Brain Research Bulletin 79 (2009) 85–88

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

The neuroprotective effects of NGF combined with GM1 on injured spinal cord neurons *in vitro*

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ARTICLE INFO

Article history:
Received 1 November 2008
Received in revised form 3 December 2008
Accepted 5 December 2008
Available online 6 January 2009

Keywords:
Nerve growth factor
Gangliosidosis
GM1
Neuroprotective agents
Neuron
Spinal cord

ABSTRACT

Monosialoganglioside (GM1) has been considered to have a neurotrophic factor-like activity. Nerve growth factor (NGF), a member of the neurotrophin family, is essential for neuronal survival, differentiation and maturation. The aim of the present study was to investigate whether co-administration of GM1 and NGF reverses glutamate (Glu) neurotoxicity in primary cultured rat embryonic spinal cord neurons. Spinal cord neurons were exposed to Glu (2 mmol/l), Glu (2 mmol/l) plus GM1 (10 mg/ml), Glu (2 mmol/l) plus NGF (10 ng/ml), Glu (2 mmol/l) plus GM1 (5 mg/ml) and NGF (5 ng/ml) and then processed for detecting intracellular concentrations of Ca^{2+}([Ca^{2+}]i) by confocal laser scanning microscopy and growth associated protein 43 (GAP43) mRNA by RT-PCR. The fluorescent intensity in Glu plus GM1 and NGF incubated neurons was the lowest as compared with that in other groups. The expression of GAP43 mRNA in Glu plus GM1 and NGF incubated neurons was the highest as compared with that in other groups. These results implicated that GM1 and NGF have synergistic neuroprotective effects on spinal cord neurons with excitotoxicity induced by Glu *in vitro*.

1. Introduction

Nerve growth factor (NGF) initially interested neurobiologists because of its effects in the developing nervous system of the survival, differentiation and maturation. It is now clear that NGF functions throughout the life of the animal with a wide repertoire of actions [9]. The expression of NGF is increased after injury of the nervous system. It is necessary in order to preserve architecture and restore function of the nervous system [12].

Monosialoganglioside (GM1) has been considered to have a neurotrophic factor-like activity by activating the Trk neurotrophin receptors [6] and plays a neuroprotective role on hippocampal neurons both *in vitro* and *in vivo* [25]. GM1 has been shown to have a stimulatory effect on neurite outgrowth and to prevent degeneration of neuronal cells and to facilitate the phenotypic recovery of spinal motor neurons during aging and after axotomy [7]. The evidence has accumulated that GM1 and NGF have synergistic effect of neurite regeneration, expression of neurofilament protein, stimulation of neuritogenesis, prevention of apoptotic death, promotion of survival and neurite outgrowth of neurons [2]. Glutamate (Glu) is an excitatory amino acid [5] and induces neuronal excitotoxicity by activating N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptors to stimulate Ca^{2+} influx [21]. Ca^{2+} is a universal second messenger that is a key component of myriad processes in all cell types. Growth-associated protein 43 (GAP43) is a membrane-bound molecule expressed in neurons. It is particularly abundant during periods of axonal outgrowth in development and regeneration of the central and peripheral nervous systems. It is known that GAP43 mRNA is expressed in the spinal cord neurons of adult rat and that GAP43 is upregulated in spinal cord neurons during regeneration [14]. The objective of the present work was to study the effects of GM1 and NGF, alone and associated, on the improvement of Ca^{2+} overloading and GAP43 mRNA expression in spinal cord neurons with excitotoxicity induced by Glu *in vitro* in order to detect synergistic effect of GM1 and NGF, and further clarify the mechanism of the synergism of GM1 and NGF.

2. Materials and methods

2.1. Spinal cord cell culture preparations

Spinal cord cell was dissected out from embryonic 15-day-old Wistar rats. The animals were obtained from the Experimental Animal Center of Shandong University of China. Spinal cord prior to establishment in culture was digested with 0.25%

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0361-9230/$ – see front matter.
doi:10.1016/j.brainresbull.2008.12.007

trypsin (Sigma) in D-Hanks solution at 37 °C for 20 min and centrifuged for 10 min at 0.8×10^3 rpm. The supernatants were removed and the pellets were resuspended in Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F-12) media (Gibco) and triturated using a sterile modified Pasteur's glass pipette. Dissociated spinal cord cells were then cultured in 24-well clusters (Costar, Corning, NY, USA) for monitoring intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$) using confocal laser scanning microscope (CLSM) or flasks (Costar, Corning, NY, USA) precoated with poly-LL-lysine for detecting GAP43 mRNA by RT-PCR. Then the cells were cultured in culture media at 37 °C with 5% CO_2 for 48 h. The composition of the culture media is D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25 μg/ml, Sigma), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.2. Exposure of glutamic acid, NGF and GM1 on spinal cord neurons

At 48 h of culture age, all spinal cord neurons both in clusters and in flasks were exposed to Glu (2 mmol/l), Glu (2 mmol/l) plus GM1 (10 mg/ml), Glu (2 mmol/l) plus NGF (10 ng/ml), Glu (2 mmol/l) plus GM1 (5 mg/ml) and NGF (5 ng/ml). Spinal cord cells were continuously exposed to growth media as control. Then all above cultures were incubated at 37 °C in a humidified 5% CO_2-air atmosphere for additional 12 h.

2.3. Double fluorescent labeling with MAP2 and DAPI

The spinal cord neurons got from rat's embryo were incubated at 37 °C 5% CO_2 for another 60 h. To define the origin of the cells, they were labeled with MAP2 and DAPI.

2.4. Ca^{2+} imaging with fluorescent probe and confocal microscopy

To assess the Ca^{2+}, the spinal cord neurons were loaded with fluo-3 acetoxymethyl ester (Fluo-3 AM, 10 mmol/l, Biotium) for 30 min at room temperature. Excess dye was removed by washing it out with 0.01 mol/l PBS. The cells were imaged in 0.01 mol/l PBS. $[Ca^{2+}]i$ was assessed by a CLSM (Zeiss LSM510). Fluo-3 AM was excited with the 480 nm of an argon ion laser, and the emitted fluorescence was measured at 530 nm. Each sample was randomly selected one visual field under the CLSM. All the spinal cord cells on the screen were monitored. By applying the computer software (Laser Scanning Microscope LSM 510 Version 2.5 SP2), the intensities of the intracellular fluorescence were measured. The intensity of the intracellular fluorescence on the screen was divided by the number of monitored cells. Then the average intensity of the intracellular fluorescence of the individual cell was obtained.

2.5. RNA extraction and reverse transcription-PCR

The mRNA levels of GAP43 were analyzed by RT-PCR. The expression of β-actin was also determined as an internal control. Total spinal cord cell RNA of each flask was isolated by TRIzol (Gibco). cDNA synthesis was performed with M-MLV reverse transcriptase. The gene-specific primers were synthesized by use of the published cDNA sequences for GAP43 and β-actin. The synthetic oligonucleotide primer sequences for GAP43 and β-actin were as follows: GAP43 5′-GGG AGA TGG CTC TGC TAC T-3′ (upper primer) and 5′-AGA CAG GGT TCA GGT GGG-3′ (lower primer); β-actin 50-ATC ATG TTT GAG ACC TTC AAC-30 (upper primer) and 50-CAT CTC TTG CTC GAA GTC CA-30 (lower primer). The predicted size of the amplified GAP43 and β-actin DNA products were 778 and 317 bp respectively. PCR amplification was performed for 35 cycles. The cycle profile included denaturation for 60 s at 94 °C, annealing for 60 s at 58 °C, and extension for 60 s at 72 °C. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products. The amplified products were analyzed by standard agarose gel electrophoresis and stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned and the electrophoresis gel images were analyzed quantitatively by using a Totallab image analysis software. The levels of GAP43 mRNA were expressed as the ratio of the gene to β-actin.

2.6. Statistical analysis

Data are expressed as mean ± S.D. Statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student–Newman–Keuls test for significance to compare the differences among various groups. Significance was accepted at $P < 0.05$.

3. Result

3.1. Definition of the spinal neurons

Double fluorescent labeling with MAP2 and DAPI, the spinal cord neurons was labeled with red fluoresce in the cytoplasm and blue fluoresce in the nuclear in the same time, and non-neurons was labeled with the blue fluoresce in the nuclear (Fig. 1). The result indicates most of the cells were neuron cells.

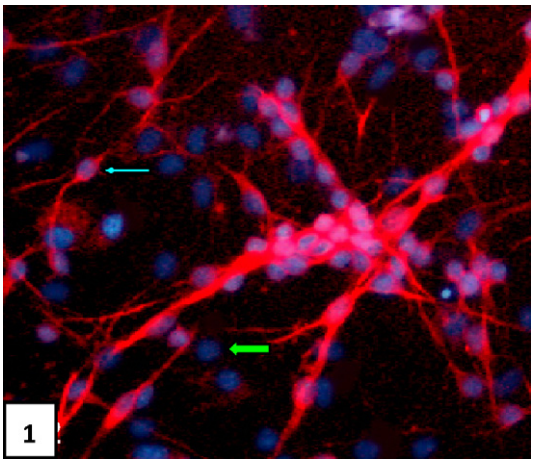

Fig. 1. Spinal cord neurons from 15-day-old embryonic Wistar rats cultured 60 h, labeled with MAP2 and DAPI. (( ) neuron cells, ( ) nucleus of non-neuron cells.)

3.2. GM1 and NGF reverse Ca^{2+} overloading caused by Glu in spinal cord neurons

The Fluo-3 AM fluorescent intensity in Glu plus GM1 and NGF incubated neurons (53.936 ± 9.945) was lower than that in Glu incubated neurons (177.416 ± 23.471) ($P < 0.001$). The fluorescent intensity in Glu plus GM1 incubated neurons (114.145 ± 18.850) was lower than that in Glu incubated neurons ($P < 0.05$). The fluorescent intensity in Glu plus NGF incubated neurons (78.108 ± 10.320) was lower than that in Glu incubated neurons ($P < 0.05$). There was no significance of the fluorescent intensity between Glu plus GM1 and NGF incubated neurons and normal control neurons (33.919 ± 9.991) ($P > 0.05$). The fluorescent intensities in Glu plus GM1 incubated neurons and Glu plus NGF incubated neurons were higher than that in normal control neurons ($P < 0.001$). The fluorescent intensity in Glu plus GM1 and NGF incubated neurons was lower than that in Glu plus GM1 incubated neurons and Glu plus NGF incubated neurons ($P < 0.05$) (Fig. 2).

3.3. Effects of GM1 and NGF on GAP43 mRNA expression in Glu incubated spinal cord neurons

The effects of GM1, NGF and GM1 plus NGF on GAP43 mRNA expression in Glu incubated spinal cord neurons were investigated by RT-PCR. As shown in Figs. 3 and 4, GM1 plus NGF promoted GAP43 mRNA expression in Glu incubated spinal neurons more obviously than that GM1 or NGF treated alone.

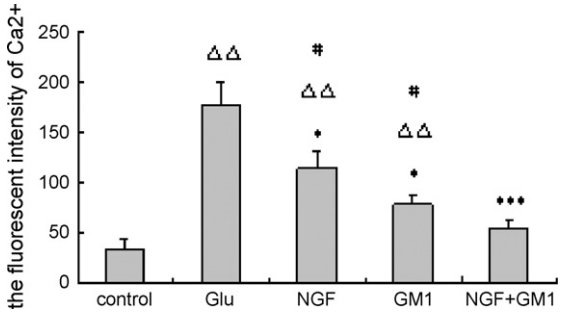

Fig. 2. The fluorescent intensity of Ca^{2+} in different groups as marked above. $^{*}P < 0.05$ vs Glu treated group. $^{***}P < 0.001$ vs Glu treated group. $\triangle\triangle P < 0.01$ vs control. $^{\#}P < 0.05$ vs Glu plus GM1 and NGF treated cells.