Role of Piezo Channels in Ultrasound-stimulated Dental Stem Cells

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Abstract

Introduction: Piezo1 and Piezo2 are mechanosensitive membrane ion channels. We hypothesized that Piezo proteins may play a role in transducing ultrasound-associated mechanical signals and activate downstream mitogen-activated protein kinase (MAPK) signaling processes in dental cells. In this study, the expression and role of Piezo channels were investigated in dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) after treatment with low-intensity pulsed ultrasound (LIPUS). **Methods:** Cell proliferation was evaluated by bromodeoxyuridine incorporation. Western blots were used to analyze the proliferating cell nuclear antigen as well as the transcription factors c-fos and c-jun. Enzyme-linked immunosorbent assay and Western blotting were used to determine the activation of MAPK after LIPUS treatment. Ruthenium red (RR), a Piezo ion channel blocker, was applied to determine the functional role of Piezo proteins in LIPUS-stimulated cell proliferation and MAPK signaling. **Results:** Western blotting showed the presence of Piezo1 and Piezo2 in both dental cell types. LIPUS treatment significantly increased the level of the Piezo proteins in DPSCs after 24 hours; however, no significant effects were observed in PDLSCs. Treatment with RR significantly inhibited LIPUS-stimulated DPSC proliferation but not PDLSC proliferation. Extracellular signal-related kinase (ERK) 1/2 MAPK was consistently activated in DPSCs over a 24-hour time period after LIPUS exposure, whereas phosphorylated c-Jun N-terminal kinase and p38 mitogen-activated protein kinase MAPK were mainly increased in PDLSCs. RR affected MAPK signaling in both dental cell types with its most prominent effects on ERK1/2/MAPK phosphorylation levels; the significant inhibition of LIPUS-induced stimulation of ERK1/2 activation in DPSCs by RR suggests that stimulation of DPSC proliferation by LIPUS involves Piezo-mediated regulation of ERK1/2 MAPK signaling. **Conclusions:** This study for the first time supports the role of Piezo ion channels in transducing the LIPUS response in dental stem cells. *(J Endod 2017;■:1–7)*

Key Words

Dental pulp cells, mitogen-activated protein kinase, periodontal cells, Piezo1, Piezo2, ultrasound

Significance

This article explores the mechanisms underlying the effects of therapeutic ultrasound on dental stem cells, which will support the further development and application of a novel, noninvasive biophysical therapy using low-intensity ultrasound therapy in dental repair and endodontics.

Following the recognition of low-intensity pulsed ultrasound (LIPUS) as a stimulant to promote bone fracture healing (1, 2), LIPUS is now also increasingly regarded as a potential adjuvant therapy in stem cell–based tissue engineering (3–6). Studies have shown LIPUS enhanced viability, proliferation, and multilineage differentiation in a variety of postnatal mesenchymal stem cell populations, and the efficacy may be modulated by signaling pathways such as extracellular signal-related kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (p38) mitogen-activated protein kinase (MAPK) (3, 7, 8). Our previous study highlighted that the proliferation of dental pulp stem cells (DPSCs) and periodontal ligament–derived stem cells (PDLSCs) was promoted by LIPUS via distinct MAPK signaling pathways (9). ERK1/2 played a critical role in LIPUS-induced DPSC proliferation, whereas p38 and c-Jun N-terminal kinase (JNK) were essential in LIPUS-stimulated PDLSC proliferation. However, the early cellular biomechanical responses and upstream factors necessary for MAPK activation by LIPUS are yet to be elucidated.

Mesenchymal stem cells, including bone marrow mesenchymal stem cells, DPSCs, and PDLSCs, are mechanosensitive and play an important role in tissue homeostasis and repair. It is understood that LIPUS generates mechanical stresses, which can affect specific cellular mechanical transduction components, such as integrins, focal adhesion complexes, membrane receptors, ion channels, and cytoskeleton components (10). Notably, after exposure to mechanical stimuli, mechanosensitive ion channels are known to be able to induce downstream signaling processes, which eventually lead to a change in cellular behavior (11).

Piezo1 and -2 proteins are transmembrane pore-forming cation channels that have recently been identified as being mechanoresponsive in numerous eukaryotic cell types (12–15). Thus, Piezo proteins represent mechanically activated channels inducing cationic currents across the cell membrane (12, 13). Their role in cellular migration, proliferation, and elongation has implicated a functional relationship of Piezo proteins with integrin activation (16, 17). Piezo1 and -2 are encoded by *FAM38A* and *FAM38B* genes, respectively, and they exhibit different expression profiles (18). Unlike Piezo1, Piezo2 is particularly highly expressed in dorsal root

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ganglia, suggesting involvement in neural system responses (16). Based on previous data, we hypothesized that Piezo1 and -2 may be important cell membrane–located mechanotransduction components expressed on dental cells and involved in activating intracellular signaling that underpins cellular responses. Therefore, in this study, we investigated the presence of Piezo1 and -2 in DPSCs and PDLSCs and their involvement in LIPUS-associated proliferation and MAPK signaling.

Materials and Methods

Cell Culture

DPSCs were obtained from the pulp tissue of incisors and PDLSCs from the periodontal ligament of molars from 6-week-old male Wistar Hann rats (weight = 120 g; Charles River Laboratories, Aston University, Birmingham, UK) as described previously (9, 19, 20). In brief, to isolate DPSCs, pulp tissue was minced into pieces of ~1 mm^3 and digested at 37°C with 0.25% (w/v) trypsin and 1 mmol/L EDTA (Gibco, Paisley, UK) for 30 minutes. After centrifugation at 900*g* for 5 minutes, the cell pellet was resuspended and seeded in alpha-minimum essential medium (α-MEM)/20% fetal bovine serum (FBS). For PDSLC isolation, the extracted periodontal tissue was incubated with 0.25% (w/v) trypsin and 1 mmol/L EDTA (Gibco, Paisley, UK) for 45 minutes at 37°C. Cell pellets were resuspended and seeded in α-MEM/20% FBS. The established DPSC and PDLSC cultures used for the experiments at passages 3 through 5 were shown to express a range of stem cell markers and had multilineage differentiation potential (9).

LIPUS Treatment

A calibrated therapeutic ultrasound device (DuoSon, SRA Developments, UK) emitting pulsed ultrasound (63-Hz repetition rate with 3.2-millisecond pulse duration) at a frequency of 1 MHz was used. An in-house custom-built silicon antireflection chamber to house 6-well culture plates was used for ultrasound irradiation (21, 22).

One day before ultrasound treatment, 10,000 cells were seeded into each well containing α-MEM/10% FBS. Cells were washed, and 4 mL fresh supplemented culture medium was added to each culture well immediately before ultrasound exposure. The following ultrasound parameters were applied based on our previous experience (9): 1 MHz 250 mW/cm^2 for DPSCs and 1 MHz 750 mW/cm^2 for PDLSCs for 5 minutes.

In ruthenium red (RR; Sigma-Aldrich, St Louis, MO) inhibition experiments, RR was freshly diluted with double-distilled water, and 20 μmol/L RR was added to cultures 5 minutes before ultrasound treatment (eg, [13]).

Western Blotting

Cells were removed from the culture plastic in ice-cold PBS using a cell scraper (Millipore, Watford, UK). After centrifugation, the cell pellet was lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) and analyzed using the Bradford assay. Twenty micrograms of protein was electrophoresed on precast 4%–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Protein was transferred from gels to a polyvinylidene fluoride membrane using the Trans-Blot transfer system (Bio-Rad, Hercules, CA). Membranes were incubated with 5% fat-free milk before incubation with rabbit polyclonal antibodies directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10,000; Abcam, Cambridge, UK), *FAM38A*/Piezo1 (1:500, Abcam), *FAM38B*/Piezo2 (1:500, Abcam), c-fos (1:1000; Cell Signaling Technology, Danvers, MA), c-jun (1:1000, Cell Signaling Technology), proliferating cell nuclear antigen (PCNA) (1:1000, Cell Signaling Technology), ERK (1:1000, Cell Signaling Technology), phospho-ERK (1:1000 Cell Signaling Technology), p38 MAPK (1:1000 Cell Signaling Technology), phospho-p38 MAPK (1:1000, Cell Signaling Technology), JNK (1:1000, Cell Signaling Technology), and phospho-JNK (1:1000, Cell Signaling Technology) overnight at 4°C. After washing with PBS, goat antirabbit immunoglobulin G (680 RD; LI-COR Biosciences, Lincoln, NE) was applied for 90 minutes at room temperature (1:5000, LI-COR Biosciences). Visualization and quantification were performed using the LI-COR Odyssey scanner and software (LI-COR Biosciences). Quantification was undertaken using ImageJ software (National Institutes of Health, Bethesda, MD) (23). The expression of Piezo1/2, PCNA, c-fos, and c-jun is presented as the GAPDH normalized ratio calculated as the target protein (PCNA/c-fos/c-jun)/GAPDH.

Enzyme-linked Immunosorbent Assay

The following specific enzyme-linked immunosorbent assays (ELISAs) were used to quantify total and phosphorylated p38, ERK1/2, and JNK p38 MAP kinase: ERK 1/2 ELISA Kit SimpleStep (ab176660), p38 MAPK alpha ELISA Kit SimpleStep (ab176664), and JNK 1/2 ELISA Kit SimpleStep (ab176662) (all from Abcam). Whole-cell extracts were collected from cultures following manufacturer's instructions. The phosphorylated ratio of MAPK pathway proteins was calculated as phospho-MAPK/total MAPK and used as a parameter of pathway activation as previously described (9).

Cell Proliferation Analysis

Cell proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation using a commercial cytochemical assay kit (Roche Diagnostics GmbH, Mannheim, Germany) (9). Cultures were labeled using 10 μmol/L BrdU label for 1 hour, rinsed with PBS, and fixed in ethanol for 20 minutes followed by immunolabeling using BrdU antibody. Images were captured using an AxioCam phase-contrast microscope (Carl Zeiss Ltd, Cambridge, UK) and analyzed with ImageJ software (9, 23). Cell counts are presented as averages from 5 fields per time point from at least 3 independent experiments.

Statistical Analysis

All data are expressed as the mean ± standard deviation and statistically analyzed using SPSS 10.0 for Windows (SPSS Inc, Chicago, IL) using 1-way analysis of variance for comparison between the control and test groups. A value of $P < .05$ was considered statistically significant.

Results

LIPUS Increased Piezo1 and Piezo2 in DPSCs

The protein expression of Piezo1 and -2 in the dental stem cells was evaluated by Western blot analysis. Both Piezo proteins were evident in DPSCs and PDLSCs (Fig. 1*A–D*). Interestingly, LIPUS significantly increased the expression of Piezo1 and Piezo2 in DPSCs at 24 hours after treatment. A modest increase in Piezo1 and a decrease of Piezo2, both nonsignificant, were observed in PDLSCs (Fig. 1).

LIPUS-stimulated Cell Proliferation Is Inhibited by the Piezo Protein Blocker RR

LIPUS-stimulated proliferation of DPSCs and PDLSCs was evidenced by enhanced BrdU incorporation and underlined by an increased expression of PCNA (Fig. 2*A*, *B*, *D*, and *E*). Moreover, the expression of the transcription factors c-fos and c-jun was increased in both dental stem cell types after LIPUS treatment (Fig. 2*A*, *B*, *D*, and *E*). The highest levels of PCNA and c-jun were observed at 24 hours after LIPUS treatment (Fig. 2*B* and *E*), whereas c-fos reached peak levels at 4 hours after LIPUS treatment (Fig. 2*B* and *E*).

The inclusion of RR, a pharmacologic blocker of Piezo channels (13), 5 minutes before LIPUS treatment resulted in a significant