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Effect of Low- and High-Intensity Pulsed Ultrasound on Collagen Post-translational Modifications in MC3T3-E1 Osteoblasts

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Abstract. Different intensities of pulsed ultrasound have distinct biological effects on bone mineralization in the process of bone fracture repair, even across a narrow range (e.g., 30–120 mW/cm^2). The aim of our study was to elucidate the effect of low-intensity (30 mW/cm^2) and high-intensity (120 mW/cm^2) pulsed ultrasound on collagen metabolism by using MC3T3-E1 osteoblasts. Of special interest was the relationship between posttranslational collagen quality and prostaglandin E_2 activity. Cells with or without a cyclooxygenase-2 inhibitor, NS398, were exposed every day for four consecutive days to high-level or low-level intensities of pulsed ultrasound. We examined the, expression patterns of cyclooxygenase-2, lysyl oxidase, telopeptidyl lysyl hydroxylase (TLH), and helical lysyl hydroxylase by real-time polymerase chain reaction analysis. Quantitative analyses of reducible immature and nonreducible mature cross-links were also performed. Ultrasound at 30 mW/cm^2 upregulated TLH messenger RNA (mRNA) expression and enzyme activity compared to the control and resulted in increased relative concentrations of telopeptidyl hydroxylysine–derived cross-links. In addition to upregulated lysyl oxidase mRNA expression, increased total reducible and nonreducible cross-links were observed by 30 mW/cm^2 exposure compared to the control. In contrast, ultrasound at 120 mW/cm^2 had no obvious effect on collagen metabolism owing to high levels of endogenous prostaglandin E_2 induced by ultrasound. Our results showed that (1) low-intensity, but not high-intensity, ultrasound may accelerate the formation of the unique molecular packing of collagen fibers conducive to bone mineralization and that (2) the high dose of endogenous prostaglandin E_2 induced by pulsed ultrasound may be detrimental to calcifiable cross-link formation.

Key words: Ultrasound — Prostaglandin E_2 — Collagen — Cross links — Lysine hydroxylation — MC3T3-E1 cells

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Different intensities of pulsed ultrasound have distinct biological effects on bone mineralization *in vitro* [1–3] and *in vivo* [4, 5]. In an experiment using a rat femur fracture model, Yang et al. [5] found that low-intensity (50 mW/cm^2) pulsed ultrasound (LIPUS) increased the average maximum torque and torsional stiffness compared to the control group, whereas higher intensity (100 mW/cm^2) pulsed ultrasound had no significant effect on fracture callus maturation. Parvizi et al. [6] also reported that calcium influx to chondrocytes was rapid and transient at low intensity (50 mW/cm^2) LIPUS, but rapid and sustained at higher ultrasound exposures (>100 mW/cm^2). These results suggest that different intensities of ultrasound may have distinct effects, even across a narrow range (e.g., 30–120 mW/cm^2, as employed in this study).

A possible mechanism of LIPUS on the promotion of fracture healing is postulated to involve mechanical stress to bone cells in the form of acoustic pressure waves [7, 8]. We demonstrated in a recent study that physiologic levels of mechanical strain to osteoblasts may regulate the formation of calcifiable collagenous matrix into a specific molecular packing arrangement through the formation of characteristic types of cross-links as mineralization begins [9]. Thus, the intensity-related, distinctive effect of LIPUS on the synthesis of calcifiable collagen may be at least partly due to the altered regulation of collagen post-translational modification. A favorable arrangement of intrafibrillar collagen molecules via the formation of a specific cross-linking pattern plays an important role in proper mineralization [10–12] and callus calcification in the fracture bone repair process [13]. The cross-linking sites in collagen molecules are mainly lysine (Lys) or hydroxylysine (Hyl) residues. Type I collagen heterogeneity is attributed chiefly to quantitative differences in the conversion of Lys to Hyl residues [14–16]. Procollagen-lysine, 2-oxyglutarate, 5-dioxigenase 1 (PLOD1, LH1) and PLOD2 (LH2) are assumed to be crucial enzymes for hydroxylation of triple helical

regions (helical lysyl hydroxylase, HLH) or telopeptides (telopeptidyl lysyl hydroxylase, TLH) of collagen molecules, respectively [17, 18]. Cross-linking is initiated only after specific Lys or Hyl residues in the telopeptides are converted extracellularly by the lysyl oxidase enzyme into the aldehydes, allysine and hydroxyallysine, respectively [14, 19]. These aldehydes then react with the Lys or Hyl residues of the triple helix to give characteristic di- and tri-functional cross-links [20].

Several studies have demonstrated that prostaglandin E_2 (PGE_2) is a crucial early mediator of bone formation that can be induced by mechanical stress as well as by therapeutic-range ultrasound [3, 7]. PGE_2 also has biphasic effects, with low concentrations of PGE_2 increasing and high doses decreasing mineralized nodule formation in osteoblasts [21–25]. Interestingly, PGE_2 is assumed to be a negative regulatory factor for lysyl oxidase, which catalyzes collagen cross-linking [26]. However, little is known about the relationship between endogenous PGE_2 production induced by LIPUS and collagen cross-link formation in osteoblasts.

The aim of this study was to examine the effect of low-intensity (30 mW/cm^2) or high-intensity (120 mW/cm^2) pulsed ultrasound on qualitative and quantitative changes in collagen cross-linking and the correlation between cross-link patterns and endogenous PGE_2 levels induced by ultrasound exposure.

Materials and Methods

Cell Culture

Murine MC3T3-E1 osteoblasts, purchased from RIKEN Cell Bank (Tsukuba, Japan), were maintained in alpha minimal essential medium ($\alpha\pm$MEM) (GIBCO, Life Technology, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal calf serum (HyClone Laboratories, Logan, UT, USA), 50 μg/mL freshly prepared ascorbate (Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin, 50 μg/mL streptomycin at 37°C in an atmosphere of 95% humidified air, and 5% CO_2 . Cells (5×10^4 cells/cm^2) were seeded into a 6-well culture plate (Falcon, Becton Dickinson Labware, Frankin Lakes, NJ, USA) and were exposed to ultrasound from day 14 postconfluence. This MC3T3-E1 cell culture system has been found to be a successful tool in the study of regulatory aspects of normal collagen post-translational controls in the bone mineralization process, in that it appears to proceed via a process similar to that observed *in vivo* [9, 11, 27]. On the basis of previous reports [10, 11], at day 14 postconfluence, our culture system generates cell multilayers surrounded by type I collagen, which closely mimics the collagen-rich extracellular matrix of normal tissue. Selection of observation points after day 14 postconfluence was supported by the following observations: (1) Parvizi et al. [6] showed that earlier treatment with ultrasound caused considerable detachment of cells; (2) cells are assumed to sense the mechanical stress through their attachment to extracellular matrix *in vivo*.

Exposure to Ultrasound

A modified clinical device, which was previously reported by Ito et al. [28] and specially designed for a 6-well tissue culture plate (Sonic Accelerated Fracture Healing System (SAFHS); Exogen Inc., NJ, USA), was used as the ultrasound exposure system. The pulsed ultrasound signal consisted of a 1.5-MHz, 200-ms burst sine wave with repeating pulsation at 1.0 kHz, delivered at an intensity of 30 or 120 mW/cm^2 spatial and temporal average (SATA). After the application of ultrasound treatments for 20 minutes per day for four consecutive days, cell layers were washed three times with phosphate-buffered saline (PBS) and collected for analyses of collagen post-translational modification. For analyses of gene expressions involved in cross-link formation, cell layers were harvested at each time-point from 0 to 24 hours after the first ultrasound exposure at day 14 postconfluence. Control (unexposed) samples were subjected to the same manipulations under the same conditions but without ultrasound exposure. In a preliminary study, we examined whether ultrasound exposure affected the temperature and pH of the condition medium. We found these parameters to be identical to those of the control culture medium.

Prostaglandin E_2 and Cyclooxygenase Inhibitor

The time-dependent accumulation of PGE_2 in medium for up to 24 hours after ultrasound exposure was determined by using a commercially available ELISA kit (Prostaglandin E_2 Immunoassay, R & D System, Minneapolis, MN, USA). To investigate whether PGE_2 production induced by exposure to pulsed ultrasound (30 or 120 mW/cm^2) was mediated by changes in cyclooxygenase (COX) activity, NS398 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA), a selective inhibitor of prostaglandin H (PGH) synthase-2 over PGH synthase-1, was used to block COX-2 activity as described by Naruse et al. [7]. NS398 was added to the medium before the 1 hour equilibration time-period to allow time for this inhibitor to diffuse into the cell layer prior to beginning the ultrasound regimen from day 14 postconfluence for four consecutive days. We used NS398 at a concentration of 1.5 μM, since the inhibitory concentration $(IC)_{50}$ range of NS398 for COX-1 is 75 μM to >100 μM and for COX-2 the IC_{50} range of NS398 is 0.1–1.77 μM [29, 30].

Total RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from harvested cell layers at each time-point from 0 to 24 hours after ultrasound exposure using TRIzol reagent (GIBCO-BRL, Grand Island, NY, USA) according to the manufacturer's protocol. Conditions for reverse transcription-polymerase chain reaction (RT-PCR) and the primer for collagen $\alpha\pm1$ (COL1A1), lysyl oxidase, telopeptidyl Lys hydroxylase (TLH, PLOD2), helical Lys hydroxylase (HLH, PLOD1), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) are described elsewhere [9]. Amplification products were run on 2.0% agarose gels and stained with ethidium bromide to visualize bands. Real-time PCR was performed to allow the relative quantification of lysyl oxidase, TLH, HLH, and GAPDH mRNA expression by using the LyghtCycler™ system with SYBR® Green I (QuantiTect SYBR Green RT-PCR kit, QIAGEN Inc, Hilden, CA, USA). FRG, as previously described [9]. The primer set for COX-2 genes was derived from Kokubu et al. [31]. Products were quantified using a standard curve that correlated each cycle number of linear-phase amplification with a value. Index mRNA levels were assessed using a threshold cycle (Ct) value. The relative expression of each target mRNA was computed from the target Ct values and the GAPDH Ct value using the standard curve method. To control for variability in amplification due to differences in starting mRNA content, GAPDH was used as internal standard. Results are given as mean ± standard deviation (SD) of six independent experiments, normalized against the mean control value.

Isolation of Cell-Matrix Fraction Containing Insoluble Collagen

Cell layers were lysed with a lysis buffer (0.1 M Tris-HCl, 0.125 M NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate) (Sigma-Aldrich), and a cocktail of protease inhibitors, including 0.5 mM phenylmethanesulfonylfluoride, 5 mM benzamidine, 2 mM pepstain A, and 1 mM leupeptin for 30 minutes at 4°C. Lysates were centrifuged at 3000 *g* for 30 minutes at 4°C and insoluble residues were washed three times with cold distilled water and then lyophilized. This lyophilized fraction was designated as the cell-matrix fraction. We carried out amino acid analysis of this insoluble fractions and found that relative amounts of glycine of this insoluble fraction were in the range of 298–310 per 1000 amino acids and > 70 residues hydroxyproline per 1000 amino acids, indicating that the insoluble fraction consists of predominant collagenous matrix. The total amount of collagen in the cell-matrix fraction was calculated by the hydroxyproline (Hyp) assay by using high-performance liquid chromatography (HPLC), based on the assumption that collagen weighs seven-and-a-half times the measured Hyp weight, with a molecular weight of 300,000 [32]. The total amount of collagen was normalized per dish and per cellular DNA. The total cellular DNA content was measured by adding fluorochrome dye Hoechst 33258 (Pharmacia Biotech, Tokyo, Japan) [33].

Pulse Labeling, for Analysis of Newly Formed Cross-Link of Collagen

For analysis of kinetics of collagen cross-link formation after ultrasound exposure, day 14 postconfluence cell cultures were labeled with 40 μCi [^{3}H] Lys (40-100 mCi/mmol, Moravec Biochemical, Inc., Brea, CA, USA) per 1 well for 12 hours before the first ultrasound exposure. This experiment followed the metabolic fate of newly formed radiolabeled cross-links. Labeling was performed in medium lacking serum and Lys but supplemented with 50 μg/mL ascorbate. The medium was then removed and replenished with fresh medium as described previously [9, 10].

Characterization of Collagen Cross-Links

The reduction of the cell layer matrix with sodium borohydride ($NaBH_4$) (Sigma-Aldrich) and subsequent quantification of cross-links was carried out by using HPLC as previously described [32]. Reducible immature cross-links (deH-DHLNL, dehydro-dihydroxylysinonorleucine; deH-HLNL, dehydro-hydroxylysinonorleucine; and deH-LNL, dehydro-lysinonorleucine) were identified and quantified according to their reduced forms (DHLNL, HLNL, and LNL, respectively). Reducible cross-links and common amino acids, such as Hyp, Hyl, and Lys, were detected with Ortho-phthalaldehyde derivatization by the postcolumn method, whereas nonreducible mature cross-links, such as pyridinoline (Pyr) and deoxypyridinoline (Dpyr), were detected by natural fluorescence. The content of each cross-link was expressed as mol/mol of collagen. In a preliminary study, we attempted to determine the concentration of histidino-hydroxymerodesmosine (HHMD), which is mainly distributed in skin, ligament, and tendon [34], in cell layers by a authenticated NaB^3H_4 labeling method [11, 35]. A very low level of HHMD was detected in control and LIPUS-exposed cultures. Kuboki et al. [11] showed the existence of a relatively low level of HHMD during the course of calcification in MC3T3-E1 cells. We used the medium including ascorbate and purified collagenous protein from cell layer matrix for analysis of the cross-links, whereas Kuboki et al. used the medium without ascorbate and analyzed the cell matrix fraction including relatively high amount of noncollagenous protein (approximately 50 to 70%) for analyses of the cross-links. Therefore, one reason for the present finding of lower concentrations of HHMD compared with the results reported by Kuboki et al. [11] and our present data may be different media conditions or extraction procedure described previously, although the implication needs further consideration.

Lysyl Oxidase Enzyme Activity

Lysyl oxidase enzyme activity was measured in cell layers and conditioned media by using murine calvaria bone collagen labeled with [^{3}H] Lys (250 μ Ci) (Moravec Biochemical, Inc., Brea, CA, USA) by using standard methods [36, 37]. Briefly, 0.5-mL aliquots of conditioned media and cell lysates (0.5 mL) extracted by 1 mL of lysis buffer (0.02 M boric acid, 4 M urea, 0.15 M NaCl, pH 8.0) were prepared after ultrasound exposure. Cell lysate was dialyzed with 0.02 M boric acid, 0.15 M NaCl, pH 8.0 for elimination of urea before assay.

Samples were analyzed in a final volume of 1.0 mL, containing 380,000 disintegrations per minute (dpm) tritiated collagen in the presence and absence of 5×10^{-4} M β-aminopropionitrile. Reactions were incubated for 90 minutes at 37°C followed by distillation under vacuum. Units were defined as disintegrations per minute released over and above β-aminopropionitrile only. All experiments were repeated six times for each biochemical analysis to confirm the reproducibility of the results.

Lys Hydroxylation of Collagen

The level of Lys hydroxylation in the $\alpha 1$ and $\alpha 2$ chains of type I collagen, including or excluding telopeptides, was analyzed according to Uzawa et al. [12] with some modifications [9]. Briefly, to estimate the level of Lys hydroxylation in the collagen molecules, including both nonhelical (telopeptides) and helical domains, cell-matrix fractions were extracted by 0.5 N acetic acid for 2 days at 4°C with gentle agitation. For the analysis of Lys hydroxylation in collagen molecules excluding telopeptides, the helical region of collagen was isolated by digesting cell-matrix fractions with pepsin (Worthington, Freehold, NJ, USA). In a preliminary study, we estimated the solubilities of the cell-matrix fractions after acid and pepsin treatment by hydroxyproline assay in both the soluble and insoluble fractions after treatments [32]. The solubilities of cell-matrix fractions after acid and pepsin treatment were 34% to 38% and 56% to 60% of cell-matrix fractions, respectively. Dried samples were subjected to 5% SDS-PAGE under nonreducing conditions, transferred onto Trans-Blot Transfer membranes (BioRad Laboratories, Inc., Hercules, CA, USA), and stained with 0.25% Coomassie Brilliant Blue (Wako, Tokyo, Japan). Visualized bands corresponding to type I collagen $\alpha 1$ and $\alpha 2$ chains were excised, hydrolyzed, and subjected to amino acid analysis by using established methods [32]. The purity and identity of the type I collagen chains were assessed by amino acid analysis of the cell-matrix fractions (relative amounts of glycine in the range of 322–333 per 1000 ammo acids and > 80 residues hydroxyproline per 1000 amino acids). The extent of Lys hydroxylation was expressed as the ratio of Hyl/(Lys + Hyl) [38].

Lysyl Hydroxylase Enzyme Activity

Determination of TLH and HLH enzyme activities was performed according to the method of Miller [39] by using murine calvaria bone protocollagen labeled with [^{3}H] Lys. Briefly, cell-matrix fractions were homogenized in lysis buffer (0.2 M NaCl, 0.1 M glycine, 50 mM dithiothreitol, 0.1% (w/v) Triton-X, and 20 mM Tris-HCl, pH 7.5) and the homogenates were analyzed in a final volume of 1.0 mL containing 220,000 dpm tritiated protocollagen. Samples were incubated for 2 hours at 30°C, after which NaCl was added to a final concentration of 0.7 M. Precipitates were collected by centrifugation at 25,000 *g* for 30 minutes. Precipitates with or without pepsin treatment were used to estimate the activity of the tritiated Hyl in the

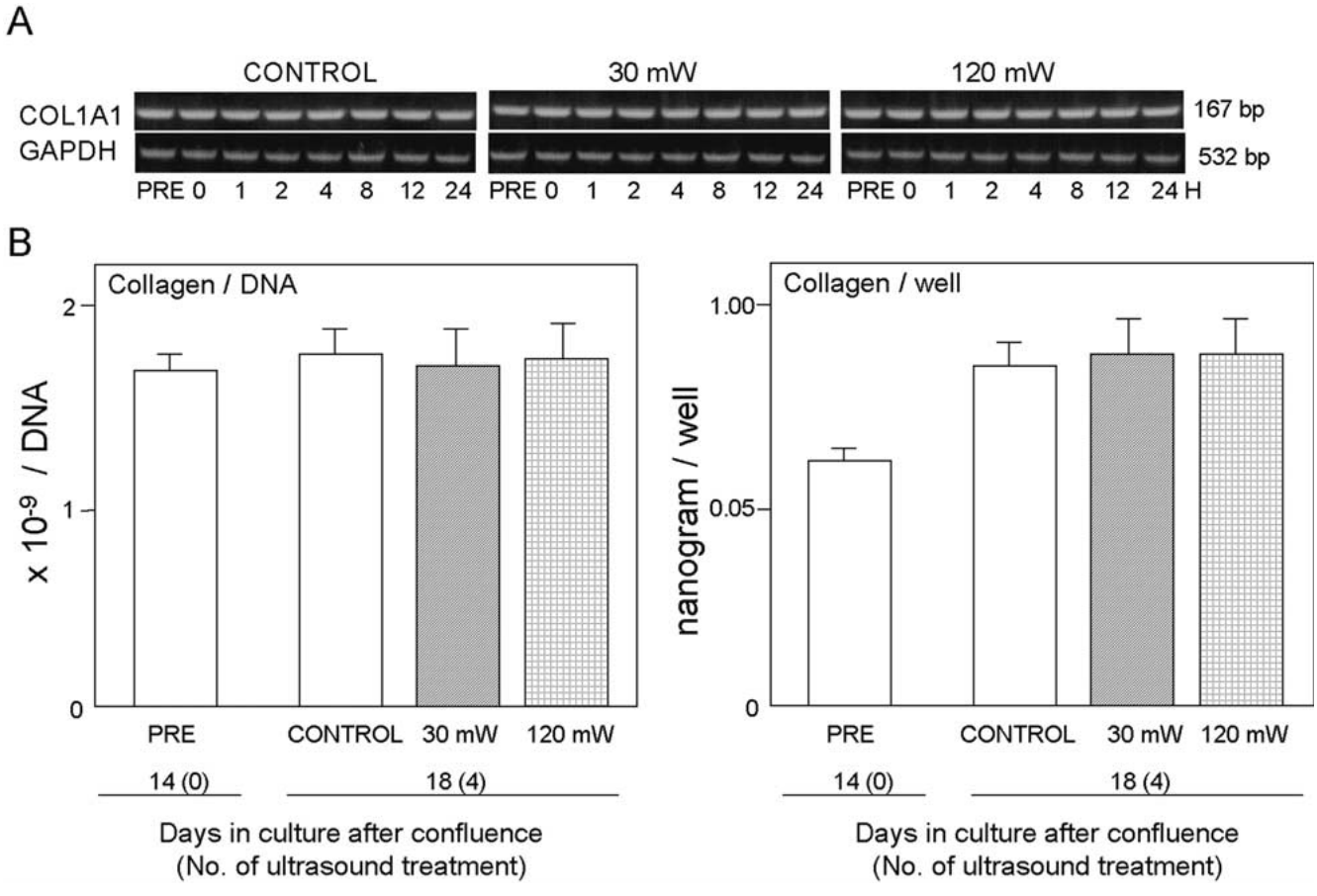

Fig. 1. Effect of pulsed ultrasound at an intensity of 30 or 120 mW/cm^2 on COL1A1 mRNA levels after 20 minutes exposure (**A**) and collagen accumulation in cell layers after 20 minutes exposure daily for four consecutive days (**B**). Total RNA was extracted at pre-exposure (PRE) and postexposure at time-points from 0 to 24 hours and subjected to RT-PCR. Amplification products were run on 2.0% agarose gels and stained with ethidium bromide to visualize bands. GAPDH amplification was used as an internal standard. Data represent the mean ± SD of six independent cultures.

presence or absence of collagen molecule telopeptides, respectively. HLH enzyme activity was estimated by determining ^{3}H-Hyl activity in the collagen helical domains, whereas TLH enzyme activity was assessed by subtracting the activity of ^{3}H-Hyl in collagen helical domains from that of the sum of nonhelical and helical domains [40]. Quantitative determination of enzyme activity as units of disintegrations per minute of Hyl was carried out by HPLC after acid-hydrolysis of the fractions according to established methods [31]. The enzymatic activities of TLH and HLH were repeated six times for each biochemical analysis.

Statistical Analysis

All experiments were repeated six times for each biochemical analysis to confirm the reproducibility of the results. Data were expressed as mean ± SD. Results were analyzed using single-factor analysis of variance (ANOVA) and Student's *t* test for unpaired samples. Statistical significance was set at $P < 0.01$.

Results

Pulsed ultrasound treatment at 30 or 120 mW/cm^2 had no obvious effect on collagen synthesis or accumulation in cell layers at both the mRNA and protein levels (Fig. 1). There were no differences in cell number, as estimated by total cellular DNA per culture, between the control cultures and ultrasound-treated cultures (data not shown).

COX-2 Gene Expression and PGE_2 Production

Figure 2A shows COX-2 mRNA expression at each time-point up to 24 hours after ultrasound exposure to cells without the COX-2 inhibitor, NS398. PGE_2 time-dependent total accumulation in media up to 8 hours after ultrasound exposure with or without the COX-2 inhibitor (Fig. 2B). Cells without COX-2 inhibition, both the 30 and 120 mW/cm^2 exposures induced significant upregulation in COX-2 mRNA expression, up to 2.9-fold and 5.9-fold higher, respectively, compared to the control cells at 2 hours (Fig. 2A). This increase in COX-2 expression was associated with a significant increase in the time-dependent accumulation of PGE_2

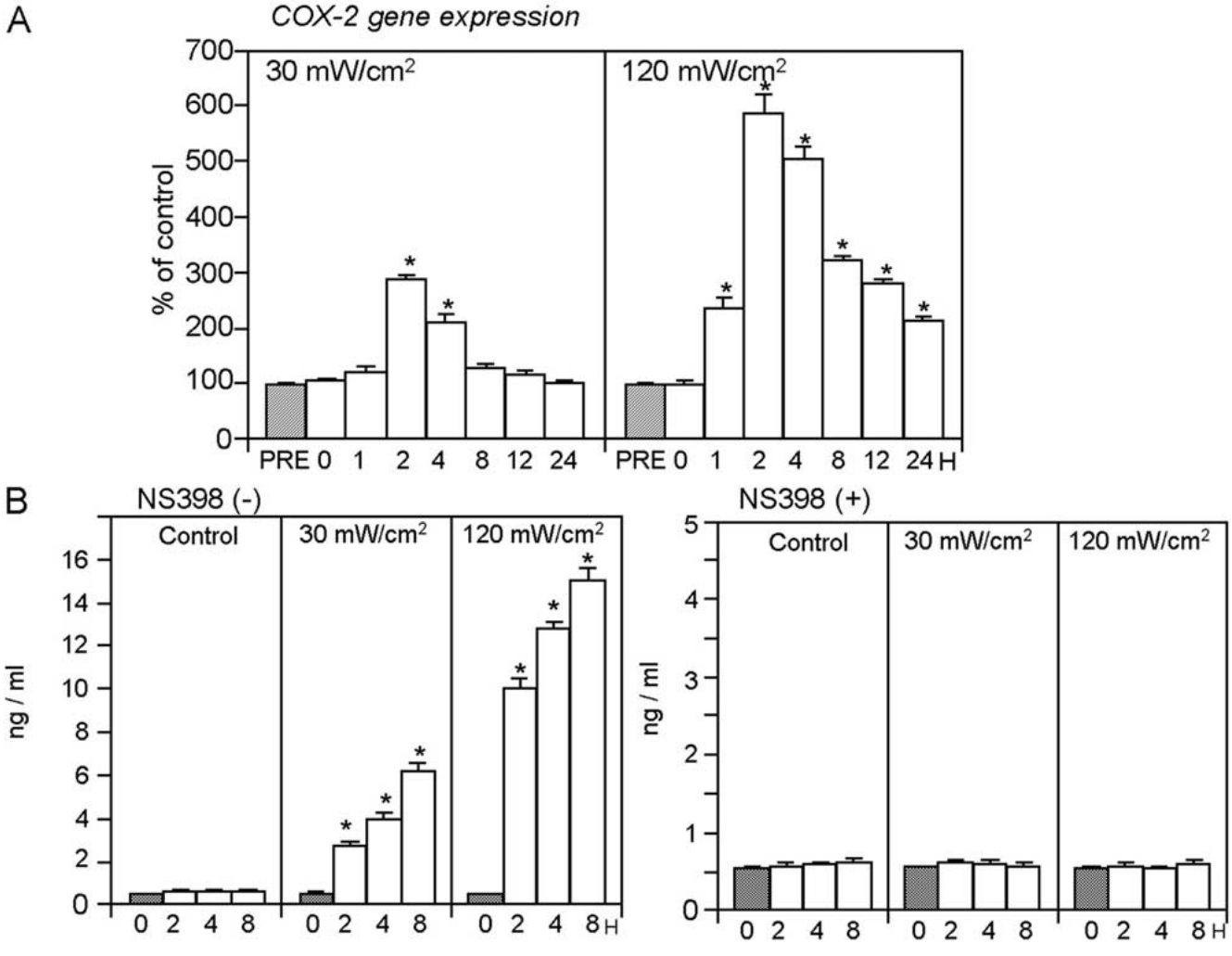

Fig. 2. Effect of pulsed ultrasound at an intensity of 30 or 120 mW/cm^2 on relative COX-2 mRNA levels (**A**) and PGE_2 concentrations in the medium from cells with or without the COX-2 inhibitor, NS398 (**B**). Messenger RNA levels of COX-2 were estimated by real-time PCR analyses at each time-point from 0 to 24 hours after ultrasound exposure. Values are expressed as mean ± SD of six independent experiments, normalized against the average control value. The asterisk represents a significant increase ($P < 0.01$) in relation to the control culture.

in medium without COX-2 inhibition, as reflected by the persistent, upregulated mRNA expression (Fig. 2B).

The time-dependent accumulation of PGE_2 in medium induced by ultrasound at 120 mW/cm^2 exposure was 3.7-, 3.2-, and 2.4-fold higher than that induced by 30 mW/cm^2 exposure at 2, 4, and 8 hours, respectively, without COX-2 inhibition (Fig. 2B). However, the increased PGE_2 accumulation following the 30 and 120 mW/cm^2 exposures was almost totally abrogated by pretreatment with NS398 at each time-point compared to cells without COX-2 inhibition (Fig. 2B), but there were no significant changes in PGE_2 production in control cultures at any time-point. In contrast, COX-1 mRNA expression was not affected by exposure to the pulsed ultrasound (data not shown).

Lysyl Oxidase and Cross-Links

Lysyl oxidase mRNA upregulation was first observed 2 hours after exposure to low-intensity (30 mW/cm^2) pulsed ultrasound, reached a peak of up to a 3.8-fold increase compared to control cells at 8 hours, and then subsided to a 1.7-fold increase compared to control cells at 24 hours without COX-2 inhibition. Although high-intensity pulsed ultrasound (120 mW/cm^2) increased lysyl oxidase mRNA expression up to 1.7-fold of the control value 2 hours after exposure, it did not affect subsequent gene expression as assessed by real time PCR analyses (Figs. 3 and 4A) without COX-2 inhibition.

Because the differences in lysyl oxidase mRNA expression levels were not great, lysyl oxidase enzyme activity and cross-link content were determined by

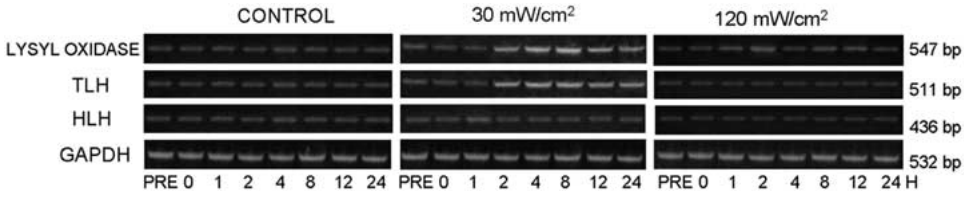

Fig. 3. Effect of pulsed ultrasound at low and high intensities (30 or 120 mW/cm^2, respectively) on lysyl oxidase, TLH, and HLH mRNA expression in cells without COX-2 inhibitor, NS398. Amplification products were run on 2.0% agarose gels and stained with ethidium bromide to visualize bands. GAPDH amplification was used as an internal standard.

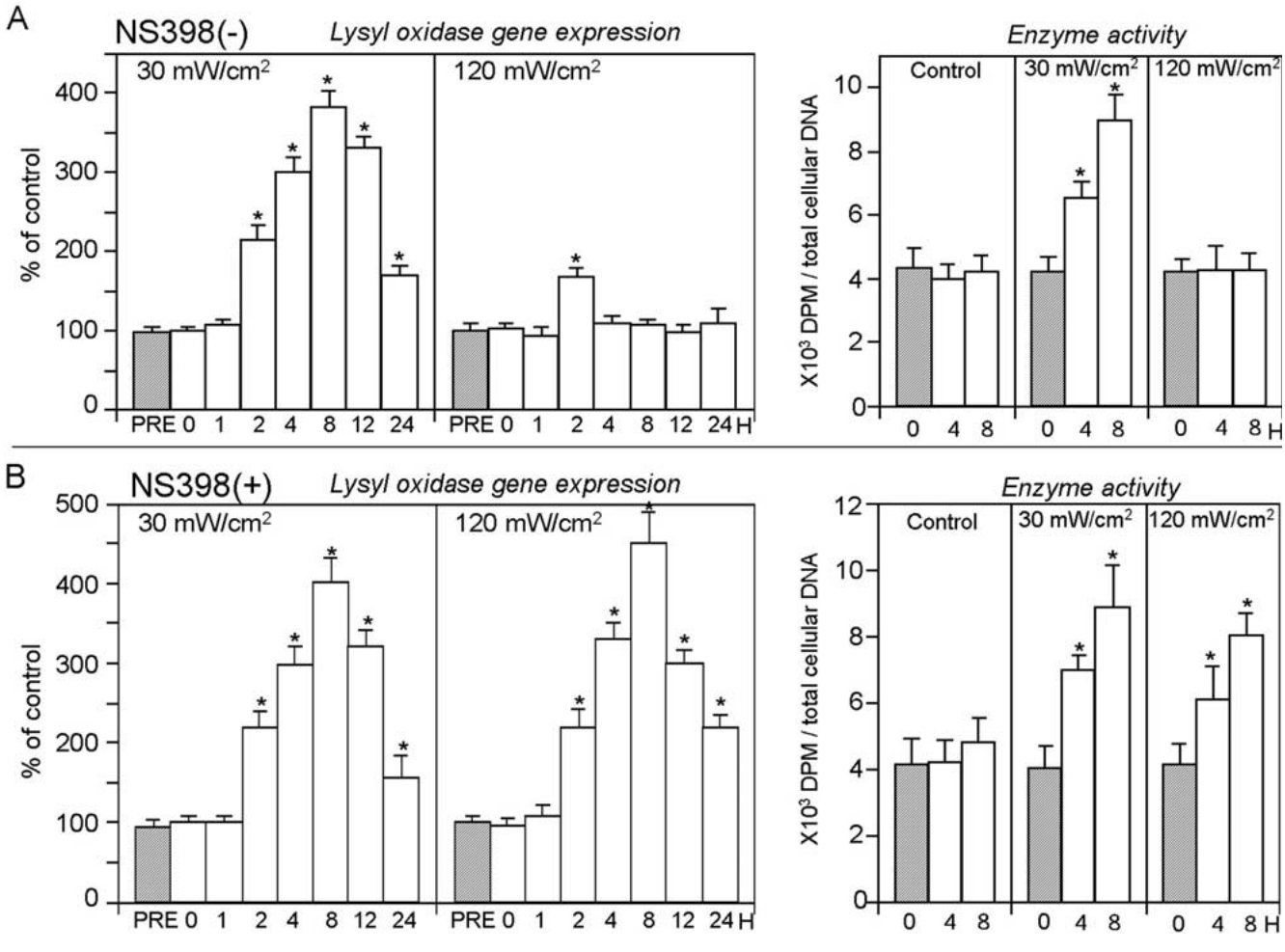

Fig. 4. Effect of pulsed ultrasound at low and high intensities (30 or 120 mW/cm^2, respectively) on relative lysyl oxidase mRNA levels and enzymatic activity in cells without (**A**) or with (**B**) the COX-2 inhibitor, NS398. Messenger RNA levels of lysyl oxidase were estimated by real-time PCR analyses at each time-point from 0 to 24 hours after ultrasound exposure. Values are expressed as mean ± SD of six independent experiments, normalized against the average control value. The asterisk represents a significant increase ($P < 0.01$) in relation to the control culture.

conventional methods. The upregulation of lysyl oxidase mRNA induced after exposure to 30 mW/cm^2 was associated with significantly increased enzyme activity, up to 1.63- and 2.12-fold higher than in control cells at 4 and 8 hours after LIPUS exposure, respectively. In contrast, 120 mW/cm^2 ultrasound exposure had no obvious effect on either lysyl oxidase mRNA expression or enzymatic activity (Fig. 4A). After 120 mW/cm^2 ultrasound exposure, lysyl oxidase mRNA expression and enzymatic activity levels dramatically increased to levels similar to those observed after 30 mW/cm^2 exposure with COX-2 inhibition. However, there were no differences in lysyl oxidase mRNA expression and enzymatic activity levels between cells with and without COX-2 inhibition after 30 mW/cm^2 exposure (Fig. 4B).

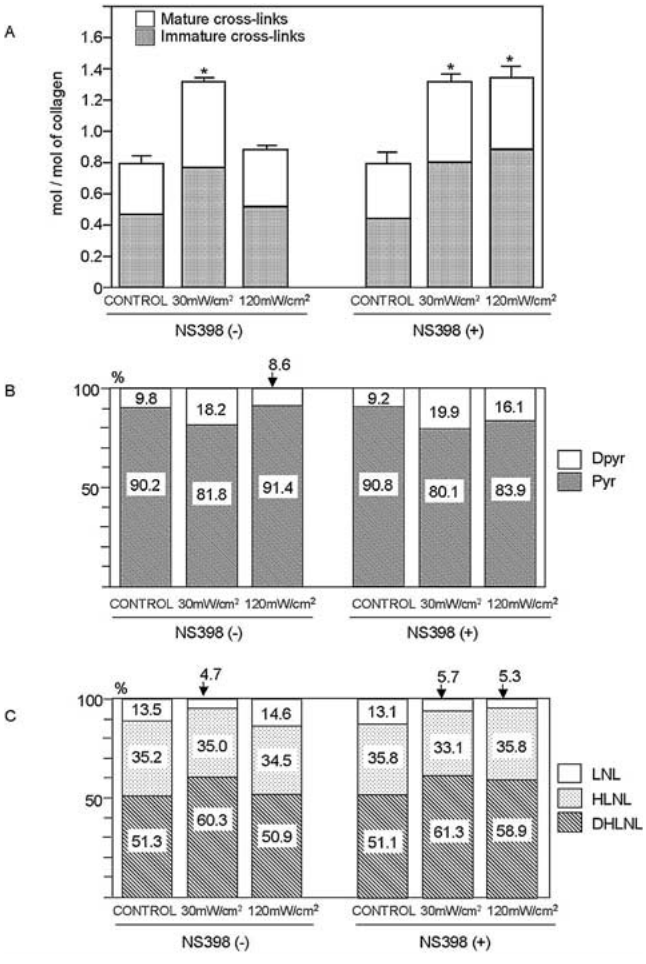

Fig. 5. (**A**) Effect of pulsed ultrasound at low and high intensities (30 or 120 mW/cm^2, respectively) on total collagen cross-links in cell layers after 20 minutes exposure daily for four consecutive days in cells with or without the COX-2 inhibitor, NS398. The total amount of cross-links in the cell-matrix fractions was determined by adding the total mature and immature cross-link contents expressed as mol/mol of collagen. Data are represented by the mean ± SD of six independent cultures. The asterisk represents a significant increase ($P < 0.01$) in relation to the control culture. Proportion of mature (**B**) and immature (**C**) cross-link contents with respect to total mature and immature cross-link contents, respectively.

It is of interest to note that, these findings were consistent with results obtained for total cross-link content (the sum of immature and mature cross-links). After 30 mW/cm^2 exposure on cells with and without COX2 inhibition, total immature cross-links increased 180% and 165%, respectively, and mature cross-links increased 146% and 167%, respectively, compared to control cells (Fig. 5A). After 120 mW/cm^2 exposure, cells pretreated with NS398 showed increased total cross-links, similar to those observed after 30 mW/cm^2 exposure, although there was no significant difference in total cross-links after 120 mW/cm^2 exposure in the absence of NS398 pretreatment (Fig. 5A).

Although we used the cells at day 14 postconfluence, there could be a significant background level of cross-links for 14 days before ultrasound treatment. Therefore, a biosynthetic study was performed to determine the metabolic fate of newly formed radiolabeled cross-links using ^{3}H-Lys from days 14 to 18 postconfluence in cells without COX-2 inhibition (results summarized in Table 1). An approximate doubling in content of mature cross-links and the half-life of reducible cross-links was observed after a 4-day exposure period (between culture days 14 and 18 postconfluence) in control cells. This physiological maturation period, which was reported by Gerstenfeld et al. [10] in osteoblasts, was consistent with that of the previously reported maturation time *in vitro*. Exposure to 30 mW/cm^2 resulted in increase in the tritium activity of reducible immature cross-links at day 15 (after 2 days from the first ultrasound exposure) and decrease in that of immature cross-links at day 18 postconfluence compared to control. On the other hand, a significant increase in the tritium activity of nonreducible mature cross-links after exposure to 30 mW/cm^2 was observed at day 18 postconfluence. There was no difference in ^{3}H-Lys incorporation to cross-links between 120 mW/cm^2 exposed culture and control culture. These results suggest that 30 mW/cm^2 exposure may stimulate the newly formed immature cross-links within 2 days and these newly formed immature cross-links were converted into mature pyridinium cross-links thereafter.

Lys Hydroxylation and Cross-Link Pattern

To elucidate the relationship between Lys hydroxylation in collagen and cross-link pattern after exposure to ultrasound, we analyzed not only lysyl hydroxylation in telopeptides and the helical portion of collagen and the proportion of various cross-links, but also LH gene expression and enzymatic activities.

In cells without COX-2 inhibition, semiquantitative RT-PCR and quantitative real-time PCR analyses showed that 30 mW/cm^2 exposure increased TLH mRNA expression (Figs. 3 and 6). The increase began 2 hours after LIPUS treatment, peaked at 4 hours with up to 2.9-fold higher mRNA levels compared to control cells, and subsided to 1.3-fold of control cells at 24 hours by real-time PCR analysis. Accompanying enzymatic activities were stimulated in a manner similar to that indicated by changes in mRNA expression. However, no differences in either TLH mRNA expression or enzyme activity were observed after 120 mW/cm^2 ultrasound exposure compared to control cells (Figs. 3 and 6A).

In contrast, TLH mRNA expression and enzyme activity in cells with COX-2 inhibition following 120 mW/cm^2 exposure were dramatically elevated, to a level similar to that observed after exposure to low-intensity (30 mW/cm^2) pulsed ultrasound (Fig. 6B). Exposure to

Table 1. Kinetics of cross-link formation

Days in culture		15	18
Numbers of ultrasound exposure		2	4
		Tritium activity incorporated from [^{3}H] lysine	
Total immature cross-links (DHLNL + HLNL + LNL)	Control	22.01	16.10
	30 mW	28.82[a]	12.31[b]
	120 mW	23.24	16.03
Total mature cross-links (Pyr + Dpyr)	Control	0.84	1.21
	30 mW	0.96	3.37[a]
	120 mW	0.87	1.40

Values are the averages of three separate determinations and represent the counts per minute for each cross-linked adduct relative to the cpm of hydroxylysine (normalized to 100) in each sample. NS398 nontreated cells were labeled with [^{3}H] lysine for 12 hours on day 13 before the first ultrasound exposure
Labeling was in medium lacking serum and lysine, and then cultures were continued in medium containing nonradioactive lysine until cell layers were harvested for analysis
DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; Pyr, pyridinoline; Dpyr, deoxypyridinoline
Significantly higher ([a]) and lower ([b]) mean than that of control culture ($P < 0.01$)

either 30 or 120 mW/cm^2 failed to change HLH mRNA expression (Fig. 3) or enzymatic activity in both cells with and without COX-2 inhibition (data not shown).

To assess the level of Lys hydroxylation in $\alpha\pm1$ and $\alpha\pm2$ type I collagen chains, first including the telopeptides and then excluding these regions after pepsin digestion, cell-matrix fractions with or without pepsin treatment were subjected to SDS-PAGE and each chain was analyzed for amino acid composition (Table 2). Compared to the control cells, exposure to 30 mW/cm^2 LIPUS resulted in significantly increased Lys hydroxylation of the intact $\alpha\pm1$ and $\alpha\pm2$ chains, including nonhelical domains (telopeptides), for both cells with and without COX-2 inhibition. Although 120 mW/cm^2 exposure also elevated Lys hydroxylation of the intact $\alpha\pm1$ and $\alpha\pm2$ chains in cells with COX-2 inhibition, no such effect was observed in the cells without COX-2 inhibition (Table 2). However, no significant differences in Lys hydroxylation levels of helical domains of $\alpha\pm1$ and $\alpha\pm2$ chains were observed between the ultrasound exposures (30 and 120 mW/cm^2) and the control cells (Table 2). These results suggest that the level of telopeptidyl Lys hydroxylation (except helical Lys hydroxylation) was elevated after exposure to low-intensity (30 mW/cm^2) pulsed ultrasound in both cells with and without COX-2 inhibition, and elevated to a similar degree in cultures exposed to high-intensity (120 mW/cm^2) pulsed ultrasound in cells after COX-2 inhibition.

The previously mentioned differences in Lys hydroxylation of $\alpha\pm1$ and $\alpha\pm2$ chains after ultrasound exposure were associated with altered TLH and HLH expression levels, such that the extent of Lys hydroxylation in the triple helical domains and nontriple helical domains correlated with the mRNA expression levels of HLH and TLH, respectively. Figures 5B and C show the proportion of immature and mature cross-links to total cross-links, whereas Table 2 shows the content of each type of cross-link. Increases were observed not only in the relative concentrations of DHLNL immature cross-links and the amount of pyridinium cross-links derived from telopeptidyl Hyl residues, but also in the relative concentrations of Dpyr distributed in calcified tissues from cells with or without COX-2 inhibition after 30 mW/cm^2 LIPUS exposure and from cells with COX-2 inhibition after exposure to high-intensity (120 mW/cm^2) pulsed ultrasound. These findings were consistent with the levels of $\alpha\pm1$ and $\alpha\pm2$ type I collagen chain Lys hydroxylation, such that Lys hydroxylation in the telopeptides was very high after 30 mW/cm^2 exposure in cells with and without COX-2 inhibition and after 120 mW/cm^2 exposure in cells with COX-2 inhibition compared to the control cells. These results suggested that the proportions of cross-link type correlated with TLH and HLH expression levels and with Lys hydroxylation of the helical and nonhelical domains of the $\alpha\pm1$ and $\alpha\pm2$ type I collagen.

Discussion

The wide ranges of ultrasound intensities at the level not only from milliwatt to watt, but also less than a hundred milliwatt have distinctive effects on the bone fracture repair process through the different mechanisms described by a number of researchers [2, 4, 5, 41]. However, there is variation not only in level of intensity, but also in frequency (1.0–3.0 MHz) pulse repetition frequency (0.5–1.0 kHz), and pulse burst width (200–500 ms) on the experimental design in these reports. In the present study, these ultrasound parameters, with the exception of intensity, were in accord with the LIPUS-exposing device (SAFHS). The device has been widely distributed for clinical use, since we attempted to determine whether different intensities of pulsed ultrasound (low-intensity,

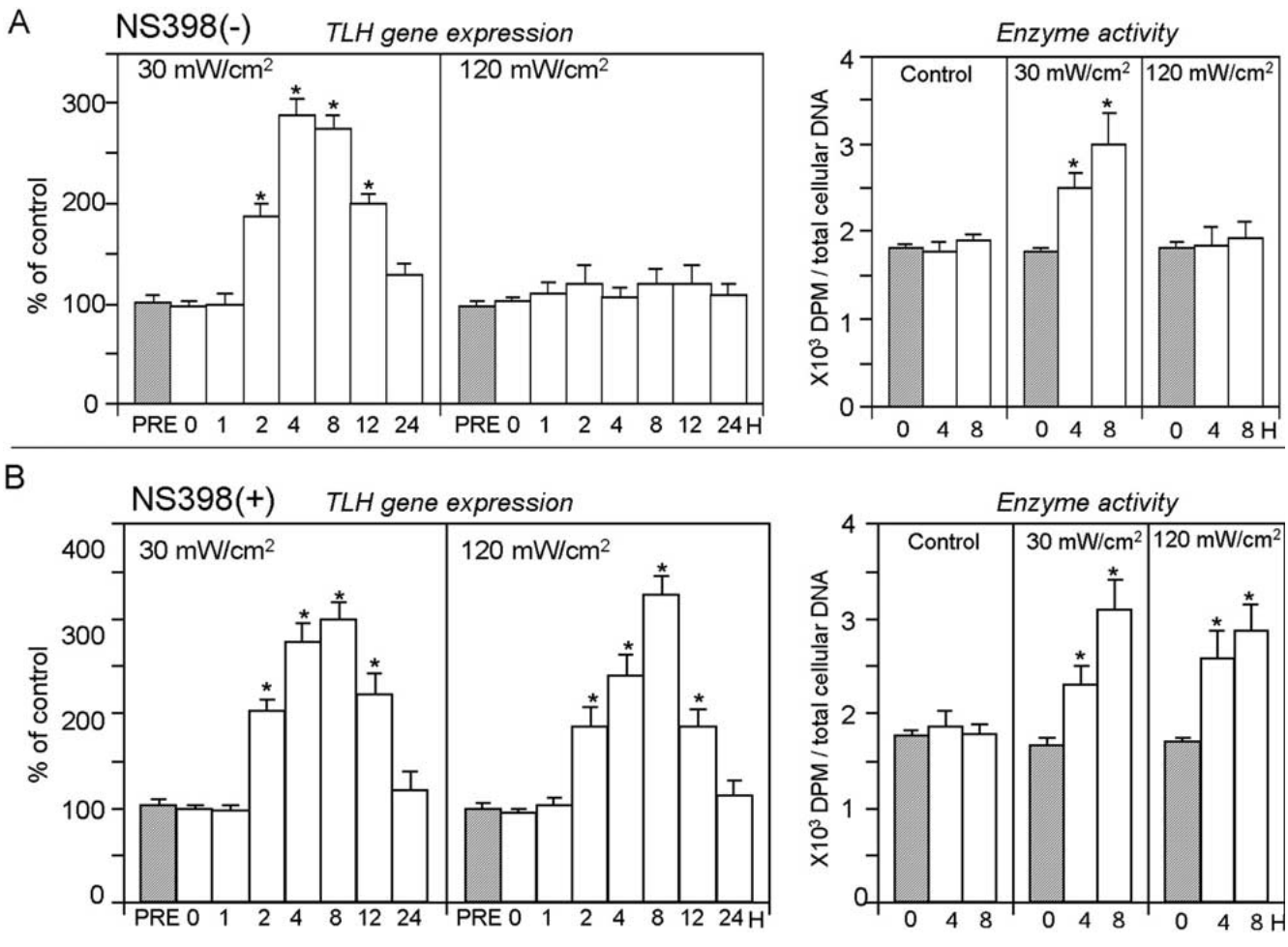

Fig. 6. Effect of pulsed ultrasound at high- and low-intensities (30 or 120 mW/cm^2) on relative TLH mRNA levels and enzymatic activity in cells without (A) or with (B) the COX-2 inhibitor, NS398. Messenger RNA levels of telopeptidyl Lys hydroxylase (TLH) were estimated by real time PCR analyses at each time point from 0 to 24 h after ultrasound exposure. Values are expressed as means ± SD of six independent experiments, normalized against the average control value. The asterisk represents a significant increase ($P < 0.01$) in relation to the control culture.

30 mW/cm^2; high-intensity, 120 mW/cm^2) based on the reports of Yang [5] and Parvizi [6] have a distinctive effect on the synthesis of calcifiable collagen matrix by using the osteoblastic MC3T3-E1 culture system.

COX-2 Expression and PGE_2 Production Induced by LIPUS

COX-1 is constitutively expressed in most cells and tissues, whereas COX-2 is induced in response to a variety of stimuli and is thought to be involved in prostaglandin production in certain stages of cell proliferation and differentiation during bone turnover [42]. Indeed, PGE_2 synthesis is promoted by the inducible COX-2 pathway. The results of our study showed that LIPUS (< 120 mW/cm^2) treatment of MC3T3-E1 cells significantly increased COX-2 mRNA expression and PGE_2 production in an ultrasound, intensity-dependent manner. The PGE_2 production induced by LIPUS exposure were totally suppressed by the presence of the COX-2-specific inhibitor, NS398. These results agree with those of Kokubu et al. [31], in that (1) upregulation of COX-2 mRNA expression in MC3T3-E1 cells began 1 hour after LIPUS exposure (pulsed sine wave of 30 mW/cm^2, 1.5 MHz repeating at 1 kHz, 20 minutes); (2) the gradual increase in COX-2 mRNA expression occurred in a postexposure, time-dependent manner; and (3) the increase was associated with the time-dependent accumulation of PGE_2 in the medium. Furthermore, Reher et al. [3] demonstrated that relative high-intensity ultrasound (pulsed sine wave of 100 to 700 mW/cm^2, 1.0 MHz, 5 minutes) compared to the therapeutic range induced an intensity-dependent increase in PGE_2 production in mandibular osteoblasts, and that NS398 completely inhibited the elevated PGE_2 synthesis. These findings suggest that the production of PGE_2 by osteoblasts, both *in vivo* and *in vitro*, may be regulated in part by LIPUS exposure with the subsequent upregulation of COX-2 gene expression.

Table 2. Lysine hydroxylation of type I collagen and cross-links in cell layer matrix

	NS398(–)			NS398(+)		
Lysine hydroxylation (%)	Control	30 mW	120 mW	Control	30 mW	120 mW
Helical domain $\alpha\pm1$(I)	23.7 [0.3]	23.9 [0.5]	23.2 [0.3]	23.5 [0.4]	24.1 [0.5]	23.7 [0.4]
$\alpha\pm2$(I)	28.1 [0.5]	27.6 [0.4]	27.7 [0.6]	28.2 [0.2]	28.0 [0.4]	28.3 [0.3]
Helical + nonhelical domain $\alpha\pm1$(I)	27.2 [0.2]	31.9 [0.4]*	27.8 [0.5]	26.5 [0.6]	30.6 [0.3]*	28.7 [0.2]*
$\alpha\pm2$(I)	30.5 [0.5]	36.2 [0.6]*	29.8 [0.4]	30.4 [0.5]	36.9 [0.4]*	33.5 [0.3]*
DHLNL	236 [12]	458 [53]*	260 [48]	226 [20]	490 [39]*	518 [52]*
Cross-links[b] HLNL	162 [25]	266 [43]*	176 [39]	158 [33]	265 [29]*	315 [48]*
LNL	62 [3]	36 [7]*	74 [10]	58 [4]	46 [4]$^{\#}$	47 [7]$^{\#}$
Pyr	298 [22]	450 [41]*	338 [40]	318 [35]	409 [29]*	386 [19]*
Dpyr	32 [2]	100 [13]*	32 [5]	34 [3]	101 [8]*	74 [11]*

[a] Hyl/(Hyl + Lys) × 100 (%)
[b] The content of cross-links is expressed as mmol/mol of collagen
Values are expressed as mean ± SD of six independent experiments
Superscripts indicate a significantly higher (*) or lower ($^{\#}$) mean than that of control culture ($P < 0.01$)
DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; Pyr, pyridinoline; Dpyr, deoxypyridinoline

Cross-Link Formation

Because the synthesis and deposition of organic matrix plays an important role in the initial steps of mineralization by providing an organic scaffold for subsequent mineral deposition, new bone mass can be acquired only by increased matrix synthesis and maturation. Our results showed that ultrasound exposure at low and high intensities (30 and 120 mW/cm^2, respectively) had no quantitative effect on collagen in cell layers in terms of mRNA and protein expression.

The accumulation of insoluble collagen is dependent on a number of factors, including the number of cells synthesizing collagen, the level of collagen synthesis, extracellular processing steps of procollagen involving procollagen C- or N-protease, and collagen cross-link formation [37]. Our results showed not only that the lysyl oxidase gene expression and enzymatic activity, but also the total amount of cross-links and ^{3}H-Lys, were significantly elevated in cells exposed to the low-intensity pulsed ultrasound (30 mW/cm^2) compared to control cells. However, these findings were not observed in cells exposed to high-intensity pulsed ultrasound (120 mW/cm^2), despite the fact that there was no obvious effect on COL1A1 expression and the amount of collagen per cellular DNA after ultrasound exposure. Therefore, cross-link formation appeared to have the most important role in insoluble collagen accumulation—more important than the amount of collagen synthesis following therapeutic levels of LIPUS exposure (30 mW/cm^2). However, the mechanism underlying the quantitative changes in cross-link formation after LIPUS exposure remains unclear.

Little is known about the regulation of the expression and activation of lysyl oxidase in osteoblasts. Knowing that PGE_2 is assumed to act as a negative regulator of lysyl oxidase [26] to determine whether LIPUS-induced PGE_2 affected cross-link formation in osteoblasts, we measured the parameters of lysyl oxidase gene expression, enzymatic activity, and the actual amount of cross-links in the presence and absence of NS398 pretreatment. Exposure to high-intensity (120 mW/cm^2) pulsed ultrasound resulted in significant reduction of these parameters in cells without COX-2 inhibition, whereas lysyl oxidase activity and cross-link formation increased to levels similar to those observed after 30 mW/cm^2 exposure in cells with COX-2 inhibition. Thus, the suppression observed with use of the 120 mW/cm^2 exposure may be attributable to the production of PGE_2. Boak et al. [26] demonstrated that relatively high levels of PGE_2 (100 nM) reduced lysyl oxidase enzyme activity, and that the addition of indomethacin abrogated the suppression of lysyl oxidase enzyme activity *in vitro*. Furthermore, PGE_2 is thought to be have a biphasic effect with low concentrations of PGE_2 increasing and high doses decreasing mineralized nodule formation in rat bone marrow cells or calvarial cells [21–25]. Despite differences in the cell types and culture condition used, our results and the results of these earlier studies suggest that high doses of PGE_2 may be detrimental to calcifiable cross-link formation. Other candidates for regulatory factors of lysyl oxidase gene expression are transforming growth factor beta (TGF-β) [25, 43], connective tissue growth factor (CTGF) [44], insulin-like growth factor I (IGF-I) [45], and basic fibroblast growth factor (bFGF) [46]. It is interesting to note that these growth factors are thought to play an important role not only in physiological fracture healing [47, 48], but also in accelerated fracture repair induced by LIPUS [7, 49]. Thus, the growth factors induced by low- or high-intensity ultrasound exposure may be candidates for the positive or negative regulatory factor of the enzymes involved in cross-link formation. However, further studies are needed to support this hypothesis.

Lys Hydroxylation and Cross-Link Pattern

Biochemical evidence has shown that increased Lys hydroxylation of telopeptides is associated with upregulation of TLH gene expression and occurs coincidentally with the onset of bone mineralization [12]. Wassen et al. [13] demonstrated in an *in vivo* study of canine callus maturation that a low Pyr/Dpyr ratio was essential for a specific pattern of molecular packing of collagen fibers during the onset of the mineralizing stage. A current supposition is that the low Pyr/Dpyr ratio may be the result of a specific pattern of molecular packing of collagen fibers unique to bone. In calcified tissue, a large proportion of the telopeptidyl Lys residues are hydroxylated, and formed to immature cross-links, such as DHLNL and HLNL, which can lead to the formation of pyridinium cross-links on maturation [9, 11, 13, 17, 32]. Although it is unclear whether the observed positive effect of low intensity ultrasound in hydroxylysine aldehyde–derived cross-links is caused by enhanced lysyl oxidase activity or TLH activity, it is assumed that both intracellular post-translational hydroxylation of telopeptide Lys by TLH and extracellular conversion of these Hyl residues to hydroxyallysine by lysyl oxidase play important roles in the synthesis of calcifiable collagen.

In the present study, we showed that elevated telopeptidyl Lys hydroxylation of type I collagen appeared to coincide with increased expression of TLH mRNA after 30 mW/cm^2 exposure in cells with and without COX-2 inhibition. This exposure resulted in elevated levels of TLH mRNA and protein as evidenced by the increased telopeptidyl Hyl derived cross-links, such as the DHLNL and pyridinium cross-links, but also by the low Pyr/Dpyr ratio caused by the relatively high levels of Dpyr compared to Pyr. Because there was no difference between cells with and without COX-2 inhibition in post-translational modifications induced by exposure to low-intensity (30 mW/cm^2) pulsed ultrasound, it appears that the physiological level of PGE_2 induced by low-intensity ultrasound may not play a crucial role in collagen post-translational controls.

We also found that downregulation of TLH gene expression and enzymatic activity after exposure to high-intensity (120 mW/cm^2) pulsed ultrasound was restored to levels similar to those of cells exposed to low-intensity (30 mW/cm^2) pulsed ultrasound by pretreatment with NS398. This finding suggested that high PGE_2 levels may affect the regulation of TLH gene expression and enzymatic activity, as well as the lysyl oxidase expression described previously. Therefore, our findings suggests that therapeutic levels of LIPUS (30 mW/cm^2) may promote the synthesis of a mature collagenous matrix as a scaffold for calcification. Further studies are necessary, however, to elucidate the particular regulatory factors of LH expression.

In summary, our findings provide evidence that low-intensity (30 mW/cm^2), but not high-intensity (120 mW/cm^2), pulsed ultrasound may accelerate the formation of the previously reported unique molecular packing of collagen fibers conducive to mineralization. In addition, our results suggest that the high dose of PGE_2 induced by high-intensity ultrasound may be detrimental to the physiological cross-link formation required for initiation of the mineralization process.

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