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Short report

Oxidative stress induces the decline of brain EPO expression in aging rats

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ABSTRACT

Brain Erythropoietin (EPO), an important neurotrophic factor and neuroprotective factor, was found to be associated with aging. Studies found EPO expression was significantly decreased in the hippocampus of aging rat compared with that of the youth. But mechanisms of the decline of the brain EPO during aging remain unclear. The present study utilized a D-galactose (D-gal)-induced aging model in which the inducement of aging was mainly oxidative injury, to explore underlying mechanisms for the decline of brain EPO in aging rats. D-gal-induced aging rats (2 months) were simulated by subcutaneously injecting with D-gal at doses of 50 mg·kg^{-1}, 150 mg·kg^{-1} and 250 mg·kg^{-1} daily for 8 weeks while the control group received vehicle only. These groups were all compared with the aging rats (24 months) which had received no other treatment. The cognitive impairment was assessed using Morris water maze (MWM) in the prepared models, and the amount of β-galactosidase, the lipid peroxidation product malondialdehyde (MDA) level and the superoxide dismutase (SOD) activity in the hippocampus was examined by assay kits. The levels of EPO, EPOR, p-JAK2 and hypoxia-inducible factor-2α (HIF-2α) in the hippocampus were detected by western blot. Additionally, the correlation coefficient between EPO/EPOR expression and MDA level was analyzed. The MWM test showed that compared to control group, the escape latency was significantly extended and the times of crossing the platform was decreased at the doses of 150 mg·kg^{-1} and 250 mg·kg^{-1} ($p < 0.05$). Also, the amount of β-galactosidase and the MDA level in the hippocampus were significantly increased but the SOD activity was significantly decreased ($p < 0.05$, 0.01 and 0.01, respectively). Similar to aging rats, the expressions of EPO, EPOR, p-JAK2, and HIF-2αin the brain of D-gal-treated rats were significantly decreased ($p < 0.05$) at 150 mg·kg^{-1} and 250 mg·kg^{-1}. Interestingly, negative correlations were found between EPOR ($r = -0.699$, $p < 0.01$), EPO ($r = -0.701$, $p < 0.01$) and the MDA level. These results indicated that aging could result in the decline of EPO in the hippocampus and oxidative stress might be the main reason for the decline of brain EPO in aging rats, involved with the decrease of HIF-2α stability.

1. Introduction

EPO production has been identified in the brain (Masuda et al., 1994). Cerebral EPO has drawn much attention as a neurotrophic and a neuroprotective factor for cerebral diseases including Alzheimer's (Maurice et al., 2014), depression (Miskowiak et al., 2010), epilepsy (Castaneda-Arellano et al., 2014) and ischemia (Nguyen et al., 2014). A few researches reported that the brain EPO expression was significantly decreased in the hippocampus of 24 months old rats (Chung et al., 2004). But the reason why EPO expression in hippocampus declined in aging was unclear.

Aging is an intricate phenomenon and many theories of aging have been proposed. Free-radical theory suggests that cumulative damage to the mitochondrion caused by reactive oxygen species (ROS) is one of causes of aging (Harman, 1956). Then we proposed a hypothesis that whether the accumulation of ROS is responsible for the decline of EPO.

To clarify the hypothesis, we utilized two aging models, natural aging (24-month-old) rat model and D-gal-induced aging rat model. Compared with natural aging model, the external interference factors of D-gal-induced aging model are lesser and the main cause of D-gal induced aging was proposed to be oxidative stress (Bei et al., 2010). Here we mainly observed behavioral defection, degree of oxidative stress impairment and the expression of HIF-2α, EPO, EPOR and p-JAK2

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expression in the two aging models to explore whether oxidative stress was involved in the decline of hippocampus EPO.

2. Material and methods

2.1. Animals and treatment

Male Sprague-Dawley rats aged either 2 or 24 months. 2 months rats weighted 200 ± 20 g, and 24 months rats weighted 500 ± 50 g. They were all obtained from the Medical Laboratory Animal Center, Chongqing Institute of TCM (Chongqing, China Certificate No. SCXK 2012.006) and housed in the Experimental Animal Center, College of Pharmaceutical Sciences, Southwest University (Chongqing, China Certificate SYXK 2014.0002). After acclimatized for one week under constant conditions of temperature (22 ± 2 °C) and humidity (50–70%) with a 12-h light–dark cycle, the 2-month-old rats were randomly divided into 4 groups, including control group, 50 mg/kg D-gal-treated group, 150 mg/kg D-gal-treated group, and 250 mg/kg D-gal-treated group ($n = 13$). In addition, the 24-month-old rats is the aging group ($n = 13$). 50 mg/kg D-gal-treated group, 150 mg/kg D-gal-treated group, and 250 mg/kg D-gal-treated group individually received subcutaneous injection of D-gal (Amresco, USA) at doses of 50 mg/kg, 150 mg/kg, and 250 mg/kg daily for 8 weeks. Both the control group and aging group were injected subcutaneously with the same volume of vehicle (0.9% saline) daily for 8 weeks. All the principles of laboratory animal care were followed in compliance with the Chinese Experimental Animals Administration Legislation.

2.2. Morris water maze

The Morris water maze (MWM) test was performed according to the procedures as described previously (Vorhees and Williams, 2006). MWM test was conducted in the 8th week, including 4-day learning and memory training and a spatial probe on day 5. The rat were trained in a black circular water tank (150 cm in diameter, 40 cm in height) and an escape platform (9.5 cm in diameter) was submerged 2.0 cm below the surface of the pool water (25 ± 5 °C) which was mixed with milk powder to obscure the platform. The pool was divided into four quadrants and the platform remained in the midpoint of one quadrant. During 4-day learning and memory training, rats were trained to search the submerged platform facing the pool wall at 4 different points. When the rat found the platform within 60 s, the time used to find the hidden plat was recorded as escape latency, otherwise, it was placed on the platform for 15 s at the end of the trial and the escape latency was recorded as 60s. On the 5th day, it was a spatial probe in which the platform was removed and the rat was allowed to swim freely for 60 s inside the pool and the number of times crossing over the platform were recorded as the times of crossing platform. All data were recorded using a computerized video and analyzed by the software of TM-200 video analysis system (Chengdu Techman Software Co.LTD, Chengdu, China).

2.3. In situ staining for β-galactosidase activity in hippocampus of brain

2.3.1. The preparation of frozen section

After the behavioral test, 3 rats in each group were anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde solution in 0.1 M PBS. The brain was divided and fixed for 24 h with 4% paraformaldehyde solution, then dehydrated in gradient manner with 10%, 20%, and 30% sucrose in 0.1 M PBS at 4 °C. After dehydration, the brain was embedded using embedding medium and then was cut into free-floating sections (10 μm) by freezing microtome (Leica 1900, Germany).

2.3.2. The detection of β-galactosidase activity

β-galactosidase activity was detected as the instructions of Senescence β-galactosidase Staining Kit (Beyotime Biotechnology, Shanghai, China). The frozen sections were fixed with fixative for 15 min at room temperature. Then they were washed three times with 0.1 M PBS (PH7.4) and incubated overnight at 37 °C in freshly prepared staining buffer. After incubation, the sections were examined with optical microscope and the pictures were analyzed by the software of Image-Pro Plus 6.0 ($n = 3$).

2.4. Measurement of MDA level and SOD activity of rat in the hippocampus

After the detection of β-galactosidase activity, the remaining 10 rats in each group were killed by anesthetizing and the brains were separated on ice. Then 10 intact brains was divided into 20 hemispheres, and 3 hemispheres were remained for western blot while the corresponding 3 hemispheres and the other 7 intact brains were used for the measurements of MDA level and SOD activity. The hippocampus used for the measurement of MDA and SOD were homogenized with ice-code saline to be 10% (w/v) homogenate. Then the homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was used to determine MDA level and SOD activity using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). MDA level and SOD activity were individually expressed as nmol/mg of protein and U/mg of protein ($n = 10$). Protein concentration was measured by the method of Bradford.

2.5. Western blot for EPO, EPOR, p-JAK2 and HIF-2a

After the hippocampus was isolated from each cerebral hemisphere, 3 hemispheres in each group were remained for western blot. The hippocampus were homogenized with Tissue Total Protein Lysis Buffer (RIPA, Beyotime Biotechnology, Shanghai, China) which containing 1 mM Phenylmethanesulfonyl fluoride (PMSF). Then the homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C and protein concentration of the supernatant was measured by the method of Bradford. Samples containing 40 μg protein were diluted with Laemmli buffer to 20 μl and boiled for 10 min at 95 °C. Then the samples were electrophoresed through 12% SDS-PAGE and transferred on PVDF membranes (Millipore, USA). The membranes were incubated with 5% non-fat milk in Tris-buffered saline with 0.1% tween-20 (TBST) for 1.5 h at room temperature (RT) to block nonspecific binding sites and subsequently incubated with antibodies against rabbit polyclonal EPO (diluted 1:200, bs-2343R, Bioss Biotechnology Inc., Beijing, China) or rabbit polyclonal EPOR (diluted 1:200, bs-1424R, Bioss Biotechnology Inc., Beijing, China) or rabbit polyclonal HIF-2α (diluted 1:200, bs-1447R, Bioss Biotechnology Inc., Beijing, China) or rabbit polyclonal JAK2 (diluted 1:500,sc-16,566-R, Santa Cruz, CA, USA) or rabbit polyclonal p-JAK2 (Tyr1007/Tyr1008) (diluted 1:500,sc-16,566-R, Santa Cruz, CA, USA) overnight at 4 °C. Then membranes were washed with TBST and incubated for 1 h at RT with horseradish peroxidase-conjugated goat *anti*-rabbit IgG secondary antibody (diluted 1:3000, bs-0295G, Bioss Biotechnology Inc., Beijing, China). After being washed with TBST, the membranes were blanketed with enhanced chemiluminescence (ECL) detection reagents (Millipore, USA), then exposed to the autoradiography film (Tatan 5200, Shanghai, China). Lastly, the average optical density was analyzed with Tatan software and the rate of optical density of aim protein and β-actin was recorded ($n = 3$).

2.6. Statistical analysis

All data were presented as mean ± SD using the SPSS 17.0 software. Group differences in the escape latency in the MWM test were analyzed by a two-way ANOVA method. Correlations between variables were determined by linear regression analyses. The other data were analyzed with one-way ANOVA and the Tukey's HSD post-hoc test was used to compare differences between two of the groups. All tests were two-sides, with significance setting at $p < 0.05$.