Neuroprotective Effects of (−)-Epigallocatechin-3-gallate on Aging Mice Induced by D-Galactose

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Aging is a complicated multifactorial process in which a progressive decline in the physiologic function of organisms occurs. Oxidative stress and reactive oxygen species (ROS) have been proposed to be major cause of aging and other aging-related neurodegenerative conditions such as Alzheimer’s disease. Aging-related oxidative damage in the brain and memory impairment mainly result from an imbalance between ROS generation and antioxidant enzyme activities. The overproduction of ROS or decrease in antioxidants in the brain can cause lipid peroxidation, nuclear and mitochondrial DNA damage, and protein oxidation and finally affect the normal functions of organisms.

D-Galactose (D-gal) is a reducing sugar normally present in the body. When present at levels greater than its normal content, it can be oxidized into aldehydes and hydrogen peroxide (H2O2) by galactose oxidase. It has been shown that D-gal-treated animal models show aging-related changes including the impairment of spatial learning and memory, object novelty preference, and locomotor activity. Other studies demonstrated that long-term subcutaneous injection of D-gal in mice induced decreased immune responses and increased cell karyopyknosis, apoptosis, and caspase-3 protein levels in hippocampal neurons. Therefore, mice continuously injected with D-gal have been extensively used for pharmacologic research on brain aging.

(−)-Epigallocatechin-3-gallate (EGCG) is a member of the catechin family and a major polyphenolic constituent of green tea. It was reported that EGCG has potent iron-chelating, antioxidant, antiinflammatory, anticancer, and neuroprotective activities. In particular, EGCG has been shown to have neuroprotective effects by elevating the α-secretase activity of amyloid precursor protein (APP) and conversion to soluble APP-α (sAPP-α) and reducing amyloid beta (Aβ)-induced neurotoxicity in a 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) mouse model, the “Swedish” APP transgenic (Tg2576) mouse model of Alzheimer’s disease, and N2a cells stably transfected with “Swedish” mutant human APP. Furthermore, some studies have recently demonstrated that the neuroprotective mechanisms of EGCG are partly due to increasing activities of antioxidant enzymes and decreasing advanced glycation endproduct (AGE)-induced damage in aged rat brain or neuronal cells.

However, no study has been performed to evaluate whether EGCG has protective effects on aging mice induced by D-gal and its mechanisms of action. In the present study, we induced the aging mice model with D-gal (150 mg/kg/d, 6 weeks subcutaneously (s.c.)) and observed whether EGCG (2 mg/kg/d or 6 mg/kg/d, 4 weeks intragastrically (i.g.)) had potent antioxidant and antiapoptotic neuroprotective effects using behavioral testing and measurements of the activities of total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px), contents of malondialdehyde (MDA) and activation and expression of proapoptotic protein caspase-3 in the hippocampus of the mice.

MATERIALS AND METHODS

Drugs and Chemicals D-Gal and EGCG were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., U.S.A. Commercial kits used for the determination of T-SOD, GSH-Px, and...
MDA levels were purchased from Jiancheng Institute of Biotechnology, Nanjing, Jiangsu Province, P. R. China; polyclonal rabbit anti-cleaved caspase-3 antibody was purchased from Cell Signaling Technology, Inc., U.S.A. Other chemicals were of analytical reagent grade purchased locally from commercial suppliers.

**Animals** Equal numbers of healthy male and female Kunming mice, aged 6—8 weeks, weighing 22—28 g, were obtained from the Experimental Animal Breeding Center of China Medical University (Shenyang, P. R. China). They were housed in plastic cages under standard conditions and allowed free access to standard laboratory food and water in a 12-h light/dark cycle. All animals were used only once. The principles of laboratory animal care were followed in accordance with the Chinese Experimental Animal Administration Legislation.

**Experimental Design** After acclimatization to the laboratory for 1 week, the mice were randomly divided into four groups (n=16 in each group): control group; d-gal model group; d-gal+EGCG 2 mg/kg/d group; and d-gal+EGCG 6 mg/kg/d group. Mice in the d-gal model group, d-gal+EGCG 2 mg/kg/d group and d-gal+EGCG 6 mg/kg/d group were subcutaneously injected with 3% d-gal at the dose of 150 mg/kg body weight once daily for 6 weeks, while those in the control group were treated with same volume of normal saline. From the third week, mice in the d-gal+EGCG 2 mg/kg/d group and d-gal+EGCG 6 mg/kg/d group were given EGCG at the dose of 2 mg/kg/d i.g. or 6 mg/kg/d i.g., respectively, after injection of d-gal. Mice in the control group and model group were administered the same volume of vehicle (distilled water). After finishing all treatments, the animals were evaluated in behavioral testing and then immediately killed to dissect the hippocampus for various experiments or stored at −80°C for later experiments.

**Locomotor Activity Testing** After the final treatment, the locomotive activity of mice was measured using a ZZ-6 spontaneous activity apparatus (Chengdu Technology & Market Co., Ltd., China), which was in a fully computerized multibox infrared-sensitive motion-detection system. The apparatus consisting of six boxes sheltered from each other was put inside a large black opaque iron box. One mouse was placed in each box; six mice were tested simultaneously for spontaneous activity counts within 10 min. All mice were subjected to locomotor activity testing.

**Behavioral Testing in the SMG-2 Water Maze** The SMG-2 water maze test was performed after the completion of all treatments. The apparatus (Chinese Academy of Medical Sciences, China) consisted of a black plexiglas rectangular tank, 73 cm in length, 42 cm in width, and 20 cm in depth. The tank included a starting point, a terminal platform, and four nonexits. There was a ladder for rest near the platform, which was the safe region. The water was filled in the maze to a depth of 12 cm and the temperature was controlled at 24±1°C. On the first day of training, each mouse was allowed to stay on the terminal platform for 30 s to recognize the location, and placed in the water facing the pool wall at the first starting point containing a nonexit. The escape latencies to find the terminal platform were recorded for each trial. If the mouse could not find the ladder within 2 min, the swimming time was designated as 2 min, and then it was guided to the ladder and left there to rest for 30 s. The pathways on day 2 and day 3 were gradually prolonged and contained two and three nonexits, respectively. The pathways for the ultimate 4 d were the same, containing three nonexits. The escape latencies to find the terminal platform for all mice in 6 days were measured and calculated.

**Measurements of the Activities of T-SOD and GSH-Px** and Contents of MDA in the Hippocampus of Mice  The hippocampi stored at −80°C were prepared as 10% (w/v) homogenates in cold normal saline and centrifuged at 4000×g for 10 min at 4°C, and then the supernatants were used for measurements of the activities of T-SOD and GSH-Px and MDA contents. All examinations were carried out using commercially available kits following the manufacturer's instructions.

**Immunohistochemical Staining of Caspase-3 and TUNEL Staining** After behavioral testing, the mice (n=6) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and transcardially perfused with normal saline followed by 4% paraformaldehyde solution. The brains were removed, postfixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin wax, and then serial coronal 5-μm sections were cut and subjected to standard hematoxylin and eosin (HE) and immunohistochemical staining. Briefly, the sections were dewaxed with xylene and rehydrated through a series of graded alcohols. Protein antigenicity was enhanced by boiling sections with microwaves in citrate buffer 10 mM (pH 6.0) for 20 min. Sections were treated with 3% H2O2 in phosphate buffer solution (PBS) 0.1 M for 20 min at room temperature to inactive endogenous peroxidase activity. After washing the sections with PBS, nonspecific blocking was performed by incubating sections with normal goat serum for 30 min at 37°C. The sections were then incubated overnight at 4°C with primary rabbit anti-cleaved caspase-3 antibody (1:100, Cell Signaling Technology, Inc., U.S.A.). As a negative control, the primary antibody was replaced with an equivalent volume of PBS. After washing three times with PBS, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200, Maixin) for 30 min at 37°C, and then in streptavidin-peroxidase conjugate (1:200, Maixin) for 30 min at 37°C. The immunoreactions were visualized by staining sections with 3'-diaminobenzidine (DAB, Sigma) for 1—3 min. Finally, the sections were counterstained with hematoxylin for 30 s. Images from the hippocampus in each section were observed under a ×400 magnification with a microscope (Leica CME) and digital sight camera (Nikon E4500, U.S.A.).

Apoptosis was detected using a TdT-mediated dUTP-biotin nick end labeling (TUNEL) Site Cell Apoptosis Detection Kit, according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Briefly, brain sections were dewaxed in xylene and hydrated in gradient alcohol, incubated in 3% hydrogen peroxide for 10 min, rinsed three times with PBS, incubated with proteinase-K for 15 min at 37°C, and rinsed three times with PBS. The TUNEL reaction was performed for 2 h at 37°C in labeling buffer containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-d-UTP. Biotinylated anti-digoxigenin antibody was then reacted with the sections for 30 min at 37°C. Apoptotic nuclei were visualized using the peroxidase-DAB reaction. The sections were then counterstained with hematoxylin. TUNEL-
positive neurons in the hippocampus were counted in three random areas of three different sections at a magnification of ×400 and expressed as the percentage of 100 neurons as the cell apoptosis index.

**Western Blot Analysis of Caspase-3** The hippocampi stored at −80°C were homogenized in ice-cold RIPA buffer solution [Tris–CHCl buffer 50 mM (pH 8.0) containing NaCl 150 mM, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.1% phenylmethylsulfonyl fluoride (PMSF)] and centrifuged at 12000 × g for 30 min at 4°C. The supernatants were collected. Protein quantification was carried out using a BCA kit (Walterson Biotechnology Inc., Beijing, China). Samples were boiled in the presence of sample buffer [10% SDS, Tris–CHCl 0.5 M (pH 6.8), 25% β-mercaptoethanol, 0.25% bromophenol blue, 50% glycerol] for 3 min at 95°C. Proteins (50 μg per lane) were separated on 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane by electrophoretic transfer. Blocking was carried out with 5% nonfat dry milk in PBS containing 0.1% (v/v) Tween-20 (PBST) for 1.5 h at room temperature. The immobilized proteins were incubated with polyclonal rabbit anti-cleaved caspase-3 antibody (1:2000, Santa Cruz Biotechnology Inc., Beverly, CA, USA) overnight at 4°C. Following 3×10-min washes in PBST, the membranes were incubated with goat horseradish peroxidase-conjugated antibody (1:2000, Santa Cruz Biotechnology, USA) for 1 h at room temperature. The membranes were again washed three times in PBST. The protein bands were visualized using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China) and exposed to Kodak X-ray film for 1—30 min. The results of protein expression were quantitatively analyzed with FluorChem V2.0 software (Alpha Innotech Corp., USA). Each density (integrated density value, IDV) of cleaved caspase-3 expression band was used as an internal control. All experiments were repeated at least two times to ensure reproducibility of the results.

**Statistical Analysis** All statistical analyses were performed using SPSS software, ver. 11.5. One-way analysis of variance (ANOVA) followed by the least significant difference method (LSD) was adopted for multiple-group comparison. Data are expressed as mean ± S.D. Statistical significance was set at p < 0.05.

**RESULTS**

**Effects of EGCG on Behavioral Changes in Aging Mice Induced by n-Gal** The spontaneous activity counts of mice showed no significance between the control group and n-gal model group (104.8 ± 54.3 vs. 90.0 ± 24.8), indicating that n-gal did not significantly affect locomotive activity. The water maze is an effective test used for the assessment of spatial learning and memory in mice. The results of the SMG-2 water maze test are shown in Table 1. The escape latencies to the terminal platform for all mice progressively increased with the prolonged pathways from day 1 to day 3 of the training trials and gradually declined from the beginning of day 3 to day 6 for the same pathways containing three nonexits. However, statistical analysis indicated that n-gal model mice had longer latencies compared with control mice [F(3, 56) = 2.638, p < 0.05; F(3, 56) = 7.303, p < 0.01; F(3, 56) = 5.475, p < 0.05; F(3, 56) = 4.046, p < 0.05; F(3, 56) = 5.903, p < 0.01]. This implies that n-gal caused significant cognitive deficits in mice. EGCG administration (2 mg/kg/d or 6 mg/kg/d) significantly (p < 0.01 and p < 0.05, respectively) shortened the prolonged escape latencies to the platform induced by n-gal, suggesting that EGCG improves the learning and memory in the aging model mice induced by n-gal.

**Antioxidant Effects of EGCG in the Hippocampus of Aging Mice Induced by n-Gal** To understand the mechanisms of the neuroprotective effects of EGCG, we measured the activities of T-SOD and GSH-Px and the MDA contents of the hippocampus. The hippocampi were homogenized in ice-cold RIPA buffer solution containing NaCl 150 mM, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.1% phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 12000 × g for 30 min at 4°C. The supernatants were collected. The activities of T-SOD and GSH-Px and the MDA contents of the hippocampus were significantly increased compared with those in the control group (2 mg/kg/d or 6 mg/kg/d) significantly (p < 0.01 and p < 0.05, respectively) decreased the contents of MDA in the hippocampus of group (11.24 ± 16.87 U/mg protein, p < 0.01; 38.12 ± 7.70 U/mg protein, p < 0.05) or 6 mg/kg/d (110.85 ± 15.90 U/mg protein, p < 0.05; 34.70 ± 3.18 U/mg protein, p < 0.01). Accordingly, Fig. 1C shows that the contents of MDA in the hippocampus of n-gal model mice significantly increased compared with those in the control group (4.27 ± 1.18 nmol/mg protein in the model group vs. 2.50 ± 1.00 nmol/mg protein in the control group, p < 0.01). The administration of EGCG 2 mg/kg/d or 6 mg/kg/d significantly decreased the contents of MDA in the hippocampus of n-gal model mice.
of MDA in the hippocampus of D-gal-treated mice to 3.05 ± 0.66 nmol/mg protein and 2.91 ± 0.77 nmol/mg protein, respectively (p < 0.05 and p < 0.01, respectively).

Effects of EGCG on Hippocampal Neuronal Injury in Aging Mice Induced by D-Gal

Karyopyknosis and neuronal loss were observed in the hippocampal region in D-gal model mice on HE staining. The administration of EGCG 2 mg/kg/d or 6 mg/kg/d significantly prevented the impairment (data not shown). We used the TUNEL staining method to detect neuronal apoptosis. Almost no TUNEL-positive cells were observed in the hippocampus of control group mice (Fig. 2). The cell apoptosis index was significantly increased in the hippocampus of mice treated with D-gal and clearly reduced in the hippocampus of mice that received EGCG 2 mg/kg/d or 6 mg/kg/d.

Effects of EGCG on Activation of Caspase-3 in the Hippocampus of Aging Mice Induced by D-Gal

To investigate further the neuroprotective mechanisms of EGCG, we also observed its effects on the activation and expression of the proapoptotic protein caspase-3 in mouse hippocampus using immunohistochemical staining and Western blot analysis. Figure 3 shows the representative staining of cleaved caspase-3 in the hippocampus of mice. The positive staining was very weak in the hippocampus of the control group mice.

However, there was a marked increase in the expression in the cleaved caspase-3 in D-gal model mice as compared with the control mice, and the administration of EGCG 2 mg/kg/d or 6 mg/kg/d significantly reversed the effect.

Western blot analysis (Fig. 4) also showed that D-gal administration induced a clear increase in cleaved caspase-3 in the hippocampus of mice [0.39 ± 0.15 in the model group vs. 0.12 ± 0.08 in the control group, F(3, 20) = 9.297, p < 0.01]. These increases were significantly ameliorated by EGCG 2 mg/kg/d or 6 mg/kg/d [0.18 ± 0.11 or 0.12 ± 0.02, F(3, 20) = 9.297, p < 0.01].

DISCUSSION

Several hypotheses have been raised regarding the mechanisms of aging. Among them, the free radical/oxidative stress theory is one of the most approved. It has been shown that overproduction of ROS or free radicals can cause an age-related increase in oxidative damage to a variety of molecules, including lipid, protein, and DNA, and result in lipid peroxidation, protein carbonyl formation, and genome instability, finally leading to aging processes. D-Gal has been demonstrated to cause cellular metabolic disturbances by reducing Na⁺,K⁺-ATPase activity, reduce immune responses, and increase oxidative stress by increasing lipid peroxidation and...
advanced glycation end products and decreasing antioxidant enzyme activities and mitochondrial function in different animal species, including mice, rats, *Drosophila*, etc., accelerating aging and degeneration. D-Gal has thus been extensively used for aging research, especially in China. In this study, we observed that subcutaneous injection of 3% d-gal at the dose of 150 mg/kg body weight once daily to mice for 6 weeks induced prolonged escape latencies to the platform in the water maze test, indicating that d-gal induced learning and memory impairment in mice, consistent with previous reports.

Many studies have shown that EGCG displays potent antioxidant properties *in vitro* and *in vivo*. Jung *et al.* reported that EGCG had a protective effect against nitric oxide (NO)-induced apoptosis in rats PC12 cells by scavenging ROS. Recently, Senthil *et al.* have reported the antioxidant effects of EGCG in aged rats. In addition, EGCG can protect several organs from lipid peroxidation injury. However, there was no previous report on the protective effects of EGCG against d-gal-induced brain injury in mice. When we investigated the protective effects of EGCG in aging mice induced by d-gal, in behavioral testing EGCG was an effective neuroprotective agent that significantly reversed d-gal induced learning and memory impairment.

To protect cells against oxidative damage induced by ROS, the antioxidant system in the body is activated and endogenous antioxidant enzymes, such as SOD and GSH-Px, can scavenge ROS or prevent their formation. ROS production can also be indirectly evaluated by analyzing MDA, a product of free radical-induced lipid peroxidation. These alterations are considered to play important roles in improving learning and memory deficits. In the present study, we also observed that d-gal caused significant impairments of antioxidant enzyme activities of T-SOD and GSH-Px and an increase in the contents of MDA in the hippocampus of mice. These results were completely consistent with those in previous studies. We found that EGCG significantly elevated the antioxidant ability of aging mice induced by d-gal by increasing the activities of SOD and GSH-Px and reduced the formation of ROS by reducing the contents of MDA in the hippocampus. Ran *et al.* have recently also reported EGCG increased SOD activity and decreased the MDA level in rats with colitis induced by acetic acid. Overall, these findings suggest that EGCG, to some extent, protects mice against oxidative stress injury to the brain induced by d-gal and improves the impairments of learning and memory in aging mice.

D-Gal also causes neuronal damage in the cerebral cortex and hippocampus of mice. In the present study, we observed...
using TUNEL staining that the cell apoptosis index was increased in the hippocampus of mice treated with D-gal, and the administration of EGCG 2 mg/kg/d or 6 mg/kg/d obviously reduced the cell apoptosis index. Caspase-3 is a major effector of apoptosis in cells, exists in the cytoplasm as an inactive precursor (procaspase-3, 32 kDa), is cleaved into the two mature subunits p17 (17 kDa) and p12 (12 kDa), and activated by the action of upstream initiator caspases. Only activated caspase-3 (cleaved caspase-3) can cause apoptosis in cells. It has been shown that oxidative stress and ROS can activate procaspase-3 and result in apoptosis and function impairment of neuronal cells and eventually brain aging. 35,36 Considering the roles of caspase-3 in the process of cell apoptosis, in this study we measured the expression of cleaved caspase-3 in the hippocampus of mice using immunohistochemistry and Western blot analysis, and the results demonstrated that D-gal significantly increases the activation of caspase-3. These effects were attenuated by the administration of EGCG. Consistent with the present results, recent studies have shown that EGCG inhibits caspase-3 activation in the spinal cord in amyotrophic lateral sclerosis model mice and in NO-treated neuronal cells. 2,3 Therefore our findings suggest that caspase-3 may be one of the main effector proteins in D-gal-induced apoptosis and that EGCG protects against D-gal-induced apoptosis by blocking the activities of caspase-3.

In conclusion, EGCG, a polyphenol constituent of green tea, has antioxidant and antiapoptotic activity. Our present study showed that EGCG protects mice against learning and memory impairments and neuronal injury induced by D-gal by increasing the activities of antioxidant enzymes and reducing the MDA content and reducing the activation of caspase-3.

REFERENCES