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Catalpol improves cholinergic function and reduces inflammatory cytokines in the senescent mice induced by D-galactose

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ABSTRACT

The neuroprotective effects of catalpol, an iridoid glycoside isolated from the fresh *rehmannia* roots, on the cholinergic system and inflammatory cytokines in the senescent mice brain induced by D-galactose were assessed. The results showed that acetylcholinesterase (AChE) activity increased in senescent mice brain and choline acetyltransferase (ChAT) positive neurons, detected by immunohistochemical staining, decreased remarkably in the basal forebrain of senescent mice. Simultaneously, muscarinic acetylcholine receptor M1 (mAChR1) expression declined in senescent mice brain by western blotting method. We also found that the contents of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and advanced glycation endproducts (AGEs) increased in senescent mice brain by ELISA method. However, administration of catalpol for 2-weeks significantly reversed the biochemical markers mentioned above. These results suggest that catalpol can exert protective effects on senescent mice brain induced by D-galactose and this effect may be due to its protective effects on cholinergic and immune impairment in mice brain. Thus catalpol is worth testing for further preclinical study aimed for senescence or neurodegenerative diseases such as Alzheimer's disease.

1. Introduction

Memory is a complex process requiring the coordination of many different regions of brain and many neurotransmitter systems. Among the various neurotransmitter systems, the cholinergic system has attracted special attention because lesions of cholinergic system can cause defects of memory (Conner et al., 2003; Ikonomovic et al., 2003, 2005). Acetylcholinesterase (AChE) and choline acetyltransferase (ChAT), the biolysis enzyme and biosynthesis enzyme for neurotransmitter acetylcholine (ACh) respectively, are presently the specific indicators for monitoring the functional state of cholinergic neurons in the central and peripheral nervous system (Oda, 1999; Zambrzycka et al., 2002). The activity of ChAT is prominently reduced in aged brains, and the degree of reduction of ChAT activity is significantly correlated with the severity of cognitive impairments (Terry and Mahadik, 2007).

Inflammation has increasingly been recognized to play an important role in the pathogenesis of aging related diseases, including Parkinson's disease and Alzheimer's disease. Inflammation-mediated neurodegeneration involves activation of microglia, the brain's resident immune cells, which produce proinflammatory and neurotoxic cytokines, including tumor necrosis factor-a (TNF-a), interleukin-1β (IL-1β), reactive oxygen species (ROS) and nitric oxide (NO), that impact on neurons to induce neurodegeneration (Sawada et al., 1989; Block and Hong, 2005). Advanced glycation endproducts (AGEs), a heterogeneous group of non-enzymatic glycation products of proteins, accumulates at an accelerated rate in circulation and various tissues. AGEs formation is a trigger for the onset of age-related disease. Recent epidemiological studies demonstrate that elevated circulating AGEs are associated with increased risk of developing many chronic diseases that disproportionally affect older individuals as AGEs increase oxidative stress and inflammation (see review Semba et al., 2010; Tian et al., 2005).

Recently, herbal medicines have been attracting a great deal of attention as alternative and supplemental medicines. Many extracts from the herbal agents have been proven to be useful in the treatment of age-associated diseases (Liu et al., 2007). *Rehmannia* is an important traditional Chinese herbal medicine which is widely used to replenish vitality, strengthen the liver, kidney, heart, and for treatment of a variety of ailments like diabetes, anemia, and urinary tract problems according to the Chinese Pharmacopoeia. There has been growing evidence that the extract from the root of *Rehmannia* possesses significant neuroprotective activity

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(Yu et al., 2006; Cui et al., 2004). Catalpol, an iridoid glycoside, was isolated from the fresh root of *Rehmannia* with column chromatography method in our laboratory. It exists broadly in many plants all over the world and has many biological functions such as anti-inflammation, promotion of sex hormones production, protection of liver damage, and reduction of elevated blood sugar, but its neuroprotective effect to our knowledge has been scarcely studied.

Our previous works have demonstrated that catalpol can protect against LPS-induced neurotoxicity on dopaminergic neurons (Tian et al., 2006). In animal models, we found that catalpol could enhance cognitive performance in transient global ischemia in gerbils and protect mice brain from oxidative damage (Li et al., 2004). Most important is that catalpol could increase presynaptic proteins and up-regulate protein kinase C (PKC), brain derived neurophic factor (BDNF) in the hippocampus of the aged rats (Liu et al., 2006). Recently, we found that catalpol could reduce oxidative stress by increasing the activities of SOD, GSH-Px and decreasing the concentration of MDA in the brain of aged mice induced by D-galactose (Zhang et al., 2007). Rodent chronically injected with D-galactose has been used as an animal aging model for brain aging or anti-aging pharmacology research. It was reported that D-galactose could impair neurogenesis in the dentate gyrus, a process similar to the natural aging in mice (Zhang et al., 2005). In the present study, we mainly focus on observation the effect of catalpol on cholinergic system and inflammatory cytokines in the brain of senescent mice induced by 6-weeks subcutaneous injection of D-galactose. The activities of AChE in brain cortex and hippocampus were determined by spectrophotometric assay, and immunoreactive cells of ChAT in brain were counted by immunohistochemical staining. In addition, the expression of muscarinic acetylcholine receptor M1 (mAChR1) in the brain of different groups were evaluated by western blotting. The contents of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and advanced glycation endproducts (AGEs) were detected by enzyme-linked immuno sorbent assay (ELISA) method.

2. Materials and methods

2.1. Reagents and drugs

D-galactose was purchased from Shanghai Yuanju Chemical-Regent Company (Shanghai, China) and dissolved in 0.9% saline at concentrations of 3%. Catalpol (purity > 90%) was separated by our lab and dissolved in physiological saline (Zhang et al., 2007). Piracetam (Pingyuan pharmaceutical factory, Shandong, China) was dissolved in physiological saline. Commercial kit used for determination of AChE and ChAT were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). ChAT and mAChR1 antibodies were purchased from Beijing Boaosen Biotechnology Incorporation. TNF-α, IL-1β ELISA kits were purchased from WUhan Boster bio-engineering limited company (Wuhan, China) and advanced glycation end products (AGEs) ELISA kit was purchased from Adlitteram diagnostic laboratories Inc. (USA).

2.2. Animals and drug administration

The Kunming mice (obtained from Experimental Animal Center, Dalian Medical University, China), half male and half female, aged 3 months, weighing 25–30 g, were housed in cages in an air-conditioned room with controlled temperature (25 ± 1 °C) for 5 days before the experiment and were maintained on a 12 h:12 h light cycle (07:00 on–19:00 off). They were allowed free access to food and water. The mice were randomly divided into four groups: control group (n = 10), model group (n = 10), catalpol group (n = 30) and piracetam group (n = 10). Except control group, mice were subcutaneously injected with D-galactose at the dose of 150 mg/kg body weight once daily for 6 weeks while those of control group were treated with same volume physiological saline. From the fifth week, catalpol group and piracetam group were subcutaneously injected with catalpol at three doses of 2.5, 5, 10 mg/kg and piracetam at the dose of 300 mg/kg once daily for 2 weeks. Control group and model group were administered with same volume physiological saline. Behavioral testing was subsequently conducted between 9:00 and 17:00 h for 7 days. Piracetam, as positive drug in the experiment, is a nootropic drug. It appears to enhance cognition and memory, slow brain aging, and ameliorate Alzheimer's syndrome, dementia, and dyslexia, among other diseases and conditions. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Dalian Medical University, Dalian, China, and conformed to the international guidelines on the ethical use of animals (NIH publications No. 80-23) revised 1996. We used another batch of mice for the immune cytokines detection, and the drug administration was completely same as above.

2.3. Preparation of brain samples

After drug administration, all mice were fasted overnight and then sacrificed by decapitation. Brains were removed carefully and quickly to 0.9% cold saline and the right hemispheres were immediately dissected cerebral cortex and hippocampus on a cold plate, weighed and homogenized with ice-cold saline and stored at −70 °C for biochemical analysis. On the day of assay, the homogenate was centrifuged at 4000 rpm for 10 min at 4 °C and the supernatant was used. The left hemispheres were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for more than 24 h. Coronal blocks were embedded in paraffin for histological evaluation. For another batch of mice, we just took the whole brain of mice for the detection of inflammatory cytokines and AGEs, the treatment procedures were complied with the assay kits.

2.4. Biochemical assays

The activities of AChE and ChAT were determined by using commercially available kits. All procedures were completely complied with the manufacturer's instructions. The activity of enzyme was expressed as units per milligram protein.

2.5. Immunohistochemistry for ChAT

Immunohistochemistry stain was carried out following the avidin–biotin–peroxidase method. All sections were treated simultaneously. After deparaffination and rehydration, sections were treated with 0.3% H_2O_2 solution and 1.5% normal goat serum to quench endodenous peroxidase activity and block non-specific binding, and then with primary antibody in a humidified chamber for 1 h at room temperature. Rabbit anti-ChAT (Beijing Boaosen Biotechnology Inc.) was diluted 1:200. After washing with PBS, The sections were further incubated respectively with biotinylated secondary antibody (goat-anti-rabbit IgG, 1:200 dilution) and an avidin-biotin-peroxidase complex (Sino-American Biotechnology Company) for 30 min, and then rinsed in PBS and subsequently exposed to diaminobenzidine (DAB) for 3 min. After the final wash, the immunostained sections were dehydrated in graded ethanol and mounted with Permount. Quantitative analysis of ChAT immunoreactivity was performed under light microscopy (magnification 400×) and the number of positive cell was counted by the Image-Pro Plus software. The average value of five slices was used for each animal and expressed as the number of cells per 1 mm^2 of basal forebrain area. Negative control sections were incubated without the primary antibody.

2.6. Western blot

Brain tissue was homogenized in lysis buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, Complete Protease Inhibitor. The homogenate was incubated on ice for 30 min, and then the suspension was sonicated on ice using three 10-s bursts at high intensity with a 10-s cooling period between each burst. The samples were centrifuged at 13,000g for 15 min at 4 °C. Protein concentrations in the supernatant were measured by the method of Bradford (Bradford, 1976). Fifty micrograms of each protein extract was separated on a 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and then probed overnight with primary antibody (mAChR1 antibody; 1:500 in TBST/5% milk). After washing with TBST, the membrane was incubated for 1 h in TBST containing the appropriate horseradish peroxidase-conjugated secondary antibody (1:200) and again washed. DAB was used to visualize the peroxidase-coated bands. Densitometric analysis was performed in Scion Image software. β-Actin normalization was performed for each sample.

2.7. Enzyme-linked immuno sorbent assay (ELISA)

The production of AGEs and release of TNF-α, IL-1β were measured with a mouse AGEs, TNF-α and IL-1β ELISA kit using ELISA method, and all procedures were completely complied with the manufacturer's instructions.

2.8. Protein assay

Protein concentration was measured by the method of Bradford (1976). Bovine serum albumin was used as standard.