J Periodont Res 2007; 42: 383–392

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00965.x

Mechanical loading and Δ^{12}prostaglandin J_2 induce bone morphogenetic protein-2, peroxisome proliferator-activated receptor γ-1, and bone nodule formation in an osteoblastic cell line

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Siddhivarn C, Banes A, Champagne C, Riché EL, Weerapradist W, Offenbacher S. Mechanical loading and Δ^{12}prostaglandin J_2 induce bone morphogenetic protein-2, peroxisome proliferator-activated receptor γ-1, and bone nodule formation in an osteoblastic cell line. J Periodont Res 2007; 42: 383–392. © Blackwell Munksgaard 2007

Background and Objective: We have previously reported that mechanical strain applied at a 1% level to an osteoblastic cell line induces the transcription of prostaglandin D_2 synthase and increases the levels of prostaglandin D_2 and its Δ^{12}prostaglandin J_2 metabolite. Mechanical strain also induces the expression of peroxisome proliferator-activated receptor γ-1 and bone nodule formation. We hypothesized that mechanical load induces bone formation via Δ^{12}prostaglandin J_2-dependent synthesis of bone morphogenetic proteins. Our goal was to investigate the molecular events involved in osteogenesis induced by mechanical loading and Δ^{12}prostaglandin J_2, namely the induction of bone morphogenetic proteins and peroxisome proliferator-activated receptor γ-1, a nuclear receptor for Δ^{12}prostaglandin J_2.

Material and Methods: Osteoblast monolayers were stretched for 1 h with a 1-h resting period and stretched for another hour at 1 Hz with 1% elongation. Cells were collected 0, 1, 6 and 16 h after stretching. Cyclooxygenase inhibitors and Δ^{12}prostaglandin J_2 were added in some experiments. Relative quantitative reverse transcriptase-polymerase chain reaction was used to examine whether the mRNA of bone morphogenetic protein-2, -4, -6, -7 and peroxisome proliferator-activated receptor γ-1 was induced. Immunohistochemistry was used to evaluate bone morphogenetic protein expression in cells.

Results: Mechanical strain significantly increased the mRNA expression of bone morphogenetic protein-2, -6, -7 and of peroxisome proliferator-activated receptor γ-1, but not of bone morphogenetic protein-4. In stretched cells, bone

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Key words: bone morphogenetic protein; cyclopentenone; mechanical loading; mouse osteoblast; Δ^{12}prostaglandin J_2; peroxisome proliferator-activated receptor

Accepted for publication September 14, 2006

morphogenetic protein-2 and peroxisome proliferator-activated receptor γ-1 expression was blocked by cyclooxygenase inhibitors, but restored by exogenous Δ^{12}prostaglandin J_2. Δ^{12}Prostaglandin J_2 significantly enhanced bone nodule formation and bone morphogenetic protein-2 expression when added alone to resting osteoblasts.

Conclusion: These results suggest that the osteoblastic biomechanical pathways that trigger bone formation involve cyclooxygenase and prostaglandin D_2 synthase activation, induction of Δ^{12}prostaglandin J_2 and its nuclear receptor, peroxisome proliferator-activated receptor γ-1, and increased expression of bone morphogenetic protein-2. These data suggest that the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1/bone morphogenetic protein-2 pathway plays an important role in osteogenesis.

The effect of mechanical loading on bone is important to the field of dentistry. Movements accompanying eating and chewing involve contact between teeth and between teeth and nutrients, resulting in mechanical forces being applied to the jaws. It is well accepted that the lack of mechanical load on bone (e.g., where one or more teeth are lost) results in alveolar bone resorption (1) and morphologic changes of mandibular condyles (2,3). In order to understand and control these destructive effects, it is important to examine the effects of mechanical strain on osteoblasts. Many bone cell culture studies have unveiled evidence that mechanical loading results in anabolic responses, such as the induction of various growth factors and of bone-specific proteins involved in osteogenesis (4–6). Mechanical strain also induces cell membrane perturbation and activation of the arachidonic acid cascade, leading to the release of prostaglandin E_2 and prostaglandin I_2 in bone cells (7). We have recently used MC3T3-E1 osteoblastic cells to study the effect of mechanical loading by 1% elongation, which was found to induce bone nodule formation (8). In this experimental model, mechanical loading was also found to promote the expression of prostaglandin D_2 synthase and the release of prostaglandin D_2 and its metabolite, Δ^{12}prostaglandin J_2; induction of the Δ^{12}prostaglandin J_2 ligand, peroxisome proliferator-activated receptor γ-1, was also reported.

In addition to having antitumor and antiviral activities (9), Δ^{12}prostaglandin J_2, a naturally occurring cyclopentenone, also has anabolic effects on osteoblasts, such as stimulating alkaline phosphatase activity, calcification, and enhancing the transcription of type I procollagen (α_1) (10–12). Prostaglandin D_2 has been reported to be the major arachidonic acid metabolite in bone marrow (13). It is also the major product of stretched osteoblasts (8). As prostaglandin D_2 and its metabolite, prostaglandin J_2, are converted to Δ^{12}prostaglandin J_2 in the presence of serum, it is plausible that some of the biological activities of prostaglandin D_2 may actually result from Δ^{12}prostaglandin J_2. However, the mechanism by which Δ^{12}prostaglandin J_2 exerts its anabolic effect in osteogenesis via the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway is currently unknown. We hypothesized that Δ^{12}prostaglandin J_2 may serve as a biochemical transduction signal resulting from mechanical stress that induces the synthesis of bone morphogenetic proteins, which are, in turn, sufficient to induce bone formation.

In the present study, we further characterized the molecular signals involved in osteoblastic nodule formation and osteogenesis. In particular, we examined the role of the prostaglandin D_2 synthase pathway and the effect of mechanical stretching on the transcriptional regulation of bone morphogenetic protein expression. We demonstrated that inhibition of cyclooxygenase blocks both peroxisome proliferator-activated receptor γ-1 and bone morphogenetic protein-2 expression. The addition of exogenous Δ^{12}prostaglandin J_2 overcomes the effect of cyclooxygenase inhibition on bone morphogenetic protein-2 expression. Δ^{12}Prostaglandin J_2 independently regulates bone morphogenetic protein-2 expression and bone nodule formation in resting osteoblasts. These experiments suggest that Δ^{12}prostaglandin J_2 synthesis is a key molecular signal that transduces mechanical strain into bone formation.

Material and methods

Cell culture

Mouse osteoblast-like cells, MC3T3-E1, were cultured in α-minimum essential medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum. Cells were conditioned to serum starvation and quiescence, as previously described (8), before the experiments were performed.

Mechanical strain application

A Flexercell Strain unit (Flexcell International Corporation, Hillsborough, NC, USA) was used to apply cyclic load with equibiaxial strain on cells. At confluence and quiescence, cell monolayers were stretched at 1 Hz (60 cycles/min) with 1% elongation for 1 h followed by a 1-h resting period, then were stretched for another 1 h. For bone morphogenetic proteins and peroxisome proliferator-activated

receptor γ-1 gene expression analysis, samples were collected immediately after stretching ($t = 0$), and 1, 6, and 18 h after stretching. Unstretched controls consisted of cell monolayers cultured in BioFlex® plates under parallel conditions and collected at the same time points as the stretched groups. During the entire strain regimen, cell monolayers were kept in an incubator at 37°C under an atmosphere of 5% CO_2. Experiments were performed in triplicate using cells from the same passage. Following the strain regimen, cells were lysed in TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and kept at −80°C until extraction of total RNA and quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis.

Cyclooxygenase inhibitors and Δ^{12}prostaglandin J_2 administration

A selective inhibitor of cyclooxygenase-1, SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethy-lpyrazole] (Calbiochem, La Jolla, CA, USA), and a selective inhibitor of cyclooxygenase-2, NS-398 [N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide] (Cayman Chemical, Ann Arbor, MI, USA), were used concurrently at a concentration of 10 μM (14). Both cyclooxygenase inhibitors were freshly prepared as 5 mg/mL stocks in dimethylsulfoxide. Δ^{12}prostaglandin J_2 (Cayman Chemical) was optimized and used at a concentration of 1.0 μM. It was prepared before use by evaporating the methyl acetate solvent and adding dimethylsulfoxide to reach a 100 mg/mL concentration. Cells were washed twice with phosphate-buffered saline and then incubated for 3 h in α-minimum essential medium containing 1% serum and both SC-560 and NS-398 (10-μm) (15). After washing the cells twice with phosphate-buffered saline, fresh medium containing 1% serum was added. Cells were then divided into two stretching groups: (i) stretching in both SC-560 and NS-398; and (ii) stretching in SC-560 and NS-398 with exogenous Δ^{12}prostaglandin J_2 (or vehicle control).

Evaluation of gene transcription in stretched osteoblasts by relative quantitative RT-PCR

The levels of bone morphogenetic protein-2, -4, -6, and -7 and of peroxisome proliferator-activated receptor γ-1 mRNA were evaluated by a relative quantitative RT-PCR assay using Ambion's QuantumRNA™ 18S rRNA internal standards kit (Ambion Inc., Austin, TX, USA). The sequences of the primers used were as follows: bone morphogenetic protein-2, 5′-TTTGGCCTGAAGCAGAGAC-3′ and 5′-GACGCTTTTCTCGTTTGTG-3′ (664 bp); bone morphogenetic protein-4, 5′-TGGAGCCATTCCGTAGTGC-3′ and 5′-GCGCCTCCTAGCAGGACTT-3′ (196 bp); bone morphogenetic protein-6, 5′-TGGGCCTCAGAAGAAGGTTG-3′ and 5′-GTTGGTGGCATTCATGTGTG-3′ (477 bp); bone morphogenetic protein-7, 5′-CTGATTGGACGGCATGGAC-3′ and 5′-AGGTGCAATGATCCAGTCCTG-3′ (264 bp); peroxisome proliferator-activated receptor γ-1, 5′-AAGCGGTGAACCACTGATATT-3′ and 5′-AAGGTGGAGATGCAGGTTCTA-3′ (317 bp); Classic 18S QuantumRNA™ Internal Standards (489 bp); and Universal 18S QuantumRNA™ Internal Standards (315 bp).

Total RNA was extracted from cell monolayers, using the TRIzol® reagent, by the single-step method described by Chomczynski & Sacchi (16). Total RNA (2.5 μg) was used for first-strand cDNA synthesis reactions using Superscript™ II RNase H-reverse transcriptase (Invitrogen™) and random hexamers (Gibco™). One microlitre of the resulting cDNA was used in the following PCR reactions. The number of cycles used for every pair of primers, and the optimum ratio of 18S primers/competimers standards (QuantumRNA™ 18S Internal Standards; Ambion Inc.), were determined in accordance with the manufacturer's protocol. The ratios of primer/competimer were as follows: bone morphogenetic protein-2 (1 : 9); bone morphogenetic protein-4 (2 : 8); bone morphogenetic protein-6 (3 : 7); bone morphogenetic protein-7 (2 : 8); and peroxisome proliferator-activated receptor γ-1 (2 : 8). The PCR reactions for bone morphogenetic protein-2, -6, and -7, and for peroxisome proliferator-activated receptor γ-1, were performed for 35 cycles of: 94°C, 45 s; 55°C, 1 min and 72°C, 1 min. The PCR reaction for bone morphogenetic protein-4 was performed for 32 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min.

Each PCR reaction (10 μL) was performed with *Taq* DNA polymerase in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Each PCR reaction product was isolated by electrophoresis, stained, digitally captured, and quantified using a Lumi-imager F1 workstation (Roche Applied Science, Indianapolis, IN, USA). The relative ratios of bone morphogenetic proteins and peroxisome proliferator-activated receptor γ-1/18S rRNA amplification products were determined from three independent samples.

Effect of Δ^{12}prostaglandin J_2 on bone morphogenetic protein-2 mRNA, protein expression, and bone nodule formation

At confluence and quiescence, cell monolayers were incubated with medium containing 1 μM Δ^{12}prostaglandin J_2, and collected after 1, 6, 18, and 24 h. Total RNA was extracted, and bone morphogenetic protein-2 mRNA expression was analyzed using relative quantitative RT-PCR, as described above. Bone morphogenetic protein-2 expression was detected in osteoblasts, after incubation with Δ^{12}prostaglandin J_2 for 24 h, by using the HISTOSTAIN™SP immunohistochemistry assay, in accordance with the manufacturer's protocol (Zymed Laboratories Inc., San Francisco, CA, USA). Negative controls omitted the primary antibody. The area of positive staining in the cell monolayer was measured by histomorphometric quantification, using image-analysis software (JAVA; Jandel Scientific, San Rafael, CA, USA) as the percentage positive area per total area. Bone nodule formation in resting osteoblasts was evaluated in cells incubated with or without Δ^{12}prostaglandin J_2

(1 μM). Throughout a 5-d stimulation period, cells were maintained in fresh medium containing 2% fetal bovine serum and 10 mM α-glycerophosphate (the medium was changed every 24 h). Observed bone nodules were quantified and photographed. Bone nodule formation was also observed in the stretched cells and resting cells incubated with cyclooxygenase inhibitors. The observation was carried out on day 10 (5 d after stretching).

Statistical analysis

Each value reported is the mean ± standard error of the mean of triplicate independent samples. Repeated-measures analysis of variance was used to determine time-dependent differences in gene expression in stretched and unstretched cells. When significant group differences were present, an unpaired Student's *t*-test was used to assess statistical significance between control and experimental groups at various time points. Differences were considered significant if the *p*-value was < 0.05.

Results

Effect of stretching on the transcription of bone morphogenetic factors

We examined whether stretching induced mRNA expression of the bone growth factors, bone morphogenetic protein-2, -4, -6 and -7, as well as a nuclear receptor, peroxisome proliferator-activated receptor γ-1, a ligand for Δ^{12}prostaglandin J_2. Figure 1 shows the levels of each mRNA, as determined by relative quantitative RT-PCR analysis, in stretched osteoblasts and unstretched controls. As shown in Table 1, the mRNA expression levels of bone morphogenetic protein-2, -6 and -7 were increased by ≈10-, 6- and 4-fold, respectively, by cellular stretching ($p < 0.05$). Furthermore, this higher level of expression was maintained for a minimum of 6 h. There was no detectable expression of these genes in unstretched osteoblasts throughout 18 h of incubation. The peroxisome proliferator-activated receptor γ-1 mRNA level also increased significantly, by 1.86-fold, immediately after stretching, and this had reduced slightly, to 0.81-fold, 6 h after stretching, compared with that of the unstretched control at the same time points ($p < 0.05$). There was no detectable expression of peroxisome proliferator-activated receptor γ-1 mRNA throughout 18 h of incubation in unstretched osteoblasts. Interestingly, we observed constitutive expression of bone morphogenetic

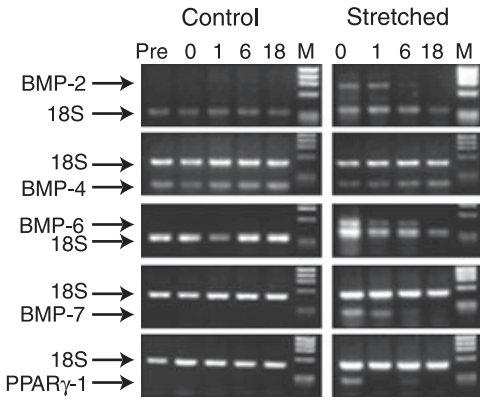

Fig. 1. Digital images of stained electrophoresis gels showing polymerase chain reaction products generated by relative quantitative reverse transcription-polymerase chain reaction of bone morphogenetic protein-2, -4, -6, -7 and peroxisome proliferator-activated receptor γ-1 mRNA at various time-points after cells were stretched. Polymerase chain reaction products from unstretched (left) and stretched (right) osteoblasts are shown. The numbers at the top of the gels represent the time in hours. 'Pre' designates baseline cells before the stretching regimen, and '0' designates cells immediately after stretching. Lane 'M' represents marker DNA ΦX174 *Hae*III fragments (Gibco, Rockville, MD, USA). Control 18S rRNA standards, amplified in the same reaction, are also denoted by arrows. One representative gel of three is shown. BMP, bone morphogenetic protein; PPARγ-1, peroxisome proliferator-activated receptor γ-1.

Table 1. The relative ratios (multiplied by a factor of 10 for presentation) of bone morphogenetic proteins and peroxisome proliferator-activated receptor γ-1 mRNA to 18S rRNA in stretched and unstretched osteoblasts at various time points after starting the stretching regimen

	Relative ratios × 10 (mean ± SEM)				
mRNA	Pre	0	1 h	6 h	18 h
BMP-2					
Unstretched	ND	ND	ND	ND	ND
Stretched	ND	10.40 ± 0.57[a]	9.47 ± 0.21[a]	6.25 ± 0.07[a]	ND
BMP-4					
Unstretched	4.54 ± 0.24	4.45 ± 0.18	4.33 ± 0.15	4.49 ± 0.18	5.77 ± 0.38
Stretched	4.56 ± 0.17	4.86 ± 0.25	4.25 ± 0.08	4.65 ± 0.17	5.67 ± 0.41
BMP-6					
Unstretched	ND	ND	ND	ND	ND
Stretched	ND	6.41 ± 0.29[a]	5.35 ± 0.27[a]	5.64 ± 0.14[a]	ND
BMP-7					
Unstretched	ND	ND	ND	ND	ND
Stretched	ND	4.03 ± 0.06[a]	2.80 ± 0.11[a]	1.40 ± 0.08[a]	ND
PPARγ-1					
Unstretched	ND	ND	ND	ND	ND
Stretched	ND	1.86 ± 0.15[a]	ND	0.81 ± 0.16[a]	ND

[a]Significant difference between control and experimental groups at $p < 0.05$.
BMP, bone morphogenetic protein; ND, not detected; PPARγ-1, peroxisome proliferator-activated receptor γ-1; Pre, baseline before the stretching regimen; 0, immediately after stretching.

protein-4 mRNA in unstretched and stretched cells, and this level of expression did not change over 18 h of incubation ($p > 0.05$). Thus, we report significant induction of bone morphogenetic protein-2, -6, -7, and of peroxisome proliferator-activated receptor γ-1 mRNA expression, in osteoblasts, as a result of cellular stretching.

Effect of cyclooxygenase inhibitors and Δ^{12}prostaglandin J_2 on gene expression

We have previously reported that osteoblastic stretching induces Δ^{12} prostaglandin J_2 synthesis and increases the expression of its ligand, peroxisome proliferator-activated receptor γ-1 (8). We sought to determine whether Δ^{12}prostaglandin J_2 (which is regulated via cyclooxygenase-1 and/or cyclooxygenase-2 plus prostaglandin D_2 synthase, as components of the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway) regulates the induction of bone morphogenetic proteins after cellular stretching. We also examined the role of cyclooxygenases in the induction of the individual mRNAs of the bone morphogenetic proteins.

In the first experiment, both cyclooxygenase-1 and cyclooxygenase-2 inhibitors (SC-560, NS-398) were incubated with MC3T3-E1 cells before, during, and after stretching to measure their effects on the induction of bone morphogenetic protein-2, -4, -6, and -7, and of peroxisome proliferator-activated receptor γ-1, by relative quantitative RT-PCR analysis. As shown in Fig. 2A(a), the pair of cyclooxygenase inhibitors suppressed the induction of bone morphogenetic protein-2 and peroxisome proliferator-activated receptor γ-1 mRNA. The inhibitors did not suppress the induction of bone morphogenetic protein-6 or -7 mRNA following stretching. The bone morphogenetic protein-4 mRNA levels were not affected by the pair of inhibitors.

We further examined whether the addition of exogenous Δ^{12}prostaglandin J_2 would overcome the suppressive effect of cyclooxygenase inhibitors on the induction of bone morphogenetic proteins and peroxisome proliferator-activated receptor γ-1. Figure 2A(b) demonstrates that suppression of the up-regulation of bone morphogenetic protein-2 and peroxisome proliferator-activated receptor γ-1 mRNA by cyclooxygenase inhibitors was nullified and restored by the addition of exogenous Δ^{12}prostaglandin J_2. However, neither cyclooxygenase inhibitors nor the addition of Δ^{12}prostaglandin J_2 had an effect on the mRNA expression of bone morphogenetic protein-6 or -7. The mRNA levels for bone morphogenetic protein-4 were not affected by Δ^{12}prostaglandin J_2 (Fig. 2B). These results suggest a role for Δ^{12}prostaglandin J_2 and cyclooxygenases in the regulation of bone morphogenetic protein-2 and peroxisome proliferator-activated receptor γ-1 following cellular stretching.

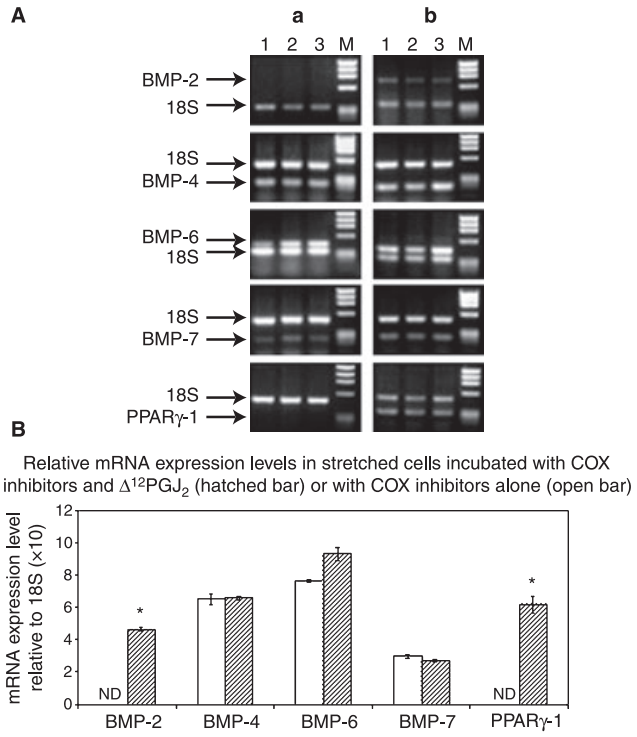

Fig. 2. The effect of cyclooxygenase-1 and -2 inhibitors on the mRNA levels of bone morphogenetic protein-2, -4, -6, and -7 in stretched osteoblasts, with or without Δ^{12}prostaglandin J_2. The relative quantitative reverse transcription-polymerase chain reaction was used to compare mRNA levels in cells immediately after stretching. (A) Relative quantitative reverse transcription-polymerase chain reaction products of bone morphogenetic protein-2, -4, -6, -7 and peroxisome proliferator-activated receptor γ-1 mRNA from cells grown and stretched in the presence of cyclooxygenase inhibitors only (a) and with added Δ^{12}prostaglandin J_2 (b). Lanes 1, 2, and 3 represent the results of triplicate experiments. 18S rRNA was amplified as an internal standard. Lane 'M' represents DNA markers. BMP, bone morphogenetic protein. (B) The relative ratios of bone morphogenetic protein-2, -4, -6, -7 and peroxisome proliferator-activated receptor γ-1 mRNA to 18S rRNA standard derived from relative quantitative reverse transcription-polymerase chain reaction analysis. The vertical axis shows the ratios multiplied by a factor of 10 for presentation. The open bars represent ratios obtained from osteoblasts grown and stretched in the presence of cyclooxygenase-1 and -2 inhibitors only. The hatched bars represent ratios obtained from stretched osteoblasts with added Δ^{12}prostaglandin J_2. The error bars represent the standard error of the mean from three independent experiments. Asterisks (*) denote a significant difference between the control and the experimental groups ($p < 0.05$). BMP, bone morphogenetic protein; ND, not detected; PPARγ-1, peroxisome proliferator-activated receptor γ-1. $\Delta^{12}PGJ_2$, Δ^{12}prostaglandin J_2.

Δ^{12}prostaglandin J_2 induces bone nodule formation and bone morphogenetic protein-2

We examined whether Δ^{12}prostaglandin J_2 promotes cell nesting and bone nodule formation in resting unstretched osteoblasts. An optimized cell-stimulating procedure was performed to measure the optimum concentration of Δ^{12}prostaglandin J_2 that promotes bone nodule formation. The results showed that after 5 d of incubation in media containing 1 μM Δ^{12}prostaglandin J_2, the number of cell nestings and bone nodules in osteoblasts increased significantly compared with those in the control osteoblasts ($p < 0.05$) (Fig. 3A,B). The number of cell nestings doubled in the experimental groups compared with the control groups, and the number of bone nodules increased from 0 to 55 (Table 2). We observed no bone nodules in the control resting cells or in the cultures incubated in the presence of cyclooxygenase inhibitors (data not shown).

We further examined, by immunostaining, the increased protein expression of bone morphogenetic protein-2 in resting unstretched osteoblasts stimulated with Δ^{12}prostaglandin J_2 (Fig. 3C,D). The percentage of positive stain for bone morphogenetic protein-2 increased significantly, from 0 to 55%, in osteoblasts stimulated with Δ^{12}prostaglandin J_2. No increase in the percentage of positive staining was observed in unstimulated control cells (Table 3). The effect of exogenous Δ^{12}prostaglandin J_2 on unstretched cells not only increased immunostaining for bone morphogenetic protein-2 but also increased the expression of bone morphogenetic protein-2 mRNA. Figure 4 shows that the level of bone morphogenetic protein-2 mRNA increased by 3.4 and 2.0-fold, respectively, 1 and 6 h after incubation with Δ^{12}prostaglandin J_2 compared with the control ($p < 0.05$). There was no detectable expression of bone morphogenetic protein-2 mRNA in the unstimulated control cells. These data further support a role for Δ^{12}prostaglandin J_2 in bone formation, independent from cell stretching.

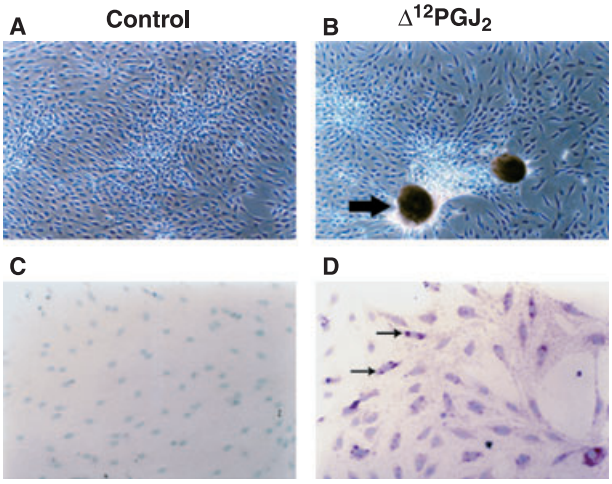

Fig. 3. Effect of Δ^{12}prostaglandin J_2 on bone nodule formation and bone morphogenetic protein-2 expression in resting unstretched osteoblasts. Cells were grown to quiescence and stimulated with Δ^{12}prostaglandin J_2 for 5 d before evaluation of bone nodules, or for 24 h before evaluating bone morphogenetic protein-2 expression by immunohistochemistry (right). Unstimulated control cells are shown on the left. Control cells show no nodule formation (A) and undetectable bone morphogenetic protein-2 expression (C). Stimulated cells show increased bone nodules (B, arrow) and significant bone morphogenetic protein-2 expression, as evident by the red color (D, arrow).

Table 2. Number of bone nodules and cell nestings per 10 cm^2 in osteoblasts stimulated with 1 μM Δ^{12}prostaglandin J_2

	Cell nesting (mean ± SEM)	Bone nodule (mean ± SEM)
Control resting cells	26.3 ± 2.0	0
Resting cells stimulated with $\Delta^{12}PGJ_2$	42.6 ± 3.0[a]	55.0 ± 3.0[a]

[a]Significant difference between control and experimental groups ($p < 0.05$).
$\Delta^{12}PGJ_2$, Δ^{12}prostaglandin J_2.

Table 3. Percentage of positive immunohistochemistry stains for bone morphogenetic protein-2 in osteoblasts stimulated with 1 μM Δ^{12}prostaglandin J_2 for 24 h

	Percentage positive BMP-2 stains (mean ± SEM)	
Group	Baseline	24 h
Control	0	0
$\Delta^{12}PGJ_2$	0	55.0 ± 3.0[a]

[a]Significant difference between control and experimental groups ($p < 0.05$).
BMP, bone morphogenetic protein.

Discussion

Numerous bone cell culture studies have reported an increased production of prostaglandins, in particular prostaglandin E_2, when osteoblasts were subjected to mechanical strain (17–20). It has been shown that prostaglandin E_2 greatly enhances the synthetic activities of osteoblasts following exposure to mechanical stress (21,22). In addition, strain enhances the expression of load-responsive genes, such as c-fos, cyclooxygenase-2, osteopontin, and matrix metalloproteinase 1B, which are involved in osteogenesis (23). However, most *in vitro* studies have not reported the production of prostaglandin D_2 and its Δ^{12}prostaglandin J_2 metabolite following mechanical loading on osteoblasts. Moreover, there is no evidence to show that strain also enhances the induction of growth factor genes, specifically bone morphogenetic proteins, in *in vitro* studies.

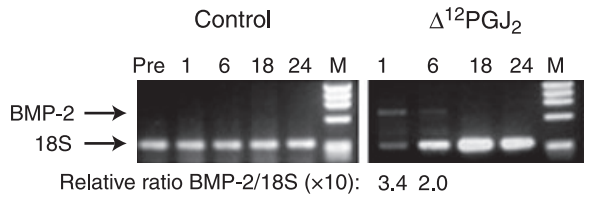

Fig. 4. Polymerase chain reaction products generated by relative quantitative reverse transcription-polymerase chain reaction analysis of bone morphogenetic protein-2 mRNA from resting osteoblasts stimulated with 1 μM Δ^{12}prostaglandin J_2. The polymerase chain reaction product from the control (left panel) and Δ^{12}prostaglandin J_2-treated cells (right panel) are shown. The numbers at the top of the gels represent the time in hours. 'Pre' designates baseline cells before the stimulation. Control 18S rRNA standards amplified in the same reaction are also denoted by an arrow. The numbers at the bottom of the right gel represent the calculated relative ratios of the bone morphogenetic protein-2 : 18S rRNA PCR product (multiplied by a factor of 10 for presentation). BMP, bone morphogenetic protein; $\Delta^{12}PGJ_2$, Δ^{12}prostaglandin J_2.

In this study, we provide evidence for the role of the prostaglandin D_2 synthase pathway and Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 signaling in the regulation of strain-induced osteoblastic nodule formation. We demonstrated the role of mechanical strain in inducing the expression of bone morphogenetic proteins, as well as the role of cyclooxygenase-1 and/or cyclooxygenase-2 in the regulation of bone morphogenetic protein-2 expression. Furthermore, the role of Δ^{12}prostaglandin J_2 in the expression of bone morphogenetic protein-2 and bone nodule formation in resting osteoblasts was also identified.

In the present study, we found that mechanical strain on osteoblasts induced the transcriptional expression of particular bone morphogenetic proteins, specifically bone morphogenetic protein-2, -6, and -7. Our previous report showed that strain also induced the secretion of Δ^{12}prostaglandin J_2, the expression of peroxisome proliferator-activated receptor γ-1, and dramatically induced the formation of cell nestings and bone nodules in osteoblastic cells (8). Together, not only do these data provide further evidence of strain-induced bone formation, they also support a role for physiological force in bone growth, development, and remodeling at the cellular level, which is critical in the maintenance of bone mass (24).

Among the factors controlling bone morphogenesis and the proliferation, differentiation, and matrix secretion of bone cells, bone morphogenetic proteins, including bone morphogenetic protein-2, -4, -6, and -7, are known to play a critical role. These bone morphogenetic proteins are sufficient to induce morphogenetic progenitor cell recruitment, which ultimately promotes bone formation (25). Studies in animal models have shown a link between bone morphogenetic proteins and mechanical loading. For example, Sato *et al.* (26) reported that mechanical tension-stress by bone lengthening with osteotomy induced mRNA synthesis of bone morphogenetic protein-2 and bone morphogenetic protein-4, but not of bone morphogenetic protein-6 or bone morphogenetic protein-7. Our results differed from this animal model study in that we observed cellular stretching of osteoblasts to induce expression of bone morphogenetic protein-2, -6, and -7 without the increase of bone morphogenetic protein-4. One surprising outcome of this study was the observation that bone morphogenetic protein-4 mRNA was constitutively expressed in unstretched cells. In addition, mechanical loading on this osteoblastic cell line had no effect on bone morphogenetic protein-4 transcription. Even though it has been shown that bone morphogenetic protein-4 is induced by mechanical loading in animal models, and that it is one of the most active progenitors in the differentiation process of primitive mesenchymal cells, immature chondrocytes and osteoblasts (27), we found no relationship between bone morphogenetic protein-4 expression and mechanical loading in our study model. These results suggest that in isolated osteoblasts (*in vitro*), bone morphogenetic protein-2, -6, and -7 are induced by applying physiological force on cells. Further comparative studies of bone morphogenetic protein-4, -6, and -7 induction *in vivo* and *in vitro* are needed to clarify these conflicting observations.

Mechanical loading on osteoblasts induces prostaglandin D_2 synthase transcription and the release of prostaglandin D_2 and its metabolite, Δ^{12}prostaglandin J_2. Mechanical loading also up-regulates the expression of peroxisome proliferator-activated receptor γ-1, a ligand for Δ^{12}prostaglandin J_2 (8). Here, we confirmed the previous observation that peroxisome proliferator-activated receptor γ-1 transcription is induced in stretched osteoblasts. Furthermore, we found evidence that these bone morphogenetic proteins, induced by mechanical stress, are regulated via the cyclooxygenase-mediated Δ^{12}prostaglandin J_2 pathway. Reports have shown that cyclooxygenase-2 inhibitors can block osteoblastic bone formation (28–31). We used a pair of cyclooxygenase-1 and cyclooxygenase-2 inhibitors at levels sufficient to block the synthesis of the major arachidonic acid metabolites of stretched cells, including prostaglandin E_2, prostaglandin D_2, and Δ^{12}prostaglandin J_2 (14). Other significant outcomes, derived from these experiments, are that only bone morphogenetic protein-2 and peroxisome proliferator-activated receptor γ-1 expression were down-regulated by cyclooxygenase inhibitors and that the effect of this inhibition was overcome by the addition of Δ^{12}prostaglandin J_2. These observations suggest that the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway plays a role in the expression of bone morphogenetic protein-2. However, the exact mechanism of Δ^{12}prostaglandin J_2 action in affecting bone morphogenetic protein-2 transcription is not known.

We previously showed that peroxisome proliferator-activated receptor γ-1 is up-regulated in stretched osteoblasts, whereas peroxisome proliferator-activated receptor γ-2 is not (8). It has been shown that fatty acids and fatty acid metabolites, such as prostaglandin D_2 and its metabolites, exert transcriptional effects through activation of nuclear receptors (peroxisome proliferator-activated receptors) and that the formed ligand receptor complex binds directly to a peroxisome proliferator-responsive element (32). The peroxisome proliferator-responsive element contains the DR-1 DNA recognition motif for this complex (33). Interestingly, the 5′ flanking sequence of the mouse bone morphogenetic protein-2 gene contains a canonical half site sequence (AGGTCA) of the peroxisome proliferator-responsive element, DR-1 (34). However, it is not known whether Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 activates this canonical half site, resulting in bone morphogenetic protein-2 expression.

The mouse bone morphogenetic protein-4 gene promoter has been shown to contain several upstream peroxisome proliferator-responsive element DR-1 elements (35). This suggests that the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 activation pathway may play a role in the expression of bone morphogenetic protein-4. However, we found no induction of bone morphogenetic protein-4 mRNA in our experiments. This suggests that the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 activation pathway plays no role in bone morphogenetic protein-4 mRNA expression in our experimental model. We also found no effect of cyclooxygenase inhibitors and Δ^{12}prostaglandin J_2 on bone morphogenetic protein-6 and -7 expression in stretched osteoblasts. These observations suggest that the induced transcriptions of bone morphogenetic protein-6 and -7, observed after mechanical stress, are controlled by mechanisms other than the cyclooxygenase-induced Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway in this cell line. It is probable that expression of these growth factors after mechanical stress is more complex.

We observed that nodules were formed in resting cells stimulated with Δ^{12}prostaglandin J_2 associated with an increase of bone morphogenetic protein-2 expression. This observation supports the results of others, that the growth factor bone morphogenetic protein-2 functions as a potent mediator in osteogenesis (36–38) and that Δ^{12}prostaglandin J_2 plays a crucial role in the regulation of osteogenesis (10–12). This observation is also in agreement with the results of Koshihara *et al*. (10,11), who reported a similar anabolic effect on osteogenesis by Δ^{12}prostaglandin J_2 in human osteoblastic cells.

The observation that cyclooxygenase inhibitors blocked stretch-induced bone nodule formation also suggests that among the bone morphogenetic proteins studied, only bone morphogenetic protein-2 plays an important role in strain-induced bone formation via the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 activation pathway. Two different findings from this study support this concept: first, that cyclooxygenase inhibitors had no effect on bone morphogenetic protein-6 and -7 expression in stretched osteoblasts; and, second, that only cell nestings (not bone nodules) were observed in the stretched osteoblasts incubated with cyclooxygenase inhibitors. These results further suggest that bone morphogenetic protein-6 and -7 play a supplemental role in mechanical load-induced bone formation.

Overall, these data suggest that cellular stretching, which induces the activation of cyclooxygenases and prostaglandin D_2 synthase, triggers bone morphogenetic protein-2 and peroxisome proliferator-activated receptor γ-1 expression and results in bone nodule formation. The observation that bone morphogenetic protein-2 was blocked by cyclooxygenase inhibitors and restored by exogenous Δ^{12}prostaglandin J_2 suggests a role of Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 in activating bone morphogenetic protein-2 expression. We observed that Δ^{12}prostaglandin J_2 induces anabolic effects on bone, as evidenced by increased bone morphogenetic protein-2 expression and bone nodule formation. These results also suggest that the clinical benefits of bone-loading in the maintenance of bone mass may be mediated via this biochemical pathway. For example, our previous study showed that mechanical load applied to bone beyond 1% cell elongation decreases the viability of osteoblasts (8). Previous studies have shown that when mechanical load induces small strains applied to whole bone (within the physiological range of ≈ 0.1 to 0.5%), these strains are amplified to $\geq 1\%$ at the level of the membrane of the osteocytes (39). Hence, 1% strain can be clinically applied to bone cells to promote bone regeneration, maintain bone mass, and activate bone morphogenetic protein-2 expression and bone nodule formation via the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway. Forces that we apply to teeth and supporting bone (e.g., in orthodontic tooth movement, implants, crowns, and bridges) should be kept within this physiologic range to activate this anabolic pathway. In addition, future technology may be able to apply cyclic load to induce bone regeneration in periodontal bony defects or any tooth-related bony lesions. Based on our studies, possible mechanisms for promoting bone formation and regeneration for clinical purposes could be activation of the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway by strain-induced nodule formation or, alternatively, through pharmaceutical means.

In conclusion, these results clarify the relationship among cyclooxygenases, Δ^{12}prostaglandin J_2, and bone morphogenetic protein-2 expression in stretched osteoblasts, and suggest that the Δ^{12}prostaglandin J_2/peroxisome proliferator-activated receptor γ-1 pathway may have a role in regulating strain-induced bone formation. However, further investigations are needed to clarify the exact role of this pathway during the anabolic induction of osteoblasts.

Acknowledgements

We thank Dr Mari Tsuzaki and Xi Yang (Department of Orthopaedics, UNC School of Medicine), and Dr Sarah Geva, Cynthia Suggs, Wallace Ambrose and Frances Smith (Dental Research Center, UNC School of Dentistry) for technical assistance. This work was financially supported by NIDCR grant P6O DE 13079, NCRR grant RR00046 and by OraPharma Inc. The authors also thank Dr Steve Pettit for invaluable editorial guidance.

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