LIPUS Enhance Elongation of Neurites in Rat Cortical Neurons through Inhibition of GSK-3β[1]

CONG REN, JIA-MOU LI*, AND XIN LIN*

Department of Orthopedics, Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China

Objective Low-intensity pulsed ultrasound (LIPUS) has been reported to enhance proliferation and to alter protein production in various kinds of cells. In the present study, we measured the neurites length after LIPUS treatment to define the effectiveness of LIPUS stimulation on neurons, and then we examined the acticity of GSK-3β to study the intracellular mechanism of neurite's outgrowth. **Methods** LIPUS was applied to cultured primary rat cortical neurons for 5 minutes every day with spatial- and temporal average intensities (SATA) of 10 mW/cm^2, a pulse width of 200 microseconds, a repetition rate of 1.5 KHz, and an operation frequency of 1 MHz. Neurons were photographed on the third day after LIPUS treatment and harvested at third, seventh, and tenth days for immunoblot and semi-quantitative RT-PCR analysis. **Results** Morphology change showed that neurite extension was enhanced by LIPUS. There was also a remarkable decrease of proteins, including p-Akt, p-GSK-3β, and p-CRMP-2, observed on the seventh and tenth days, and of GSK-3β mRNA expression, observed on the seventh day, in neurons treated with LIPUS. **Conclusion** LIPUS can enhance elongation of neurites and it is possible through the decreased expression of GSK-3β.

Key words: LIPUS; Rat cortical neurons; GSK-3β

INTRODUCTION

In recent years, data on the therapeutic effects of ultrasounds have been accumulating. So far, it has been reported that LIPUS enhances cell proliferation and alters protein production in various kinds of cells such as endothelial cells, osteoblasts, chondrocytes, and fibroblasts[1-3], but there is little information on the response of neurons to LIPUS irradiation. Some studies have indicated that LIPUS has positive effects on axonal regeneration during *in vivo* peripheral nerve injury trials[4-5] and that its stimuli on the injured sciatic nerve can increase the number of nerve fibers compared to that of untreated injured nerves in rats[6]. Thus, treatment with LIPUS is likely to be advantageous to a curative effect on the regeneration of neuronal axons. However, the mechanism of such events is unknown.

There are two main types of ultrasound effects: thermal and nonthermal. Both types are thought to first "injure" the cells, resulting in their growth retardation, and then to initiate a cellular recovery response characterized by an increase in protein production[7]. Compared to high-intensity continuous ultrasound, LIPUS is much lower in intensity (10 mW/cm^2, SATA) and has unique characteristics such as pulsed waves, which are regarded as nonthermogenic and nondestructive[8].

Intracellular mechanisms that enhance neurite outgrowth evidently require the reorganization of the neurite cytoskeletons including the microtubules and actin filaments[9]. Recently, a cytoskeletal-related signaling pathway: PI 3-kinase/Atk/glycogen synthase kinase (GSK-3)/collapsin response mediator protein (CRMP-2) was reported to be important for the outgrowth of neurite, with GSK-3 being a central regulator[10-11]. GSK-3 is a multifunctional serine/threonine kinase found ubiquitously in eukaryotes[10] and it plays key roles for various biological processes, such as the canonical Wnt signaling pathway, microtubule dynamics, and astrocyte migration[12-13]. GSK-3 phosphorylates at least have four types of microtubule-associated proteins (MAPs), CRMP-2[11], tau[10], adenomatous polyposis coil gene product[14-15] (APC), and MAP1B[16-17]. It modulates axial orientation during the development, differentiation, and neurite outgrowth in neurons through phosphorylation of these MAPs[10-11,18-19]. Some research have proved that the local inhibition of GSK-3 effectively enhance neurite/axon elongation[20] whereas overexpression of GSK-3 could impair neurite/axon elongation[21].

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[2]Correspondence should be addressed to Xin LIN and Jia-Mou LI. Tel: 86-010-67016611. E-mail: lis@bjmu.edu.cn

Biographical note of the first author: Cong REN, male, born in 1980, MD. Beijing Tiantan Hospital, Capital Medical University, majoring in surgery.

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During peripheral nerve regeneration, some factors, such as BDNF, NT3, and laminin, locally activate the PI3-kinase/Akt/GSK-3 pathway and inhibit GSK-3, which favors neurite elongation[20].

In the present study, we measured the length of neurites to examine whether LIPUS is effective for the elongation of the neuronal processes. Then we examined the change in the activity and the mRNA expression of GSK-3β to determine the intracellular mechanism of neurite outgrowth following irradiation by LIPUS.

MATERIALS AND METHODS

Cell Culture

Cortical neurons isolated from the brain of Wistar rats were bought from ScienCell Research Laboratories (San Diego, USA). These cortical neurons were subcultured with a density of 20 000 cells/1.6 cm^2 in poly-L-lysine coated 6-well plates (Costa, USA) for immunoblot and semi-quantitative RT-PCR analysis, and a density of 100 cells/0.32 cm^2 in poly-L-lysine coated 96-well plates (Costa, USA) for the measurement of neurite length. The cells were cultured in neuronal medium (3 mL medium per well in the 6-well plates and 0.1mL per well in the 96-well plate; ScienCell Research Laboratories, San Diego, CA, USA) in a humidified atmosphere of 5% CO_2 in air at 37 ℃. The medium was refreshed every 3 d.

Ultrasound Treatment

A LIPUS-therapeutic apparatus, Nexson-The-P41 was constructed according to instructions from Nexus Biomedical Devices (Hangzhou, China). There were two LIPUS probes in the apparatus, both of which generated LIPUS with a SATA intensity of 10 mW/cm^2, pulse width of 200 microseconds, repetition rate of 1.5 kHz, and an operation frequency of 1 MHz. The LIPUS was applied to the cultured cortical neurons after 24 h in culture through the bottom of the 6-well plates via a coupling gel (Smith & Nephew, Oklahoma, CA, USA) and was administered for 5 min every day during the span of this experiment (Fig. 1). Ultrasound signals from this generator were detected by a hydrophone system (Model OS-111; Hewlett-Packard, Japan), and the wave amplitudes of the signals passing through the tube wall were more than 90%, which resulted in more than 85% energy irradiated (data not shown). Control samples were prepared in the same manner with the exception of no LIPUS treatment.

Neurite Length Measurement Protocol

Cultured cortical neurons in 96-well plates were randomly divided into two groups: the LIPUS-treated group and the control group. After being subcultured for 24 h, the LIPUS treatment began and was administered for 5 min every day. On the third day, both the LIPUS-treated and control groups were photographed 2 h after the treatment. A Nikon Diaphot inverted microscope with a Nikon Plan 20× objective (Nikon, Tokyo, Japan) coupled to a video camera was used to obtain cell images (Carl Zeiss, Germany). Images of at least 200 neurons for each group were obtained. For each neuron, we measured its longest neurite with the software Image-Pro Plus 6.0 (Media Cybernetics, USA).

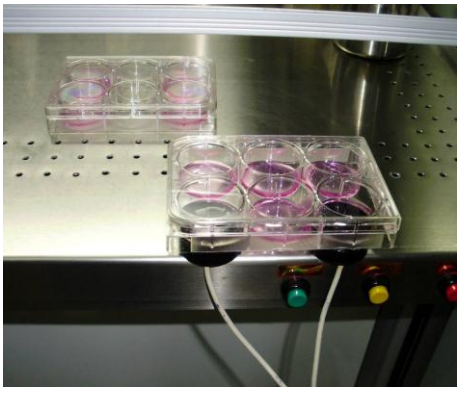

FIG. 1. Experimental apparatus for applying low- intensity pulsed ultrasound (LIPUS) which generated LIPUS with a SATA intensity of 10 mW/cm^2, pulse width of 200 microseconds, repetition rate of 1.5 KHz, and an operation frequency of 1 MHz. LIPUS irradiated neurons with two probes 24 h after in culture. A six-well plate was placed on the probes. LIPUS was transmitted to culture plate via an interpositioning ultrasound gel.

Western Blot Analysis

For Western blot analysis, the treated and untreated cultured cells were harvested at third, seventh, and tenth days. LIPUS group cells were harvested 2 h after the last LIPUS treatment. Whole cell extracts were prepared by boiling the cells in lysis buffer (2% SDS; 10% glycerol; 10 mmol/L Tris, pH 6.8; 100 mmol/L DTT) for 10 min. Proteins were separated by electrophoresis on 4%-12% Bis-Tris gels (Novex; Invitrogen, Carlsbad, CA, USA). Separated proteins were then transferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in PBS, pH 7.4, and 0.1% Tween 20 (PBS-Tween) for 1 h at room temperature. The membranes were incubated with primary antibodies diluted in 5% BSA overnight at 4 ℃. The blots were washed in PBS-Tween and then incubated with diluted secondary antibodies (HRP, 1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Reactive proteins were visualized

with SuperSignal West Pico chemiluminescence's reagent (Pierce Biotechnology, Rockford, IL, USA) followed by exposure to x-ray film.

The primary antibodies used for the Western blot analysis were as follows: rabbit anti-GSK-3β antibody (21001-1; Signalway Antibody, Pearland, TX, USA), rabbit anti-phospho GSK-3β (Ser 9) antibody (11002-1; Signalway Antibody), rabbit anti-CRMP-2 antibody (SC-30228, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-phospho CRMP-2 (Thr 514) antibody (9397, Cell Signaling Technology, Beverly, MA, USA), goat anti-Akt antibody (SC-1618; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-phospho-Akt (Ser 473) antibody (SC-101629; Santa Cruz Biotechnology, Santa Cruz, CA, USA). All of these primary antibodies were polyclonal and used at a dilution of 1:500. Mouse anti-β actin polyclonal antibody (SC-81178, 1:1000, Santa Cruz, CA, USA) was used at a dilution of 1:1 000. As secondary antibodies, HRP-conjugated goat anti-rabbit (SC-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA), donkey anti-goat (SC-2020; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-mouse (SC-2005; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:10 000.

Semi-quantitative RT-PCR Analysis

For RT-PCR analysis of GSK-3β gene expression, neurons were cultured in two 6-well plates. One of the plates was irradiated by LIPUS for 7 d (5 min/day; 10 mW/cm^2); the other was the control group without LIPUS treatment. Cultured cells were harvested 2 h after the last irradiation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the instruction manual, then resuspended in diethylpyrocarbonate (DEPC)-treated water. The extracted RNA was used to synthesize first strand cDNA with the PrimeScript™ RT-PCR Kit (Takara Biotechnology, Dalian, China) according to the kit's manual. Aliquots of synthesized cDNA were added to PCR mixtures containing sense and antisense primers (0.1 μmol/L each) for GSK-3β, dNTP mixture (0.2 mmol/L of each dNTP), 1.5 mmol/L $MgCl_2$, and rTaq DNA polymerase (1 unit) (Takara Biotechn ology, Dalian, China). The primers for GSK-3β were 5′-AGCCAGTGCAGCAGCCTTCAG C-3′ for the sense strand and 5′-TCTCCTCGGACCA GCTGCT TTG-3′ for the antisense strand. The primers for β-actin were 5′-GAGCTACGAGCTGCC TGACG-3′ for the sense strand and 5′-CCTAGAA GCATTTGC GGTGG-3′ for the antisense strand. The PCR products were electrophoretically separated in a 2% agarose gel and then visualized and photographed with an imager (Alhpa-imager™ 2200; Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical Analysis

All values are expressed as mean±SE. Differences between LIPUS group and control group were assessed with the Student's *t* test. Statistical significance was established at a value of $P<0.01$.

RESULTS

Effect of LIPUS Treatment on Neurite Outgrowth

There are no significant difference in morphology between LIPUS-treated group and control group except the length of neurites. In both LIPUS-treated group (Fig. 2a) and control group (Fig. 2b), there were many neurons with 2-7 processes; some were thick fibers, or some were thin fibers with varicosities. We measured the length of 200 neurites in each group and most neurite measured have a length between 50 μm to 80 μm. Data showed that compared with control group, neurites in LIPUS-treated group were significant longer [(73.14 ±8.32) μm vs. (68.18 ±8.96) μm, $P<0.01$](Fig. 2c).

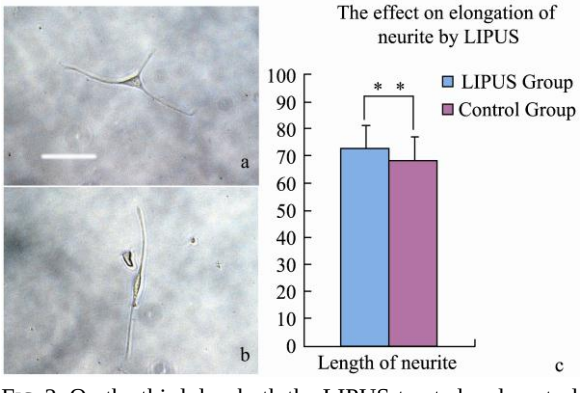

FIG. 2. On the third day, both the LIPUS-treated and control groups were photographed with a Nikon Plan 20× objective coupled to a video camera. In both LIPUS-treated group (Fig. 2a) and control group (Fig. 2b), there were many neurons with 2-7 processes; some were thick fibers, or some were thin fibers with varicosities. Bar=50 μm. Images of at least 200 neurons for each group were obtained. For each neuron, we measured its longest neurite with the software Image-Pro Plus 6.0. Most neurite measured have a length between 50 μm to 80 μm. Data showed that neurites in LIPUS-treated group were significant longer than that in control group [(73.14 ±8.32) μm *vs.* (68.18 ±8.96) μm, $P<0.01$] (Fig. 2c).

Changes in Protein Activity Related to the Cytoskeletal-signaling Pathway Caused by LIPUS Treatment

To investigate changes in protein activity related

to cytoskeletal-signaling pathway caused by LIPUS, total proteins were extracted on the third, seventh, and tenth days following daily LIPUS treatment and their acticity were examined using Western blot analysis (Fig. 3). On the third day, there was no significant difference in protein levels between the control and LIPUS groups. However, on the seventh and tenth days after irradiation by LIPUS, the levels of p-Akt, GSK-3β, p-GSK-3β, and p-CRMP-2 were decreased in the LIPUS group compared to the controls. On the tenth day, a remarkable decrease of p-Akt, p-GSK-3β, and p-CRMP-2 were observed while it appeared that GSK-3β were slightly decreased.

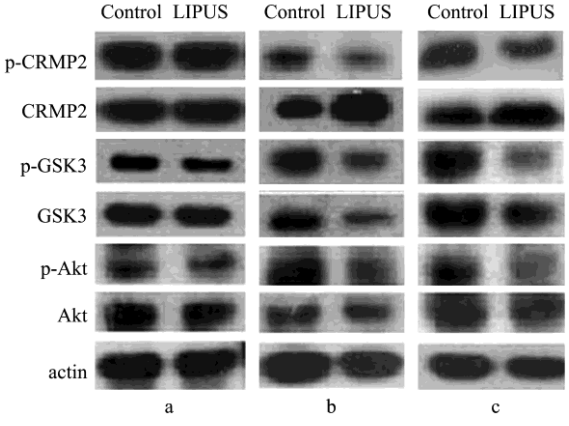

FIG. 3. Total proteins were extracted on the third, seventh, and tenth days following daily LIPUS treatment and their activity were examined using Western blot analysis. (a) On the third day, there was no significant difference in protein levels between the control and LIPUS groups. (b) On the seventh day, the levels of p-Akt, GSK-3β, p-GSK-3β, and p-CRMP-2 were decreased in the LIPUS group compared to the controls. (c) On the tenth day, a remarkable decrease of p-Akt, p-GSK-3β, and p-CRMP-2 were observed while it appeared that GSK-3β was slightly decreased. The β-actin in each lane served as an internal control.

Change in mRNA Expression of GSK-3β Caused by LIPUS

The mRNA expression of GSK-3β in the cultured neurons following LIPUS treatment was examined using a semi-quantitative RT-PCR. For this analysis, the LIPUS-treated cultured neurons on the seventh day were selected as they showed a significant decrease in their mRNA levels compared to the control. Data from analysis of the imager indicated mRNA of GSK-3β decreased about 4 folds [(1.001 ±0.017) *vs*. (0.627 ±0.037), $P<0.001$] (Fig. 4). As a result, mRNA expression of GSK-3β was also decreased on the seventh days compared to the control.

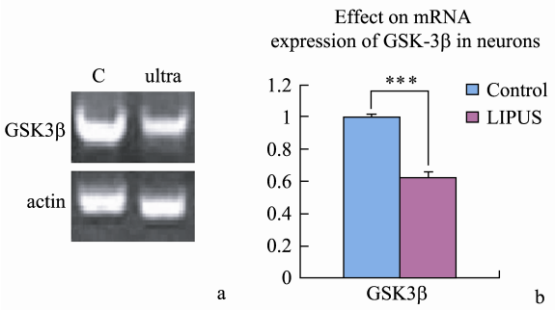

FIG. 4. Expression of GSK-3β in neurons was evaluated by semiquantitative RT-PCR. Neurons were irradiated for 7 d and harvested 2 h after the last LIPUS irradiation. The right lane represents the experimental mRNA expression, and the left lane corresponds to the control mRNA expression (Fig. 4a). Data showed that expression of GSK-3β decreased about 4 folds [(1.001 ±0.017) *vs*. (0.627 ±0.037), $P<0.001$] (Fig. 4b). The β-actin in each lane served as an internal control.

DISCUSSION

In the present study, we attempted to investigate how processes of the cultured neurons were extended under the influence of LIPUS. Morphological changes revealed that LIPUS could effectively enhance elongation of neurites after three days of treatment compared to the control group. However, we failed to measure the length of neurites on the seventh or tenth day because after the fifth day, most neurites reached another neurite and, consequently, the growth of those neurites stopped. Although the mechanism by which LIPUS affects the neuronal processes is likely to be complex, the regulation of the cytoskeleton is crucial for the proper growth cone motility[22]. To clarify the intracellular mechanism of this effect, we examined the proteins related to the cytoskeletal-related signaling pathway to determine whether the proteins in the cultured neurons were changed following the LIPUS treatment.

During nerve regeneration, GSK-3β is locally inhibited by some factors at the growth cone through the PI3-kinase/Akt/GSK-3β signaling pathway which favors neurite outgrowth[18]. The overexpression of active GSK-3β blocks neurite growth in cultured neurons[21]. In the PI3-kinase/Akt/GSK-3β/CRMP-2 pathway, active Akt inhibits GSK-3β through phosphorylation at Ser 9 and GSK-3β inhibits CRMP-2 though phosphorylation at Thr 514. If LIPUS enhances neurite elongation though this pathway, the phosphorylation of GSK-3β should be up-regulated and the activity of GSK-3β should be inhibited. However, in this research, the activity of GSK-3β was inhibited by LIPUS and the

phosphorylation of GSK-3β by Akt was inhibited, too. This conflict of results revealed that LIPUS enhances neurite outgrowth through the down-regulation of GSK-3β activity but not through the PI3-kinase/Akt/GSK-3β pathway. Therefore, we employed semi-quantitative RT-PCR to examine the mRNA of GSK-3β. The results of the semi-quantitative RT-PCR revealed that the expression of GSK-3β mRNA decreased after LIPUS irradiation on the seventh day. From these findings, we postulate that when neurons are irradiated by LIPUS, an unknown intracellular mechanism may be activated as a response to this "injury" and, consequently, neurons reduce the mRNA expression of GSK-3β. The decrease of GSK-3β activity comes from reduced expression, but not through the PI3-kinase/Akt/GSK-3β signaling pathway.

The reduced expression is a kind of global inhibition of GSK-3β that has a complex effect on neurite elongation. It favors neurite elongation at a low level of inhibition whereas it impairs neurite elongation at a high level of inhibition[21]. Strong global GSK-3β inhibition results in excessive microtubule stability all along the neurite shaft due to the inhibition of MAP1B, which eliminates dynamic microtubules, and the abnormal distribution of APC that stabilizes microtubules all along the neurite shaft. In this case, there was no pool of dynamic microtubules at the growth cone, which are necessary for growth cone advancement, and no localization of APC to microtubule plus ends[20].

In our research, significant morphological changes were found on the third day whereas significant changes in the activity of GSK-3β were found on the seventh and tenth days. We postulate that daily treatment of LIPUS would result in neurons' response accumulation. Morphological changes were observed on the third day when the inhibition of GSK-3β is not significant enough to be found. Since overly strong global inhibition of GSK-3β impairs neurite elongation, whether LIPUS could impair neurite elongation needs further study.

LIPUS enhances neurite elongation in rat cortical neurons, indicating that LIPUS could be a potential application for clinical treatment of nerve regeneration in both the central and peripheral nervous systems. The intracellular mechanism also indicates that LIPUS has the same action as the neurotrophic factors, laminin and LiCl. Compared to other pharmacological inhibitors of GSK-3β, LIPUS has some advantages: (1) LIPUS is considered to be nontoxic, thus it has a wide margin of biologic safety; (2) It directly irradiates target neurons and does not affect other tissues; and (3) The decreased expression comes from a response of neurons and is not affected by the metabolism or blood brain barrier. However, further investigation is required to identify an accurate and continuous application of LIPUS treatment to achieve constant and reproducible results prior to clinical use.

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