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Shockwaves Induce Osteogenic Differentiation of Human Mesenchymal Stem Cells Through ATP Release and Activation of P2X7 Receptors

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Abstract

Shockwave fractures treatment promotes bone healing of nonunion fractures. In this study, we investigated whether this effect could be due to adenosine 5'-triphosphate (ATP) release-induced differentiation of human mesenchymal stem cells (hMSCs) into osteoprogenitor cells. Cultured bone marrow-derived hMSCs were subjected to shockwave treatment and ATP release was assessed. Osteogenic differentiation and mineralization of hMSCs were evaluated by examining alkaline phosphatase activity, osteocalcin production, and calcium nodule formation. Expression of P2X7 receptors and c-fos and c-jun mRNA was determined with real-time reverse transcription polymerase chain reaction and Western blotting. P2X7-siRNA, apyrase, P2 receptor antagonists, and p38 MAPK inhibitors were used to evaluate the roles of ATP release, P2X7 receptors, and p38 MAPK sig naling in shockwave-induced osteogenic hMSCs differentiation. Shockwave treatment released significant amounts (~7 μM) of ATP from hMSCs. Shockwaves and exogenous

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Disclosure of Potential Conflicts of Interest
None of the authors has any conflicts of interest to report.

ATP induced c-fos and c-jun mRNA transcription, p38 MAPK activation, and hMSC differentiation. Removal of ATP with apyrase, targeting of P2X7 receptors with P2X7-siRNA or selective antagonists, or blockade of p38 MAPK with SB203580 prevented osteogenic differentiation of hMSCs. Our findings indicate that shockwaves release cellular ATP that activates P2X7 receptors and downstream signaling events that caused osteogenic differentiation of hMSCs. We conclude that shockwave therapy promotes bone healing through P2X7 receptor signaling, which contributes to hMSC differentiation.

Keywords

Shockwaves; Human mesenchymal stem cells; Osteogenic differentiation; ATP; p38 MAPK; c-Fos; c-Jun

Introduction

Clinical and experimental evidence indicates that shockwave therapy enhances osteogenic activity [1–4]. Despite numerous in vitro and in vivo studies to define the processes underlying this phenomenon, the precise mechanisms by which shock-waves promote bone healing and osteogenesis have remained unclear. Adenosine 5’-triphosphate (ATP) release in response to mechanical stimulation or tissue injury may play an important role in the regulation of bone formation.

In recent years, purinergic signaling through cellular release of ATP and autocrine/paracrine stimulation of P2-type purinergic ATP receptors has been found to regulate a wide range of physiological processes [5]. The P2 receptor family comprises seven structurally related P2X receptor subtypes and eight different P2Y receptor subtypes, which are G protein-coupled receptors [5–8]. P2X receptors can facilitate influx of extracellular Ca^{2+} and are therefore critical initiators of cell activation in many cell types [5]. Osteoblasts express several types of P2 receptors [9]. Activation of P2 receptors in osteoblasts by ATP has been shown to increase the intracellular Ca^{2+} concentration [10]. P2 signaling also modulates ERK 1/2 and p38 MAPK signaling pathways and c-fos expression in osteoblasts [11]. Stimulation of P2X7 receptors can result in the activation of osteoblasts [9] and the enhancement of cell functions [11]. P2X7 receptors are the most abundantly expressed subtype of the ionotropic P2X receptors, and a growing body of evidence indicates that human osteoblasts as well as several other populations of primary human bone-derived cell (hBDC) types express functional P2X7 receptors [9–11]. Based on this evidence, we speculated that shock-waves may cause ATP release and activation of P2X7 receptors and that this purinergic signaling process may bring about the beneficial effects observed with shockwave therapy by inducing osteogenic differentiation of human mesenchymal stem cells (hMSCs).

Extracellular ATP can activate p38 MAPK by triggering P2X7 receptors [12]. Activation of p38 MAPK plays an important role in modulating hMSC osteogenic differentiation and has been shown to induce c-Fos and c-Jun expression [13–15]. Clear evidence indicates that the expression of c-Fos and c-Jun increases during osteogenic differentiation of undifferentiated hMSCs [16–18]. c-Fos and c-Jun are cellular proto-oncogene products belonging to the

immediate early gene family of transcription factors. Members of the Fos family dimerize with c-Jun to form the activator protein 1 (AP-1), a transcription factor that upregulates transcription of a diverse range of genes involved in cell proliferation, differentiation, and adaptation of cells to cope with various environmental stimuli [19–21]. Based on this evidence, we wondered whether shockwave-induced ATP release may activate P2X7 receptors of hMSC and whether this process might affect osteogenic differentiation by inducing p38 MAPK and c-Fos and c-Jun signaling.

We have previously found that shockwave treatment of T cells causes mechanical stimulation that rapidly releases cellular ATP and stimulates purinergic signaling which promotes T-cell activation [22]. In this study, we could show that shockwave treatment of hMSCs causes a similar increase in extracellular ATP concentrations and the activation of p38 MAPK signaling via of P2X7 receptors. Our data indicate that this sequence of events results in c-Fos and c-Jun activation and osteogenic differentiation of hMSCs. These findings shed light on possible mechanisms by which shockwave therapy promotes bone healing in patients with nonhealing bone fractures.

Materials And Methods

Materials

Apyrase, dimethyl sulfoxide, pyridoxal phosphate 6-azophenyl 2’,4’-disulfonate (PPADS), 2’-deoxy N6-methyladenosine 3’,5’-bisphosphate (MRS 2179), and ATP were from Sigma (St. Louis, MO, http://www.sigmaaldrich.com), while 1-[N, O-bis(5-isoquinolinesulfonyl) *N*-methyl-L-tyrosyl] 4-phenylpiperazine (KN-62), suramin, and SB203580 were from BioSource International Inc. (Camarillo, CA, http://www.xxx). Dulbecco's modified Eagle's medium, heat-inactivated fetal bovine serum (FBS), and Iscove's modified Dulbecco's medium (IMDM) were from GIBCO (Invitrogen Corporation, Tulsa, OK, http://www.invitrogen.com). Oligofect-amine was from Life Technologies (Gaithersburg, MD, http:// www.lifetech.com), and a KDE-2001 Extracorporeal Shockwave Lithotripter was from Beijing Zhongke Jian An Meditech Co. (Beijing, China, http://www.xxx) A Precision Acoustics membrane hydrophone was purchased from Precision Acoustics (Dorchester, Dorset, U.K., http://www.xxx). Polystyrene round-bottom tubes were from Falcon (Becton-Dickson Co., Franklin Lakes, NJ, http://www.bd.com). Ultrasound transmission gel was purchased from Pharmaceutical Innovations (Newark, NJ, http://www.xxx), and an ATP Bioluminescence Assay Kit was obtained from Calbiochem (San Diego, CA, http://www.emdbiosciences.com). A temperature-controlled luminometer was from Luminoskan, Labsystems (Helsinki, Finland, http://www.xxx) and a PhosphoPlus p38 MAP Kinase Antibody Kit was obtained from Cell Signaling Technology Inc. (Boston, MA, http://www.cellsignal.com). Tris/glycine polyacrylamide gradient electrophoresis gels were from Novex (San Diego, CA, http://www.xxx), polyvinylidene difluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA, http://www.millipore.com), and LumiGLO chemiluminescent reagent was from Cell Signaling Technology. Each reagent was dissolved in tyrode solution and applied to the tissue culture bath superfusion.

Isolation, Culture, Characterization, and Phenotype of hMSCs

Bone marrow aspirates (50 ml) were obtained from the iliac crest of 23 different healthy volunteer donors after informed consent and approval by the institutional ethics committee of the First Hospital of Jilin University was obtained. The aspirate was diluted 1:3 in IMDM. After density-gradient centrifugation at 750 × *g* for 20 minutes, the mononuclear cell layer was obtained from the interface, washed twice, suspended, and plated in 75-cm^2 flasks (1.6 × 10^5 cells per cm^2) in IMDM supplemented with L-glutamine and Hepes (25 mM) and with gentamicin (50 μg/ml), 10% FBS, and 2% Ultroser G serum supplement (Pall Corporation, Port Washington, NY, http://www.xxx) and incubated at 37° C in a humidified atmosphere containing 95% air and 5% CO_2. After reaching confluence, adherent cells were harvested by incubating cells with 0.05% trypsin and 0.02% EDTA for 10 minutes at 37° C. Cells were washed with Hanks' balanced saline solution (HBSS) without calcium and magnesium supplemented with 10% FBS and then resuspended in the complete IMDM medium described above. The resulting cell population was referred to as primary culture (P0). This population was propagated by plating the cells at a density of 10^4 cells per cm^2 in 100-mm dishes (secondary culture; P1). This population was used for the in vitro differentiation and other experiments described below and for determination of growth kinetics. Expansion of the cells was obtained with successive cycles of trypsinization and reseeding. A total of 23 donors took part in the research study. Because of initial technical difficulties, the cells of nine of these donors were successfully passaged to a state in which they could be used for the study. Nevertheless, successfully passaged hMSC populations were no longer suitable for our studies once they had been passaged for more than five times.

For flow cytometric analyses of the morphologically homogeneous population of hMSCs (P1), the following conjugated monoclonal antibodies were used: CD34-fluorescein isothiocyanate (FITC), CD45-FITC, CD105-FITC (Ancell, Bayport, MI, http://www.xxx), and CD73-phycoerythrin (PE) (BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com/index_us.shtml). After trypsin-EDTA treatment as described above, hMSCs were washed in HBSS and 10% FBS, resuspended in flow cytometer buffer consisting of Cell WASH solution (0.1% sodium azide in phosphate-buffered saline; Becton Dickinson) containing 2% FBS. Cells were stained with conjugated monoclonal antibodies at room temperature for 15 minutes and washed and fixed in 1% paraformaldehyde. Nonspecific fluorescence and morphologic parameters of the cells were determined by incubation of cell aliquots with iso-type-matched mouse monoclonal antibodies. After incubation, cells were washed and stained with 7-amino-actinomycin in order to exclude dead cells from analysis. Flow cytometric acquisition was performed using a BD FACSort instrument (Becton Dickinson), and data were analyzed using dot-plot biparametric diagrams using Cell Quest software (Becton Dickinson).

After 4 days in culture, freshly harvested bone marrow cells were adherent (Fig. 1A, left image). After 14 days, a morphologically homogeneous population of fibroblast-like cells with >90% confluence was observed (Fig. 1A, right image). At this time, these primary cell cultures (P0) were trypsinized and passaged. After the first passage (P1), cell cultures grew exponentially and were passaged weekly. Flow cytometric analysis of these cells was used to assess the purity of the hMSC cultures. Cells positive for CD73 and CD105 were judged

to be hMSCs [23–26]. There was no detectable contamination with hematopoietic cells as judged by the absence of CD34 [27] and CD45 [28], which are markers of the hematopoietic lineage (Fig. 1B). These data indicated a morphologically homogeneous population of hMSCs.

Shockwave Treatment of Cell Cultures

A KDE-2001 Extracorporeal Shockwave Lithotripter was used for all studies [22]. The instrument settings and detailed procedures have been described previously [22]. Briefly, shockwaves were generated by underwater spark discharge from an electrode located at the focus of a hemi-ellipsoid immersed in de-gassed water jacketed by a UV-resistant outer membrane [22]. The propagation waves were focused toward the center of the test tubes containing target cells. Low-density shockwaves (LDSWs) at a frequency of 50 Hz were generated at a generator voltage of 8.5 kV and a capacitance of 0.3 μF. The positive pressure of the shockwaves generated was determined to be 30 ± 1.9 MPa [22]. Cells (1×10^6 cells per ml) were suspended in a 2-ml polystyrene round-bottom tube containing 0.5 ml IMDM pH 7.4. The cells were exposed to 0, 50, 100, or 150 LDSW impulses at an energy flux density of 0.18 mJ/mm^2 as indicated. The duration of the LDSW treatment was 10–20 minutes depending on the number of impulses applied. An ultrasound transmission gel was used as contact medium between the apparatus and the target test tube. The apparatus was covered with a UV-resistant membrane in order to avoid UV light exposure of the cells. After the shock-wave treatment, cell numbers and viability were determined using a hemocytometer and trypan blue exclusion assay.

Cell Viability Assay

Cell viability was assayed using trypan blue dye exclusion. After hMSCs (10^6 per ml) were treated as described in the different experiments below, the cells were cultured in 1 ml IMDM in 12-well plates. Cell viability was determined 5 minutes after plating. Briefly, aliquots of 20 μl of these cell suspensions were mixed with 20 μl of a 0.4% trypan blue solution (GIBCO, Invitrogen) and incubated for 3 minutes at room temperature. Then a drop of the trypan blue/cell mixture was applied to a hemocytometer (Haimen Tianlong Scientific Instruments, Jiangsu, China, http://www.xxx). The hemocytometer was placed onto the stage of a binocular microscope, and unstained (viable) and stained (nonviable) cells were separately counted and percentages of viable cells were calculated.

Preparation of Cell Membrane and Cytosolic Protein Samples

After treatment of hMSCs (10^6 cells per ml) with shockwaves (0.18 mJ/mm^2 for 100 impulses) or extracellular ATP (1 μM ATP for 5 minutes), cells were washed with ice-cold Dulbecco's phosphate-buffered saline (KD Medical, Columbia, MD, http://www.xxx) and resus-pended in 200 μl of 20 mM Tris-HCl (pH 7.4) in Eppendorf tubes containing protease inhibitor mixture (Sigma). The cell suspensions were sonicated three times with a pulse of 6 seconds each time, and the mixtures were transferred into Beckman ultracentrifuge tubes and centrifuged at 200,000×*g* for 1 hour with a Beck-man ultracentrifuge to separate cytosolic (supernatants) and membrane fractions (pellets). After ultracentrifugation, the pellets were resuspended in 200 μl of 20 mM Tris-HCl (pH 7.4) containing protease

inhibitor mixture (Calbiochem) and 1% Triton X-100. The pellet suspensions were placed on ice for 1 hour and, 100 μl of lysis buffer containing 1% Triton X-100 was added to each sample. The mixtures were sonicated three times with a pulse of 6 seconds each time; the sonicated mixtures were subjected to ultra-centrifugation (200,000 *g* for 1 hour) again to obtain supernatants containing Triton X-100-soluble membrane protein fractions.

Western Blotting

The phosphorylation of p38 MAP kinase of hMSCs was measured with the PhosphoPlus p38 MAP kinase antibody kit (Cell Signaling Technology). Briefly, hMSCs (10^6 cells per ml) were subjected to shockwave treatment at 0.18 mJ/mm^2 for 0, 50, 100, 150, 200, or 250 impulses and cultured in a 12-well plate with 1 ml per well of IMDM medium containing 10% FBS for 45 minutes. Then cells were placed on ice, centrifuged, resuspended in 100 μl of ice-cold SDS sample buffer containing 100 mM dithiothreitol and lysed by boiling for 5 minutes. The cell lysates were separated on 8%–16% Tris-glycine polyacrylamide gradient gels (Novex). Lysed proteins were transferred onto PVDF membranes (Immobilon-P; Millipore), and these membranes were subjected to immunoblotting with phospho-specific antibodies that recognized the phosphorylated (on Thr180/Tyr182) form of p38 MAPK, or to immunoblotting with antibodies recognizing both the active and inactive forms of p38 MAPK. Activated p38 MAPK or total p38 MAPK on PVDF membranes were detected with X-ray film using LumiGLO chemiluminescence reagent (Cell Signaling Technology) according to the protocol provided by the manufacturer. Band intensities on the X-ray film were analyzed with commercially available graphic software (Image Tool 3.00) purchased from The University of Texas Health Science Center in San Antonio, and ratios between activated and total p38 MAPK were used to calculate p38 MAPK activation.

The membrane and cytosolic protein preparations prepared as described above were separated on 8%–16% Tris-glycine polyacrylamide gradient gels (Novex). The separated proteins were transferred onto PVDF membranes (Immobilon-P; Millipore), and the membranes were incubated with mouse anti-β-actin antibodies (Biomig, San Diego, CA, http://www.xxx) or rabbit anti-P2X7 receptor antibodies (Alomone Labs). The membranes were washed, incubated with horseradish peroxidase-conjugated goat secondary antibody (Bio-Rad), and protein quantities were detected with LumiGLO chemiluminescence reagent. Band intensities on X-ray film were analyzed with imaging software (Image Tool 3.00), and ratios between control and shockwave-treated or ATP-treated samples were used to estimate increases in protein expression.

ATP Release Assay

ATP concentrations in the culture supernatant of hMSCs were determined with a commercially available ATP bioluminescence assay kit from Calbiochem as previously described [22]. Briefly, cells were adjusted to a density of 5×10^6 per ml in IMDM without fetal calf serum and allowed to rest for 3 hours at 37° C. Then the cells were exposed to LDSWs in the presence of 100 μM suramin, which blocks hydrolysis of extracellular ATP at this concentration [12, 22]. Cells were placed on ice, centrifuged at 12,000 × *g* for 5 minutes, and supernatants (50 μl per well) were transferred to a 96-well plate. Luciferase reagent dissolved in IMDM was then added at a volume of 50 μl per well, and the 96-well

plate was placed in a temperature-controlled luminometer to determine ATP concentrations based on luminescence signals that were compared to results with standard solutions of known ATP concentration. Results shown are representative of three different experiments and expressed as mean ±SD.

P2X7 Receptor Silencing

hMSCs were transfected with small interfering RNA (siRNA) to silence P2X7 receptor expression. A P2X7 receptor-specific siRNA construct and a nontargeting control siRNA construct were obtained from Dharmacon (Lafayette, CO, http://www.xxx). The siRNA constructs were introduced using Oligofectamine Reagent (Life Technologies) per manufacturer's protocol. P2X7 receptor-specific siRNA (P2X7R-siRNA) and nontargeting control siRNA was used at final concentrations of 140 nM. Briefly, Oligofectamine was mixed gently, diluted in serum-free medium, and incubated for 10 minutes at room temperature. P2X7R-siRNA or control siRNA was mixed with diluted Oligofectamine reagent. The mix ture was incubated for 20 minutes at room temperature. While complexes formed, the growth medium was removed from the cells and the cells were washed once with serum-free medium. Following this, cells were incubated with siRNA in serum-free medium and collected after 24, 48, or 72 hours for real-time reverse transcription polymerase chain reaction (RT-PCR) or immunocytochemistry to verify effective downregulation of P2X7 receptor expression (Supporting Information Fig. 1) and to determine shockwave-induced p38 MAPK phosphorylation or osteogenic differentiation as described below.

P2X7 Receptor Expression and C-Fos and C-Jun mRNA Transcription

We assayed mRNA transcript levels of P2X7 receptors, c-fos and c-jun in hMSCs before and 45 minutes or 1 hour after exposure to shockwaves or extracellular ATP using real-time RT-PCR (ABI Prism7300, Applera Corporation, Applied Biosystems, Norwalk, CT, http://www.appliedbiosystems.com) as indicated. The comparative Ct method for relative quantification of gene expression (ΔΔCt method) was used, and gene expression levels were normalized to gene expression of β-actin (as a housekeeping gene control) using the following equation: P2X7 mRNA normalized expression = $2^{[Ct(\beta\text{-actin mRNA}) - Ct(\text{P2X7 receptor})]}$; c-fos mRNA normalized expression = $2^{[Ct\beta\text{-actin mRNA}) - Ct(\text{c-fos mRNA})]}$; c-jun mRNA normalized expression = $2^{[Ct(\beta\text{-actin mRNA}) - Ct(\text{c-jun mRNA})]}$. P2X7 mRNA, c-fos mRNA, and c-jun mRNA were assessed using the primer sets (Supporting Information Table 1).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes on ice, blocked with 1% bovine serum albumin (BSA) in PBS, and stained with rabbit anti-P2X7 receptor antibody overnight at 4° C (1:100 in blocking buffer; Alomone Labs). Washed cells were then incubated with goat anti-rabbit Alexa 555 antibody (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for 1 hour at room temperature (diluted 1:2,000 in blocking buffer), washed again, and placed on coverslips in mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). As a control, cells were stained in the absence of primary antibody. Immunofluorescence and

bright-field images were captured using a Leica DMIRB fluorescence microscope (Leica Microsystems, Wetzlar, Germany, http://www.xxx) equipped with a Hamamatsu ORCA II camera (Hamamatsu Photonics, Hamamatsu, Japan, http://www.xxx).

Determination of Osteogenic Differentiation

In order to confirm alkaline phosphatase (ALP) expression, hMSCs with or without shockwave treatment were cultured for 6 days and analyzed with EnzoLyte pNPP Alkaline Phosphatase ELISA Assay Kit (AnaSpec, San Jose, CA) according to the supplier's instructions. Briefly, hMSCs (1×10^5cells per well) in 96-well plates were incubated at 4° C overnight with peptide conjugate (PP-BSA) at a concentration of 10 μg/ml in coating buffer. Cells were washed and 200 ll per well of blocking buffer was added. Then cells were incubated for 1 hour at room temperature, washed, and 100 μl per well diluted antibody solution was added. Cells were incubated at room temperature for 1 hour on a plate shaker, washed, and 100 μl of diluted secondary antibody was added and incubated at room temperature for another hour with shaking. Finally, 100 μl per well of *p*NPP (*p*-nitrophenyl phosphate) reaction mixture was added, incubated for 20 minutes in the dark, and absorbance at 405 nm was read using a Synergy HT multiplate microplate reader (BioTek Instruments).

The amount of osteocalcin protein secreted into culture medium was assessed with a commercially available ELISA kit (Biomedical Technologies, Stoughton, MA) 30 days after shock-wave treatment of hMSCs. To measure the formation of bone nodules, hMSCs subjected to shockwave treatment were cultured for 30 days, fixed in 70% ethanol, treated with a 40 mM Alizarin Red staining (AR-S) solution (pH 4.2) for 10 minutes to stain calcium deposits, and the stained cell cultures were photographed. For quantification, AR-S was extracted with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate buffer (pH 7.0), and AR-S concentrations were determined by measuring absorbance at 540 nm using a multiplate spectrophotometer (BioTek Instruments) and an AR-S standard curve.

Statistical Analysis

Data are presented as mean ± SE or mean ± SE. Differences between groups were evaluated by Student's *t* test or ANOVA as indicated. Differences were considered significant at $p < .05$.

Results

Shockwave Treatment Releases ATP from hMSCs

After four passages, hMSCs were subjected to shockwave treatment and viability, and ATP release were assayed. Viability of cells subjected to <200 shockwave impulses remained at >95% when examined immediately after shock-wave treatment (Fig. 1C). However, cells exposed to 200 www.StemCells.com impulses showed significantly decreased viability, which was paralleled by a dose-dependent release of ATP (Fig. 1D). Ecto-apyrases, ecto-ATPases, and ecto-5'-nucleotidases found on the cell surfaces of many cell types can rapidly hydrolyze extracellular ATP [29]. In order to inhibit the breakdown of released ATP by these enzymes, suramin was added at a concentration of 100 μM, which blocks ATP

hydrolysis [12, 22, 30]. Taken together with the viability data shown above, we conclude that ATP is released into the extracellular space primarily in response to cell damage, and that the released ATP can be rapidly hydrolyzed by nucleotidases of hMSCs.

Shockwave Treatment Activates p38 MAPK Signaling in hMSCs

Our previous work has shown that shockwave-induced ATP release activates p38 MAPK in Jurkat T cells [22]. Therefore, we studied whether shockwave treatment affects p38 MAPK activation in hMSCs. We observed considerable phosphorylation of p38 MAPK at a maximum of 100 shock-waves impulses (Fig. 2A).

In order to determine whether ATP release is responsible for p38 MAPK activation, we added increasing concentrations of exogenous ATP to hMSCs. At concentrations ranging from 0.1 to 1 μM, ATP induced phosphorylation of p38 MAPK, while ATP concentrations >1 μM resulted in increasingly attenuated p38 MAPK phosphorylation (Fig. 2B). Taken together with the findings shown above, these results suggest that shockwave-induced p38 MAPK activation is at least in part due to the release of cellular ATP from hMSCs.

P2X7 Receptors Mediate Shockwave- and ATP-Induced p38 MAPK Activation

Extracellular ATP influences bone formation and resorption through P2 receptors, which may involve the activation of P2X7 receptors [31, 32] and of p38 MAPK [33, 34]. Therefore, we investigated the role of such purinergic signaling mechanisms in shockwave-induced p38 MAPK activation using apyrase an enzyme that hydrolyzes extracellular ATP [12], P2X7R-siRNA to silence P2X7 receptor expression, or the P2 receptor antagonists MRS-2179 (P2Y1 receptors), PPADS (nonselective P2 antagonist), and KN-62 (P2X7 receptor antagonist) [12, 35]. hMSCs treated with these agents were subject to shockwave treatment (100 impulses at 0.18 mJ/mm^2), and p38 MAPK activation was determined. Apyrase, PPADS, P2X7R-siRNA, and KN-62 caused a significant reduction in p38 MAPK phosphorylation (Fig. 2C). The same agents also blocked p38 MAPK activation in response to exogenous ATP (1 lM), suggesting that activation of P2X7 receptors by ATP plays an important role in the activation of p38 MAPK in hMSCs subjected to shockwave treatment (Fig. 2D). Although other purinergic receptors are likely to contribute, the results with P2X7R-siRNA suggest that P2X7 receptors play a major role in the response of hMSCs to shockwave treatment.

Shockwave Treatment Induces C-Fos and C-Jun Transcription Via P2X7 Receptors and p38 MAPK

We used real-time RT-PCR to determine transcription of cfos and c-jun mRNA in hMSCs at 0, 15, 30, 45, and 60 minutes after shockwave treatment with 100 impulses at 0.18 mJ/mm^2 or after stimulation with exogenous ATP (1 μM). Both treatments increased the abundance of c-fos and c-jun mRNA in hMSCs with similar kinetics, reaching peak mRNA levels within 45 minutes after cell stimulation (Fig. 3).

Next we explored the roles of extracellular ATP, P2X7 receptors, and p38 MAPK signaling in c-fos and c-jun transcription. hMSCs were subjected to shockwave (100 impulses at 0.18 mJ/mm^2) or ATP (1 μM) treatment in the presence or absence of agents to target P2X7

receptor and p38 MAPK signaling. We found that apyrase, silencing of P2X7 receptors, blocking of P2X7 with KN-62 or PPADS, or inhibition of p38 MAPK activation with SB203580 greatly decreased shockwave- and ATP-induced transcription of c-fos and c-jun (Fig. 4). Failure of MRS-2179 to block c-fos or c-jun mRNA transcription (Fig. 4) or p38 MAPK phosphorylation (Fig. 2D) suggests that P2Y1 receptors have no role in the underlying purinergic signaling response.

Shockwaves Induce Osteogenic Differentiation of hMSCs Via P2X7 Receptor Signaling

In order to test whether shockwave or extracellular ATP treatment can induce osteogenic differentiation, we measured ALP activity, OC production, and bone-nodule formation of hMSC cultures. ALP activity and OC production increased dose-dependently in response to shockwave treatment (100 impulses at 0.18 mJ/mm^2) or stimulation with 1 μM exogenous ATP (Fig. 5). Shockwave treatment and ATP stimulation induced the formation of bone nodules as evidenced by microscopy and AR-S (Fig. 5C).

Next, we tested whether osteogenic differentiation of hMSCs is mediated by P2X7 receptor and p38 MAPK activation. hMSCs were treated with various drugs that interfere with these signaling processes and subjected to shockwave treatment (100 impulses at 0.18 mJ/mm^2) or stimulation with ATP (1 μM). Silencing of P2X7 receptor expression or treatment with apyrase, KN-62, PPADS, or SB203580 significantly reduced ALP expression, osteocalcin formation, and AR-S of bone nodules (Fig. 6). These data indicate that ATP release, stimulation of P2X7 receptors, and activation of p38 MAPK are required for osteogenic differentiation of hMSCs by shockwave treatment.

P2X7 Receptor Expression and Function in hMSCs

Many cell types of stromal origin show P2X7 receptor expression [36]. We used real-time RT-PCR analysis and immunocytochemical examination to investigate the expression of P2X7 receptors by hMSCs. We found that hMSCs express P2X7 receptors and that shockwaves and ATP treatment induces rapid upregulation of the expression of P2X7 receptors within 1 hour after stimulation (Fig. 7). Taken together with the findings above, these data imply that P2X7 receptors as well as changes in P2X7 receptor expression may influence differentiation of hMSCs in response to shockwave treatment. This conclusion is supported by the facts that silencing of P2X7 receptors decreased p38 MAPK activation, c-Fos and c-Jun expression, as well as osteogenic differentiation.

Discussion

Despite the widespread use of shockwave therapy for orthopedic procedures, the underlying mechanism of action by which shockwaves improve bone healing has remained unclear [37, 38]. Histological studies provide evidence that shockwave treatment stimulates osteogenesis, but clinical and experimental studies supporting this notion have been lacking [37–39]. In this study, we investigated whether shockwaves elicit hMSC responses that could explain the beneficial clinical findings others and we have made with shockwave therapy of nonunion long bone fractures. Our results demonstrate for the first time that hMSCs express functional P2X7 receptors and that shockwave treatment induces osteogenic differentiation

by stimulating these P2X7 receptors by the release of cellular ATP. Apparently, hMSCs can spontaneously release cellular ATP resulting in average extracellular ATP concentrations of 3–7.5 nM in the culture supernatants [40, 41]. These ATP concentrations are too low to activate P2X7 receptors. We found that shockwave treatment results in the release of higher ATP concentrations (up to ~6.9 ISCUSSIONM) and that these concentrations are sufficient to activate P2X7 receptors and p38 MAPK signaling, which we found to be important steps leading to osteogenic differentiation of hMSCs. In vivo shockwave treatment likely results in even higher ATP levels by triggering ATP release from cell within the treatment area. We found that removal of extracellular ATP or inhibition of P2X7 receptors prevented shockwave-induced p38 MAPK activation, osteogenic differentiation and transcription of cfos, c-jun. SB203580, an inhibitor of p38 MAPK prevented shockwave-induced downstream events leading to osteogenic differentiation. Taken together, these findings imply that ATP release, stimulation of P2X7 receptors, and activation of p38 MAPK are involved in the upstream signaling pathways that induce c-Fos and c-Jun expression and osteogenic differentiation.

P2X7 receptors are abundantly expressed on the surface of bone cells [32, 42] and P2X receptors can function as ATP-gated, nonselective ion channels that are permeable to Na^+, K^+, and Ca^{2+} [5, 12, 43]. P2X7 receptors play an important role in the regulation of bone formation and resorption [44]. ATP signaling through P2X7 receptors is necessary for mechanically induced release of prostaglandins from bone cells and subsequent osteogenesis [45]. These processes may thus be downstream consequences triggered by shockwave therapy. MAPKs also play vital roles in bone formation [46]. One member of the MAPK family is p38 MAPK that is preferentially activated by distinctive stimuli such as ultraviolet irradiation, reactive oxygen species, and cellular and environmental stresses [47]. Although G protein-coupled receptors are also known to play a role in the activation of p38 MAPK [47–49], our findings indicate that P2X7 receptors are responsible for the p38 MAPK activation in hMSCs subjected to shockwave treatment or exogenous ATP stimulation.

c-Fos has a leucine-zipper DNA binding domain and a transactivation domain at the C-terminus; and together with c-Jun, c-Fos forms the transcription factor AP-1 [19, 50]. AP-1 upregulates transcription of genes containing the TPA DNA response element (5’-TGAG/CTCA-3’) to which AP-1 binds via a basic amino acid region, while the dimeric structure is formed by a leucine zipper [19, 50]. Our investigations with P2X7 receptor antagonists and p38 MAPK inhibitors suggest that the activation of P2X7 receptors by shockwave treatment elicits p38 MAPK signaling in hMSCs and that p38 MAPK activation leads to c-Jun and c-Fos expression. The increasing expression of c-Jun and c-Fos suggests increased AP-1 expression. P2X7 receptors thus seem to induce secondary signaling responses that are likely to include calcium signaling, p38 MAPK activation, and upregulation of AP-1.

Our results show that excessive shockwave or ATP treatment can attenuate hMSCs responses (Fig. 5). This may be because of the desensitization of P2X7 receptors [51]. Another possibility could be that P2X7 receptors, which differ from other P2X receptor subtypes, can act either as ion channels or as pores depending on the duration and intensity of agonist stimulation. At high ATP concentrations, P2X7 receptors act as pores that could contribute to cell death [52]. It is possible that shockwave treatment (>100 impulses) may

result in local ATP concentrations near the cell surface that are high enough to induce P2X receptor pore formation. When measured in bulk culture medium, much lower ATP concentrations are found due to dilution of the ATP that is released from treated cells. Conversely, the concentrations of exogenously added ATP is higher in bulk medium than the local concentrations at the cell surface, because ATP at the cell surface is subject to hydrolysis by ectonucleotidases anchored to the cell surface.

It is now widely accepted that extracellular nucleotides acting via cell surface receptors such as P2X7 are important local modulators of bone cell function [9]. In response to the activation of P2X7 receptors, osteoblasts undergo proliferation and osteoblast-mediated bone formation [32]. Expression of P2X7 receptors was originally thought to be restricted to cells of hemopoietic origin, in which it has been implicated in various cell functions including apoptosis and the release of proinflammatory cytokines [53, 54]. However, it has also been found that human osteosarcoma cell lines and several populations of primary hBDCs express P2X7 receptor mRNA and protein [9, 55]. We found that hMSCs express P2X7 receptors and that shockwave treatment results in extracellular ATP concentrations that can activate these receptors. We also found that shockwaves and exogenous ATP promote transcription and expression of P2X7 receptors and that a series of downstream signaling responses cause osteogenic differentiation of hMSCs. ATP release increased with increasing pulses of shockwave treatment. It is possible that shockwaves induce mechanical cell deformation and opening of mechano-sensitive release channels that contribute to ATP release. However, our viability data (Fig. 1) suggest that at least some of the extracellular ATP generated in our experiments was due to loss of cell integrity.

Much published evidence indicates that shockwave treatment enhances the healing and repair of bone fractures [1, 39, 41, 56–58]. Since the late 1980s, shockwaves have been used to increase the ossification of long-bone pseudarthrosis [56], and consequently a number of studies have focused on possible mechanisms that could explain these beneficial effects [2–4]. The majority of these studies demonstrated osteogenetic effects of shockwaves [2–4]. Our findings provide strong evidence for the notion that shockwave induce osteogenic differentiation of hMSCs via direct mechanisms involving P2X7 receptor signaling. We found that extracellular ATP resulting from shockwave treatment activates P2X7 receptors and downstream responses that lead to osteogenic differentiation of hMSCs. Spontaneous release of ATP from hMSCs has been shown to regulate cell fate and proliferation [43]. We found that shockwave treatment increased P2X7 receptor expression, which may serve as a feed forward mechanism that further promotes bone formation and healing of nonunion fractures.

Conclusion

In summary, our study demonstrates that ATP release and signaling via P2X7 receptors promote hMSC osteogenic differentiation. These findings support previous reports that demonstrated that extracellular ATP can enhance proliferation and mineralization of osteoblasts by activation of P2X7 receptors [57, 59]. Thus, our data suggest that shockwave-induced ATP release from hMSCs and activation of P2X7 receptors may cause hMSCs osteogenic differentiation and osteoblasts proliferation as well as mineralization, which may

enhance bone healing in patients with nonunion fractures. Our data suggest that this is a probable, but not the only possible, mechanism by which shockwave therapy promotes bone healing. It is likely that shockwave treatment induces ATP release from many cell types in addition to hMSCs. In patients, it is possible that ATP release at the site of shockwave treatment is generated by cells other than hMSCs and that ATP may contribute to bone healing through direct as well as indirect effects on hMSCs. The in vitro experiments in this study cannot elucidate which cell types other than hMSCs might be involved in the therapeutic effects of shockwave treatment. Therefore, additional in vivo work is required to determine how shockwave treatment and ATP signaling affect the recruitment of hMSCs to fracture sites and how hMSCs differentiation and additional processes contribute to bone formation in response to shockwave therapy. While ATP release may promote additional processes that contribute to bone healing, we believe that our work underscores the therapeutic potential of shockwave therapy for the treatment of orthopedic diseases such as bone fractures, nonunions, and pseudarthrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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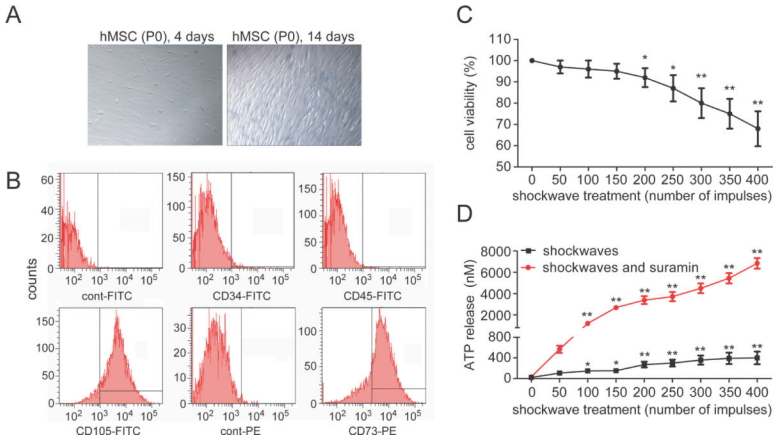

Figure 1.

Shockwave treatment causes rapid ATP release and reduces hMSC viability. (**A**): Adherent hMSCs in a primary culture (P0) on days 4 and 14 after plating of freshly isolated bone marrow cells. (**B**): After passaging hMSCs four times (P4), their phenotype was analyzed by flow cytometry to distinguish hematopoietic ($CD34^+$, $CD45^+$) and mesenchymal ($CD73^+$, $CD105^+$) lineages. (**C**) After four passages, hMSCs (5×10^6 per ml) were exposed to shockwaves using the indicated impulse numbers. Cell viability was monitored with trypan blue 5 minutes after shockwave treatment (data in mean ± SD, $n = 6$, *, $p < .05$; **, $p < .01$; Student's t test). (**D**): ATP release was determined with a commercially available ATP bioluminescence assay. Cells were allowed to rest for 3 hours at 37° C. Then the cells were exposed to shockwave treatment (0.18 mJ/mm^2) in the absence or presence of suramin (100 μM) to reduce ATP degradation. Data are representative of three different experiments and values are expressed as mean ± SD. Asterisks indicate statistically significant differences compared to cell not subjected to shockwave treatment; *, $p < .01$; **, $p < .001$; Student's *t* test. Abbreviations: FITC, fluorescein isothiocyanate; hMSC, human mesenchymal stem cell; PE, phycoerythrin.

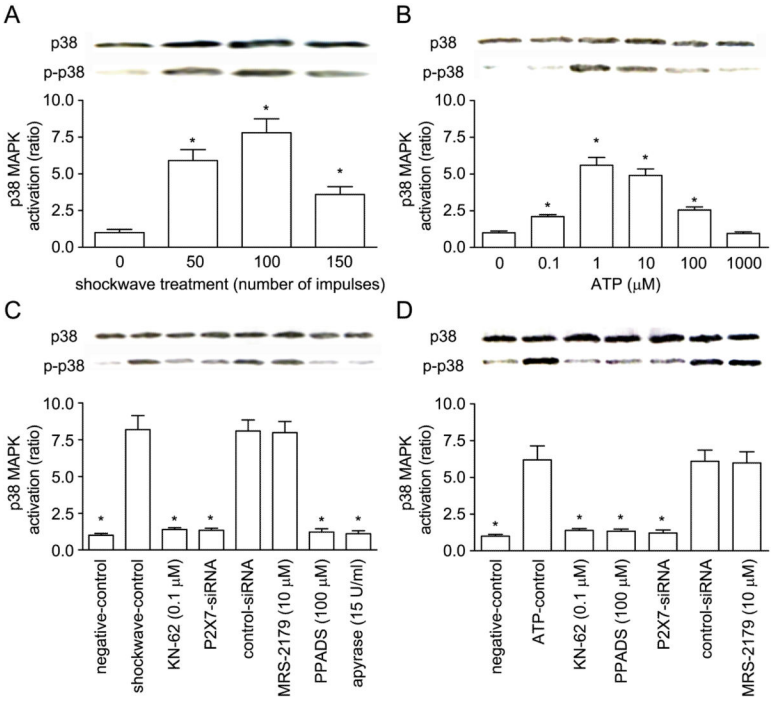

Figure 2.

Shockwave treatment activates p38 MAPK via P2X7 receptor stimulation. (**A, B**): Shockwaves and exogenous ATP dose-dependently induce p38 MAPK activation. (A): After shockwave treatment (0.18 mJ/mm^2) with indicated impulse numbers, human mesenchymal stem cells (hMSCs) (10^6 per ml, P4) were cultured for 30 minutes, and p38 MAPK activation was determined by immunoblotting with antibodies that recognize the active phosphorylated form of p38 MAPK (p-p38) and antibodies that recognize both active and inactive p38 MAPK (pan-p38 MAPK; p38). Gray intensities were analyzed and ratios between activated and pan-p38 MAPK are calculated, and data were normalized by control nontreated group and present in the corresponding graphs. (B): hMSCs (10^6 per ml) were treated with the indicated concentrations of exogenous ATP for 5 minutes to simulate sample handling as was used for shockwave treatment. Then the cells were plated and cultured in the presence or absence of ATP for 30 minutes and p38 MAPK activation was determined. (**C, D**): Purinergic signaling via P2X7 receptors contributes to p38 MAPK activation. hMSCs were subjected to shockwave treatment (0.18 mJ/mm^2 for 100 impulses; panel (C) or extracellular ATP (1 μM ATP for 5 minutes; panel (D)) in the absence or presence of a nonspecific P2 receptor antagonist (PPADS), specific antagonists of P2X7 (KN-62), P2Y1 receptors (MRS-2179), or apyrase, and p38 MAPK activation was determined. In order to silence P2X7 expression, cells were pretreated with small interfering RNA (siRNA) targeting P2X7 receptors or with control siRNA for 3 days prior to shockwave or ATP treatment. Representative Western blots of six different experiments are shown, and data were averaged in the corresponding bar graphs (n = 6, mean ± SD, *, p < . 01; Student's t test, compared to shockwave or ATP controls).

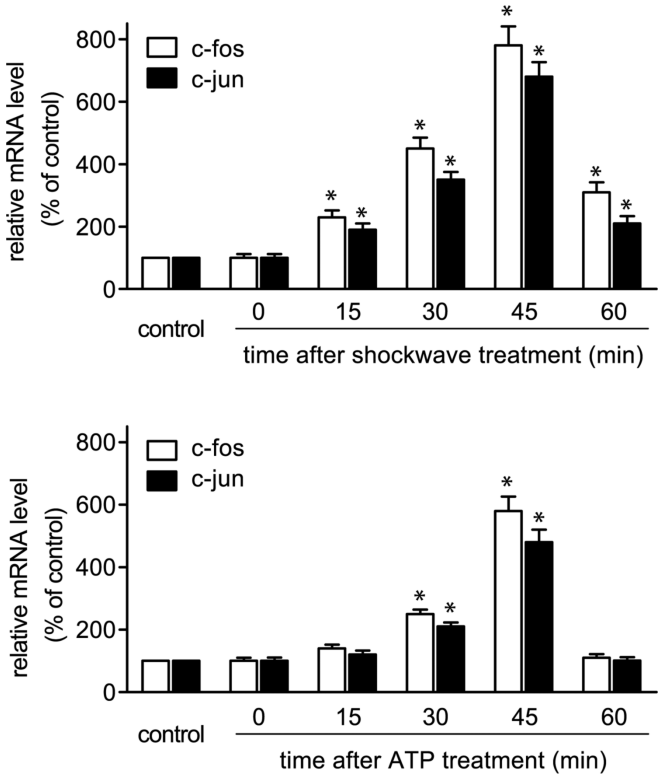

Figure 3.

Shockwaves and ATP induce c-fos and c-jun mRNA transcription. (**A, B**): The levels of c-fos or c-jun mRNA in human mesenchymal stem cells (hMSCs) were determined by real-time reverse transcription polymerase chain reaction at indicated time points after treatment of cells with shockwaves (0.18 mJ/mm^2 for 100 impulses; panel A) or exogenous ATP (1 μM; panel B). β-actin was used as internal control, and data were normalized relative to unstimulated controls. Data are shown as means ± SE; $n \geq 3$; *, $p < .05$; two-tailed unpaired Student's *t* test, compared to unstimulated controls.

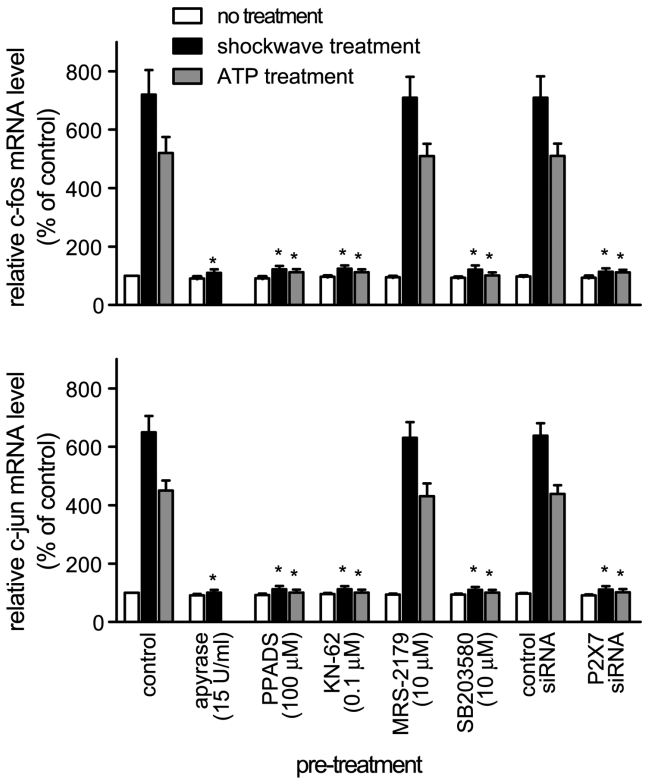

Figure 4.

Shockwaves and ATP induce c-fos and c-jun transcription via P2X7 receptors and p38 MAPK activation. Human mesenchymal stem cells were pretreated with the indicated agents and stimulated with shockwaves (0.18 mJ/mm^2 for 100 impulses) or exogenous ATP (1 μM) as described in Figure 2, and c-fos (upper panel) and c-jun (bottom panel) mRNA levels were determined 45 minutes later using real-time reverse transcription polymerase chain reaction. β-actin was used as internal control, and results were normalized by unstimulated controls. Data represent mean ± SE, $n \geq 3$, *, $p < .05$, two-tailed unpaired Student's *t* test, compared to positive controls.

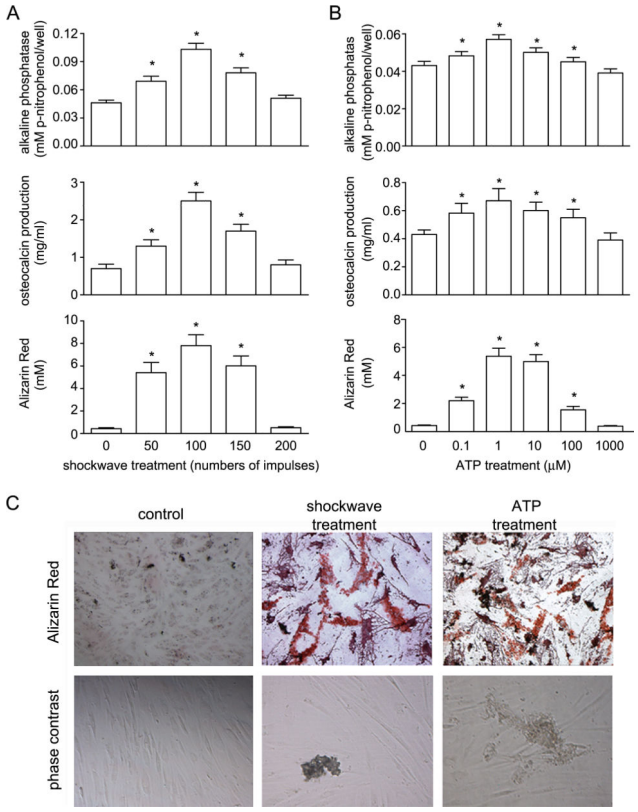

Figure 5.

Shockwave or ATP treatment induces osteogenic differentiation of human mesenchymal stem cells (hMSCs). (**A**): hMSCs were subjected to shockwave (0.18 mJ/mm^2) and then cultured for 14 or 30 days until analysis of alkaline phosphatase activity (14 days), osteocalcin (OC) protein production (30 days), and formation of bone nodules assessed by Alizarin Red staining (30 days). (**B**): Cells were stimulated by addition of exogenous ATP at the indicated final assay concentrations. After 5 minutes, the cells were plated and cultured for 14 or 30 days until analysis of alkaline phosphatase activity (14 days), OC protein production (30 days), and formation of bone nodules (30 days). Data represent means ± SE; *, $p < .05$; $n \geq 3$; two-tailed unpaired Student's *t* test, compared to unstimulated controls. (**C**): Untreated hMSCs (control) or hMSCs treated with shockwaves or ATP were cultured for 30 days, and bone nodule formation was analyzed by phase contrast microscopy (bottom images; magnification × 100) and bright-field microscopy of Alizarin Red stained cells (top images; magnification ×40).

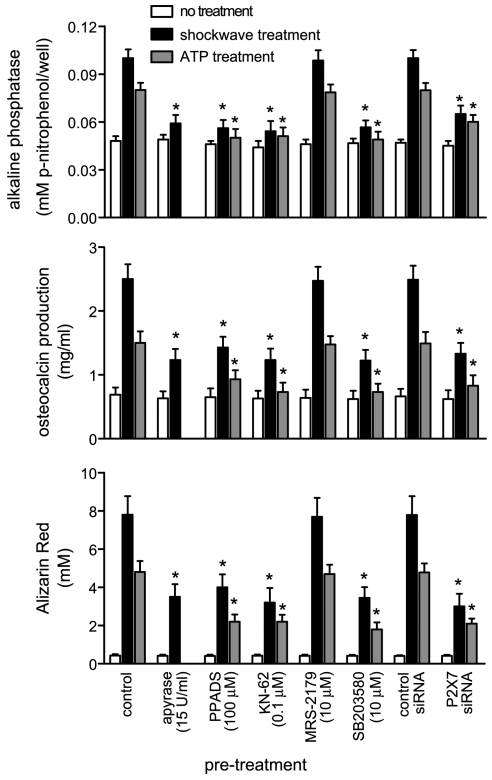

Figure 6.

P2X7 receptors and p38 MAPK signaling are involved in osteogenic differentiation of human mesenchymal stem cells (hMSCs) in response to shockwave and ATP treatment. P2X7 receptor expression was silenced or purinergic signaling events were blocked as described in Figure 2 and then hMSCs were stimulated with shock-wave treatment (100 impulses at 0.18 mJ/mm^2) or with exogenous ATP (1 μM) and alkaline phosphatase activity, osteocalcin protein expression, and formation of bone nodules was tested as described in Figure 5. Data represent mean ± SE. *, $p < .05$; $n \geq 3$; two-tailed unpaired Student's *t* test, compared to positive controls.

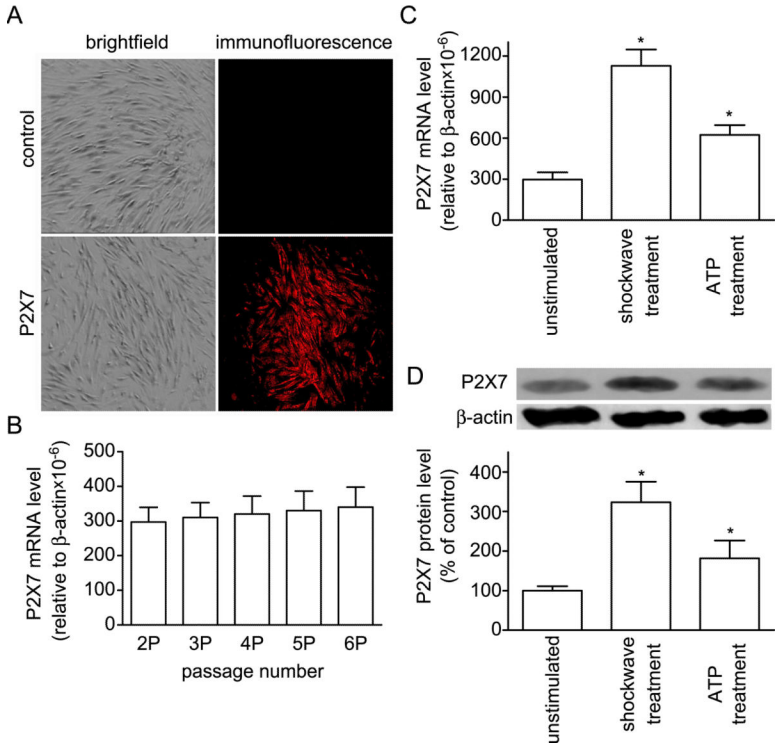

Figure 7.

Shockwaves induce P2X7 receptor expression. (**A**): Expression of P2X7 receptor in human mesenchymal stem cells (hMSCs) (P4) was assessed by immunofluorescence staining with P2X7 receptor antibodies followed by fluorescence-labeled secondary antibodies. Control slides were processed in the same fashion, except that P2X7 receptor antibodies were omitted (up images). They were examined under a magnification of 400. (**B, C**): P2X7 receptor mRNA levels of hMSCs after repeated passages (panel B) or after shockwave or ATP treatments (panel C) were determined with real-time reverse transcription polymerase chain reaction. Cells were exposed to shockwave stimulation (100 impulses at 0.18 mJ/mm^2) or treatment with exogenous ATP (1 μM) and P2X7 receptor mRNA expression was determined after 1 hour and expressed relative to β-actin control values. (**D**): P2X7 receptor protein levels in hMSC membrane preparations and β-actin levels in hMSC cytoplasm preparations were estimated by immunoblotting with P2X7 or β-actin antibodies and gray values were compared and expressed by normalizing to unstimulated controls. Data represent mean ± SE. *, $p < .05$; $n \geq 3$; two-tailed unpaired Student's *t* test, compared to unstimulated controls.