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Transcranial ultrasound stimulation promotes brain-derived neurotrophic factor and reduces apoptosis in a mouse model of traumatic brain injury

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ABSTRACT

Background: The protein expressions of brain-derived neurotrophic factor (BDNF) can be elevated by transcranial ultrasound stimulation in the rat brain.
Objective: The purpose of this study was to investigate the effects and underlying mechanisms of BDNF enhancement by low-intensity pulsed ultrasound (LIPUS) on traumatic brain injury (TBI).
Methods: Mice subjected to controlled cortical impact injury were treated with LIPUS in the injured region daily for a period of 4 days. Western blot analysis and immunohistochemistry were performed to assess the effects of LIPUS.
Results: The results showed that the LIPUS treatment significantly promoted the neurotrophic factors BDNF and vascular endothelial growth factor (VEGF) at day 4 after TBI. Meanwhile, LIPUS also enhanced the phosphorylation of Tropomyosin-related kinase B (TrkB), Akt, and cAMP-response element binding protein (CREB). Furthermore, treatment with LIPUS significantly decreased the level of cleaved caspase-3. The reduction of apoptotic process was inhibited by the anti-BDNF antibody.
Conclusions: In short, post-injury LIPUS treatment increased BDNF protein levels and inhibited the progression of apoptosis following TBI. The neuroprotective effects of LIPUS may be associated with enhancements of the protein levels of neurotrophic factors, at least partially via the TrkB/Akt-CREB signaling pathway.

Introduction

Traumatic brain injury (TBI) triggers a complex cascade of apoptotic events that cause delayed secondary injury processes [1]. Clinically, cleavage of caspase-1, caspase-3, and caspase-8 were observed in human brain after TBI, suggesting activation of caspase-dependent apoptosis [2,3]. It has been shown that caspase inhibitors were protective against TBI [4]. Furthermore, the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway plays a crucial role in regulating cell survival. Activation of Akt involves phosphorylation on both Thr308 and Ser473, and then *p*-Akt functions through its kinase activity. Activated Akt phosphorylates several downstream proteins to prevent apoptosis. Thus, the activation of Akt signaling may be a useful strategy for protection of the injured brain [5].

Neuroprotection is a potential approach for the treatment of brain injuries, but no therapeutic agents have been shown to be effective for TBI in clinical trials [6]. At the same time, a lack of sufficient pharmacokinetic analysis to determine the optimal doses and therapeutic windows for therapeutic agents may limit potential proof of their clinical efficacy [7]. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, which plays

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an important role in the survival of existing neurons, the differentiation of new neurons, and synaptic plasticity [8,9]. BDNF is involved in neuroprotection, neuronal repair, and functional recovery after TBI [10,11]. Glial cell line-derived neurotrophic factor (GDNF) is a small protein that has been found to improve survival, promote neuronal differentiation, and reduce apoptotic cells following TBI [12]. Vascular endothelial growth factor (VEGF) significantly augments neurogenesis and angiogenesis after TBI [13]. However, exogenous BDNF administration following TBI does not protect against behavioral or histological deficits [14]. Moreover, assuring the delivery of neurotrophic factors to the injured regions of the brain has been problematic.

The injured brain activates self-protective mechanisms to counteract cerebral damage and promote neuronal survival [15]. Tropomyosin-related kinase B (TrkB) signaling is activated by binding to BDNF, which is one of the important protective mechanisms induced by brain damage and a key regulator of neuronal survival [16]. PI3K/Akt and Erk signaling pathways are the major TrkB-mediated survival pathways that promote neuronal survival and protect against apoptosis [17]. BDNF/TrkB signaling can enhance further BDNF induction through cAMP-response element binding protein (CREB), a key transcription factor for BDNF production via PI3K/Akt or Erk signaling [18,19]. These data suggest the modulation of BDNF/TrkB signaling have a therapeutic role in brain injury.

Ultrasound (US) can induce bioeffects by acting as high intensity or low intensity energy as it propagates through tissues in pulsed or continuous waves [20,21]. It has been demonstrated that low-intensity pulsed US (LIPUS) could be a powerful neuromodulation tool [22,23]. Experimental studies indicated that LIPUS has neuroprotective effects against cerebral damages in terms of myelin loss and apoptosis induced by $AlCl_3$ through enhancement of neurotrophic factors [24,25]. Evidence suggests that elevated levels of BDNF in the brain have protective effects against TBI [26]. Meanwhile, an increase in neurotrophic factors such as VEGF can improve functional outcomes and reduce lesion volume in TBI [13]. Therefore, the goal of the present study was to investigate whether LIPUS stimulation could promote the TrkB downstream PI3K/Akt or Erk pathways and increase endogenous BDNF levels and to determine whether LIPUS is protective against apoptosis in a mouse model of TBI.

Materials and methods

Animals and surgical procedures

All procedures were approved according to guidelines stipulated by the Animal Care and Use Committee of National Yang Ming University. The TBI model was induced by controlled cortical impact (CCI) injury in mice as described previously [27]. Male C57BL/6 J mice (8 weeks old, about 22–25 g in weight) were intraperitoneally anesthetized with sodium pentobarbital (65 mg/kg; Rhone Merieux, Harlow, UK) and placed in a stereotaxic frame. A 5 mm craniotomy was performed over the right parietal cortex, centered on the coronal suture and 0.1 mm lateral to the sagittal suture, and injury to the dura was avoided. Injury was produced by a pneumatic piston with a rounded metal tip (2.5 mm in diameter) that was angled at 22.5° to the vertical so that the tip was perpendicular with the brain surface at the center of the craniotomy. A velocity of 4 m/s and a deformation depth of 2 mm below the dura were applied. The bone flap was immediately replaced and sealed, and the scalp was sutured closed. Mice were placed in a heated cage to maintain body temperature while recovering from anesthesia. Sham-operated mice received craniotomy as described before, but without CCI; the impact tip was placed lightly on the dura before sealing the wound. After the trauma or sham surgery, animals were housed under the conditions mentioned above.

Pulsed ultrasound apparatus

The pulsed ultrasound setup was similar to that used in our previous study [28]. LIPUS exposures were generated by a 1.0-MHz, single-element focused transducer (A392S, Panametrics, Waltham, MA, USA) with a diameter of 38 mm and a radius of curvature of 63.5 mm. The half-maximum of the pressure amplitude of the focal zone had a diameter and length of 3 mm and 26 mm, respectively. The transducer was applied with a duty cycle of 5% and a repetition frequency of 1 Hz. The transducer was mounted on a removable cone filled with deionized and degassed water whose tip was capped by a polyurethane membrane, and the center of the focal zone was about 2.0 mm away from the cone tip. The mice were anesthetized with isoflurane mixed with oxygen during the sonication procedure. The sonication was precisely targeted using a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The acoustic wave was delivered to the targeted region in the injured cortical areas. LIPUS was applied for a sonication time of 5 min at an acoustic power of 0.51 W (corresponding to a spatial-peak temporal-average intensity (I_{SPTA}) of 528 mW/cm^2) 5 min after TBI and subsequently daily for a period of 3 days. Mice were sacrificed for analysis at 1 or 4 days. The intensity of the LIPUS exposures was selected based on data from our previous studies [25,29].

Histological evaluation

Four days following TBI, mice were sacrificed by transcardial perfusion with phosphate-buffered saline (PBS), and then the tissues were fixed with 4% paraformaldehyde. Brains were collected and post-fixed in 4% paraformaldehyde overnight and transferred to PBS containing 30% sucrose for cryoprotection. Coronal sections were cut in a cryostat at 10 μm from the level of the olfactory bulbs to the visual cortex and used for immunohistochemistry. Double immunofluorescence was performed by simultaneous incubation of either anti-BDNF (1:100; Sanata Cruz) or anti-phospho-TrkB (1:200; Cell Signaling, MA, USA) with anti-neuronal nuclei antigen (NeuN, neuronal marker; 1:100; Millipore, Billerica, MA, USA) overnight at 4 °C. Sections were then washed, incubated with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1:400; Molecular Probes, Eugene, OR, USA) for 2 h, observed under a fluorescence microscope, and photographed.

Quantification of double staining

Double immunofluoresence labeling of neurons (NeuN) and BDNF was quantified on three consecutive sections from the injury core at the level of 0.74 mm from the bregma. The number of positive cells was counted in an area of 920 × 860 μm^2 in 8–10 non-overlapping fields immediately adjacent to the cortical contusion margin using a magnification of × 200 as previously described [30]. The total number of NeuN-positive or NeuN-BDNF-double-label cells was expressed as the mean number per field of view. Analysis was performed by two experimenters who were blinded to all animal groups. Inter-rater reliability was within 10%.

Western blotting analysis

One and 4 days after TBI, a 4-mm coronal section was taken from the injured area over the parietal cortex and then homogenized by T-Per extraction reagent supplemented with the Halt Protease Inhibitor Cocktail (Pierce Biotechnology, Inc.). Lysates were centrifuged and the supernatants were harvested, and protein