

Distinct Anabolic Response of Osteoblast to Low-Intensity Pulsed Ultrasound

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ABSTRACT

Low-intensity pulsed ultrasound, a form of mechanical energy transmitted as high-frequency acoustical pressure waves, provides noninvasive therapeutic treatment for accelerating fracture repair and distraction osteogenesis. Relatively young osteoblasts respond to ultrasound by transiently upregulating message levels of immediate-early genes as well as that of osteocalcin and insulin-like growth factor I (IGF-I). Osteocytes derived from newborn rat tibia and calvaria responded to a lesser extent only in *c-fos* and cyclooxygenase-2 (*COX-2*) messages. Compared with the stretched osteocytes, which use stretch-activated and parathyroid hormone (PTH)-potentiated Ca^{2+} influx as an entry route to the protein kinase A (PKA) signal transduction pathways, there was no evidence of Ca^{2+} internalization by any of the cells tested on exposure to the ultrasound. On the other hand, inhibitors of p38 mitogen-activated protein kinase (MAPK) and upstream phosphoinositide 3-kinase (PI3K) blocked *COX-2* and osteocalcin upregulation by the ultrasound-exposed ST2, murine bone marrow-derived cells. This is distinct from the aforementioned osteocytic response to low-frequency stretching and implies the involvement of integrins. Our findings suggested that accelerated fracture repair and distraction osteogenesis by the low-intensity pulsed ultrasound depend, at least in part, on the stimulation of osteoblastic cells at relatively early stages of osteogenic lineage. Bone is under control of multiple regulatory mechanisms so that diverse physical forces can be reflected to the microenvironment of each cell, in turn, to the entire bone. (J Bone Miner Res 2003;18:360–369)

Key words: therapeutic ultrasound, insulin-like growth factor I, osteoblast, osteocyte, PI3K

INTRODUCTION

CELLS IN BONE are equipped with mechanisms to sense diverse physical forces and transduce signals for adjustment of their microenvironment.⁽¹⁾ Our in vitro studies of osteocytic stretch-sensing mechanisms showed that certain types of mechanical stress are sensed only by certain stages of osteogenic cells.^(2,3) We have identified parathyroid hormone (PTH)-potentiated Ca^{2+} influxes into the osteocytic cell processes, which use protein kinase A (PKA) signaling pathways for downstream anabolic responses such as production of insulin growth factor I (IGF-I) and osteocalcin.⁽³⁾ The upregulation of mRNA levels of these molecules occurred in a manner similar to that of typical immediate-early genes, *c-fos* and *COX-2*.⁽⁴⁾ The upregulation was dependent on the presence of extracellular Ca^{2+} and was inhibited by

Gd^{3+} , suggesting the involvement of stretch-activated (SA) cation channels. Ca^{2+} influx has been mainly localized along the cell processes in rat young osteocytes.⁽³⁾ Presence of secondarily driven Ca^{2+} channels such as the voltage-operated L-type channel⁽⁵⁾ or $\text{Na}^+/\text{Ca}^{2+}$ exchangers^(6,7) has suggested that multiple ion channels might be functioning in osteocytic processes in collaboration to propagate mechanical stimuli to the entire bone.⁽⁸⁾

In search of mechanotransduction pathways unique to osteoblasts, and of crosstalk among signaling pathways, we studied the anabolic response of osteogenic cells to low-intensity pulsed ultrasound. It provides a noninvasive therapeutic treatment for fracture repair and distraction osteogenesis.^(9,10) Our goal in the present study was (1) to determine target cells in the bone cell lineage and (2) to elucidate sensory and signal transduction mechanisms. Compared with the extremely low frequency of stretching deformation (1/3 Hz) used to stimulate osteocytes in our

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previous studies, pressure pulses of the sonic accelerated fracture healing system (SAFHS) send high frequency sine waves of 1.5 MHz in pulses of 200 μ s each, at a pulsing frequency of 1.0 kHz. When intact bone is mechanically loaded experimentally, strain of relatively high intensity, 1000–4000 μ E, was reported necessary to induce bone formation.^(11,12) Lower intensity, however, is compensated by the strain frequency^(13,14) or by the strain rate when the frequency is relatively low.⁽¹⁵⁾ While it is entirely artificial, SAFHS provides an effective mechanical stimulation to the cells in fracture callus,⁽¹⁶⁾ probably because of the high frequency. In this article, we have examined anabolic responses in rat long bone- and bone marrow-derived primary cells as well as murine bone marrow-derived ST2 cells to evaluate their responses in relation to the stages of osteogenic cell differentiation.

MATERIALS AND METHODS

ST2 cells

We have preliminarily characterized anabolic responses of ST2 cells to SAFHS.⁽¹⁷⁾ Stromal ST2 cell line of murine bone marrow origin was established by Ogawa et al.⁽¹⁸⁾ as a cell line that supports differentiation of murine T cells, and was obtained from the RIKEN Cell Bank (Tsukuba, Japan). ST2 cells or bone marrow cells cultured in the presence of ascorbate or dexamethasone (Dex) were differentiated successfully for studies of osteoblastic cells.^(19–22) Cells within the fifth or sixth subculture were plated in 6-well dishes at 1×10^5 cells/cm² (9.4-cm²/well) 24–48 h before exposure to ultrasound. Alpha minimum essential medium (α -MEM) was used with supplements, 10% FBS (Gibco, Invitrogen Corp., Carlsbad, CA, USA), and the above-mentioned antibiotics mixture (Gibco) in the presence or absence of 25 μ g/ml ascorbate-2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or 10^{-8} M dexamethasone (Sigma, St. Louis, MO, USA) for 1 week. Medium was replaced with 2 ml fresh medium 24 h before the experiment.

Preparation of osteoblastic and osteocytic cells from rat long bone

The following experimental protocols were approved by either Kanagawa Dental College or Kitasato University School of Medicine Animal Care Committee. Osteoblastic, as well as osteocytic, cells were enriched by sequential collagenase digestion as reported earlier.⁽²³⁾ Pieces of 3-day-old rat femur and tibia bone collars were split, stripped carefully of most periosteal soft tissues, and washed by repeated pipetting to remove bone marrow cells. After the first brief digestion with 0.75 mg/ml collagenase (Wako Pure Chemical Industries, Ltd.), cells released by the second and third digestions (20 minutes each) at 37°C were collected and cultured as osteoblasts. After each sequential release of osteoblastic cells, strips of long bone fragments were flushed by repeated pipetting with Ca²⁺/Mg²⁺-free PBS (Nissui Pharmaceutical, Tokyo, Japan); PBS (–) and the cells released were combined with the digests and inoculated at a density of 5×10^4 cells/cm² in the 6-well plate (Falcon, BD Biosciences, Bedford, MA, USA). α -MEM was used with supplements, 10% FBS (Gibco), and antibiotics

mixture (Gibco). To prepare osteocytic cells, residual bone strips were further cut into 1- to 2-mm³ pieces, incubated with collagenase again, washed with PBS (–), and placed in a culture dish. Some of the released cells and outgrowth of flat cells, distinct from fibroblastic cells, were used as sources of osteocytic cells after they reached confluence. Final cell density of these cells was less than one-tenth of the osteoblasts, approximately 2000–5000/cm².

Bone marrow-derived cells

Six-week-old Wistar rats were killed, their femora were excised and cleaned, and the epiphyses were removed. Marrow contents were flushed out with α -MEM squirted through a 20 G \times 11/2" needle, and single cells were isolated in suspension by repeated pipetting. Cells from each femur, 2×10^7 cells, were cultured in a 25-cm² flask (Falcon) in α -MEM containing 10% FBS (Gibco) and antibiotics in a humidified atmosphere of 5% CO₂ in air at 37°C. After a 24-h incubation to allow cells attach to the bottom of the plate, nonadherent cells were removed by rinsing the plate with PBS (–). Cultures were maintained for another week. After reaching 70% confluence, the cells were detached by treatment with 0.05% trypsin and replated either in 75-cm² flasks (Falcon) or in 6-well plates (9.4-cm²/well) at a density of 5.3×10^4 /cm². Cells were cultured for 2 weeks before the experimental use. Dexamethasone (1×10^{-8} M from a 1-M stock solution in ethanol) or 0.25 mM ascorbate 2-phosphate (Asc 2-P) was added to differentiate these cells.

Exposure to ultrasound

Ultrasound was generated with the transducer (effective area for each well is 3.88 cm²) for the SAFHS (Smith & Nephew Inc., Memphis, TN, USA) operated at 1.5-MHz frequencies in a pulsed-wave mode.⁽¹⁷⁾ Medium was changed 24 h before the exposure of cultures to the ultrasound and care was taken not to disturb cells before the experiment. Unless otherwise described, cells were exposed to the ultrasound for 20 minutes and harvested after another 20 minutes. At 40 minutes, significant upregulation of messages were observed in our previous experiments.⁽¹⁷⁾ To preincubate Asc 2-P-treated ST2 cells with inhibitors, 5000-fold stock solution of the highest experimental concentrations was prepared in DMSO, and 250 μ l of the 5-fold solution in the complete medium was added gently to every milliliter medium to make a final concentration. LY294002, SB203580 (SB202190), and PD98059 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). NS398 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA) was dissolved to DMSO (16 mM) and diluted to 16 μ M with the complete medium to make a final concentration of 2 μ M.

Reverse transcription-polymerase chain reaction analysis

Total RNA from the culture was extracted using ISOGEN from Nippon Gene Co. (Tokyo, Japan). Conditions for reverse transcription-polymerase chain reaction (RT-PCR) and primers used were listed elsewhere^(4,17) except for mu-

rine glucocorticoid receptor GR⁽²⁴⁾ and BMP-4⁽²⁵⁾ primers. Egr-1 primers were designed from the reported sequence⁽²⁶⁾; Egr-1 sense, 5'-CGCTGCAGATCTCTGACC-CGTTCG-3' and antisense, 5'-TGCCACTGTTG-GGTGCGGGCTCCAG-3'. For the relative quantification of COX-2, IGF-I, and osteocalcin mRNA by real-time PCR, the LyghtCycler system was used with SYBR Green I and other reagents provided by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Except for GAPDH⁽²⁷⁾ and murine COX-2⁽²⁸⁾ primers prepared for conventional PCR were used. Total RNA (1 µg) from the ultrasound and vehicle experiments was reverse transcribed and 5% of the RT reaction was amplified in a quantitative real-time PCR with the GAPDH primers. The product was quantified using a standard curve that correlated each cycle number at which the amplification of the product was in the linear phase with a value. This value was then normalized to the value of the standard GAPDH. Amount of cDNA subjected to relative quantification was determined so that the crossing cycle number of GAPDH product curve is 20. Experimental samples of such GAPDH values were examined for each message with standards, which were derived from serially diluted control samples. Measurements were repeated at least three times to determine statistical significance by Student's *t*-test (*p* < 0.05).

Measurement of cytosolic calcium, [Ca²⁺]_i, and [Ca²⁺]_i imaging in ultrasound-exposed cells

Cytosolic calcium was measured in single cells as described previously.⁽³⁾ ST2 cells cultured for 1–2 days on glass coverslips were washed with PBS and loaded with 5 µM fura-2/AM for 1 h at 25°C in Ringer's solution (138 mM NaCl, 5.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 20 mM HEPES buffer, pH 7.3 with NaOH). After two washes with the Ringer's solution, the measuring chamber and an ultrasound probe were mounted to a microfluorometric system. Ultrasound was emitted, and images (F340 and F360) were collected at wavelengths of 340 and 360 nm. The ratio of F340/F360, constructed after background image subtraction, disclosed subcellular [Ca²⁺]_i localization. The average [Ca²⁺]_i values were calculated from calibration curve as described.⁽³⁾

Prostaglandin E₂ assay

Prostaglandin E₂ (PGE₂) produced by ST2 cells plated at 10⁵ cells/well was determined using an ELISA (Assay Designs Inc., Ann Arbor, MI, USA). After the exposure to the ultrasound, cells were incubated to collect medium at each time point. Original medium was replaced with 2 ml of fresh medium, and cells were further incubated before harvesting. Medium samples were centrifuged to remove debris and stored in portions at –80°C until the assay. Concentration of PGE₂ in 50 µl of the supernatant was determined from a standard curve of serially diluted PGE₂.

Western blot

Immediately after the 20-minute exposure of ultrasound or set incubation periods, the culture wells were rinsed in ice-cold PBS (–), and the cells on each slide were lysed in

400 µl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue). Duplicate gels were run simultaneously to facilitate probing for COX-2 on a blot and staining for total protein. A 10-µl aliquot of each sample was fractionated on a 4–12% SDS-PAGE gel at 50 mA/gel. After electrophoresis, the fractionated proteins were transferred to Immobilon-P (Millipore Corp., Bedford, MA, USA) using plate electrode Trans-Blot system (Bio-Rad Laboratories, Hercules, CA, USA) for 30 minutes, and probed for COX-2 using 1:1000 dilutions of polyclonal rabbit antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Using Immuno Star Western Blot Detection Kit (Wako Pure Chemical Industries, Ltd.), blotted filters were incubated for 1 minute with chemiluminescence substrate and exposed to ECL film (Amersham Pharmacia Biotech, Buckinghamshire, UK) up to 2 minutes. The bands on the gel were digitized using the CCD camera-based Image Acquisition System (ATTO Corp., Tokyo, Japan) and analyzed densitometrically using the Scion Image analysis software (Scion Corp., Frederick, MD, USA). A representative result was presented.

RESULTS

Response of bone- and bone marrow-derived primary osteogenic cells to SAFHS

Rat long bone-derived primary cells at different stages of differentiation and Dex-treated bone marrow-derived cells were compared in Figs. 1 and 2 and in Tables 1 and 2. In OB1 cells, transient expression of immediate-early genes, *c-fos* and COX-2, as well as changes in other genes such as IGF-I (up-), ALP (down-), and osteocalcin (upregulation) were confirmed 20 minutes after the 20-minute exposure to the ultrasound (Fig. 1; Table 1). Osteocyte response to SAFHS was relatively small in *c-fos* and COX-2 messages and was insignificant in other messages such as IGF-I and osteocalcin. Osteocytes derived from newborn rat calvaria were equally less responsive (data not shown). OB2 cells, which showed response mainly in *c-fos* and COX-2, were cultured longer than OB1 osteoblasts. Overall responsiveness decreased in the order of OB1, OB2, and OCY, along with the bone cell differentiation. Bone marrow-derived adherent cells without any treatment (bone mineral content [BMC]) were essentially insensitive to SAFHS. Significant differences were detected by relative quantification with the LyghtCycler system in the following messages: COX-2, IGF-I, and osteocalcin in OB1, and COX-2 in OB2 and OCY (Table 1). Alkaline phosphatase and *c-fos* were not quantified.

In the bone marrow-derived cells, which were forced to differentiate into osteoblasts by incubation with Dex for 1 week, basal message levels of osteocalcin and ALP were significantly upregulated. On the other hand, response of the osteoblastic cells to the ultrasound was inapparent at 1 week except in *c-fos*, and to a lesser extent, COX-2 (Fig. 2, 1W). In fact, Dex by itself was not inhibitory to the stimulation, but provoked a significant response at 1 day in both IGF-I and osteocalcin (Fig. 2, 1D). As summarized in Table 2, significant differences were detected in 1D (IGF-I and os-

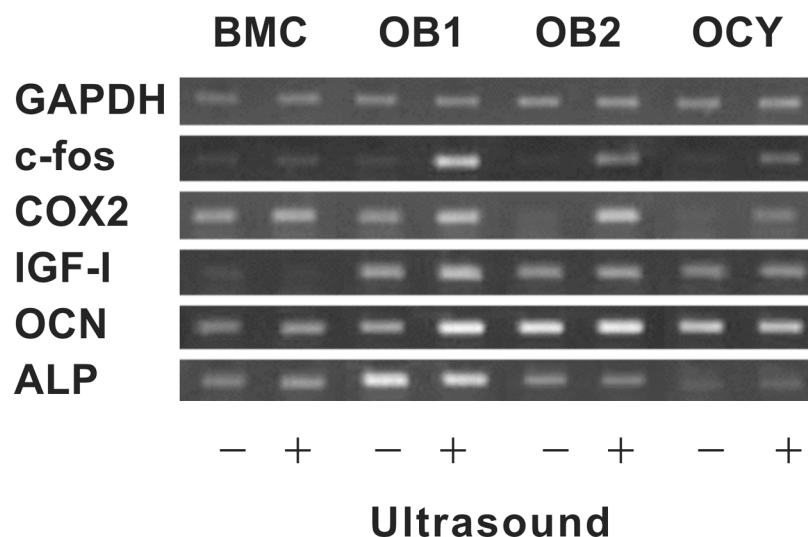


FIG. 1. mRNA levels of immediate early genes and other bone proteins after 20-minute exposure of rat bone marrow-derived cells (BMC) and the primary long bone cells (OB1, OB2, and OCY) to low-intensity pulsed ultrasound. Adherent BMC cells were cultured for 3 weeks before the experiment. OB1 (osteoblastic cell 1) were released from bone chips by collagenase digestion as described in the Materials and Methods section and cultured for 1 week before the experiment. OB2 cells were cultured for another 2 weeks. OCY (osteocytic cells) were prepared as described in the Materials and Methods section and cultured for 4 weeks before the experiment. After the 20-minute exposure and 20-minute incubation, total RNA was extracted and subjected to RT-PCR as described in the Materials and Methods section. GAPDH was used as an internal standard. +, samples exposed to the ultrasound; -, vehicles not exposed to ultrasound under otherwise identical conditions.

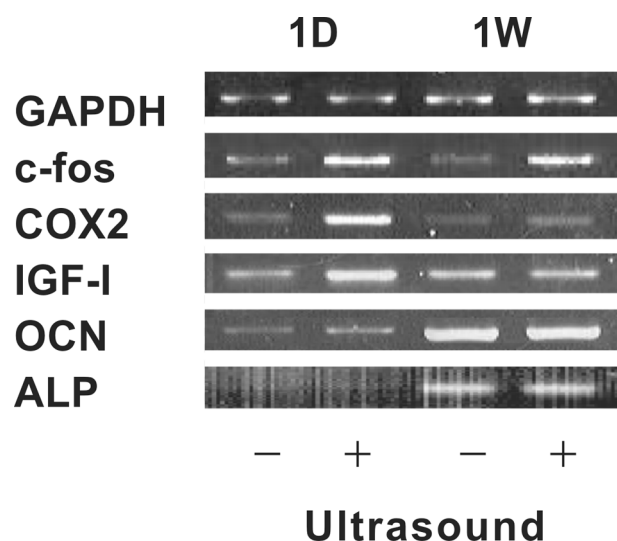


FIG. 2. Differential response of Dex-differentiated rat bone marrow-derived cells to the low-intensity pulsed ultrasound. BMC cells were prepared as described in Fig. 1 and treated with Dex at 1×10^{-8} M for either 1 day (1D) or 1 week (1W) before the exposure to the ultrasound. After the 40-minute experiment as in Fig. 1, total RNA was extracted and subjected to RT-PCR as in Fig. 1. +, samples exposed to the ultrasound; -, vehicles not exposed to ultrasound under otherwise identical conditions.

TABLE 1. UPREGULATION OF MESSAGES IN BONE-DERIVED CELLS EXPOSED TO LOW-INTENSITY PULSED ULTRASOUND

Gene	BMC	OB1	OB2	OCY
COX2	NS	2.8 ± 0.6	7.9 ± 1.3	3.4 ± 0.7
IGF-I	NS	3.2 ± 0.5	NS	NS
OCN	NS	4.1 ± 1.0	NS	NS
GAPDH	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1

Alkaline phosphatase and *c-fos* were not quantified.
NS, no significant difference.

TABLE 2. DIFFERENTIAL RESPONSE OF BONE MARROW-DERIVED CELLS TO LOW-INTENSITY PULSED ULTRASOUND AFTER 1-DAY AND 1-WEEK TREATMENT WITH DEXAMETHASONE

Gene	1D	1W
COX2	10.6 ± 2.3	2.8 ± 0.7
IGF-I	6.4 ± 2.3	NS
OCN	3.0 ± 0.5	NS
GAPDH	1.0 ± 0.1	1.0 ± 0.1

Alkaline phosphatase and *c-fos* were not quantified. See Fig. 2 for the detail.
NS, no significant difference.

teocalcin as well as COX 2) and in 1W (COX 2 alone) by the relative quantification method described in Table 1.

ST2 cells and SAFHS

ST2 cells of murine bone marrow origin were treated with ascorbate or Dex so that we were able to study osteoblastic cells in their response to the ultrasound (Fig. 3; Table 3). One-week incubation with Dex significantly lowered the basal level of COX-2. Dex, here again, was not inhibitory at all in the response of ST2 cells, at least to this particular mechanical perturbation. Besides, typi-

cal messages of osteoblastic phenotype, such as ALP and osteocalcin, which were induced either by ascorbate 2-phosphate or Dex, were further upregulated in response to the ultrasound. Response to the ultrasound seems to be characteristic to the osteoblastic cells, excluding immediate-early genes such as *Egr-1* or *c-fos*, which were always responsive regardless of the differentiation status in osteogenic lineage. No changes were detected in the levels of BMP-4 and glucocorticoid receptor messages, which may alter the responsiveness of cells indirectly. Excluding *Egr-1* and *c-fos* messages, significant differences were detected only in +Asc 2-P and +DEX by the relative quantification (See Table 3).

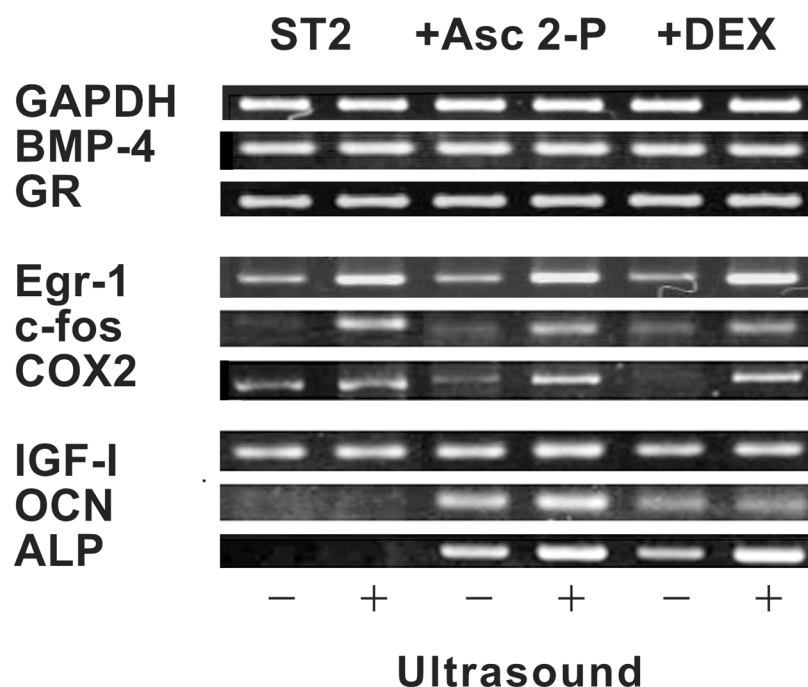


FIG. 3. Response of Dex- or ascorbate-differentiated ST2 cells to the low-intensity pulsed ultrasound. Cells were prepared as described in the Materials and Methods section and treated for 1 week either with dexamethasone at 1×10^{-8} M (DEX) or with 0.25 mM ascorbate 2-phosphate (Asc 2-P) before the exposure to the ultrasound. After the 40-minute experiment as in Fig. 1, total RNA was extracted and subjected to RT-PCR. +, samples exposed to the ultrasound; -, vehicles not exposed to ultrasound under otherwise identical conditions. Messages were grouped in three types: unresponsive, immediate early gene, and variably responsive types.

TABLE 3. UPREGULATION OF MESSAGES IN DIFFERENTIATED ST2 CELLS EXPOSED TO LOW-INTENSITY PULSED ULTRASOUND

Gene	ST2	Asc 2-P	DEX
COX2	2.1 ± 0.3	4.0 ± 0.6	9.5 ± 3.2
IGF-I	NS	1.9 ± 0.4	NS
OCN	NS	2.6 ± 1.0	NS
GAPDH	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.0

Egr-1, *c-fos*, and ALP were not quantified.
NS, no significant difference.

PGE₂ and COX-2 as mediators of signals in the ultrasound-stimulated ST2 cells

As we have suggested previously, COX-2 mRNA may appear in a biphasic manner in ST2 cells on exposure to ultrasound.^(17,29) In Fig. 4A, the only band that seems to be almost totally abrogated is COX-2 at 8 h in the NS398-treated cells. NS398, a selective inhibitor of PGHS₂, which is an inducible form of prostaglandin G/H synthase, did not reduce bands at earlier time points. To examine the actual release of PGE₂, medium was collected periodically after the 20-minute exposure to ultrasound. A significant release of PGE₂ was detected in the replaced fresh medium by ELISA at 30 minutes and around 6 h in the absence of added substrate, arachidonic acid (Fig. 4B). Further determination of the enzyme activity (Fig. 4B) and the protein by Western blotting (Fig. 4C) confirmed the cumulative effect; enzyme levels increased up to 8 h and was somewhat reduced by 24 h.

Possible signal transduction pathways leading to the anabolic message induction

First, we tested the extracellular Ca²⁺ influx, which is significant in the stretched osteocytes.⁽³⁾ None of the cells

tested in the osteoblast lineage, osteoblastic cells (rat and mouse, data not shown), osteocytes (rat and human, data not shown), or ST2 cells demonstrated any response on exposure to the ultrasound. Figure 5 shows the results of both ST2 cells and ascorbate-differentiated ST2 cells. In both cases, control experiment that involved stretching with hypoosmotic Ringer's solution induced Ca²⁺ influx. The increase in the intracellular Ca²⁺ concentration, [Ca²⁺]_i, was approximately 50% of rat osteocytes (dotted line) in the stretched ST2 cells. Second, we tested the efficacy of metabolic inhibitors in the mRNA levels of ascorbate-differentiated ST2 cells (Fig. 6). Specific and selective inhibitors of p38 MAPK, SB203580 (SB), and SB202190 (data not shown), and a specific inhibitor of upstream effector, PI3K, LY294002 (LY) resulted in distinct modulation profiles, whereas PD98059 (PD), an inhibitor of MAPK kinase-1 and -2 (MEK) did not have any significant effect on the ultrasound-induced message upregulation. Namely, COX 2 and osteocalcin (OCN) messages were multiplied by 3.6 ± 0.9 and 2.5 ± 0.6 in control ST2 cells, whereas the two messages were similarly multiplied by 2.9 ± 0.6 and 1.8 ± 0.5 in the cells treated with PD98059 (PD; Table 4). It should be noted that inhibitor alone might have some effect on message levels.

DISCUSSION

There is no doubt that mechanical stimuli play critical roles to maintain healthy skeleton. Cells in bone are equipped with mechanisms to sense diverse physical forces and transduce signals for adjustment of their microenvironment.^(30,31) To date, however, it is unclear as to exactly which forms of mechanical stimuli trigger bone formation or prevent bone loss in vivo and which types of bone cells are responsible. Some candidates include shear stress or

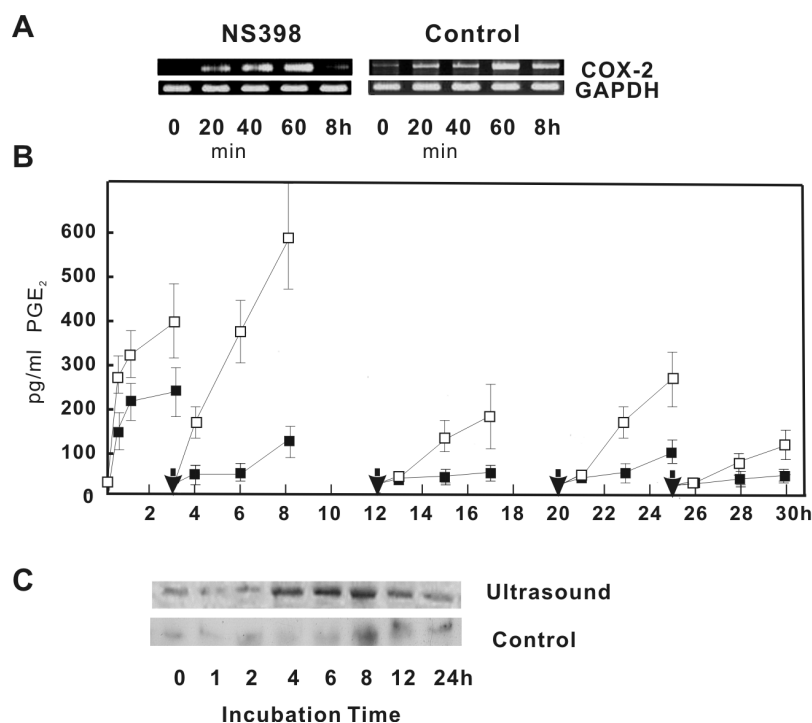


FIG. 4. COX-2 messages, PGE₂ release, and the enzyme protein in the ultrasound-exposed ST2 cells. (A) Upregulation of COX-2 mRNA level by the ultrasound in the presence and absence of NS398. Experimental ST2 cells including the control sample (time 0) were pretreated with 2 μ M NS398 for 10 minutes before the exposure. Cells were harvested at 20 minutes, which was immediately after the exposure, 40 minutes, 1 h, and 8 h. All other conditions were similar to those in Fig. 3. RT-PCR experiments were carried out as in Fig. 1. (B) The released PGE₂ into the fresh medium was assayed without adding substrate, arachidonic acid, in cultures. Medium collected at each time point after the 20-minute ultrasound exposure was subjected to ELISA as described in the Materials and Methods section. Medium was replaced with the fresh medium at the points marked with arrows. \square , ST2 cells exposed to ultrasound; \blacksquare , control cells. (C) The enzyme protein in ST2 cells was determined in a similar set of cultures with and without the exposure to the ultrasound up to 24 h. Western blotting was carried out as described in the Materials and Methods section.

streaming potentials sensed by osteoblasts,^(32–36) osteoclasts,⁽³⁷⁾ and osteocytes^(38–41); deformation of cells in osteoblasts and osteocytes^(3,42,43); and cytoskeletal reaction in osteoblasts and osteocytes.^(44–46)

Our in vitro studies of osteocytic stretch-sensing mechanisms showed that bone cells respond differently to specific types of mechanical stress depending on stages of differentiation.⁽²⁾ We have reported PTH-potentiated Ca²⁺ influxes, which use PKA signaling pathways for downstream anabolic responses such as the production of IGF-I and osteocalcin.^(3,4,47) The upregulation of mRNA levels occurred in a manner similar to that of immediate-early genes, *c-fos* or *COX-2*.⁽⁴⁾ This upregulation was dependent on the presence of extracellular Ca²⁺, which suggests the involvement of stretch-activated (SA) cation channels. Ca²⁺ influx, visualized in fura-2 preloaded rat “young” osteocytes, has been localized along the cellular processes, not the cell body.⁽³⁾ Our results further suggested that multiple ion channels might be interacting to process and transduce mechanical stimuli in osteocytes.⁽⁸⁾

We then searched for mechanotransduction pathways unique to osteoblastic cells. SAFHS, a fracture healing system using sonication, has been reviewed as a device that reduces the time frame of fracture healing by 38% in human tibial diaphysis and distal radius.^(9,10) Although SAFHS seems to facilitate the overall process of fracture repair,⁽¹⁶⁾ the underlying mechanisms by which this is achieved in individual processes are not well understood. Azuma et al.⁽¹⁶⁾ reported an interesting observation that the maximum torque of the ultrasound-treated femur was significantly greater than that of the controls while no accompanying changes, neither in hard callus area nor in the BMC, were present. Such a qualitative improvement by SAFHS implies

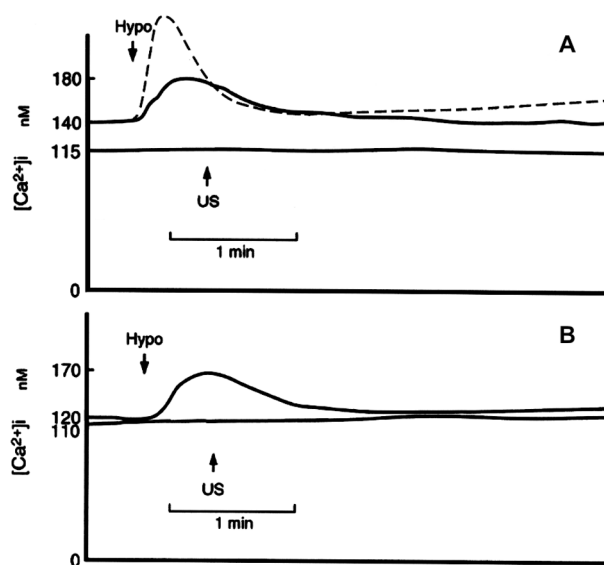


FIG. 5. Time-based plot of the intracellular Ca²⁺ concentration in ST2 cell exposed to the pulsed ultrasound. Result was compared with that of the cells exposed to hypotonic Ringer’s solution for stretching experiment. [Ca²⁺]_i of single cells was measured as described in the Materials and Methods section. ST2 cells (A) without any treatment with differentiating agent and (B) with ascorbate 2-phosphate were subjected to the mechanical stimulation to detect Ca²⁺ influxes. No Ca²⁺ influx was observed in either cell in response to the pulsed ultrasound. Peak [Ca²⁺]_i increased from the basal value in the stretched ST2 was lower (40–50 nM in both A and B) than that of control rat osteocyte, approximately 100 nM (dotted line in A). Arrows in the figure represent time points when hypotonic exposure (Hypo) or the transmission of pulsed ultrasound (US) was started. Tracings are representative of at least three experiments for each condition.

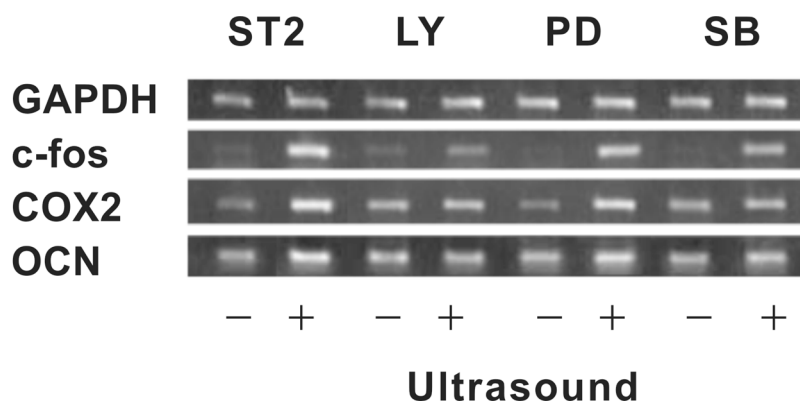


FIG. 6. Effect of various agents on upregulated message levels of Asc 2-P-treated ST2 cells by the exposure to the pulsed ultrasound. Experiments were performed as described in Fig. 3, except that cells were challenged in the presence of a certain agent. RT-PCR analysis of *c-fos*, COX-2, and osteocalcin messages was performed after 10-minutes preincubation with each agent, which was followed by the 20-minute exposure to the ultrasound and 20-minute incubation. Control samples (–) were harvested after a similar 10-minute preincubation with each agent followed by a 40-minute incubation without the exposure to the ultrasound. Final concentration of LY294002 (LY) and PD98059 (PD) was 2 μ M, and for SB203580 (SB) was 1 μ M. Compared with the upregulated mRNA levels in control ST2, no such differences were apparent in the presence of inhibitors except with PD98059.

TABLE 4. EFFECT OF AGENTS ON UPREGULATED LEVELS OF MESSAGES IN ASC 2-P-TREATED ST2 CELLS EXPOSED TO LOW-INTENSITY PULSED ULTRASOUND

Gene	ST2	LY	PD	SB
COX2	3.6 \pm 0.9	2.9 \pm 0.6	NS	NS
OCN	2.5 \pm 0.6	1.8 \pm 0.5	NS	NS
GAPDH	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.0	1.0 \pm 0.1

c-fos was not quantified. See Fig. 6 for the detail.

NS, no significant difference.

the enhanced osteoblastic activity, but not the induction of cell proliferation. Our result in Fig. 1 suggests that the osteoblasts at 1 week are early osteoblasts expressing higher alkaline phosphatase and at 3 weeks, late osteoblasts expressing higher levels of osteocalcin. Increased osteocalcin expression by the exposure in the early osteoblast perhaps means that ultrasound induces differentiation. It should be noted that a large part of the given energy remitted by SAFHS in vivo does not penetrate the intact bone. Therefore, the effect detectable in osteoblastic cell culture systems must be prevailing when compared with the in vivo results.^(17,48,49) The difference has to be explained by a large proportion of cells exposed to the ultrasound in culture.

In our earlier study using ST2 cells, biphasic upregulation of mRNA levels of bone proteins such as IGFs and osteocalcin were detected after 20-minute exposure to ultrasound.⁽¹⁷⁾ Together with our result in Fig. 4, it is suggested that ultrasound stimulates early PGE₂ production, which in turn induces late COX-2 mRNA expression followed by other messages at 24 h. PGE₂ seems to be an essential molecule responsible for the amplifying, long-lasting effect of mechanical stimulation. While amplification of PGHS2 by PGE₂ has been reported in osteoblastic cell lines,⁽²⁹⁾ Chow and Chambers⁽⁵⁰⁾ demonstrated the biphasic expression of IGF-I mRNAs in an in vivo “pinned tail” experiment. Our results confirmed the immediate PGE₂ release in

ST2 cells as well as the increased enzyme protein at 6–12 h. On the other hand, we detected that the TGF- β 1 message level does not elevate in the exposed ST2 cells⁽¹⁷⁾ and that PTH is not synergistic to the ultrasound (H Sekiya and Y Mikuni-Takagaki, unpublished observation, 2002). These results agree with the previous report by Ryaby et al.,⁽⁵¹⁾ which Warden et al.⁽⁹⁾ reviewed lately as an inconclusive negative effect of SAFHS on osteogenesis. We have presented in this article, however, that anabolic response to the ultrasound is distinct from that interactive with PTH. None of the osteoblastic or osteocytic cells showed any sign of extracellular Ca²⁺ influxes induced by the ultrasound as shown in Fig. 5. Apparently, Ca²⁺/PKA pathway, which is potentiated by PTH and plays an essential role in stretched osteocytes, is not involved. Instead, response of osteoblasts to the ultrasound was modulated by inhibitors of p38 MAPK and of upstream effector, PI3K. We believe that is probably why SAFHS is extremely effective in accelerating bone maturation in distraction osteogenesis, which applies static tensile force.⁽⁵²⁾ In addition to stimulating stretch-sensitive cells by stretching callus, exposure to the ultrasound activates osteoblastic population responsive to ultrasound. A regulatory role of PI3K activity in mechanical response has been studied in endothelial cells but not extensively.⁽⁵³⁾ Therefore, the confusion might have arisen because messages such as COX-2 and IGF-I are similarly upregulated in response to both types of mechanical stimulation we have described in osteocytic and osteoblastic cells, respectively.

In primary rat bone- and bone marrow-derived cells, OB1 fraction of high ALP expression showed greater response, whereas OB2 and osteocytes were much less responsive to the pulsed ultrasound. Limited response in osteocytes was observed regardless of the origin of bone. In another line of experiment, bone marrow-derived cells were treated with dexamethasone to differentiate osteoblastic cells. When treating cells, which are not originally re-

sponsive to the ultrasound, with Dex for 1 day and for 1 week, cells first acquired responsiveness to the ultrasound and then lost it. With the established line, ST2, cells also responded to the pulsed ultrasound only after the exposure to agents used to develop ST2 cells into osteoblasts. Without such agents, SAFHS did not upregulate COX-2 much from the basal level, which was relatively high to begin with. Again, it is likely that osteoblasts, but neither the precursor cells nor mature osteoblasts are the target cells of SAFHS among the osteogenic cell populations.

In patients with steroid-induced osteoporosis, lowered PGE₂ level in the bone cells may play an important role in the pathogenesis.⁽⁵⁴⁾ As mentioned earlier, it is likely that Dex does not inhibit anabolic response to the ultrasound but induce differentiation of osteoblast resulting in reduced response. It is recognized that osteoblasts are quite heterogeneous.⁽⁵⁵⁾ Slightly different responses to Dex between 1W (bone marrow cells) in Fig. 2 and +DEX (ST2 cells) in Fig. 3 may be reflecting that aspect of bone cell differentiation. Although treating ST2 cells with Dex and vitamin D₃ together may create fully differentiated osteoblastic cells, complication is expected in studying mechanical responses with such cells. Induction of COX-1 or inhibition of NF- κ B ligand 2, which may modulate the response, has been reported.^(56,57) In any case, certain mechanical stimulus is likely to provoke anabolic response of relatively young osteoblasts at whatever level of COX-2 they may be. Our result provides not only a basis for exercise programs for such patients, but also a clue for mechanical manipulation.

Regarding the functional pathways involving PI3K, it has been reported that the type 1 IGF-I receptor; IGF-IR is important. When the concentrations of insulin receptor substrate 1 (IRS-1) are high, the signal is mitogenic and anti-apoptotic, while, in the absence of IRS-1, the IGF-IR sends a differentiation signal, which leads to cell differentiation.^(58,59) They suggested that the mitogenic signal of the IGF-IR depends largely on the activation of the PI3K. Phosphorylation by PI3K determines cell proliferation/differentiation. As presented in Fig. 6, the anabolic effect of the ultrasound was not inhibited at all by PD98059. It is likely that the activation of ERK, typically leading cells to proliferation,^(60,61) is not involved, while the p38-dependent bone cell differentiation pathway is accelerated. Association of p38, but not of ERK, has also been suggested in the induction of COX-2 in mesangial cells exposed to chloride.⁽⁶²⁾ This particular choice of discretionary route may be consistent with the characteristic features of accelerated fracture healing by the ultrasound: promotion of osteogenic differentiation but not of cell proliferation.^(9,16) The responses we observed are likely reflection of the osteogenic phase in the SAFHS-accelerated fracture repair. Effects of growth factors on cell differentiation also pointed to the regulatory role of PI3K,⁽⁶³⁾ which is a key molecule in cell proliferation, apoptosis, and differentiation. Downstream regulatory pathways have been reviewed in the integrin-stimulated cells⁽⁶⁴⁾ and in FAK signaling complex in myocardium mechanotransduction.⁽⁶⁵⁾ In endothelial cells, PI3K-dependent NO generation and the phosphorylation of NO synthase have been studied as a consequence of fluid shear stress.^(66,67) Further characterization of PI3K-related

upstream/downstream pathways in shear stress-activated bone cells will shed light on the complex nature of mechanical stimulation of bone formation and prevention of bone loss. Although anti-apoptotic effect of the ultrasound was not emphasized in this article, our preliminary screening with a DNA array kit (SuperArray Inc., Bethesda, MD, USA) uncovered a significant difference between the experimental and control ST2 cells in related messages. Further studies will elucidate another aspect of SAFHS signaling in the anabolic exploitation.

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