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Research article

Recombinant insulin-like growth factor binding protein-4 inhibits proliferation and promotes differentiation of neural progenitor cells

Hanxu Niu, Rongbin Gou, Qunyuan Xu, Deyi Duan*

Department of Neurobiology, Beijing Center of Neural Regeneration and Repair, Beijing Institute for Brain Disorders, Laboratory of Neurodegenerative Disorders of the Ministry of Education, Capital Medical University, Beijing, 100069, China

HIGHLIGHTS

- Blockade of IGF-IR in NPCs inhibits cell growth and enhances neuronal differentiation.
- Exogenous IGFBP4 inhibits proliferation and enhances neuronal differentiation of NPCs.
- IGFBP4 decreases Akt activation without affecting Erk and p38 phosphorylation in NPCs.
- IGFBP4 affects proliferation and differentiation of NPCs via IGF-IR signaling pathway.

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ABSTRACT

Insulin-like growth factor (IGF) is involved in regulating many processes during neural development, and IGF binding protein-4 (IGFBP4) functions as a modulator of IGF actions or in an IGF-independent manner (e.g., via inhibiting Wnt/β-catenin signaling). In the present study, neural progenitor cells (NPCs) were isolated from the forebrain of newborn mice to investigate effects of IGFBP4 on the proliferation and differentiation of NPCs. The proliferation of NPCs was evaluated using Cell Counting Kit-8 (CCK-8) after treatment with or without IGFBP4 as well as blockers of IGF-IR and β-catenin. Phosphorylation levels of Akt, Erk1, 2 and p38 were analyzed by Western blotting. The differentiation of NPCs was evaluated using immunofluorescence and Western blotting. It was shown that exogenous IGFBP4 significantly inhibited the proliferation of NPCs and it did not induce a more pronounced inhibition of cell proliferation after blockade of IGF-IR but it did after antagonism of β-catenin. Akt phosphorylation was significantly decreased and phosphorylation levels of Erk1, 2 and p38 were not significantly changed in IGFBP4-treated NPCs. Excessive IGFBP4 significantly promoted NPCs to differentiate into astrocytes and neurons. These data suggested that exogenous IGFBP4 inhibits proliferation and promotes differentiation of neural progenitor cells mainly through IGF-IR signaling pathway.

1. Introduction

It has well been established that insulin-like growth factor (IGF) system plays an essential role in the normal growth and development of the brain [11]. IGF-I exerts pleiotropic effects on neural stem cells and mature neurons in the developing brain and neurogenesis, axon remodeling and de novo synaptogenesis in the embryonic and adult brain [11,14]. High affinity IGF binding proteins (IGFBPs), designated IGFBP1 through IGFBP6, have been proposed to inhibit the biological actions of IGFs by hindering their binding to IGF receptors in most circumstances [1,14], or to enhance IGF actions in certain conditions [1]. Some IGFBPs were also reported to have IGF-independent actions which are mediated by interaction with cell surface 'receptors' (such as integrins, pertussis toxin-sensitive and –insensitive G-protein coupled receptors) or nuclear hormone receptors [1]. One IGF-independent action of IGFBP4 was identified to promote cardiogenesis of induced pluripotent stem cells through inhibiting β-catenin signaling [18]. It was demonstrated that IGFBPs are relevant in development and maturation of the brain. The inhibitory effects of IGFBPs on the brain growth and the number of oligodendrocytes, astrocytes and neurons have been extensively investigated in transgenic mice that over-express IGFBP1-3,5,6 [14]. However, the roles of IGFBP4 in the brain remain to be elucidated.

In the present study, the effects of IGFBP4 on the proliferation and differentiation of primary neural progenitor cells (NPCs) were

* Corresponding author.
E-mail address: duandy@ccmu.edu.cn (D. Duan).

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observed and its IGF-dependent or -independent actions were evaluated after treatment of NPCs with blockers of IGF-IR and β-catenin. The phosphorylation levels of Akt, p38 and Erk molecules were analyzed by Western blotting.

2. Materials and methods

2.1. Suspension culture of NPCs [6]

Newborn mice were dissected in a laminar flow hood and the brain was transferred to a 35 mm dish containing cold PBS with penicillin-streptomycin (500 UI/ml–500 μg/ml) (Merck Millipore, Darmstadt, Germany). The cortex and hippocampus were dissected under stereomicroscope, then minced mechanically in DMEM/F12 (Gibco-Invitrogen, Carlsbad, CA). Tissue fragments were further dissociated by gentle trituration to release single cells. The cell suspensions were centrifuged at 1000 rpm for 5 min, filtered through a 100-μm filter. Cells were then transferred to 6-well plates and maintained at 37 °C in DMEM/F12 growth medium supplied with 2% B27 serum-free supplements (Gibco-Invitrogen), 2 mM L-glutamine (Gibco-Invitrogen), 20 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, MN), 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems), and penicillin-streptomycin (100 UI/ml–100 μg/ml) [19]. Culture medium was changed and cells passaged every 3-4 days. NPCs at passage 4 were used in all experiments. The care and handling of the newborn mice was approved by the Beijing Municipal Administration Office for Laboratory Animals (approval number SCXK(Jing)2009-0007).

2.2. Adherent monolayer culture of NPCs

An adherent monolayer of NPCs was cultured on substrates of poly-D-lysine (PDL) and laminin (R&D System, Gaithersburg, USA). PDL-laminin coated culturewares were prepared according to the Technical Manual Version 2.1.0 (2016) for In Vitro Proliferation and Differentiation of Mouse Neural Stem and Progenitor Cells Using NeuroCult (STEMCELL Technologies Inc, Vancouver, BC, Canada. Data available from: https://www.stemcell.com). The suspension cultures (free-floating neurospheres) of NPCs were harvested into a 15 ml polypropylene tube and centrifuged at 1000 rpm for 5 min. The cell pellets were suspended in 1 ml fresh medium, triturated several times until a single cell suspension was achieved, and then filtered through a 40 μm filter. The cell suspensions were then seeded onto the pre-prepared PDL-laminin coated culturewares for cell proliferation test or immunocytochemistry.

2.3. Cell proliferation

Dissociated single cell suspensions (10^5/ml) were inoculated into a PDL-laminin coated 96-well plate (100 μl/well) in triplicate or quintuplicate and cultured in a humidified incubator (37 °C, 5% CO2). The culture medium was supplemented with the following reagents: a neutralized antibody against IGFBP4 (4 μg/ml, R&D Systems, Minneapolis, MN), IGFBP4 (0.5 μg/ml, R&D Systems) [17], the half maximal inhibitory concentration of AG1024 (AG1024 IC50, dissolved in DMSO at a final concentration of 7 μM, Calbiochem, Merck Millipore, Billerica, MA, USA) [7], AG1024 (20 μM), IGFBP4 + AG1024/AG1024 IC50, FH535 (dissolved in DMSO at 20 μM, Tocris Bioscience, Avonmouth, Bristol, UK) [5,9], and IGFBP4 + FH535. Treatment with DMSO only was used as control. The cultures were fed with new medium every three days. Each day, 10 μl of CCK-8 solution was added to each well and incubated for 4 h. The absorbance was measured at 450 nm using a microplate reader (ELx808, Biotek Instruments, Winooski, VT, USA). The experiments were repeated three times.

2.4. Cell differentiation

Dissociated single cells were seeded onto PDL-laminin coated culturewares and cultured in DMEM/F12 differentiation medium with withdrawal of bFGF and EGF, and supplemented with 2% B27, 2 mM l-glutamine, 10% fetal bovine serum, and penicillin-streptomycin (100 UI/ml–100 μg/ml) [19]. The cells grown on the coated glass coverslips placed in 24-well plates were used to evaluate the number of GFAP- and MAP2-positive cells by fluorescent immunocytochemistry, and the cells on the coated 90-mm Petri dishes were collected to analyze the expression of GFAP and MAP2 by Western blotting.

2.5. Immunocytochemistry

The adherent monolayer cultures of NPCs on the coated glass coverslips were fixed by immersing in 4% paraformaldehyde for 30 min. The endogenous peroxidase was quenched by 1% hydrogen peroxide in 50% ethanol for 30 min at room temperature. After washes in PBS, the coverslips were blocked for 1 h at room temperature in a blocking solution (5% goat serum in PBS), followed by incubation at 4 °C overnight with the following antibodies: a mouse monoclonal antibody to Nestin (1:200; Abcam, Cambridge, UK), rabbit anti-IGFBP4 (1:200; Santa Cruz, CA, USA), rabbit anti-GFAP (1:1000; Abcam) and anti-MAP2 (1:500; Abcam). The cells were then incubated at room temperature for 2 h with goat anti-rabbit secondary antibodies conjugated with Fluor-488 (1:250; EarthOx, San Fransico, CA, USA) or a goat anti-mouse secondary antibody conjugated with Alexa Fluor-594 (1:250; EarthOx) in the dark. The coverslips were coverslipped with fluorescent mounting medium (Dako) and the fluorochrome labeled antibody was visualized and photographed under a fluorescent microscope (Olympus IX71) equipped with an Olympus camera (DP73).

The number of GFAP- and MAP2-immunoreactive cells was determined related to the number of DAPI-stained nuclei. Approximately 200 cells were counted within randomly selected visual fields through fluorescence microscopy at 40× magnification.

2.6. Western blotting

An adherent monolayer of NPCs was cultured on PDL-laminin coated 90 mm dishes with or without IGFBP4 for 5 d. The cells were collected and lysed for 30 min at 4 °C with a 360-μ1 lysis buffer containing 50 mM Tris (pH7.2), 150 mM NaCl, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 0.1% leupeptin. The cell lysates were harvested and centrifuged at 4 °C for 10 min at 12,000*g* to collect supernatant protein extracts. The concentration of total protein was tested using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein from each lysate (10 μg) were loaded and size-fractionated by SDS polyacrylamide gel electrophoresis at a constant voltage of 120 V at 4 °C. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (0.2 μm; Millipore, Bedford, USA) at a constant current of 100 mA for 3 h at 4 °C. The membrane was rinsed for 10 min with Tris-buffered saline supplemented with 0.05% Tween 20 (TBST, pH 7.4) followed by non-specific binding with a blocking solution (10% non-fat dry milk in TBST). The blocked membrane was probed with antibodies raised against GFAP (1:10000; Abcam); MAP2 (1:1000; Abcam); Akt, p38 and Erk1,2, phospho-Akt (*p*-Akt), *p*-p38, and *p*-Erk1,2 (1:1000 each, CST, Danvers, MA, USA) overnight at 4 °C. After wash three times (10 min each) in TBST, the membrane was incubated with a secondary goat anti-rabbit IgG or a goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000 each; EarthOx) for 2 h at room temperature. Finally, the blots were developed by use of a SuperEnhanced chemiluminescence detection kit (Applygen Technologies