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Original Articles

Enhancement of Neurotrophic Factors in Astrocyte for Neuroprotective Effects in Brain Disorders Using Low-intensity Pulsed Ultrasound Stimulation

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ABSTRACT

Background: Astrocytes play an important role in the growth and survival of developing neurons by secreting neurotrophic factors.
Objective: The goal of this study was to investigate how low-intensity pulsed ultrasound (LIPUS) stimulation directly affects brain astrocyte function.
Methods: Here, we report that LIPUS stimulation increased protein levels of BDNF, GDNF, VEGF, and GLUT1 in rat brain astrocytes as measured by western blot analysis. Histological outcomes including demyelination and apoptosis were examined in rats after administration of aluminum chloride ($AlCl_3$).
Results: At the mechanistic level, integrin inhibitor (RGD peptide) attenuated the LIPUS-induced neurotrophic factor expression. The data suggest that neurotrophic factor protein levels may be promoted by LIPUS through activation of integrin receptor signaling. In addition, LIPUS stimulation protected cells against aluminum toxicity as demonstrated by an increase in the median lethal dose for $AlCl_3$ from 3.77 to 6.25 mM. In *in vivo* histological evaluations, LIPUS significantly reduced cerebral damages in terms of myelin loss and apoptosis induced by $AlCl_3$.
Conclusions: The results of this study demonstrate that transcranial LIPUS is capable of enhancing the protein levels of neurotrophic factors, which could have neuroprotective effects against neurodegenerative diseases.

Introduction

Ultrasound (US) can be transmitted into a target tissue and produce physiological change through thermal or nonthermal effects [1–3]. Low-intensity pulsed US (LIPUS) has been known to accelerate bone and tissue regeneration following injury [4,5]. In past studies, kidneys, monocytes, and osteoblasts treated with focused US (FUS) have shown significant increases in vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which are known to promote hippocampal neurogenesis [6,7]. FUS combined with microbubbles has been shown to stimulate hippocampal neurogenesis in mice [8]. Previous studies have also indicated that LIPUS has positive effects on axonal regeneration in damaged nerves [9,10]. Transcranial pulsed US is capable of stimulating intact brain circuitry and promoting levels of brain-derived neurotrophic factor (BDNF), an important regulator of long-term memory [11]. These findings suggest the potential for broad applications in neuroscience, including the enhancement of neurotrophic factor levels via LIPUS, which could have beneficial effects against degenerative brain diseases.

In recent years, neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), have presented some of the greatest public health challenges to the world's aging populations. Various studies have shown, however, that BDNF has great potential for the treatment of AD [12,13]. Meanwhile, glial

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cell line-derived neurotrophic factor (GDNF), another neurotrophic factor, has been identified as the most suitable candidate for the treatment of PD [14,15]. Furthermore, a growing body of evidence suggests that FUS-induced blood–brain barrier (BBB) disruption may be a useful tool for delivering such neurotrophic factors directly into the central nervous system [16,17], and increased levels of BDNF and GDNF may lead to neuronal regeneration and a strong trophic effect on the dopaminergic system, respectively. On the other hand, exogenous BDNF and GDNF could also have possible side effects such as a pro-epileptic effect and cerebellar damage, respectively [18,19]. Moreover, AD may be aggravated by a breakdown of the BBB in some patients.

Microvascular length is reduced in neurodegenerative diseases (such as AD, for example) [20,21], and the transport of energy substrates across the BBB and the clearance of potential neurotoxins from the brain would be decreased due to these vascular reductions. Recent FDG (18-fludeoxyglucose)-PET (positron emission tomography) imaging studies have demonstrated that individuals with mild cognitive impairment have significantly reduced glucose utilization prior to neurodegeneration [22,23]. In addition, the protein expression of glucose transporter 1 (GLUT1) in brain capillaries is decreased in AD [20,24]. These findings suggest that a continuous shortage in metabolic activity at the BBB occurs due to GLUT1 deficiency.

The purpose of this study was to investigate how LIPUS directly affects brain astrocyte function. The results indicate that LIPUS stimulation increased the protein expression of neurotrophic factors and GLUT1 in cultured astrocytes. Furthermore, our results revealed that LIPUS has protective effects against aluminum-mediated brain damage in an animal model. This mechanism suggests a new therapeutic strategy for the treatment of neurodegenerative diseases.

Materials and methods

Astrocyte cell cultures

A supply of rat brain astrocyte cells (RBACs) (CTX TNA2) was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The cells were grown on a six-well plate (each well diameter = 34.8 mm; Corning, NY, USA) in 95% air-5% CO_2 with Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA), which was supplemented with 10% fetal bovine serum (FBS; Biological industries, Kibbutz Beit Haemek, Israel), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco, New York, USA) (pH adjusted to 7.6). Two different cell densities were prepared for subsequent experiments: a cell density of 1×10^5 cells/well for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, and a cell density of 1×10^6 cells/well for western blotting analysis.

Animal preparation

All procedures were performed according to the guidelines and approved by our Animal Care and Use Committee. Male Sprague–Dawley (SD) rats weighing from 280 to 300 g were used in this study. Before LIPUS stimulation, each animal was anesthetized in the prone position by inhalation of 2% isoflurane in 2 L/min oxygen, and the body temperature was maintained at 37 °C using a heating pad. The rat heads were mounted on a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA), and the top of the cranium was shaved for LIPUS stimulation. In one experimental protocol, normal rats were used to evaluate the protein expression of neurotrophic factors 4 h after LIPUS stimulation. In another experimental protocol, the effects of LIPUS on rats treated daily with aluminum chloride ($AlCl_3$, 100 mg/kg; oral administration) for 42 days were assessed via biodistribution of ^{11}C-PIB and TUNEL staining. Twenty-four SD rats were randomized into four groups, each with 6 animals: a control group, a LIPUS group, an $AlCl_3$ group, and an LIPUS + $AlCl_3$ group. The control group animals were treated with vehicle. Animals in LIPUS group were treated with LIPUS daily for 49 days. In the $AlCl_3$ group, the animals received $AlCl_3$ (100 mg/kg; oral administration) daily for 42 days. The effects of LIPUS on the rats treated with $AlCl_3$ (100 mg/kg; oral administration) daily for 42 days were assessed in the LIPUS + $AlCl_3$ group. LIPUS was applied daily for 49 days from 7 days before $AlCl_3$ administration began and lasted until the animals were sacrificed on day 42 of the $AlCl_3$ administration.

Pulsed ultrasound apparatus

In *in vitro* experiments, the LIPUS was generated by a 1-MHz plane piezoelectric transducer (A394S-SU, element diameter = 29 mm; Panametrics, Waltham, MA, USA) with 50 ms burst lengths at a 50% duty cycle and a repetition frequency of 10 Hz. In *in vivo* experiments, the LIPUS was generated by a 1-MHz focused piezoelectric transducer (A392S; Panametrics, Waltham, MA, USA) with 50 ms burst lengths at a 5% duty cycle and a repetition frequency of 1 Hz. The focused transducer was mounted on a removable cone filled with deionized and degassed water, the tip of which was capped by a polyurethane membrane, with the center of the focal zone placed about 5.7 mm away from the cone tip. The focused transducer was positioned using the stereotaxic apparatus in order to direct the acoustic beam to the desired region (3.5 mm posterior and 2.5 mm lateral to the bregma) of the brain. A function generator (33220A, Agilent Inc., Palo Alto, USA) was connected to a power amplifier (500-009, Advanced Surgical Systems, Tucson, AZ) to create the US excitation signal. A power meter/sensor module (Bird 4421, Ohio, USA) was used to measure the input electrical power. The spatial-peak temporal-average intensities (I_{SPTA}) over the plane and focused transducer head were 110 mW/cm^2 and 528 mW/cm^2, respectively, and were measured with a radiation force balance (RFB, Precision Acoustics, Dorset, UK) in degassed water. In the *in vitro* experiments, LIPUS was transmitted from the plane transducer to the bottom of the cell culture plate. In the *in vivo* experiments, LIPUS was transmitted from the top of the rat brain. US transmission gel (Pharmaceutical Innovations, Newark, NJ, USA) was used to cover the area between the transducer and the plate or the brain in order to maximize the transmission of the ultrasound. Astrocyte cells and each rat hemisphere were treated with multiple LIPUS stimulations by triple sonications. The duration of each sonicaton was 5 min and there was an interval of 5 min between the two sonications. Thus, the total sonication time of LIPUS stimulation was 15 min.

Cell growth and cell viability measurements

LIPUS treatment was started 15 h after the initiation of each cell culture. Cell growth was assessed by MTT assay after LIPUS stimulation. This method is based on MTT progress to form a corresponding formazan product. After incubation of the cells with 200 μl of 5 mg/ml MTT for 4 h at 37 °C under 95% air-5% CO_2, the cells were then dissolved in 1 ml of DMSO and the absorption was quantified by measuring at 570 nm using a spectrophotometer.

$AlCl_3$ (Acros Organics, New Jersey, USA) was dissolved in phosphate buffered saline (PBS) and was made freshly at the beginning of each experiment. The amount of Al was measured from the standard curve prepared with Al standard solution. Various doses (0, 2, 4, 6, and 8 mM) of $AlCl_3$ were added to RBACs 4 h after LIPUS stimulation, and then cell viability was assessed by MTT assay 24 h after the $AlCl_3$ treatment.