lead to the decreased expression of memory-related protein, deterioration of learning and memory function, and pathological alterations of astrocytes which might be associated with the increased expression of inflammation-related gene (Tian et al. 2005; Lu et al. 2006, 2007, 2009; Lei et al. 2008; Wu et al. 2008). A substantial amount of evidence has demonstrated that ROS and AGEs have been implicated in the pathological processes of age-related disease such as diabetes, arteriosclerosis, nephropathy, infection, and Alzheimer’s disease (Tian et al. 2005; Cai et al. 2006; Lei et al. 2008; Lu et al. 2009; Srikanth et al. 2009). Especially, the latest reports suggest that AGEs binding to its receptor for advanced glycation end products (RAGE) in many cell types induces pathophysiologi cal cascades linked to the downstream activation of NF-κB and other signaling pathways that lead to ROS generation and certain proinflammatory responses (Mallidis et al. 2007). Suppression of RAGE ligand binding using soluble RAGE or neutralizing antibodies has been shown to prevent various pathological events in a range of cells (Mallidis et al. 2007).

In the present study, we explored whether UA has a protective effect against d-gal-induced inflammatory response in mouse prefrontal cortex. We further investigated the potential mechanism underlying its action.

Materials and Methods

Animals and Administration

Thirty-two 10-week-old male Kunming strain mice were purchased from the Branch of National Breeder Center of Rodents (Shanghai). Prior to experiments mice had free access to food and water and were kept under constant conditions of temperature (23 ± 1 °C) and humidity (60%). Eight mice were housed per cage on a 12-h light/dark schedule (lights on 08:30–20:30). After a week of adaptation, mice were divided randomly into 4 groups. Group 1 and group 4 served as vehicle control group receiving only vehicle (1% Tween-80) orally at the same dose. The dosage of d-gal and UA in groups 1 and 4 served as vehicle control group receiving only vehicle (1% Tween-80) by oral gavage for 8 weeks, and the mice of groups 1 and 4 received daily subcutaneous injection of d-gal (Sigma-Aldrich, MO) at the dose of 50 mg/(kg day) for 8 weeks, respectively. At the same time, mice in groups 2 and 3 received daily UA (Sigma-Aldrich) of 10 mg/(kg day) in distilled water containing 0.1% Tween-80 (dH2O/0.1% Tween-80) by oral gavage for 8 weeks, and the mice of groups 1 and 2 were given distilled water containing 0.1% Tween-80 (dH2O/0.1% Tween-80) orally at the same dose. The dosage of d-gal and UA in the experiments agreed with our previous report (Lu et al. 2007). Before experiment, the mean body weights of each group were also examined: control group: 30.44 ± 2.15 g; d-gal group: 30.58 ± 2.57 g; d-gal + UA group: 31.12 ± 2.71 g; UA group: 30.63 ± 2.32 g. After 8-week administration, the mean body weights of each group were also examined: control group: 35.25 ± 2.64 g; d-gal: 36.41 ± 3.05 g; d-gal + UA: 36.88 ± 3.26 g; UA: 35.48 ± 2.49 g. The administration of d-gal and/or UA does not significantly influence on the body weight change in the mice. All experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals and were approved by the respective university committees for animal experiments. After the behavioral testing, mice were sacrificed and brain tissues were immediately collected for experiments or stored at −70 °C for later use.

Behavioral Tests

Step-through Test

The step-through passive avoidance apparatus consisted of an illuminated chamber (11.5 × 9.5 × 11 cm) attached to a darkened illuminated chamber (11.5 × 9.5 × 11 cm) attached to a darkened...
chamber (23.5 × 9.5 × 11 cm) containing a metal floor that could deliver footshocks. The 2 compartments were separated by a guillotine door. The illuminated chamber was lit with a 25-W lamp. The step-through test was performed as described previously (Lu et al. 2006).

MWM Test
The Morris water maze (MWM) test was performed as described previously (Lu et al. 2006; Wu et al. 2008). The experimental apparatus consisted of a circular water tank (100 cm in diameter, 35 cm in height), containing water (23 ± 1 °C) to a depth of 15.5 cm, which was rendered opaque by adding ink. A platform (4.5 cm in diameter, 14.5 cm in height) was submerged 1 cm below the water surface and placed at the midpoint of one quadrant. The pool was located in a test room, which contained various prominent visual cues. The test was performed as described previously. Each mouse received 4 training periods per day for 4 consecutive days. Latency to escape from the water maze (finding the submerged escape platform) was calculated for each trial. On day 5, the probe test was carried out by removing platform and allowing each mouse to swim freely for 60 s. The time that mice spent swimming in the target quadrant (where the platform was located during hidden platform training) was measured. For the probe trial, the number of times crossing over the platform site of each mouse was also measured and calculated. All data were recorded with a computerized video system.

Preparation of Tissue Samples
Tissue Homogenates
For biochemical studies performed as described previously (Lu et al. 2006, 2007, 2009), animals were deeply anesthetized and sacrificed. Two hemispheres were separated and weighed on ice. Left prefrontal cortex was homogenized in 1/5 (w/v) 50 mM (pH 7.4) ice-cold Tris-buffered saline (TBS) solution containing a protease inhibitor cocktail (Sigma-Aldrich) with 10 strokes at 1200 rpm in a Potter homogenizer. Homogenates were centrifuged at 12,000 × g for 10 min to obtain the supernatant. Supernatant aliquots were used to determine brain AGES, ROS, and protein carbonyl levels.

For western blot analysis performed as described previously (Lu et al. 2006, 2009; Wu et al. 2008), right prefrontal cortex was homogenized in 1/5 (w/v) 50 mM (pH 7.4) ice-cold Tris-buffered saline (TBS) solution containing a protease inhibitor cocktail (Sigma-Aldrich) with 10 strokes at 1200 rpm in a Potter homogenizer. Homogenates were centrifuged at 12,000 × g for 10 min to obtain the supernatant. Supernatant aliquots were used to determine brain AGES, ROS, and protein carbonyl levels.

Biochemical Analysis
Assay of ROS
ROS was measured as described previously, based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to 2′,7′-dichlorofluorescein (DCF) (Shinomol and Muralidhara 2007; Lu et al. 2009). Briefly, 96-well plates were coated with 100 μL/well of 3 μg/mL AGE-bovine serum albumin in coating buffer overnight at 4 °C. Wells were washed 3 times with 150 μL washing buffer (phosphate-buffered saline [PBS], 0.05% Tween-20, 1 mM NaCl), then blocked with 1% normal goat serum in 100 μL PBS for 2 h at 37 °C. After washing, 50 μL of 1:10 diluted samples in dilution buffer (PBS, 0.02% Tween-20, 1 mM NaCl, 1% normal goat serum) and 50 μL anti-AGE polyclonal antiserum in dilution buffer (1:2000) were added. Plates were incubated at room temperature for 2 h with gentle agitation on a horizontal rotary shaker. Wells were washed, 100 μL alkaline phosphate conjugated 2nd antibody in dilution buffer (1:2000) was added, and the plates were incubated at 37 °C for 1 h. They were then washed 6 times as above, 100 μL p-nitrophenyl phosphate substrate was added to each well. After 60 min, optical density (OD) at 405 nm was measured using a microplate reader (Wallac 1420 Multiscan, Turku, Finland).

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Oxidative Stress Marker: Protein Carboxyls
Protein carbonyl content was measured as a marker of oxidative damage to proteins (Tsai et al. 1998; Kujoth et al. 2005). They were determined the hydrazone derivatives between 360 and 390 nm (ε = 22,000 M−1 cm−1) according to the method described earlier (Shinomol and Muralidhara 2007; Lu et al. 2009). Data are expressed as nmol carbonyls/mg protein.

AGE Enzyme-Linked Immunosorbent Assay
Quantitative measurement of AGES was performed by AGE-enzyme-linked immunosorbent as described previously (Song et al. 1999; Lu et al. 2009). Briefly, 96-well plates were coated with 100 μL/well of 3 μg/mL AGE-bovine serum albumin in coating buffer overnight at 4 °C. Wells were washed 3 times with 150 μL washing buffer (phosphate-buffered saline [PBS], 0.05% Tween-20, 1 mM NaCl), then blocked with 1% normal goat serum in 100 μL PBS for 2 h at 37 °C. After washing, 50 μL of 1:10 diluted samples in dilution buffer (PBS, 0.02% Tween-20, 1 mM NaCl, 1% normal goat serum) and 50 μL anti-AGE polyclonal antiserum in dilution buffer (1:2000) were added. Plates were incubated at room temperature for 2 h with gentle agitation on a horizontal rotary shaker. Wells were washed, 100 μL alkaline phosphate conjugated 2nd antibody in dilution buffer (1:2000) was added, and the plates were incubated at 37 °C for 1 h. They were then washed 6 times as above, 100 μL p-nitrophenyl phosphate substrate was added to each well. After 60 min, optical density (OD) at 405 nm was measured using a microplate reader (Wallac 1420 Multiscan, Turku, Finland).

Collection of Brain Slice
The mice were perfused transcardially with 25 mL of normal saline (0.9%). The brain tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) or acetic acid 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 (Leica, CA, Germany). Coronal sections (12 μm) from cryofixed tissue were collected on 3-aminopropyl-trimethoxysilane-coated slides (Sigma-Aldrich) and stored at −70 °C.

Immunofluorescence Staining
All immunofluorescence staining or double immunostaining was performed on cryofixed sections cut and mounted as described above.
The double staining was performed by simultaneously adding both first antibodies, followed by overnight incubation at 4°C. In addition to the above described antibodies, the following antibodies were used: rat anti-CD11b (1:100, Serotec, Oxford, UK); rabbit anti-RAGE antibody (1:100, Abcam); chicken anti-GEAP antibody (1:500, Abcam); and mouse anti-NF-kB p65 (1:50, Santa Cruz Biotechnology, CA). The secondary antibodies (Texas red-conjugated anti-rabbit 1:100, fluorescein isothiocyanate [FITC]-conjugated anti-rat 1:150, FITC-conjugated anti-chicken 1:350, FITC-conjugated anti-mouse 1:100, Santa Cruz Biotechnology, CA) were applied sequentially after being washed in PBS. The specificity of the staining was assessed by omitting the primary antibody.

Double-labeled specimens were analyzed with a Zeiss Axioskop 40 microscope equipped for both light and fluorescence microscopy (Carl Zeiss, Oberkochen, Germany). The images were taken with a CCD camera (CoolSNAP Color, Photometrics, Roper Scientific, Inc., Trenton, NJ) connected to a microscope (40× objective) and processed and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD). For analysis, plaque areas were excluded and the number of stained cells in 0.01 mm² was estimated by blind manual counting of the region located at a consistent position per section.

Statistical Analysis
All statistical analyses were performed using the SPSS software, version 11.5. Group differences in the escape latency in the MWM training task were analyzed using 2-way analysis of variance (ANOVA) with repeated measures, the factors being treatment and training day. The other data were analyzed with one-way ANOVA followed by Newman–Keuls or Tukey’s honestly significant difference post hoc test. Data were expressed as means ± standard deviation. Statistical significance was set at P < 0.05.

Results

UA Reverses Cognitive Impairment of D-gal-Treated Mice

Step-through Passive Avoidance Task

The effect of oral administration of 10 mg/(Kg day) UA to D-gal-treated mice for 8 weeks on the step-through latencies is shown in Figure 1. In the acquisition trial, the initial latencies did not differ significantly among the 4 groups (F(3,28) = 0.171, P > 0.05; Fig. 1). But mice that were treated with D-gal at the dose of 50 mg/(Kg day) for 8 weeks showed a significant reduction of step-through latencies in the 24-h retention trial (F(3,28) = 6.793, P < 0.01). Our results also showed that the latencies in D-gal-treated mice received daily 10 mg/(Kg day) UA for 8 weeks were markedly lengthened as compared with D-gal-treated mice (F(3,28) = 6.793, P < 0.05). The shortened latencies induced by D-gal were restored to near-normal level after oral administration of UA. There was no significant difference in step-through latencies between the control group and the UA group. The result suggested that no obvious neural toxicity in mice treated with UA alone was found.

MWM Test

The MWM is one of the most widely used behavioral tasks designed to study the psychological processes and neural mechanisms of spatial learning and memory. In the present study, all groups of mice improved their performance as indicated by the decreased escape latencies across successive days (Fig. 2A). Mice showed significant difference in mean latencies between training days (F(3,112) = 30.315, P < 0.001; Fig. 2A) and between treatments (F(3,112) = 11.346, P < 0.001) but no interaction between the factors day and treatment (F(3,112) = 1.118, P > 0.05).

Two-way ANOVA showed that D-gal–treated mice had longer escape latencies compared with control mice (P < 0.001). This result indicated that D-gal treatment impaired the spatial learning and memory ability. A comparison between the D-gal group and the UA/D-gal group showed that UA could shorten escape latencies of D-gal–treated mice (P < 0.001). These results indicated that UA could improve spatial learning and memory in the D-gal–treated mice, and there was no significant difference in spatial learning and memory ability between the control group and the UA/D-gal group. There was also no significant difference between the control group and the UA group. Note that mice of every treatment started at the same level of performance (no significant individual effect was observed for the first 4 trials of day 1).

A probe test, in which the platform was removed, was accomplished on day 5. The control, UA, and UA/D-gal groups spent more time swimming in the target quadrant (where the platform was located; no significant difference was observed among the 3 treated groups), while the D-gal group spent less time in the target quadrant (F(3,28) = 4.422, P < 0.05 vs. control group; Fig. 2C). Similar results were obtained from the former platform crossings experiments. The D-gal–treated mice crossed over the platform less frequently than the controls (F(3,28) = 4.46, P < 0.01; Fig. 2B). No significant difference between the control and the UA/D-gal group was found, as well as the difference between the control group and the UA group.

UA Decreases AGEs, ROS, and Protein Carbonyl Levels in the Prefrontal Cortex of D-gal–Treated Mice

Recent reports have revealed that D-gal can induce AGEs, ROS, and protein carbonyl production resulting in brain aging and neurodegeneration (Lu et al. 2006, 2007, 2009; Wu et al. 2008). The prefrontal cortex is a structure within the brain that plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness. But it was more susceptible to various neurotoxic agents such as ROS, α-gal, and β-amyloid (Beglopoulos et al. 2004; Sathyasai Kumar et al. 2007; Lu et al. 2009). Our results (Fig. 3) showed that D-gal administration significantly increased AGEs, ROS, and protein carbonyl levels in the prefrontal cortex (F(AGEs(3,8)) = 21.498, P < 0.01; F(ROS(3,8)) = 31.25, P < 0.01; F(protein carbonyl(3,8)) = 22.862, P < 0.001) as compared with the control group. The data indicated that elevated oxidative stress in the prefrontal cortex of D-gal–treated mice was one potential reasons of the behavioral...
deficit. Whereas, UA could decrease AGEs, ROS, and protein carbonyl levels and improve behavioral deficit in the prefrontal cortex of d-gal-treated mice ($F_{AGE}(3,8) = 21.498, P < 0.05$; $F_{ROS}(3,8) = 31.25, P < 0.01$; $F_{protein~carbonyl}(3,8) = 22.862, P < 0.05$). But, the UA/d-gal group was not reached normal level as compared with the control group ($F_{AGE}(3,8) = 21.498, P < 0.05$; $F_{ROS}(3,8) = 31.25, P < 0.05$; $F_{protein~carbonyl}(3,8) = 22.862, P < 0.05$). No significant difference was found between the control group and the UA group.

**UA Reduces the Number of Activated Microglia Cells (CD11b-Stained Cell), Activated Astrocytes (GFAP-Stained Cell), and RAGE-Positive Cells and Decreases the Expression of CD11b, GFAP, and RAGE in the Prefrontal Cortex of d-gal-Treated Mice**

Glial cells are the most abundant cell types in the central nervous system. There are 3 types of glial cells: astrocytes, oligodendrocytes, and microglia. Recently, evidence shows that microglia cells and astrocytes activation are being considered as a pathological hallmark in neurodegenerative disorders (Wu et al. 2002; Dauer and Przedborski 2003; Gao et al. 2003; Benner et al. 2004). CD11b and GFAP are specific markers for activated microglia cells and astrocytes, respectively. RAGE, a multiligand receptor in the immunoglobulin family, is expressed in tissues and is upregulated with ongoing glycooxidation and tissue damage. Latest reports indicate that AGEs binding to its receptor (RAGE) leads to NF-κB activation which allows its translocation to the nucleus, where it regulates the expression of a large number of genes, including TNF-α, IL-1β, and RAGE (Singh et al. 2001; Evans et al. 2002). In a further study, RAGE expression has been shown to be colocalized with activated microglial cells and astrocytes in neurodegenerative disease (Takeda et al. 1998; Lue et al. 2001).

In this study, d-gal administration significantly increased the number of CD11b-positive microglia cells and GFAP-positive astrocytes in mouse prefrontal cortex as compared with the control group ($F_{CD11b}(3,8) = 148.846, P < 0.001$; $F_{GFAP}(3,8) = 49.772, P < 0.001$; Fig. 4A,B). Similarly, d-gal administration also significantly increased the number of RAGE-positive cells in mouse prefrontal cortex as compared with the control group ($F_{RAGE}(3,8) = 52.355, P < 0.001$; Fig. 4A,B). Moreover, RAGE-positive cells colocalized with activated microglial cells and astrocytes in the prefrontal cortex of d-gal-treated mice (Fig. 4A).

But, oral administration of UA to d-gal-treated mice for 8 weeks could significantly reduced the number of CD11b-positive microglia cells and GFAP-positive astrocytes and decreased the number of RAGE-positive cells in the prefrontal cortex ($F_{CD11b}(3,8) = 148.846, P < 0.001$; $F_{GFAP}(3,8) = 49.772, P < 0.01$; $F_{RAGE}(3,8) = 52.355, P < 0.01$; Fig. 4A,B). Furthermore, the semi quantitative western blot analysis of CD11b, GFAP, and RAGE expression was carried out in prefrontal cortex of each group (Fig. 4C). Similar results were obtained from this semiquantitative analysis (Fig. 4D). The expression of CD11b, GFAP, and RAGE in the prefrontal cortex of d-gal-treated mice was significantly upregulated as compared with that in the prefrontal cortex of control group ($F_{CD11b}(3,8) = 80.23, P < 0.001$; $F_{GFAP}(3,8) = 34.741, P < 0.001$; $F_{RAGE}(3,8) = 71.637, P < 0.001$). Whereas, these increases were significantly attenuated in the presence of UA at the dose of 10 mg/(Kg day) ($F_{CD11b}(3,8) = 80.23, P < 0.001$; $F_{GFAP}(3,8) = 34.741, P < 0.01$; $F_{RAGE}(3,8) = 71.637, P < 0.001$). But, these protein levels in the prefrontal cortex of UA/d-gal group were not restored to normal level ($F_{CD11b}(3,8) = 80.23, P < 0.01$; $F_{GFAP}(3,8) = 34.741, P < 0.05$; $F_{RAGE}(3,8) = 71.637, P < 0.01$). There was no significant difference between the control group and the UA group.

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**Figure 2.** MWM test (n = 8). All values are expressed as mean ± standard deviation. (A) Mean latency in the hidden platform test. (B) The number of crossings over the exact location of the former platform. *P < 0.05 versus control group; #P < 0.05 versus d-gal group. (C) Comparison of the time spent in target quadrant on day 5 (where the platform was located during hidden platform training). *P < 0.05 versus control group; #P < 0.05 versus d-gal group. (D) The number of crossings over the exact location of the former platform and the number of crossings over the exact location of the former platform.
present study, we evaluated the activation of NFκB and result in neurodegeneration (Salminen et al. 2009). In the line with aforesaid result, western blot analysis showed that UA significantly decreased the expression of NFκB p65 in cytoplasm (Fcytoplasm(3,8) = 77.478, P < 0.001; Fig. 5C). Oral administration of UA significantly inhibited the nuclear translocation of NFκB p65 as demonstrated by immunofluorescence staining (Fig. 5A). In the study of NFκB p65 protein levels in nuclei or in cytoplasm was not restored to normal level (Fnucleo(3,8) = 266.674, P < 0.05; Fcytoplasm(3,8) = 77.478, P < 0.001; Fig. 5C). But the NFκB p65 protein levels in nuclei or in cytoplasm was not restored to normal level (Fnucleo(3,8) = 266.674, P < 0.05; Fcytoplasm(3,8) = 77.478, P < 0.001; Fig. 5C). There was no significant difference between the control group and the UA group (Fig. 5C).

UA Reduces the Expression of Inflammatory Markers in the Prefrontal Cortex of d-gal-Treated Mice

We further examined the expression of inflammatory markers, including COX-2, iNOS, IL-1β, IL-6, and TNF-α, in the prefrontal cortex of each group by western blot analysis (Fig. 6). d-gal administration induced significant increases of COX-2, iNOS, IL-1β, IL-6, and TNF-α levels in mouse prefrontal cortex as compared with those in the prefrontal cortex of control group (FcoxA(3,8) = 24.787, P < 0.001; Finos(3,8) = 38.675, P < 0.001; fil(3,8) = 73.573, P < 0.001; fil12(3,8) = 102.117, P < 0.001; finf(3,8) = 23.729, P < 0.001). Interestingly, oral administration of UA could significantly attenuate these increases in the prefrontal cortex of d-gal group (FcoxA(3,8) = 24.787, P < 0.001; finos(3,8) = 38.675, P < 0.001; fil(3,8) = 73.573, P < 0.001; fil12(3,8) = 102.117, P < 0.001; finf(3,8) = 23.729, P < 0.001). But, these protein levels in the prefrontal cortex of UA/d-gal group were not restored to normal level (FcoxA(3,8) = 24.787, P < 0.001; finos(3,8) = 38.675, P < 0.001; fil(3,8) = 73.573, P < 0.001; fil12(3,8) = 102.117, P < 0.001; finf(3,8) = 23.729, P < 0.001). No significant difference was found between the control group and the UA group.

Discussion

Previous evidence shows that d-gal is a nerve poison that reacts readily with the free amines of amino acids in proteins and peptides and induces AGEs formation (Lu et al. 2006, 2007; Lei et al. 2008; Wu et al. 2008). It is well known that AGEs are a harmful posttranslational protein modification and a trigger of excess ROS and abnormally high oxidative stress, which will accelerate the aging process (Baynes and Thorpe 1999; Brownlee 2001; Srikantan et al. 2009). On the other hand, a growing body of evidence suggests that the interaction of AGEs with its cell surface receptors, such as RAGE, mediates NFκB activation which will increase the production of inflammatory markers and enhance microglia cells and astrocytes activation, ultimately resulting in diabetes mellitus, atherosclerosis, and neurodegenerative disorders (including Alzheimer’s disease; Bierhaus et al. 2005; Srikantan et al. 2009). So the AGES/RAGE/NFκB axis is an attractive drug target for clinical interventions in these chronic disease states. In particular, NFκB pathway is a critical player for these disease actions of nutritional oxidative stress and metabolic inflammation (Cai 2009). In the present study, our results demonstrated that d-gal injection could significantly increase AGES, ROS, and demonstrated by the intense green fluorescence localized in the cell nuclei (Fig. 5A) and significantly decreased the expression of NFκB p65 in cytoplasm (Fcytoplasm(3,8) = 77.478, P < 0.001; Fig. 5C).

UA Inhibits NFκB Activation through Inhibiting NFκB p65 Nuclear Translocation in the Prefrontal Cortex of d-gal-Treated Mice

NFκB, a family of DNA-binding proteins, is activated under physiological and pathological conditions and involved in regulating many aspects of cellular activity, in stress, injury, and especially in pathways of the immune response. The survey evidence shows interaction between AGES and its receptor (RAGE) could activate NFκB-mediated inflammatory pathway and result in neurodegeneration (Salminen et al. 2009). In the present study, we evaluated the activation of NFκB p65 and its nuclear translocation by immunofluorescence and western blot (Fig. 5). d-gal administration significantly increased the nuclear translocation of NFκB p65 (Fnucleo(3,8) = 266.674, P < 0.001) as compared with those in the prefrontal cortex of control group. But the NFκB p65 protein levels in nuclei or in cytoplasm was not restored to normal level (Fnucleo(3,8) = 266.674, P < 0.05; Fcytoplasm(3,8) = 77.478, P < 0.001; Fig. 5C). There was no significant difference between the control group and the UA group (Fig. 5C).
protein carbonyl levels in mouse prefrontal cortex and induce behavior deficit which agreed with our recent reports (Lu et al. 2006, 2007, 2009; Wu et al. 2008). In addition, we also found that D-gal administration induced a significant increase in the number of RAGE-positive cells and the expression of RAGE in the prefrontal cortex of D-gal-treated mice (n = 3). Values are averages from 3 independent experiments. All values are expressed as mean ± standard deviation. (A) Representative immunofluorescence staining for CD11b, GFAP, and RAGE in mouse prefrontal cortex (×400 magnification). (B) Quantitative analysis of the number of CD11b, GFAP, and RAGE-positive cells. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; ###P < 0.01, ####P < 0.001 versus D-gal group. (C) Representative immunoblot for CD11b, GFAP, and RAGE in mouse prefrontal cortex. (D) Fold change in relative density of CD11b, GFAP, and RAGE-protein bands. The relative density is expressed as the ratio CD11b, GFAP, and RAGE/β-actin, and the vehicle control is set as 1.0. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; ##P < 0.01, ###P < 0.001 versus D-gal group.

Figure 4. UA reduces the number of CD11b-stained cells, GFAP-stained cells, and RAGE-positive cells and decreases the expression of CD11b, GFAP, and RAGE in the prefrontal cortex of D-gal-treated mice (n = 3). Values are averages from 3 independent experiments. All values are expressed as mean ± standard deviation. (A) Representative immunofluorescence staining for CD11b, GFAP, and RAGE in mouse prefrontal cortex (×400 magnification). (B) Quantitative analysis of the number of CD11b, GFAP, and RAGE-positive cells. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; ##P < 0.01, ###P < 0.001 versus D-gal group. (C) Representative immunoblot for CD11b, GFAP, and RAGE in mouse prefrontal cortex. (D) Fold change in relative density of CD11b, GFAP, and RAGE-protein bands. The relative density is expressed as the ratio CD11b, GFAP, and RAGE/β-actin, and the vehicle control is set as 1.0. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; ##P < 0.01, ###P < 0.001 versus D-gal group.
Figure 5. UA inhibits NF-κB activation through inhibiting NF-κB p65 nuclear translocation in the prefrontal cortex of d-gal-treated mice (n = 3). Values are averages from 3 independent experiments. All values are expressed as mean ± standard deviation. (A) Representative immunofluorescence detection of NF-κB p65 in mouse prefrontal cortex (×400 magnification). (B) Representative immunoblot for NF-κB p65 in the cytoplasmic and nuclear extracts of prefrontal cortex tissue. (C) Fold change in relative density of NF-κB p65 protein bands. The relative density is expressed as the ratio NF-κB p65/β-actin or NF-κB p65/β-tubulin, and the vehicle control is set as 1.0. *P < 0.05, ***P < 0.001 versus control group; ###P < 0.001 versus D-gal group.

Figure 6. UA reduces the expression of inflammatory markers in the prefrontal cortex of d-gal-treated mice (n = 3). Values are averages from 3 independent experiments. All values are expressed as mean ± standard deviation. (A) Representative immunoblot for COX-2, iNOS, IL-1β, IL-6, and TNF-α in mouse prefrontal cortex. (B) Fold change in relative density of COX-2, iNOS, IL-1β, IL-6, and TNF-α protein bands. The relative density is expressed as the ratio COX-2, iNOS, IL-1β, IL-6, and TNF-α/β-actin, and the vehicle control is set as 1.0. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; #P < 0.05, ##P < 0.01, ###P < 0.001 versus D-gal group.
brain degeneration. Similarly, recent reports demonstrate that D-gal induces astrocyte dysfunction and inflammatory response (Tian et al. 2005; Lei et al. 2008). In addition, dietary D-gal could promote energy imbalance, which plays a very important role in degenerative disorders, in mouse brain, and energy imbalance is also promoted by metabolic inflammation (Zhang, Zhang, et al. 2008; Zhang, An, et al. 2008). Therefore, our findings indicate that dietary D-gal, as a cause of oxidative stress and metabolic inflammation, could result in a decline of behavioral performance by NF-κB activation.

UA is a natural pentacyclic triterpene and is well known to possess a wide range of biological functions, such as antioxidative, anti-inflammation, and anticancer activities (Lu et al. 2007; Ikeda et al. 2008; Lee et al. 2008). Recently, evidence reveals that UA inhibits lipopolysaccharide-induced inflammatory response through inhibiting IL-8 production, NF-κB activation, and iNOS mRNA expression (Lee et al. 2008). Interestingly, it was reported that UA activated NF-κB for releasing proinflammatory mediators in nonstimulated mouse Mφ (You et al. 2001; Ikeda et al. 2007). It suggests that UA shows anti-inflammatory and proinflammatory activities that are dependent on the biological status of cells and tissues. So, we assessed the effect of UA on the inflammatory response induced by D-gal through AGEs/RAGE/NF-κB pathway in mouse prefrontal cortex. Our results suggested that UA could reduce the number of activated microglia cells (CD11b-stained cell), activated astrocytes and RAGE-positive cells, decrease the expression of CD11b, GFAP and RAGE, inhibit NF-κB activation, and reduce the expression of inflammatory markers in the prefrontal cortex of D-gal-treated mice, which attenuated D-gal-induced inflammatory response. On the other hand, biochemical data analysis indicated that UA administration could alleviate D-gal-induced oxidative stress by scavenging and prevention of free radical generation in the prefrontal cortex of D-gal-treated mice, which was consistent with our previous studies (Lu et al. 2007). All of these effects of UA will help reverse behavior abnormality and restore brain damage. At the same time, our experimental results also show that UA may be a drug candidate that cures diabetes mellitus, atherosclerosis, and neurodegenerative disorders induced by AGEs/RAGE/NF-κB pathway activation. In the future research, we will consider these experimental models to assess the effect of UA and further explore its mechanism through which UA may exert these multiple beneficial activities.

In conclusion, UA could attenuate D-gal–induced inflammatory response in the prefrontal cortex of D-gal–treated mice through decreasing AGEs, ROS, and protein carbonyl levels and inhibiting AGEs/RAGE/NF-κB-mediated inflammatory response. Signaling schematic diagram for neuroprotective effects of UA against D-gal–induced inflammatory response is shown in Fig. 7.

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Notes
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References


