Abstract. Glucocorticoids (GCs) are known to alter neuronal plasticity, impair learning and memory and play important roles in the generation and progression of Alzheimer’s disease. There are no effective drug options for preventing neuronal injury induced by chronic GC exposure. Ginsenoside Rg1 (Rg1) is a steroidal saponin found in ginseng. The present study investigated the neuroprotective effect of Rg1 on neuroinflammation damage induced by chronic dexamethasone (5 mg/kg for 28 days) exposure in male mice. Our results showed that Rg1 (2 and 4 mg/kg) treatment increased spontaneous motor activity and exploratory behavior in an open field test, and increased the number of entries into the new object zone in a novel object recognition test. Moreover, Rg1 (2 and 4 mg/kg) treatment significantly alleviated neuronal degeneration and increased MAP2 expression in the frontal cortex and hippocampus. Additionally, inhibition of NLRP-1 inflammasomes was also involved in the mechanisms underlying the effect of Rg1 on GC-induced neuronal injury. We found that Rg1 (2 and 4 mg/kg) treatment increased the expression of glucocorticosteroid receptor and decreased the expression of NLRP-1, ASC, caspase-1, caspase-5, IL-1β and IL-18 in the hippocampus in male mice. The present study indicates that Rg1 may have protective effects on neuroinflammation and neuronal injury induced by chronic GC exposure.

Introduction

Alzheimer’s disease (AD) is a progressive and neurodegenerative disorder with dementia resulting from the combination of both genetic and environmental risk factors (1). Several studies have linked psychosocial stress and stress hormone levels, such as glucocorticoids (GCs), with AD generation and development (2,3). Clinical studies have shown that plasma cortisol levels are significantly increased in AD and mild cognitive impairment patients, and dysfunction of the hypothalamus-pituitary-adrenal (HPA) axis is associated with a higher risk of AD (4). Furthermore, it has been reported that the rate of dementia progression is correlated with the plasma levels of GCs in AD patients (3). GCs are known to alter neuronal plasticity, impair learning and memory, produce atrophy in several areas of the brain, reduce hippocampal dendritic complexity (5,6), and promote hippocampal cell death (7). Yet, the precise mechanisms by which they contribute to AD remain to be fully elucidated (8).

Currently, there are no effective drugs for preventing neuronal damage induced by chronic GC exposure. Ginseng has been safely used to improve health conditions, and delay senescence for more than 2,000 years in China. Ginsenosides are the major active components in ginseng. It has been reported that ginsenosides have neuroprotective effects in animals and humans (9,10). Ginsenoside Rg1 (Rg1) is a steroidal saponin which is abundantly found in ginseng. Recent studies have revealed that Rg1 (2.5-10 mg/kg) prevents spatial learning and memory impairment, and may be a useful agent for neurodegenerative disorders such as AD (11). However, the protective effect of Rg1 on neuronal inflammation damage induced by chronic GC exposure and the underlying mechanisms have not yet been fully elucidated. Moreover, mifepristone (RU486) is a synthetic glucocorticosteroid receptor (GR) antagonist (12). RU486 has been used as a tool to study the mechanisms of steroid receptor function (13). Recent studies have reported that RU486 may act as a neuroprotective agent against excitotoxicity and traumatic brain injury (14,15). In the present study, RU486 was used as a positive control.
Dexamethasone (DEX) is a type of synthetic glucocorticoid. DEX (5 mg/kg) is often used to mimic GC-induced neurodegenerative diseases (16,17). Our most recent study showed that chronic DEX (5 mg/kg) exposure for 28 days significantly induced neurodegeneration and increased neuroinflammation via NLRP-1 inflammasome activation (18). A previous study by us also demonstrated that Rg1 protects against learning and memory impairments and ROS oxidative damage in chronic restraint stress-exposed mice (19). ROS oxidative stress is a method by which to activate NLRP-1 inflammasomes. In the present study we hypothesized that Rg1 inhibits the activation of NLRP-1 inflammasomes and attenuates neuronal damage induced by chronic DEX exposure. Our results indicated that Rg1 has protective effects against neuronal inflammation damage induced by chronic DEX (5 mg/kg, 28 days) exposure.

Materials and methods

Animals and treatment. All experiments were carried out according to protocols approved by the Committee on the Care and Use of Laboratory Animals at Anhui Medical University. Adult male ICR mice (25-30 g, Grade II) were obtained from the Center for Laboratory Animals of Anhui. The mice were housed in groups of four to six with unrestricted access to food and water and a 12-h light/dark cycle.

Animals were randomly divided into six groups (10 mice in each group): control group, DEX (5 mg/kg) exposure group, DEX (5 mg/kg) + RUG86 (5 mg/kg) treatment group and DEX (5 mg/kg) + Rg1 (1, 2, 4 mg/kg) treatment groups. DEX (Sigma, St. Louis, MO, USA) was dissolved in alcohol at a concentration of 500 mg/ml to be used as a stock solution and diluted in normal saline (NS) at a concentration of 0.5 mg/ml before use. RU486 (Beijing Zizhu Pharmaceutical Co., Ltd., Beijing, China) was prepared by dissolving RU486 in distilled water at a concentration of 0.5 mg/ml. The Rg1 (content of Rg1 >98%, obtained from the Institute of Pharmaceutical Research of Xiehe Medical University of China) was dissolved in distilled water at concentrations of 0.1, 0.2 and 0.4 mg/ml. In the DEX, RU486 and Rg1 treatment groups, the mice were injected subcutaneously (s.c.) with DEX (5 mg/kg/day) for 28 days. In the control group, the mice were injected (s.c.) with NS with an equal volume of alcohol. The RU486 (5 mg/kg) group was treated with RU486 solution (0.5 mg/ml) intragastrically (i.g., 0.1 ml/10 g) for 28 days. The Rg1 (1, 2, 4 mg/kg) groups were treated with Rg1 (0.1, 0.2 and 0.4 mg/ml) intragastrically (i.g., 0.1 ml/10 g) for 28 days. The control group and DEX exposure received equivalent volumes of distilled water for 28 days.

Open field test. The open field test was performed to study the effects of Rg1 on DEX exposure-induced motor activity and exploratory behavior impairments. It is often used to detect the locomotor behavior, anxiety and exploration of animals (20). The open field test apparatus (ShangHai Biowill Co., Ltd., Shanghai, China) consisted of a computer-tracking cage (60x60x50 cm) which was separated as 9 squares (1 central and 8 peripheral) by two vertical lines and transverse lines as previously described (21). The mouse was placed in the cage for 2 min in each testing period. The ANY-maze Behavioral Tracking Software (Stoelting Co., Wood Dale, IL, USA) was used to record the path of its motor activity and exploration for 3 min. The number of line crossings, the moving distance (m), the mean moving speed (m/sec) and the number of standing up events (indicates exploratory behavior) were calculated by the software to represent the motor and exploration behaviors (22).

Novel object recognition (NOR) test. The NOR test is usually used to evaluate recognition memory (23). The open field apparatus and the ANY-maze Behavioral Tracking system are used to detect the NOR task. The NOR test consists of three phases: the habituation phase, the familiarization phase, and the test phase. In the habituation phase, the mice are placed in an empty arena to adapt to the environment. The next day, the mice are placed in the familiar arena with two identical objects (a1, a2) (Fig. 2A). Twenty-four hours after familiarization, the mice are placed in the open field with (a1) the familiar object and (b) a novel object to examine long-term recognition memory. The number of entries to a2 zone and the time exploring the a2 zone in the familiarization phase, the number of entries to b zone, and the time exploring the b zone in the test phase were recorded to evaluate the effects of Rg1 on long-term recognition memory impairments.

Histological examination. The mice were anesthetized by injection of 3.5% chloral hydrate (i.p., 0.1 ml/10 g body weight). The brains (n=5) were carefully removed and fixed in 4% paraformaldehyde. The brain tissues were embedded in paraffin and sliced into 5-µm sections using a section cutter (Leica, Mannheim, Germany). The sections were stained with hematoxylin and eosin (H&E) and the neuronal morphology in the frontal cortex and hippocampus CA1 and CA3 was observed by a microscope (Olympus IX71; Olympus, Tokyo, Japan).

Immunohistochemistry. The immunohistochemical analysis was performed as previously described (18). The brain sections were deparaffinized and incubated with 3% hydrogen peroxide and then blocked with non-immune goat serum. The sections were washed with PBS and incubated in the mouse anti-MAP2 antibody (1:200; ab11268; Abcam, Cambridge, UK) overnight at 4°C. The next day, the sections were washed 3 times with PBS, and incubated in the secondary antibody for 1 h. Then the sections were washed and visualized by using an ABC kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The sections were mounted and examined by a light microscope (Olympus IX71; Olympus). The positive neurons were stained brown. Image-Pro Plus 6.0 analysis system was used to analyze the mean optical density in the frontal cortex, hippocampus CA1 and CA3 by an observer blinded to the experimental protocol.

Immunoblot analysis. The immunoblot method was performed according to our previous description (18). The total protein in the frontal cortex and hippocampus was extracted. Equal amounts (40 µg) of protein were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBS-T for 1 h at room temperature. The membranes were washed with TBS-T 3 times, and incubated with primary antibodies for NLRP-1, ASC, caspase-1, caspase-5, IL-1β, IL-18, glucocorticoid receptor (GR) and β-actin (1:1,000) overnight at 4°C. The antibodies for ASC...
(BS2215), caspase-1 (BS1730), caspase-5 (BS7048), IL-1β (BS3506) and GR (BS6617) were provided by Bioworld Technology, Inc. (St. Louis Park, MN, USA). The NLRP-1 antibody (ab16091) was provided by Abcam. The IL-18 antibody (sc-6179) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The dilution of all primary antibodies was at 1:500. On the next day, the membranes were washed 3 times with TBS-T and incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h. After the membranes were extensively washed, the proteins were detected using the ECL kit (Amersham Biosciences, Little Chalfont, UK). The protein bands were visualized using Chemi Q4800 Mini Imaging System (Bioshine, Shanghai, China), and Image J Software [National Institutes of Health (NIH), Bethesda, MD, USA] was used for densitometry and quantification of the protein bands. The immunoreactive band intensities were normalized to its corresponding band of β-actin.

Statistical analysis. All data are reported as mean ± SD. Statistical differences were performed using SPSS 17.0. The data were analyzed by one-way ANOVA followed by Bonferroni’s post hoc test for between-group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of Rg1 on motor and exploratory behavior impairments in mice induced by chronic DEX exposure. In the present study, the open field test was used to examine motor and exploratory behavior. The results showed that DEX exposure significantly decreased the motor activity and exploratory behavior in male mice. DEX exposure for 28 days significantly decreased the total moving distance (m), the mean moving speed (m/sec), the number of line crossings, and the number of standing up movements (Fig. 1A-D, P<0.01). Rg1 (2 and 4 mg/kg) treatment significantly increased the total m, the mean m/sec, the number of line crossings, and the number of standing up events in the male mice which were decreased by chronic DEX exposure (Fig. 1A-D, P<0.05 or P<0.01).

Effects of Rg1 on chronic DEX-induced NOR in mice. The NOR test results showed that, in the familiarization phase, DEX exposure for 28 days had no significant effects on the number of entries into the a2 zone and the exploring time in the a2 zone, which is the novel object zone (b zone) in the test phase. While in the test phase, DEX exposure significantly reduced the number of entries into the new object zone (b zone) and the exploring time in the b zone. Compared with the DEX exposure group, RU486 and Rg1 (2 and 4 mg/kg) treatment increased the number of entries into the b zone and the exploring time in the b zone in the final test phase (Fig. 2B and C, P<0.05 or P<0.01).

Effects of Rg1 on chronic DEX-induced neuronal damage in the hippocampus and frontal cortex. The H&E staining showed that no significant neuronal abnormalities were observed in the frontal cortex and hippocampus CA1 and CA3 in the control group (Fig. 3A). While in the DEX treatment group (Fig. 3B), the neurons showed significant nuclear condensation and acidophilia degeneration. Compared with the DEX exposure group, RU486 and Rg1 (2 and 4 mg/kg) treatment increased the number of entries into the b zone and the exploring time in the b zone in the final test phase (Fig. 2B and C, P<0.05 or P<0.01).

Effects of Rg1 on MAP2 expression in the frontal cortex and hippocampus in mice. The results showed that MAP2 expres-
Figure 2. Effects of ginsenoside Rg1 (Rg1) on novel object recognition in chronic DEX-exposed mice (NOR test). (A) The diagram of the NOR test. (B) The number of entries to the object zone. (C) The exploring time in the object zone. In the familiarization phase, DEX exposure for 28 days had no significant effects on the number of entries into the a2 zone and the time in the a2 zone. While in the test phase, DEX exposure significantly reduced the number of entries into the new object zone (b zone) and the time in the b zone. Compared with the DEX exposure group, RU486 and Rg1 (2 and 4 mg/kg) treatment increased the number of entries into the b zone and the time in the b zone. Results are expressed as the mean ± SD, n=10, *P<0.05, **P<0.01 compared with the control group; #P<0.05, ##P<0.01 compared with the DEX exposure group. DEX, dexamethasone; NOR, novel object recognition.

Figure 3. Effects of ginsenoside Rg1 (Rg1) on neuronal injury in the frontal cortex and hippocampus in chronic DEX-exposed mice (H&E staining, x400). Compared with the (A) control group, (B) DEX treatment for 28 days accelerated neuronal injury. Compared with the DEX treatment group, RU486 and Rg1 (2 and 4 mg/kg) treatment alleviated the neuronal degeneration (C, E and F). (A) Control group; (B) DEX group; (C) DEX+RU486 group; (D) DEX+Rg1 (1 mg/kg) group; (E) DEX+Rg1 (2 mg/kg) group; (F) DEX+Rg1 (4 mg/kg) group. DEX, dexamethasone.
sion was abundant in the frontal cortex and hippocampus in the control group (Fig. 4A and G). In the DEX treatment group, the MAP2 expression was significantly decreased in the frontal cortex and hippocampus (Fig. 4B and G, P<0.01). Compared with the DEX treatment group, RU486 and Rg1 (2 and 4 mg/kg) significantly increased the expression of MAP2 in the frontal cortex and hippocampus in the control group (A). Chronic DEX exposure significantly decreased MAP2 expression in the frontal cortex and hippocampus (B). Compared with the DEX exposure group, treatment with RU486 and Rg1 (2 and 4 mg/kg) significantly increased MAP2 expression in the frontal cortex and hippocampus. Results are expressed as mean ± SD, n=6. **P<0.01 compared with the control group; #P<0.05, ##P<0.01 compared with the DEX exposure group. DEX, dexamethasone.

Effects of Rg1 on GR expression in the frontal cortex and hippocampus. We further detected GR expression in the frontal cortex and hippocampus brain tissue by immunoblot analysis. The results revealed that GR expression was abundant in the hippocampus and cortex in the control group (Fig. 5). In the DEX treatment group, GR expression was significantly decreased (Fig. 5, P<0.01). Compared with the DEX treatment group, RU486 and Rg1 (2 and 4 mg/kg) significantly increased the expression of GR in the frontal cortex and hippocampus (Fig. 5, P<0.05 or P<0.01).

Effects of Rg1 on expression levels of NLRP-1, ASC, caspase-1, caspase-5, IL-1β and IL-18 in the frontal cortex and hippocampus. To confirm whether Rg1 can regulate...
NLRP1 inflammasome activation, which is involved in chronic DEX-induced hippocampal neuron damage, we further detected the expression of NLRP-1, ASC, caspase-1, caspase-5, IL-1β and IL-18 in the frontal cortex and hippocampus brain tissue by immunoblotting. The results showed that DEX exposure for 28 days significantly increased the expression of NLRP-1 and ASC (Fig. 6, P<0.05 and P<0.01), caspase-1 and caspase-5 (Fig. 7, P<0.05 and P<0.01) and IL-1β and IL-18 (Fig. 8, P<0.01). Compared with the DEX treatment group, RU486 and Rg1 (2 and 4 mg/kg) treatment decreased the expression of NLRP-1 and ASC (Fig. 6, P<0.05), caspase-1 and caspase-5 (Fig. 7, P<0.05 and P<0.01) and IL-1β and IL-18 (Fig. 8, P<0.01 or P<0.05). These data indicate that Rg1 decreased the activation of NLRP1 inflammasomes activated by chronic DEX exposure.

Discussion
A growing number of reports suggest that neuronal inflammation is a contributing factor to neurodegenerative diseases, such as AD (24-26). It has been reported that inflammasomes play an important role in many neurodegenerative diseases such as Parkinson's disease (PD) and AD (27). The present study was designed to investigate the effects of the ginseng extract ginsenoside Rg1 on neuron inflammation damage in the frontal cortex and hippocampus in mice following chronic DEX exposure. In the present study, the results showed that Rg1 (2 and 4 mg/kg) and RU486 (GR antagonist) treatment increased spontaneous motor activity and exploratory behavior in an open field test, and increased the number of entries into the new object zone in the NOR test. Rg1 (2 and 4 mg/kg) and RU486 significantly protected against neuronal damage induced by chronic DEX exposure. Rg1 (2 and 4 mg/kg) and RU486 were able to decrease the expression of NLRP-1 inflammasomes and attenuate the neuronal damage in the frontal cortex and hippocampus in mice. Additional, Rg1 and RU486 treatment increased GR expression and decreased IL-1β and IL-18 expression in brain tissue. The results suggest that Rg1 may inhibit chronic DEX exposure-induced neuronal inflammation, which may prevent the generation and progression of AD.

Growing evidence has linked high levels of GCs with neurodegenerative diseases, such as vulnerability to depression, AD and PD (28,29). Numerous studies have shown that chronic high concentrations of GCs have adverse effects on neurons, and even cause hippocampal neuronal death (3,5,6). DEX is a type of synthetic glucocorticoid. It has been reported that chronic exposure to DEX (5 mg/kg) can induce learning and memory impairment and mimic GC-induced neurodegenerative diseases (16,17,30). In our previous studies, we found that both chronic DEX (5 mg/kg) exposure and restraint stress induced cognition impairments and neuronal damage in the hippocampus and cortex in mice (19,31). Our most recent study revealed that DEX (5 mg/kg) exposure for 28 days significantly increased expression of NLRP-1 and accelerated neurodegeneration in the hippocampus and frontal cortex in...
mice (18). However, currently, there are no effective drugs for preventing chronic GC exposure-mediated neuronal damage. It was found that ginsenoside Rg1 attenuated the inflammation, neuronal apoptosis and alleviated the cognitive impairment in an AD model (32,33). Our previous study also showed that Rg1 could protect against learning and memory impairments and ROS oxidative damage in chronic restrain stress-exposed mice (19). Yet, it is still unclear whether Rg1 could alleviate chronic GC-induced cognitive impairment and neuronal inflammation damage.

Behavioral tests play an important role in evaluating the ability of recognition in animal models of cognitive dysfunction (34). The open field test is generally used to evaluate locomotor activity and spontaneous exploration in a novel environment (35). In the present study, we observed that chronic DEX-exposed animals showed impaired motor and exploration activities such as reduced number of lines crossed and stand up events in the open field test. Rg1 (2 and 4 mg/kg) significantly increased the number of lines crossed and stand up movements of the mice. The NOR test is also widely used to investigate memory alterations. As Ennaceur (36) reported, animals were placed to an arena with a familiar and a novel object. He found that the animals approached frequently and spent more time exploring the novel object than the familiar one. In the present study, we found that DEX exposure for 28 days significantly decreased the entering number and the exploring time in the novel object zone. Compared with the DEX treatment group, RU486 and Rg1 (2 and 4 mg/kg) treatment increased the entering number and the exploring time in the novel object zone. These results suggest that Rg1 may alleviate the cognitive dysfunction induced by chronic GC exposure.

As a cytoskeletal protein, MAP2 is a marker of structural integrity in neurons (37). It has been observed that there is...
a significant reduction in MAP2 in the cortex and hippocampus in elder rats (38). The present study showed that DEX (5 mg/kg) exposure for 28 days significantly induced neuronal damage; the neurons showed significant acidophilia degeneration and nuclear condensation in the cortex and hippocampus CA1, CA3, and the expression of MAP2 was also significantly reduced in the frontal cortex and hippocampus. Treatment with Rg1 (2 and 4 mg/kg) and RU486 significantly alleviated the histological changes, increased the expression of MAP2, and attenuated the neuronal injury in the frontal cortex and hippocampus CA1 and CA3.

Neuroinflammation has emerged as an important cause of the cognitive decline during aging and AD (25,26,39). GCs are commonly used as anti-inflammatory and immunosuppressive agents in the clinic. However, research has showed that GCs also have a proinflammatory action (40,41). The GR, as a member of the nuclear receptor superfamily, plays a key role in regulated the actions of GCs (42). GR contributes to the regulation of proinflammatory molecules at the level of gene transcription (43). Sun et al (13) reported that LPS treatment slightly downregulated GR expression. Rg1 treatment significantly increased the GR expression compared to the LPS group. The GR antagonist RU486 inhibited the neuroprotective effects of Rg1, indicating that the anti-inflammatory effects of Rg1 are dependent on GR. Du et al (44) reported that Rg1, a novel GR agonist of plant origin, possesses GC and estrogen-like activities and can effectively inhibit acute and chronic inflammation, but it does not cause an adverse reaction as noted with DEX. In the present study, we found that DEX (5 mg/kg) exposure for 28 days significantly downregulated GR expression and increased IL-1β and IL-18 expression in the hippocampus and frontal cortex in mice. Rg1 and RU486 increased GR expression and reduced IL-1β and IL-18 expression in the hippocampus and frontal cortex in mice. The results suggest that both Rg1 and RU486 can increase GR expression and are involved in anti-inflammatory effects in chronic DEX-exposed mice. However, the mechanisms through which Rg1 inhibits the downregulation of GR expression induced by DEX are still unclear and warrant further study.

The inflammasome-associated pathway plays an important role in the pathogenesis of neurodegenerative diseases. The NLRP1 inflammasome is a multi-protein complex which consists of NLRP1, the adaptor ASC and caspase-1 (45). NLRP1 inflammasomes mediate activation of caspase-1 which promotes cleavage of mature proinflammatory cytokines from pro-IL-1β and pro-IL-18 into IL-1β and IL-18 (46). The adaptor ASC is a critical component of inflammasomes by linking NLRPs to caspase-1 activation (47). Therefore, activation of inflammasomes provides a molecular platform for caspase-1 activation which promotes IL-1β and IL-18 release (48,49). Caspase-5 is also a proinflammatory cysteine protease. Caspase-5 together with caspase-1 are components of the NLRP1 inflammasome complex and enhance activation of caspase-1 (50). The present study showed that DEX exposure for 28 days significantly increased the expression levels of NLRP-1, caspase-1, caspase-5, ASC, IL-1β and IL-18 in the hippocampus and frontal cortex brain tissue. Rg1 and RU486 significantly decreased the expression levels of NLRP-1, caspase-1, caspase-5, ASC, IL-1β and IL-18 in the hippocampus and frontal cortex brain tissue. These data suggest that Rg1 may suppress neuroinflammation and inhibit chronic DEX exposure-induced inflammation injury in the hippocampus and frontal cortex.

In summary, the present study suggests that chronic GC exposure induces neurodegeneration and NLRP-1 inflammasome activation in the hippocampus and frontal cortex. Rg1 protects against the neuroinflammation and neuronal damage induced by chronic DEX exposure. Additionally, the inhibition of NLRP-1 inflammasomes was involved in the action mechanisms of Rg1 in this experimental model. However, this study only offered an experimental basis for Rg1 in the treatment of chronic DEX exposure, and other related molecular mechanisms of Rg1 in regards to DEX exposure warrant further investigation.

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