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Glutamine synthetase plays a role in D-galactose-induced astrocyte aging in vitro and in vivo

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ABSTRACT

Astrocytes play multiple roles in physiological and pathological conditions in brain. However, little is known about the alterations of astrocytes in age-related changes, and few aging models of the astrocytes in vitro have been established. Therefore, in the present study, we used D-galactose (D-Gal) to establish astrocyte aging model to explore the alterations of astrocytes in brain aging. We also used ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra to verify the metabolic changes in the cerebral cortex of mice injected with D-gal. The results showed that D-gal (55 mM) treatment for 1 week induced senescence characteristics in cultured cortical astrocytes. Real-time PCR and western blot analysis showed that the levels of glutamine synthetase (GS) mRNA and protein were strikingly decreased in the cultured senescent astrocytes, and the senescent astrocytes showed less resistance to the glutamate-induced gliotoxicity. The impairments of glutamate–glutamine cycle and astrocytes were also found in the cerebral cortex of mice treatment with D-gal (100 mg/kg) for 6 weeks, and the level of GS mRNA was also found to be reduced markedly, being consistent with the result obtained from the senescent astrocytes in vitro. These results indicate that astrocyte may be the predominant contributor to the pathogenic mechanisms of D-gal-induced brain aging in mice, and GS might be one of the potential therapeutic targets of the aged brain induced by D-gal.

1. Introduction

Aging is an extremely complex multifactorial process associated with physiological decline, and it has always been the hot spot carried out by scientists all over the world (Weinert and Timiras, 2003). In the past researches, animal models provided valuable research tools for the study of aging mechanisms and aging related diseases. The way that accelerates aging in rodents by long-term administration of D-galactose (D-gal) was first proposed by scientists in China (Wang et al., 2009), and it is gradually accepted as a rodent model for the study of aging mechanisms and anti-aging researches in Asia (Chen et al., 2006; Ho et al., 2003; Long et al., 2007; Lu et al., 2007; Xu et al., 2009). However, D-gal-induced cell aging model in vitro has rarely been studied, and the aging related changes and the underlying mechanisms are still unclear.

Astrocytes are the most abundant cell type in the brain, and play multiple roles in physiological and pathological conditions (Chen and Swanson, 2003). Although the current data collectively suggest that dysfunctions of astrocytes are implicated in various age-related neurodegenerative diseases, such as Parkinson's disease, multiple sclerosis, and Alzheimer's disease, astrocytes have received much less attention than neuronal alterations in age-related changes (Mansour et al., 2008; Seifert et al., 2006). So it is very urgent and meaningful to explore the role of astrocytes in the pathogenic mechanisms of brain aging. So far little is known about the changes of astrocytes during the aging process, and few aging models of astrocytes in vitro have been established, except the astrocytes cultured for 90 days in vitro (DIV) which show senescence characteristics and have been used as naturally aged astrocytes (Pertusa et al., 2007). However, the culture process takes as long as 90 days. Thus it is necessary to establish methods to induce aging in astrocytes in vitro much more easily and rapidly. It has been reported that chronic intraperitoneal injection of D-gal for 2 weeks induced astrocyte

Abbreviations: D-Gal, D-galactose; NMR, nuclear magnetic resonance; CNS, central nervous system; GS, glutamine synthetase; DIV, day in vitro; GFAP, glial fibrillary acidic protein; TEM, transmission electron microscopy; $\Delta\Psi$m, mitochondrial membrane potential; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TCA cycle, tricarboxylic acid cycle; mtDNA, mitochondrial DNA; GABA, γ-aminobutyric acid; SAMP8, senescence-accelerated-prone 8 mice.

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activation in mice (Wu et al., 2011). However, whether exposure to D-gal can induce a senescent phenotype of astrocytes in vitro has not been documented.

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS), and is crucial for brain function such as learning and memory. Elevated extracellular glutamate level has been shown to occur in the pathologies of Alzheimer's disease, Parkinson's disease, stroke and other age-related diseases. Because of the lack of a glutamate-metabolizing enzyme in an extracellular space, the released glutamate needs to be cleared from the extracellular space mainly by the astrocytic glutamate transporters, and is converted to glutamine by glutamine synthase (GS) which is localized chiefly in astrocytes. However, whether the aged astrocytes still have the ability to maintain glutamate homeostasis in the brain and its underlying mechanisms is unclear.

In this study, we established a D-gal-induced astrocyte aging model with the exploration of the aging-related changes in cultured cortical astrocytes. In addition, we also used ^{1}H NMR spectra to verify the metabolic changes in the cerebral cortex of mice injected with D-gal for six weeks and its underlying mechanisms.

2. Materials and Methods

2.1. Reagents

D-Galactose, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), methanol, propidium iodide (PI), Hoechst 33342, and lactic acid were from Sigma (St. Louis, MO, USA). Penicillin, streptomycin, L-glutamine, trypsin, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were from GIBCO-BRL (Grand Island, NY, USA). Senescence β-Galactosidase Staining Kit, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), BCA Protein Assay Kit, mitochondrial membrane potential assay kit with JC-1 and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, and Cy3-labeled goat anti-mouse IgG were bought from Beyotime Institute of Biotechnology (Nanjing, China). SYBR Premix EX TaqTM and PrimeScript RT reagent kit were from TakaRa Biotechnology (Dalian) Co., Ltd (Dalian, China). Rabbit anti-GS antibodies were bought from Kangchen Biotechnology (Shanghai, China). Mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) was bought from Cell Signaling Technology, Inc. (China).

2.2. Animals and Treatment

All experiments using animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J male mice of the age of 10–12 weeks (22–30 g) were used (N = 49), and they were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China, approval No. SCXK 2010–0002). The mice were randomly separated into two groups by body weight. After a 1-week acclimation period, the mice were given one of the following daily intraperitoneal injections for 6 weeks: (1) 0.9% saline at 1 mL/kg as vehicle control; and (2) D-gal at 100 mg/kg. Body weight of the mice was monitored during the experiment as a general measure of health. Mice were sacrificed at the end of treatment, and the brains were quickly removed for experiments or stored at −70 °C for later experiments.

2.3. Preparation of Samples and Acquisition of ^{1}H NMR Spectra

The brain cortical tissue was weighed and ground using an electric homogenizer with ice-cold methanol (4 mL/g) and distilled water (0.85 mL/g) at 4 °C and the mixture was vortexed. The supernatant was extracted and lyophilized for about 24 h. The metabolite mixture obtained was then weighed and dissolved in 0.6 mL of 99.5% D_2O for NMR spectroscopy. All ^{1}H NMR experiments were carried out on a Bruker AVANCE III 600 MHz NMR spectrometer, with a spectral width of 12,000 Hz. The acquisition time was 2.65 s per scan, and an additional 10-s relaxation delay was used to ensure full relaxation. The number of scans was 256. The spectra were zero-filled to 64 K, and an exponential line-broadening function of 0.3 Hz was applied to the free induction decay prior to Fourier transformation. All spectra were corrected manually for phase and baseline and referenced to the chemical shift of the methyl peak of lactate (CH_3, 1.33 ppm) using Topspin (v2.1 pl4, Bruker Biospin, Germany).

Using trimethylsilyl-propionic-2,2,3,3d_4-acid (TSP) as the internal reference, the metabolite concentrations were determined from the spectra and normalized to the weight of the freeze-dried metabolite mixture. The concentrations of metabolites were shown in the unit of mmol/kg wet tissue weight.

2.4. Cell Culture and Induction of Astrocyte Senescence

Primary cultures of cortical astrocytes were prepared from cortices of newborn Sprague–Dawley rats as previously described (Shen et al., 2010). In brief, the cerebral cortices were trypsinized (0.25%) for 20 min at 37 °C, and then the dissociated cells were seeded onto poly-D-lysine-coated 75 cm^2 flasks. Cells were cultured in high glucose (4.5 g/L) DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. The cultures were maintained at 37 °C under >90% humidity and 5% CO_2. On days 10–11, the confluent cultures were shaken overnight to minimize microglia contamination. The remaining monolayers of astrocytes were trypsinized and replated at a density of 2.5×10^4 cells/cm^2 onto 96- or 6-well plates, or culture flasks. More than 95% of the cultured cells were astrocytes as identified by immunofluorescent staining for glial fibrillary acidic protein (GFAP). Related to the induction of astrocyte senescence, the day 10–11 astrocytes were replated onto poly-D-lysine-coated 25 cm^2 flasks with the same culture medium supplemented with 55 mM D-gal. Four days after subculture the cells were trypsinized and replated onto 96- or 6-well plates, or culture flasks, and then the astrocytes were exposed to D-gal. Experiments were carried out three days after the cells were seeded.

2.5. β-Galactosidase Staining

Cellular senescence assay was performed by Senescence β-Galactosidase Staining Kit according to the manufacturer's instructions. Briefly, cells were washed twice with PBS and fixed in 4% PFA for 15 min. After being washed with PBS for three times, the cells were incubated with β-galactosidase detection solution at 37 °C overnight. β-Galactosidase is a marker which can identify senescent cells in culture (Dimri et al., 1995; Itahana et al., 2007). Senescent astrocytes stained with a blue color were observed using a microscope (Nikon, Tokyo, Japan) and digitally photographed with a ColorView camera.

2.6. Light Microscopy and Transmission Electron Microscopy (TEM)

Morphological changes were observed under a light microscope, and photographed in 24 cells cultured with or without D-gal. For TEM analysis, the control and D-gal treated cells were harvested by trypsinization, washed with PBS and pre-fixed in 2.5% glutaraldehyde at 4 °C overnight. After washing three times with PBS, the cells were fixed in 1% OsO_4 for 2 h. Then dehydrated the fixed cell specimen in an ethanol gradient (40–100%) for 10 min at each step, and embedded in Epon 812. Ultrathin sections were double-stained using uranyl acetate and alkaline lead citrate. Ultimately, the materials were examined and photographed with a transmission electron microscope (Hitachi-7500).