Biochemical and Biophysical Research Communications **268,** 216–220 (2000)
doi:10.1006/bbrc.2000.2094, available online at http://www.idealibrary.com on IDEAL®

Anabolic Response of Mouse Bone-Marrow-Derived Stromal Cell Clone ST2 Cells to Low-Intensity Pulsed Ultrasound

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Received December 13, 1999

The effects of 20-min exposure to low-intensity, pulsed ultrasound were investigated in ST2 cells of bone marrow stromal origin. They responded to ultrasound with elevated levels of IGF mRNAs, osteocalcin, and bone sialoprotein mRNAs. The upregulated expression of these messages appeared in a biphasic manner, with the first peak resistant to the protein synthesis inhibitor cycloheximide, and a second peak that was eliminated by NS398, an inhibitor of the inducive prostaglandin G/H synthase (cyclooxygenase-2). A cumulative effect of mechanical loading called the memory effect, which has been observed *in vivo,* can be explained from such a biphasic anabolic reaction mediated by prostaglandins. The upregulation of IGF or osteocalcin mRNAs can be observed even at 24 h after the initiation of the 20-min exposure to ultrasound. Our results suggest that this low-intensity, pulsed ultrasound, which has been clinically used to accelerate the healing processes of fractured bone, induces a direct anabolic reaction of osteogenic cells, leading to bone matrix formation.

Key Words: **mechanical loading; ultrasound; osteocalcin; IGF; ST2 cells.**

Medical applications of ultrasound in general and its effects on the enhancement of fracture healing have been well documented. In a recent, comprehensive review by Hadjiargyrou *et al.* (1), the authors documented transmission of the mechanical energy of ultrasound in tissues in the form of high frequency acoustic pressure waves. Ultrasound is used in various intensities; therapeutic or surgical applications are of intensities as high as 300 W/cm^2, causing heat in tissues. For diagnostic purposes, much lower magnitudes (1–50 mW/cm^2) are applied, facilitating the imaging of organs, fetuses and bone mass. The intensity level of 30 mW/cm^2, used to accelerate fracture healing, is considered neither thermal nor destructive. One successful application in humans by Xavier and Duarte demonstrated that 20 min a day of ultrasound exposure to nonunion sites resulted in a healed nonunion in 70% of the experimental cases, in a relatively short period of time (2). By histological and radiographic means, it was demonstrated that pulsed ultrasound accelerated healing of rabbit fibular osteotomies and the filling of cortical bone defects in the rabbit femur (3). Recently, in a rabbit model of midshaft fibular osteotomy, a 20-min exposure per day of low intensity pulsed ultrasound (200 μs burst of 1.5 MHz sine waves repeated at 1 kHz) with an intensity of 30 mW/cm^2 was shown to be most conducive to recovery of the fractured bone in torsional strength (4). Studies by Ito *et al.* further supported the earlier findings that a 200 μs pulse and a 1 kHz repetitive frequency are optimal parameters for the healing of fractures (5).

While the augmentation of fracture healing by ultrasound is well documented, the underlying mechanisms of action still remain unclear. The mechanotransduction pathways involved in cellular responses to ultrasound are largely unknown except that recent results by Kokubu *et al.* present the involvement of cyclooxygenase-2 (COX-2), an inducive prostaglandin G/H synthase (PGHS2) (6). Ryaby first reported Ca influx into both cartilage and bone cells, as well as enhanced TGF-β synthesis and elevated activity of ad-

Abbreviations used: IGF(s), insulin-like growth factor(s); NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; COX, cyclooxygenase; PGHS, prostaglandin G/H synthase; BSP, bone sialoprotein.

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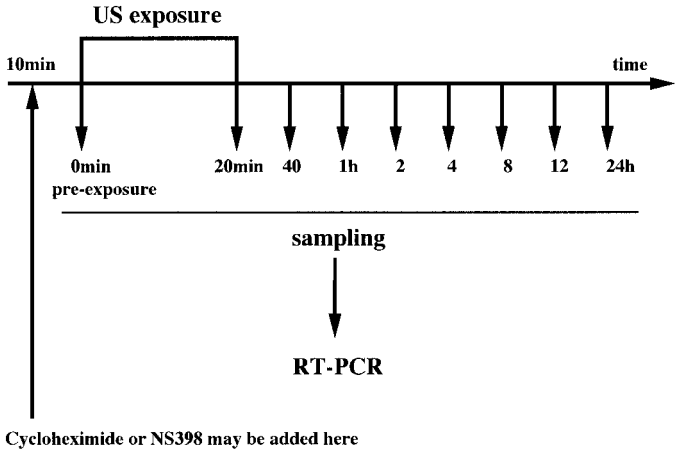

FIG. 1. Outline of the experimental procedure.

enylate cyclase in osteoblastic cell lines (7). Wu *et al.* have reported chondrocyte response by the elevated aggrecan mRNA (8). Another recent *in vivo* analysis of fracture healing showed that osteopontin mRNA level was significantly higher upon the exposure to ultrasound in rat femoral fractures (9).

The aim of our study is to compare anabolic responses toward ultrasound and those toward mechanical stretching, the latter which have been documented more extensively. In ST2 cells of bone marrow stromal origin, mRNAs of bone matrix proteins, growth factors as well as immediate-early response genes were examined during and after the 20-min exposure to the ultrasound in consideration of the induction mechanisms.

MATERIALS AND METHODS

ST2 cells. Bone marrow stromal cell line ST2 was obtained from RIKEN cell bank (Tsukuba, Japan). Compared to MC3T3-E1 cells, ROS17/2.8 cells and C3H10T1/2 cells that did not exhibit any significant elevation of osteocalcin mRNA levels, ST2 cells were extremely sensitive to stretching (Kawasaki, Mikuni-Takagaki, Takaoka, and Ebara, submitted).

Exposure to ultrasound. ST2 cells within 5th or 6th subculture were plated in 6-well dishes at 1×10^5 cells/cm^2 24–48 h prior to the exposure to ultrasound. Medium was changed 24 h before the exposure to the ultrasound. Care was taken not to disturb cells prior to the experiment. NS398, a nonsteroidal anti-inflammatory drugs (NSAID) from BIOMOL Research Laboratories, Inc. was dissolved to DMSO (16 mM), diluted to 16 μM with the medium, warmed, and 143 μl was added gently to 1 ml medium to make a final concentration 2 μM and 0.0125% DMSO. Cycloheximide (Sigma C7698) was dissolved similarly to DMSO at 1 mM, diluted with medium to make 50 μM, and 250 μl was added gently to 1 ml medium to make a final concentration 10 μM in 0.02% DMSO. Stock solution of 0.5 M EGTA, Ethylene Glycol-bis (β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid (Sigma E4378) adjusted to neutral pH, was directly dissolved into the medium to make 5 mM. Protocols for the ultrasound treatment and the inclusion of modulators were summarized in Fig. 1. Ultrasound was generated with the transducer (effective area for each well is 3.88 cm^2); the Sonic Accelerated Fracture Healing System (SAFHS, Exogen, Inc.) being operated at 1.5 MHz frequency in a pulsed-wave mode (0.2 s pulse burst width with repetition frequency of 1 kHz at the intensity of 30 mW/cm^2). Control samples were prepared in the same manner, without exposure to the ultrasound.

RT-PCR analysis. Total RNA from the culture was extracted at each time point using ISOGEN from Nippon Gene Co. (Tokyo, Japan) using the manufacturer's instructions. Each RT reaction mixture contained 1 μg total RNA, 50 pmol random 9-mer and 200 U Superscript II reverse transcriptase (GIBCO BRL) in a total volume of 20 μl. After denaturation of mRNA at 70°C for 10 min, the reactions were preincubated for 10 min at 25°C and incubated at 42°C for 50 min. After denaturation at 70°C for 15 min, portions; 0.2–1 μl of the RT product cDNA were amplified using Ready To Go PCR Beads (Pharmacia Biotech AB. Uppsala, Sweden) containing ~1.5 U *Taq* DNA polymerase. Unless otherwise noted, 12.5 pmol primers, sense and antisense, were added and the following profile was used: 1 cycle at 94°C for 3 min, followed by set cycles of: 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min. All primers used are listed in Table 1.

TABLE 1

Oligodeoxynucleotide Primers Used for PCR

Target cDNA	Primer sequence (5′-3′)	Product size (bp)	PCR cycles
GAPDH[a]	5′: CACCATGGAGAAGGCCGGGG 3′: GACGGACACATTGGGGGTAG	418	18
c-fos (32)*	5′: CTTTGGATCCGATCTGTCCGTCTCTAGTGC 3′: TTGCAAGCTTGTCGGTGGGCTGCCAAAATA	471	28
TGF-β1[b]	5′: AAGTGGATCCACGAGCCCAA 3′: GCTGCACTTGCAGGAGCGCA	245	20
Osteocalcin[b]	5′: TCTGACAAACCTTCATGTCC 3′: AAATAGTGATACCGTAGATGCG	198	28
IGF-I (33)*	5′: CAAGCCCACAGGCTATGGC 3′: TCTGAGTCTTGGGCATGTCAG	179	28
IGF-II (34)*	5′: CAGCGGCCTCCTTACCCAACT 3′: GAGGTAGACACGTCCCTCTCG	321	30
BSP (35)*	5′: CTACTTTCTTTATAAGCATG 3′: CATCTCCAGCCTTCTTGGGC	308	30

[a,b] Reported primer sequences in Ref. 30 or 31 were used, respectively.
* Primers were constructed according to the reported sequences.

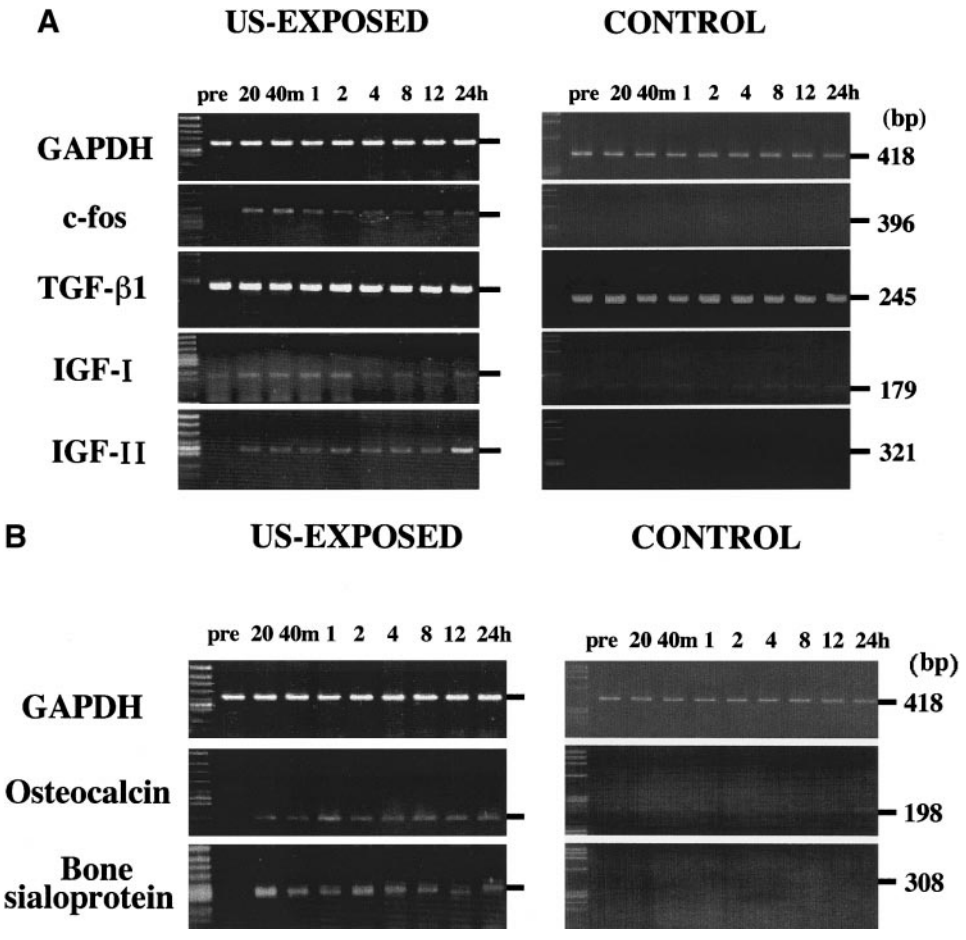

FIG. 2. Messenger RNA levels of c-fos, growth factors, and bone matrix proteins after 20-min exposure of ST2 cells to low-intensity, pulsed ultrasound. Total RNA was extracted at time points depicted in Fig. 1: 1, 0 min (control); 2, 20 min; 3, 40 min; 4, 1 h; 5, 2 h ; 6, 4 h; 7, 8 h; 8, 12 h; 9, 24 h, subjected to RT-PCR with primers listed in Table 1 as described under Materials and Methods. In both A and B of electrophoretic patterns, GAPDH was used as an internal standard. Vehicles were not exposed to ultrasound under otherwise identical conditions. Sizes of amplimers for GAPDH, c-fos, TGF-β1, IGF-I and II (A) and GAPDH, osteocalcin, and BSP (B) are indicated on the right.

RESULTS

Transient expression of an immediate-early gene, c-fos, was confirmed immediately after the 20-min exposure to ultrasound, and showed the highest value at 40 min which subsequently subsided by 4 h (Fig. 2A). The elevated transient expression of cox-2 message was also observed (data not shown). In addition to these typical immediate early genes, elevated mRNA levels were observed in many bone proteins such as IGF-I (Fig. 2A), osteocalcin and bone sialoprotein, BSP (Fig. 2B). These messages are apparently expressed during the 20-min ultrasound exposure and exhibit broad appearance thereafter. Osteopontin and IGF-II seem to be already expressed to some extent prior to the exposure, and is not clear whether they are upregulated immediately after the exposure. TGF-β does not seem to respond to the ultrasound at all. All the upregulated bone protein mRNAs (not c-fos) have second peaks at 24 h or earlier (Figs. 2A and 2B).

To examine whether the initial appearance of osteocalcin mRNA is of immediate-early expression of the osteocalcin gene or not, cycloheximide was added prior to the ultrasound exposure (Fig. 3). While the initial elevation at 20 and 40 min occurred regardless of the presence or absence of cycloheximide, an inhibitor of protein synthesis, the drug significantly lowered later mRNA levels.

The second peak of osteocalcin at 8 h was then examined for its sensitivity to NS398. Figure 4 shows that the upregulated transcription returns to the pre-exposure level while the first peak at 1 h was not affected at all by the presence of NS398.

DISCUSSION

This study described that ultrasound upregulated osteocalcin and IGF-I mRNAs in a biphasic manner in ST2 cells of bone marrow origin. In our previous re-

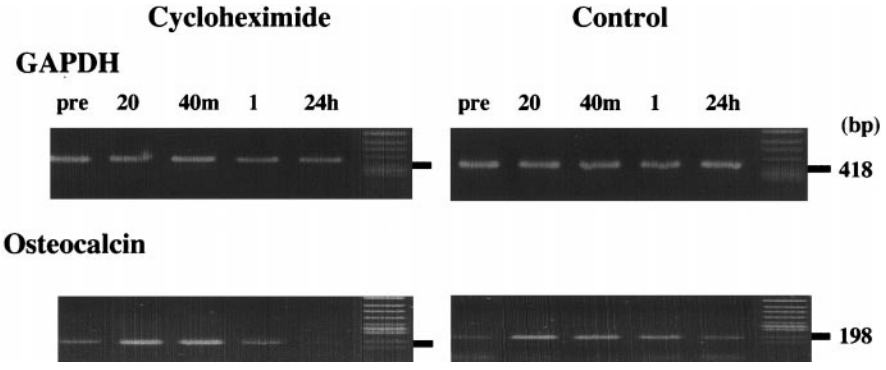

FIG. 3. Effect of cycloheximide on the upregulation of osteocalcin mRNA level by the ultrasound. ST2 cells were exposed to ultrasound after 10-min pretreatment with 1 mM cycloheximide (see Fig. 1). RT-PCR experiments were carried out as in Fig. 1, and osteocalcin and GAPDH amplimers were electrophoresed.

ports we demonstrated that young osteocytes, dendritic bone-derived cells, transduce the signals of mechanical loading. This mechanotransduction in bone is of a complex nature, being influenced by many modulators such as PTH, prostanoids and extracellular Ca^{2+} (10–12). Subsequent anabolic responses include an early response of cAMP secretion and late responses such as the production of IGF-I and osteocalcin proteins (13). Upregulation of c-fos, cox-2, i-nos, IGF-I and osteocalcin message levels were also reported (14). To date, however, it is unclear as to exactly which form of mechanical stimuli triggers these responses. Some candidates are: shear stress or streaming potentials possibly sensed by the cell surface machinery (15–18), direct deformation of cells possibly transmitted through ion channels (11, 19, 20) or cytoskeletal reaction (21, 22). The precise pathways of mechanical signal transduction are not characterized either. To delineate the distinct responses of osteoblastic cells to stretching, several bone cell lines such as MC3T3 E1, 10T1/2, ST2, and ROS17/2.8 cells were examined by Northern blot analysis of mRNAs of such proteins as osteocalcin, osteopontin, osteonectin, alkaline phosphatase and BMP-4, bone morphogenic protein-4 (23). It should be noted that the ST2 cell line was the only sensitive cell line among the above four in terms of anabolic reaction to strain at a physiological level. Bone marrow cells, which differentiate to chondrocytes and osteoblasts, are certainly involved in the processes of fracture healing (24, 25).

In this report, experiments were done using ST2 cells with pulsed ultrasound, instead of stretch loading, as the method of anabolic stimulation. The noninvasive nature of ultrasound provides for many advantages in practical applications. Provided that ultrasound has anabolic effects, local stimulation of bone formation will become possible with or without the aid of anabolic drugs such as PTH whose efficacy is potentiated by mechanical stress (11, 12).

During a 24-h period after the exposure to ultrasound in the first 20 min, we first examined mRNA levels of an immediate-early gene, c-fos, and other bone protein genes such as osteocalcin, osteopontin, BSP as well as those of IGF-I, II and TGF-β. The initial elevation of osteocalcin mRNA observed at 20 and 40 min is of immediate-early expression since cycloheximide, an inhibitor of new protein synthesis, did not affect the mRNA level (Fig. 3). Second, the effects of an NSAID

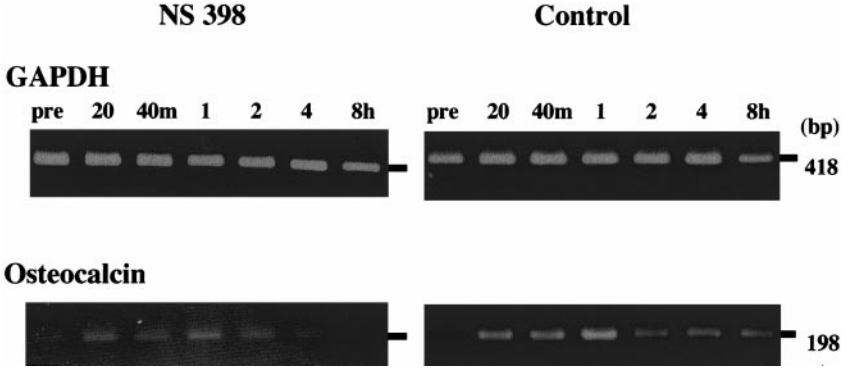

FIG. 4. Effect of NS398 on the upregulation of osteocalcin mRNA level by the ultrasound. ST2 cells were pretreated with 2 mM NS398 instead of cycloheximide. All other conditions were similar to those in Fig. 3.

(nonsteroidal anti-inflammatory drug) NS398, which is a specific inhibitor of inducive type PGHS2 (COX-2) encoded by the cox-2 gene, were tested on the messages described in the first experiment. By exposing MC3T3-E1 cells to ultrasound under the same conditions, Kokubu *et al.* reported that upregulation of cox-2 gene accompanied by the release of PGE_2 (6). Our results further confirm previous findings that mechanical loading in the physiological range can induce bone formation in two different ways: by direct anabolic reactions synthesizing matrix proteins such as osteocalcin (13, 14), and by indirect effects on osteoblastic cells through increased IGFs, PGHS and NOS which are known to lead to proliferation of osteoblasts and bone formation (26, 27). Namely, the upregulation of osteocalcin and IGFs by low-intensity pulsed ultrasound also occurs in a biphasic manner (Figs. 2–4). Interestingly, these second peaks seem to be dependent on the preceding PGHS2 expression, as shown in Fig. 4. It is likely that PGHS2 is inhibited by NS398 included in the medium during the incubation period, so that not as much prostanoids are synthesized to amplify the anabolic effect by inducing secondary PGHS2 and bone proteins. Therefore, the secondary induction of osteocalcin at 24 h, for example, is minimal. Amplification of PGHS2 has been reported by Raisz *et al.* in osteoblastic cell lines (28). A previous report by Chow and Chambers demonstrated a similar *in vivo* expression of mRNAs for IGF-I in a biphasic manner. In their experiment, indomethacin did not affect an early expression of c-fos mRNA while eliminating the upregulated transcription of IGF-I mRNA at 8 h (29).

In this article, we have clearly demonstrated that the low-intensity pulsed ultrasound induced anabolic responses of a nature similar to that in physically loaded bone. That the noninvasive ultrasound functions as a kind of osteogenic mechanical stimulus suggests many potential clinical applications other than fracture healing.

ACKNOWLEDGMENTS

We are grateful to T. Tominaga, M.D., for his editorial assistance. We also thank K. Kokubu and R. Shinntani for their technical assistance. This investigation was supported in part by grants-in-aid from the Ministry of Science, Education and Culture of Japan to Y.M.-T. and M.I.

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