ULTRASOUND INTENSITY DEPENDENT PROSTAGLANDIN E2 PRODUCTION HAS A BI-PHASIC EFFECT ON COLLAGEN POST-TRANSLATIONAL CONTROLS IN MC3T3-E1 OSTEOBLASTS

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[Introduction] Low intensity pulsed ultrasound (LIP-US) has been shown to accelerate fracture healing (1). One mechanism by which bone formation is accelerated by LIP-US is thought to involve the transmission through tissues of mechanical stress produced by ultrasound-mediated high frequency acoustic pressure waves. Prostaglandin E2 (PGE2) is a crucial early mediator in mechanically induced bone formation. Interestingly, it has also been shown that PGE2 is a regulatory factor of lysyl oxidase (LOX), which catalyzes collagen crosslinking (2). We recently showed that proper mineralization induced by mechanical strain may be regulated by formation of a matrix with a specific molecular packing arrangement as mineralization begins (3). The aim of this study is to examine the effect of low- and high-intensity pulsed US on collagen post-translational modifications in murine MCT3-E1 osteoblasts, in particular both qualitative and quantitative changes in crosslinking and the correlation between cross-link patterns and PGE2 levels induced by US.

[MATERIALS AND METHODS] Cell culture: MC3T3-E1 cells were seeded in 6-well tissue culture plates at 6 x 10^4 cells/cm^2 and maintained in alpha-MEM supplemented with 10% fetal bovine serum and ascorbate (50 μg/ml). The cells were used for the experiment 14 days after seeding, at the time of confluence. Pulse chase labeling: For analysis of the kinetics of collagen cross-link formation, cells were labeled with tritium-lysine (40 μCi/mmol) for 24 h on day 13. US exposure: Cells were exposed to low (30 mW/cm^2) or high (120 mW/cm^2) intensity pulsed US for 20 min (with signal consisting of a 200 microsecond burst of 1.5 MHz sine waves repeating at 1kHz) daily for four consecutive days. Control cultures were subjected to the same manipulations under the same conditions without LIP-US exposure.

COX2, lysyl oxidase, lysyl hydroxylases gene expressions: Real time PCR was performed to allow the relative quantification of cyclooxygenase-2 (COX-2), LOX, telopeptidyl lysyl hydroxylase (TLP, equivalent of lysyl hydroxylase (LH)), and GAPDH mRNA expression using the LightCycler® PCR system at each time point after US exposure from 0 to 24 h (3). Prostaglandin E2 and COX2 inhibitor, NS398: PGE2 released from MC3T3-E1 cells after the exposure to low- or high-intensity pulsed US was determined using a commercially available ELISA kit. To investigate whether the production of PGE2 induced by US exposure was mediated by changes in COX2 activity, NS398 was used to block COX-2 activity.

TLH, HLH, and LO enzyme activities: TLH, HLH, and LO enzyme activities were determined using standard methods (3).

Cross-links of collagen: The analysis of reducible cross-links, such as dihydroxy-lysinonorleucine (DHLNL), hydroxylysinoonorleucine (HLNL), and lysinoonorleucine (LNL), and nonreducible cross-links, such as pyridinoline (Pyr) and deoxypyridinoline (Dpyr) in the cell layer matrix was performed on a single column HPLC (4).

[RESULTS] In the NS-398 non-treated cells, the 30 or 120 mW/cm^2 exposure induced significant up-regulation in COX-2 mRNA expression up to 2.9-fold and 5.9-fold higher than that of the control at 2 h, respectively, which was associated with a significant parallel increase in the production of PGE2. The above-mentioned increases in COX-2 gene expression and the production of PGE2 after US exposure were almost totally abrogated by pretreatment with NS-398. The up-regulation of LOX mRNA began at 2 h after the 30mW exposure reached approximately 3.8 times control levels at 8 h, and declined to 1.7 times control levels by 24 h in both NS-398 non-treated and treated cells, while the 120mW exposure had no effect on the LOX expression in NS-398 non-treated cells. Correspondingly, change in lysyl oxidase enzyme activity paralleled the change in expression of this gene at each time point. NS-398 pretreatment restored the levels of lysyl oxidase mRNA and its enzyme activity to a similar extent after either the 120mW exposure or the 30mW exposure (Fig. 1). These results were consistent with the total amount of collagen cross-linking (reducible + nonreducible cross-links). Furthermore, a comparison of tritium-lysine incorporation in various cross-links revealed that the conversion of reducible cross-links into nonreducible forms was accelerated by 30mW stimulation. In the NS-398 non-treated cells, TLH mRNA expression began to increase at 2 h after the 30 mW exposure, peaked (reaching 2.9 times control levels) at 4 h, and fell to 1.3 times control levels by 24 h; TLH enzyme activity levels followed the trend in its mRNA expression. On the other hand, HLH mRNA expression did not differ significantly from that of the controls. The reducible and nonreducible cross-link patterns were consistent with these results, namely, the proportions of DHLNL, HLNL, and pyridinium cross-links after 30mW stimulation were elevated compared to those found in the control.

[DISCUSSION] PGE2 is thought to have a dose-dependent biphasic effect, with low PGE2 doses increasing and high doses decreasing mineralized nodule formation in rat bone marrow cells or calvarial cells (5). We show that pulsed US exposure (less than 120 mW/cm^2) to MC3T3-E1 cells can significantly increase COX-2 mRNA expression and the production of PGE2 in an US intensity-dependent manner. PGE2 is assumed to be a candidate negative regulator of lysyl oxidase. Therefore, to determine whether PGE2 induced by low- or high-intensity pulsed US affects cross-link formation in osteoblasts, we measured LOX gene expression, its enzyme activity, and the actual amount of cross-links with tritiated lysine incorporation (5). We show that the 30mW exposure, unlike the 120 mW exposure, resulted in elevated occupancy rate of telopeptidyl hydroxyllysine-derived cross-links (such as DHLNL and pyridinium cross-links), elevated occupancy rate of highly hydroxylated Lys in telopeptides of type I collagen (which appeared to be synchronized with rising TLH gene expression), and low Pyr/Dpyr ratio. These results are the biochemical evidence that low-intensity, but not high-intensity, pulsed US may accelerate formation of the previous reported unique molecular packaging of collagen fibers conductive to mineralization (6). Additionally, our results suggest that the high dose of PGE2 induced by high-intensity US may be detrimental to physiological cross-link formation required for the initiation of the mineralization process.

REFERENCES: