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The Biological Effects of Low Intensity Pulsed Ultrasound in Osteoblasts

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Abstract

Osteoblasts, as one of the major cells in bone tissue, play an important role in the repair of bone injury. Low-intensity pulsed ultrasound LIPUSis widely used in the treatment of bone injury due to its characteristics of non-invasive safety, concentrated energy and strong penetrability. As a new physical factor, LIPUS can not only shorten the treatment time, but also significantly improve the biomechanical properties of bone. The mechanism of LIPUS to repair bone injury includes the ability to promote proliferation and differentiation of osteoblasts, and inhibit the inflammatory responses of bone tissues. This article reviews the biological effects of LIPUS in osteoblasts.

Keywords Low intensity pulsed ultrasound; Osteoblast; Biological effects

Introduction

Osteoblasts, as the major functional cells in bone formation, are responsible for the synthesis, secretion and mineralization of bone matrix. The bone is continuously remodeling throughout the lives. The process of bone remodeling includes the change of the internal cellular components in order to maintain the structure and the suitable bearing capacity of bone tissue in different mechanical environments. The imbalance of osteoclasts and osteoblasts during bone injury is responsible for the bone resorption. Low intensity pulsed ultrasound (LIPUS) is a form of ultrasound that delivered at a much lower intensity (<3W/cm2) than traditional ultrasound energy, and it is typically used in orthopedic rehabilitation because of its bone protective effects [1]. It has been approved by the FDA as a clinical therapy to promote bone fracture healing. Especially in recent years, LIPUS has been widely used in the rehabilitation medicine of bone injuries. Experiment evidences have shown that LIPUS can promote the healing of fractures, accelerate the regeneration of soft tissues and inhibit the inflammatory responses of bone tissues [2-4]. Bone repair is a dynamic process that includes early osteogenesis, chondrogenesis, bone remodeling and ossification, in which osteoblasts play a crucial role. Here we review the biological effects of LIPUS on osteoblasts.

BMPS

Bone morphogenetic proteins (BMPs), as members of transforming growth factor-β (TGF-β) superfamily, is a kind of polypeptide growth factor involved in bone development. More than 20 different BMP isomers have been found so far, in which BMP-2, BMP-4 and BMP-7 are believed to play an important role in fracture repair process. Huang et al. found that LIPUS therapy can up-regulate the expression of BMP-2, 4 and 7 during the bone repair process of human mandible fracture [5]. BMPs can be combined with osteoprogenitors to increase the transcription of bone genes such as *RUNX2* and ultimately enhance the differentiation of osteoblasts. Suzuki et al. demonstrated that the expression of BMPs in rat osteoblast ROS17/2.8 cell line was significantly increased after treatment with LIPUS [6,7]. Experimental group was daily treated with LIPUS for twenty minutes, and the expression of BMP-2, 4, 7 cells was significantly higher than the control group after seven days culture. BMPs need to bind to specific receptors to play the role in promoting osteogenic differentiation, and this study further showed that the expression of BMP receptors in osteoblasts of the experimental group was also significantly up-regulated. In addition, the phosphorylation of *Smad1* was observed 5 minutes after LIPUS exposure which is a signal molecule in the downstream of BMP receptors, and the activation of *Smad1* can be blocked by specific inhibitors of BMPs. Another study showed that calvaria osteoblasts of neonatal mice express significantly higher BMP-2, *Smad1* and *Smad5* one day after LIPUS stimulation than before treatment [8].

IGF-1

Insulin-like growth factor-1 (IGF-1) is one of the most abundant growth factors stored in the bone. It can not only stimulate survival, proliferation, differentiation, and matrix production in cultured osteoblast cells, but also enhance alkaline phosphatase (ALP) activity, osteocalcin secretion and collagen synthesis of bone marrow stromal cells [9]. It has been reported that *IGF-1* was positively correlated with bone mineral density and high peak bone mass. The *IGF-1* gene knockout mice showed impairments of osteogenic differentiation and decline of bone formation, suggesting that the expression level of *IGF-1* is closely related to fracture risk [10]. Naruse et al. applied LIPUS to rat osteoblasts and detected that *IGF-1* was up-regulated after 20 minutes of exposure [11]. *IGF-1* mediates the expression of *Osx* gene, which is an osteoblast-specific transcription factor necessary for bone formation, performs at downstream of *Runx2* and induces osteoprogenitors to differentiate into mature osteoblasts [12]. It has been reported that during the second week of LIPUS stimulation, the expression of *Osx* in the ROS 17/2.8 cells is significantly increased [6]. Insulin receptor substrate-1 (*IRS-1*) participates in the *IGF-1* signaling pathway and is an indispensable part of bone rebuilding, growth and repair under mechanical stimulation and mediates process of osteoblast differentiation [13]. Gusmao et al. demonstrated that on the 7th day of LIPUS exposure, rat osteoblasts express higher *IRS-1* compared to the control group [14]. However, the expression of *IRS-1*

started to decrease after 7 days, indicating that LIPUS exposure is a non-cumulative effect.

PGE2/*COX-2*

Prostaglandin (PG), which induces bone formation, is an essential part of bone remodeling under mechanical stimulation. Prostaglandin E2 (PGE2), as a member of the prostaglandin family, is an important signaling molecule secreted by osteoblasts. It has been demonstrated that LIPUS influences the osteoblast proliferation and differentiation by increasing the secretion of PGE2 *in vitro* [15]. Saini et al. found that LIPUS can regulate the shear force of MLO-Y4 cells, and induce the synthesis of prostaglandin H synthase (PGHS-2) and PGE2 [16]. Cyclooxygenase-2 (*COX2*) is closely relative to inflammatory response of prostaglandin production. It is the rate-limiting enzyme in the reaction of PGE2 production and acts as a key molecule in biological effects of LIPUS. Ultrasound stimulation increases the release of PGE2 by up-regulating the expression of *COX-2* in osteoblast. Tang et al. reported that increased secretion of PGE2 and *COX-2* was found in MC3T3-E1 osteoblasts after 20 minutes of ultrasonic stimulation [17]. Nevertheless, pretreated osteoblasts with *COX-2*-specific inhibitors can significantly reduce the PGE2 production. Further studies confirmed that ultrasonic stimulation increased the expression of integrins on the membrane of osteoblasts and activated FAK, PI3K, Akt, ERK and NF-kB signal pathways, leading to the increase of *COX-2* expression, which in turn affected the mineralization of osteoblasts.

NO/iNOS

Nitric oxide (NO) is a highly active free radical associated with many biological processes. NOS enzyme catalyzes substrate arginine to produce NO. Inducible nitric oxide synthase (iNOS), another important enzyme in bone metabolism, is activated by stimulation on cells and catalyzes the production of nitric oxide. NO and iNOS was found to be important contributors to bone formation in response to mechanical loading. It has been reported that LIPUS exposure for 20 minutes significantly increased the NO secretion in MLO-Y4 osteoblast-like cells [15,18]. However, the up-regulation of NO caused by ultrasonic stimulation can be counteracted by iNOS inhibitor, confirming that the increase of NO is regulated by iNOS. iNOS expression in osteoblasts stimulated by LIPUS also involves some signaling pathways, for example, Hou et al. demostrated that ultrasound enhanced the binding of p65 and p50 to NF-kB site by activation of Ras/Raf-1/MEK/Erk signaling pathway in cultured mouse preosteoblasts, resulting in the transactivation of iNOS expression and NO production, thus enhances the osteogenic effect of osteoblasts [19].

Calcium channel

Calcium is an indispensable ion of various cellular physiological activities, playing an important role in maintaining normal nerve signal conduction and the bioelectrical potential on cell membrane. The opening of calcium channels is one of the earliest reactions of cells to mechanical loading. Extracellular calcium mobilizes into cell via the ion channels on cell membrane, where further triggering the release of intracellular calcium store. Zhang et al. showed that the intracellular free calcium concentration increased after the application of pulsed ultrasound to murine preosteoblastic MC3T3-E1 cells, moreover, the change can be blocked by intracellular calcium chelators [20]. Intracellular calcium concentration is closely related to osteoblast mineralization. There are experiment evidences suggesting that intracellular calcium concentration and mineralization of the experimental rat MC3T3-E1 osteoblasts group are significantly increased after eight days of continuous LIPUS stimulation [21]. The up-regulation of calcium in osteoblasts is also involved in the skeleton recombination, cell migration and focal adhesion in response to mechanical loading. LIPUS-type mechanical stimulation regulates calcium channel signaling in bone marrow stromal cells through the *RhoA* / *ROCK* pathway, which is considered to be the main regulatory pathway of cytoskeleton [22]. The cytoskeleton bears the force applied to the cell, so it also represents a kind of mechanical sensitive structures, and the recombination of the cytoskeleton is the direct response of the cell to the external mechanical exposure.

Integrins

Integrins is a family of transmembrane cell adhesion molecules consisting of an alpha and a beta chain. Integrins accumulate on the plasma membrane to form focal adhesions and directly bind to integrin subunit molecules as well as to intracellular protein of paxillin, ultimately integrins and cytoskeleton can organically linked together. Integrins can activate transduction of extracellular signal to intracellular such as mechanical stimulation, which could affect the cell growth, differentiation, adhesion, migration and proliferation, etc. Integrin is a key player in the transduction of ultrasound signals. It has been shown that integrin α5β protein expression remarkable rised in the mandibular and calvarial osteoblasts after stimulated by LIPUS [23]. LIPUS-type mechanical stimulation activates integrin-related signaling pathway, Kusuyama et al. reported that LIPUS promotes the differentiation of BMSCs into osteoblasts through the integrins/ROCK-Cot/Tpl2-MEK-ERK signaling pathway [24]. The integrins/ERK1/2 pathway is another important signaling pathway in mechanotransduction through which LIPUS regulates the activity of transcription factors and affects cellular processes including cell proliferation, migration and extensive gene regulation [25].

Integrins are involved in signal transduction between inside and outside of cells. In addition, direct communication between cells also plays a crucial role in mechanotransduction. Gap junctions, which regulate the exchange of intercellular ion and small molecules, allowing metabolic cooperation between adjacent cells and control cell differentiation and growth, are considered as important pathways for intercellular communication. In bone system, gap junctions are the central signal transduction of biophysical stimulation. Sena et al. showed that gap junctions and cell to cell communication were improved between rat bone marrow mesenchymal stem cells after application of LIPUS, and inhibition of gap junctions attenuated phosphorylation of Erk1/2, p38 and activity of alkaline phosphatase (ALP) induced by LIPUS exposure, which could affect the differentiation of bone marrow mesenchymal stem cells into osteoblasts [26].

Discussion and Conclusion

The biological response produced by LIPUS is very complex. Different biological effects can be caused directly or indirectly by sound waves. The response of various types of cells to LIPUS stimulation involves several signaling pathways or their synergistic effects. Researchers have studied LIPUS from cells to small animal models, and subsequently to large and functional animal models. However, the difference of physiological conditions *in vitro* and *in vivo* significantly affects the biological effects of LIPUS. In *in vitro* model, cell proliferation, differentiation and matrix production can be obtained under optimal conditions by artificial control. But *in vivo*,

bone repair involves cells from different sources (such as bone cells, stem cells, immune cells and macrophages) and needs to coordinate the synthesis of bone tissue under adverse conditions (such as nutrient reduction, accumulation of excrement and destruction of tissue structure). Therefore, the biological effects of LIPUS in *in vivo* model may be completely different from that in the optimal conditions *in vitro*. Bone repair is a dynamic process involving inflammation, ossification, bone formation and bone remodeling. Osteoblasts, bone-forming cells, are active during all stages of the repair process. Many studies have proved that LIPUS can significantly promote ossification of osteoblasts. However, the mechanism of LIPUS includes not only the cytokines, mechanotransduction, extracellular environment, but also thermal effects, angiogenesis, immune responses and synergistic effects between several signaling pathways. Based on the complex biological effects, further studies are needed to distinguish roles of LIPUS on osteoblasts at different stages of bone repair. It is believed that the biophysical mechanism of LIPUS stimulation to osteoblasts will be elucidated with further researches, which will provide new theoretical basis and clinical ideas for the treatment of bone repair.

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