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Low intensity pulsed ultrasound accelerates macrophage phagocytosis by a pathway that requires actin polymerization, Rho, and Src/MAPKs activity

Shaoxia Zhou [a,*], Max G. Bachem [a], Thomas Seufferlein [b], Yiping Li [c], Hans J. Gross [a], Andreas Schmelz [d]

[a] Department of Clinical Chemistry, University of Ulm, 89081 Ulm, Germany
[b] Department of Internal Medicine I, University of Ulm, 89081 Ulm, Germany
[c] Department of Pathology, Preclinical school, Southeast University, 210009 Nanjing, China
[d] Department of Traumatology, University of Ulm, 89075 Ulm, Germany

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Abstract

Phagocytosis is an essential event in the complex process of tissue repair. Here we examined the effect of low intensity pulsed ultrasound (US), which promotes fracture and wound healing, on phagocytosis by mouse macrophage cell line J774A.1 and human monocyte-derived macrophages. First, 10 to 40 min low intensity pulsed US increased uptake of serum opsonized *E. coli* by J774A.1 cells during a 50 min phagocytosis period. In addition, when the *E. coli* exposure time was varied between 35 to 80 min, the maximum increase in phagocytosis was observed in the first 35 min upon US exposure. In parallel, US induced robust actin polymerization in a time dependent manner in J774A.1 cells, showing the peak effect 30 min after stimulation. Interestingly, a low concentration of cytochalasin D (0.25–0.5 μM) prevented US-induced phagocytosis of *E. coli*. Furthermore, we demonstrated US enhanced activation of RhoA. Blocking its downstream effector Rho associated kinase (ROCK) with Y27632 abrogated US-induced phagocytosis. We also show that US induced activation of ERK and p38 MAPK. Pretreatment of the cells with the corresponding inhibitors PD98059 and SB203580 reduced US-induced phagocytosis. In addition, activity of tyrosine kinase Src was required for US-induced phagocytosis. Here Src represents an upstream activator of ERK and p38 MAPK. Depolymerization of actin by cytochalasin D prevented US-induced Src, ERK, and p38 activation. Our data provide a new insight into the cellular and molecular mechanisms by which low intensity pulsed US promotes tissue repair.
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Keywords: Low intensity pulsed ultrasound; Phagocytes; Signal transduction; Actin; MAPK; Src; Rho

1. Introduction

Low intensity pulsed ultrasound (US), a form of mechanical energy that is transmitted through and into tissues as pulsed acoustic pressure waves, has been clinically used as a non-invasive supplementary therapy to promote fracture healing and wound healing [1–5]. In addition, high success rates have been shown in healing non-union fractures [6,7]. The mechanisms by which US promotes tissue repair are largely unknown.

Tissue repair is a complex procedure, classically defined by three overlapping phases − inflammation, tissue formation, and tissue remodeling. Macrophages are pivotal for initiating the first stages of healing, after which they control and direct the process until the repair is completed [8]. Macrophages play a major role in the inflammatory and debridement processes, which are largely carried out by phagocytosis and the consequent generation of reactive radicals, enzymes, and more important cytokines and growth factors. Our previous work and others' have demonstrated that low intensity pulsed US promotes cell proliferation of human skin fibroblasts [9], and matrix synthesis by chondrocytes [10,11] and osteoblasts [12]. These effects may contribute to tissue repair during US-promoted fracture healing and wound healing. To date little is known about the effect of low intensity pulsed US on phagocytosis.

* Corresponding author. Tel.: +49 731 50067535; fax: +49 731 67506.
E-mail address: shaoxia.zhou@uniklinik-ulm.de (S. Zhou).

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Macrophages display functional diversity in response to their biochemical microenvironmental conditions, such as cytokines, chemokines, and soluble immunoregulatory factors [13]. Macrophages and monocytes are also sensitive to biomechanical stimulations. The mechanical environment such as stress, strain, and pressure may also play a role in regulation of macrophage functions [14–17]. Different mechanical stimuli present differential effects on phagocytosis. Increased pressure (20 mm Hg), mimicking the edema during inflammatory tissue pressure, has been reported to stimulate phagocytosis by phorbol 12-myristate 13-acetate-differentiated THP-1 macrophages and monocyte-derived macrophages [15,16,18] through activating p38 and/or inhibiting focal adhesion kinase-ERK pathways. A recent report from the same group further demonstrated that the Akt2-mTOR-p70S6K pathway plays a central role in pressure-induced macrophage phagocytosis [18]. However, Mattana and coworkers reported that cyclic mechanical strain suppressed the uptake of aggregated IgG by mouse macrophages J774.16 cells [17]. Therefore, distinct mechanical impacts are likely to induce different effects on macrophages. We hypothesized that low intensity pulsed US, a modulated acoustic mechanical stress might increase the activity of macrophages, in particular phagocytosis, subsequently promoting tissue repair processes.

To demonstrate this hypothesis, we first examined the effect of low intensity pulsed US on phagocytosis of macrophages by using serum opsonized FITC-labelled *E. coli*. We demonstrate here that US can accelerate phagocytosis by J774A.1 mouse macrophages and human monocyte-derived macrophages. Furthermore, we investigated the possible mechanisms mediating this effect.

2. Materials and methods

2.1. Reagents

Phospho-ERK (Thr202/Tyr204) mAb (E10), p42 MAPK mAb (3A7), phospho-p38 MAPK (Thr180/Tyr182) mAb (28B10), p38 MAPK mAb (5F11), and phospho-Src (Tyr416) Ab were from Cell Signalling Technology (Beverly, MA). Rho activation assay kit and anti-Src mAb (GD11) were from Upstate Technology (Lake Placid, NY). Cytochalasin D, trypan blue, and β-tubulin mAb (2-28-33) were from Sigma (Saint Louis, MO). MEK1 inhibitor PD98059, p38 MAPK inhibitor SB203580, and Src kinase family inhibitor PP1 were from Calbiochem-Novachem (La Jolla, CA); the Rho associated kinase (ROCK) inhibitor Y27632 from Tocris Cookson (Ellisville, MO). Alexa Fluor 488 phalloidin and FITC-labelled *E. coli* (K-12) were from Molecular Probes (Eugene, OR).

2.2. Cells

Mouse macrophage cell line J774A.1 was purchased from Cell Lines Service (Eppelheim, Germany), and maintained in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

Mononuclear cells were isolated from buffy coats of healthy donors (supplied by the Red Cross Blood Bank, Ulm, Germany) by Ficoll-Hypaque Plus density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). Monocytes were differentiated into macrophages by cultured in bioFolie bags (Vivascience, Goettingen, Germany) with X-VIVO 10 medium (Cambrex, Veriers, Belgium) containing 10% FCS at 37 °C in 5% CO_2 for 7 days. Thereafter, monocytes in the bioFolie bags were harvested and resuspended in RPMI 1640 medium with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Then suspension of monocytes were seeded in 24-well plates coated with fibronectin (2 μg/ml, EMP Genetech, Ingolstadt, Germany) and incubated in RPMI 1640 free of FCS at 37 °C for 2 h. Adherent macrophages were obtained by removing nonadherent cells with two washes.

2.3. Stimulation with US

To stimulate the cultured cells with US, a modified SAFHS® apparatus (Model 2A, Exogen Inc., Piscataway, NJ) was used, which produces a 1.5 MHz ultrasound wave, 200 μs pulse modulated at 1 kHz, with an average output intensity of 30 mW/cm^2. To enable cultured cells to be treated by ultrasound, six ultrasound transducers are fitted on a plastic frame, and connected to the control panel of the signal generator via six independent cables. The operation of the transducers was checked before each experiment. The plates were placed on ultrasound transducers using a coupling gel. When the experiments were performed with 24-well plates, only 6 wells were used. The untreated plates were always put in a separate incubator for control purposes.

2.4. Assay for phagocytosis

FITC-labelled *E. coli* were used for phagocytosis. Pooled human serum was collected and stored in small aliquots at −20 °C prior to use. Serum was heated at 56 °C for 45 min to inactivate complement when required. Opsonization was performed by incubation of *E. coli* with 50% serum at 37 °C for 45 min before phagocytosis assay. J774A.1 macrophages were seeded in 24-well culture plates (1.5×10^5 cells/well) and serum-free starved overnight after 24 h. *E. coli* were added to the cells as 1:15 (cell: *E. coli*) and incubated with cells at 37 °C for the indicated times. At various time points, phagocytosis was stopped by setting the cells on ice. Free *E. coli* were removed by washing the cells with cold PBS, and the extracellular fluorescence was quenched by trypan blue (2 mg/ml) for 5 min. Cells were then washed and fixed in 4% paraformaldehyde/PBS at room temperature for 10 min. Nuclei were counter stained with Hoechst 33258 (2 μg/ml) for 10 min. In the case of human monocyte-derived macrophages, phagocytosis assay was performed 2 h after nonadherent cells were removed.

To quantify phagocytosis, fluorescent images were visualized (20× objective) and captured using an Olympus IX81 Motorized Inverted Research Microscope coupled with an F-view II camera (Olympus GmbH, Hamburg, Germany). The average intensity of FITC and DAPI per image measured with Cell R Imaging Software for Life Science Microscopy, Version 1.2 (Olympus Biosystems GmbH, Planegg, Germany) is corresponding to the number of internalized *E. coli* and macrophage nuclei, respectively. The phagocytosis was assessed according to the ratio of average FITC intensity to DAPI intensity per image; at least 6 images per condition were assessed. The data were presented as the fold increase above the control.

2.5. Rho activation assay

The activation of Rho was determined by affinity precipitation using a Rho activation assay kit. J774A.1 macrophages were lysed with Mg^{2+} Lysis/Wash Buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM sodium fluoride, 1 mM Na_3VO_4 and 1 mM PMSF. The cell lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C. Aliquots of the cell lysates (1 mg of protein in 1 ml) were mixed with 30 μl of the Rhotekin RBD-agrose slurry and the mixture was rotated for 45 min at 4 °C. The beads were washed 3 times with 1 ml of Mg^{2+} Lysis/Wash Buffer. Beads were dried with a Hamilton syringe, resuspended in 40 μl of 2× Laemmli reducing sample buffer, and the suspension was heated for 5 min at 95 °C. Proteins were separated on 12.5% SDS-PAGE and detected by Western blotting using 3 μg/ml of monoclonal anti-Rho. Loading controls were taken from each lysate sample prior to affinity precipitation.

2.6. Detection of actin with fluorescence microscopy

J774A.1 cells grown on glasscoverslips in 6-well culture plates were washed with PBS twice and fixed with 4% paraformaldehyde/PBS for 15 min. After

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