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Prostaglandin D_2 pathway and peroxisome proliferator-activated receptor γ-1 expression are induced by mechanical loading in an osteoblastic cell line

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Objective: The hypothesis underlying the current study was that the arachidonic acid cascade, specifically activation of the prostaglandin (PG) D_2 pathway in osteoblasts, is an anabolic signal induced by mechanical loading.

Background: Previous studies have shown that mechanical loading of osteoblasts triggers cyclooxygenase (COX)-2, PGE_2 and prostacyclin (PGI_2) synthesis. Since modest mechanical loading of osteoblasts promotes bone formation, we sought to determine whether mechanical stress activates the osteoblastic PGD_2 pathway resulting in the synthesis of osteogenic cyclopentenones, including $\Delta^{12}PGJ_2$.

Methods: Osteoblast monolayers were stretched using a Bioflex apparatus at a frequency of 1 Hz with 1% elongation. Cells and cell media were collected at various time points: 5, 10, 15, 30 min; and 1, 4, 16, 24 h. RNA was extracted for quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). In certain experiments, cells were pre-labeled with ^{14}C arachidonic acid prior to stretching. Radiolabeled metabolites in cell media were identified by reverse-phase high performance liquid chromatography (RP-HPLC). Osteoblasts were evaluated for an induction in bone nodule formation by stretching.

Results: Mechanical strain significantly increased mRNA expression of COX-1, COX-2, PGD_2 synthase and peroxisome proliferator-activated receptor (PPAR) γ-1, but not of PPARγ-2 as compared to control unstretched cells ($p < 0.05$). Mechanical loading stimulated the release of PGE_2, PGD_2 and the PGD_2 metabolite $\Delta^{12}PGJ_2$. Mechanical strain resulted in the induction of bone nodules.

Conclusions: This report indicates that mechanical loading of osteoblasts results in activation of PGD_2 and the concomitant expression of transcription factor PPARγ-1 mRNA. The coordinated synthesis of $\Delta^{12}PGJ_2$, a natural ligand for

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PPARγ-1, with the increased expression of PPARγ-1, suggests that biomechanical transduction pathways that initially involve the activation of cyclooxygenases may also involve the activation of the $\Delta^{12}PGJ_2$–PPAR pathway.

Mechanical loading plays an important role in maintenance of bone mass and structures around the teeth and in bone remodeling (1, 2). Studies show that a reduction in physiological forces acting on the mandible leads to atrophic changes of these structures (3, 4). It is also known that mechanical loading cause bone to change its material properties (5), and that physical forces such as tension, compression and shear stress have anabolic effects on bone at the cellular level (6). For example, mechanical loading induces arachidonic acid metabolism in bone, especially the induction of prostaglandin (PG) E_2 and prostacyclin (PGI_2) (7–9). However, the effect of mechanical stress on the induction of PGD_2 and its metabolites in osteoblasts has not been defined. Certain cyclopentenone prostaglandins, synthesized by PGD_2 synthase, may play an important role in osteoblastic differentiation and activation. Exogenous administration of PGD_2 to cultured osteoblasts, for instance, induces anabolic effects by stimulating extracellular matrix secretion and calcification (10). It is not known whether these anabolic effects are due to PGD_2 or to the spontaneous dehydration products PGJ_2 and $\Delta^{12}PGJ_2$, which are the final PGD_2 metabolites in the human body (11–13).

Reports show that $\Delta^{12}PGJ_2$ stimulates alkaline phosphatase activity and calcification of human osteoblastic cells. The potency of $\Delta^{12}PGJ_2$ is comparable to that of 1-α,25-dihydroxyvitamin D3, whereas other PGD_2 metabolites, such as PGJ_2 or 15-deoxy-$\Delta^{12,14}$-PGJ_2, have no effect (10, 14). Although PGD_2 is a major arachidonic acid metabolite found in bone marrow where its level exceeds that of PGE_2 (15), its role in maintaining bone mass is unknown. In an *in vivo* study, subcutaneous administration of a slow-release preparation of PGD_2 in ovarectomized rats demonstrated that PGD_2 prevents osteopenia. This observation suggests that the stimulatory effect of PGD_2 on bone formation is mediated by either PGD_2 or its metabolites (16). Since both PGD_2 and PGJ_2 are converted to $\Delta^{12}PGJ_2$ in the presence of serum, the pathway leading to their synthesis may play a regulatory role in osteogenesis, either in physiological hormonal maintenance of bone or in response to mechanical loading.

PGD_2 metabolites such as $\Delta^{12}PGJ_2$ and 15-deoxy-$\Delta^{12,14}$-PGJ_2 bind to intranuclear peroxisome proliferator-activated receptors (PPARs) rather than cell surface receptors. They also up-regulate gene expression by binding to PPAR responsive elements on DNA (17, 18). The major isoforms of PPARs have been identified as PPARα, β and γ in human cells (19–21). Interestingly, human osteoblastic cell lines express significant levels of PPARγ and PPARβ (22). Both PGJ_2 metabolites 15-deoxy-$\Delta^{12,14}$-PGJ_2 and $\Delta^{12}PGJ_2$ activate the PPARγ receptor (17, 18). However, to date, there is no evidence that the osteogenic action of PGD_2 metabolites is mediated by PPAR activation and there are no data that examine the mechanism of osteoblastic mechanical stress on the activation of the PGD_2 pathway that results in bone formation.

The goal of this study was to test the hypothesis that mechanical loading by strain application to cultured osteoblasts could induce PGD_2 synthase, as well as PGD_2 and $\Delta^{12}PGJ_2$ syntheses. We also sought to determine whether tension induced osteoblastic expression of PPARγs that are the putative nuclear transcription factors for these prostaglandins. In this report we show that equibiaxial stretching of osteoblasts induced bone nodule formation associated with an activation of PGD_2 synthase, resulting in release of $\Delta^{12}PGJ_2$ and increased expression of PPARγ-1 mRNA. These results suggest that the $\Delta^{12}PGJ_2$–PPARγ-1 pathway may play an important role in strain-induced osteogenesis.

Material and methods

Cell culture

Murine osteoblast-like cells, MC3T3-E1, were maintained in α-minimum essential media (Gibco, Rockville MD, USA) supplemented with 10% fetal bovine serum (FBS). Cells were conditioned to serum starvation and quiescence prior to performing experiments. Cells were seeded in type-I collagen coated, six-well BioFlex® plates (Flexcell International Corporation, Hillsborough, NC, USA) with a density of 2×10^5 cells/well in 2 ml of media and grown until they reached confluence (day 3). At that point, 1 ml of media was removed and replaced with 1 ml of fresh serum-free α-minimum essential medium, resulting in an effective concentration of 5% fetal bovine serum. Twenty-four hours later, 1 ml of media was aspirated and replaced with 1 ml of fresh serum-free α-minimum essential medium resulting in an effective concentration of 2.5% fetal bovine serum. The following day, the cells were washed with 1 × phosphate-buffered saline and the media replaced with α-minimum essential medium containing 1% fetal bovine serum. Cells were incubated an additional 24 h before performing the experiments.

Mechanical strain application and sample preparation

Optimized mechanical loading procedure for the induction of prostaglandins — An optimized cell stretching procedure was performed to minimize cell death and promote bone nodule formation. First, the effect of 1%, 3% and 5% elongation on cell viability was examined. Stretching the osteoblasts at 1% elongation was the maximal level to maintain cell viability at greater than 90%. At 1% elongation, the level used for all further experiments, bone nodule formation was induced.

A Flexercell Strain unit (FX-4000T™, Flexcell International Corporation) was used to apply a cyclic load that exerted equibiaxial strain on cells. At confluence and quiescence, cell monolayers were stretched at 1 Hz (60 cycles/min) with 1% elongation for periods of 5, 10, 15, 30 min, and 1, 4, 16, 24 h. Immediately after stretching, samples were collected for analysis of cyclooxygenase (COX)-1, COX-2, PGD_2 synthase and PPARs gene expression (by evaluation of mRNA levels).

Media from cell cultures was collected in siliconized coated glass tubes and stored at −80°C for later extraction and reverse-phase high performance liquid chromatography (RP-HPLC). Unstretched controls consisted of cell monolayers cultured in BioFlex® plates under parallel conditions but not subjected to substrate deformation. During the entire strain regimen, cell monolayers remained in the humidified incubator at 37°C with 5% CO_2. All 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 stored at −80°C for later total RNA extraction.

Stretched and unstretched control cell groups were examined for nodule formation after maintaining cells for 2 days in fresh media with 2% fetal bovine serum supplemented with 10 mM of β-glycerophosphate (media was changed every 24 h). The number of nodules was quantified microscopically (Nikon Eclipse TE300, Southern Micro Instruments, Marietta, GA, USA).

Relative quantitative reverse transcription-polymerase chain reaction evaluation of gene expression in stretched osteoblasts

The expressions of COX-1, COX-2, PGD_2 synthase, PPARγ-1 and PPARγ-2 were evaluated by a relative quantitative (RQ) reverse transcriptase–polymerase chain reaction (RT–PCR) assay using Ambion's QuantumRNA™ 18S Internal Standards Kit (Ambion Inc., Austin, TX, USA). The sequences of the primers used are shown in Table 1. They are specific for mouse and optimized by Primer Express software (Applied Biosystems, Foster City, CA, USA). The cycling parameters, linear range and the optimal 18S primer/competimer ratio for each primer pair were determined as suggested by the manufacturer. The level of mRNA for each gene of interest was normalized with the level of 18S rRNA in the same RT–PCR reaction. Total RNA was extracted from cell layers using TRIzol® reagent by the method of Chomczynski and Sacchi (23). Total RNA (2.5 μg) was used for first-strand cDNA synthesis reactions using ™ II RNase H⁻ reverse transcriptase (Invitrogen) primed with random hexamers (Gibco®) in a 20 μl reaction. Then 1 μl of the resulting cDNA was used for the following PCR reactions. The conditions of the RQ RT–PCR reactions and ratios of each pair of primers are further characterized in Table 1. Ten μl PCR reaction were performed with *Taq* DNA polymerase in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) and evaluated by 1.5% agarose gels electrophoresis. The relative intensity of each band was determined on a Lumi-Imager F1 work station (Roche Molecular systems) and plotted as the relative abundance of COX-1, COX-2, PGD_2 synthase, PPARγ-1 and PPARγ-2 product against 18S rRNA amplification product. Positive controls for PPARγ-2 were conducted in 15-deoxy-$\Delta^{12,14}$-PGJ_2 induced MC3T3-E1 cells. Each set of RT–PCR evaluations derived from three separate cultures.

Eicosanoid extraction and reverse-phase high performance liquid chromatography analysis

Prior to the experiment, osteoblastic cells were labeled with 3.7 kBq of [^{14}C]arachidonic acid (Amersham Biosciences Inc., Piscataway, NJ, USA) overnight and 4 h prior to stretching. Prostanoids were extracted using an ODS-silica column [C_{18} Sep-Pak® cartridge (Waters Corp., Milford, MA, USA)], according to the method described by Powell (24) and others (25).

Table 1. Polymerase chain reaction (PCR) oligonucleotide primer sequences, amplification product sizes and conditions

Primer	Sequence	Size (bp)	Condition	Ratio	Cycle
COX-1	5′-TGTTCAGCTTCTGGCCCAACAGCT-3′ 5′-AGCGCATCAACACGGACGCCTGTT-3′	304	94°C, 1 min; 55°C, 2 min; 72°C, 3 min	4:6	30
COX-2	5′-GGTGGAAAAACCTCGTCCAGA-3′ 5′-AGTCTGGAGTGGGAGGCACTT-3′	282	94°C, 1 min; 55°C, 1 min; 72°C, 1 min	2:8	35
PGD_2 synthase	5′-CAGCGTTGGAGCAATGTCAA-3′ 5′-TGTGGTGCTGCAGATATCCC −3′	236	94°C, 45 s; 55°C, 45 s; 72°C, 1 min	2:8	35
PPARγ-1	5′-AAGCGGTGAACCACTGATATT-3′ 5′-AAGGTGGAGATGCAGGTTCTA −3′	317	94°C, 45 s; 55°C, 1 min; 72°C, 1 min	2:8	35
PPARγ-2	5′-TGCTGTTATGGGTGAAACTCT-3′ 5′-GGTGGAGATGCAGGTTCTACT-3′	348	94°C, 45 s; 55°C, 1 min; 72°C, 1 min	3:7	35
Classic 18S QuantumRNA™ Internal Standards		489			
Universal 18S QuantumRNA™ Internal Standards		315			

COX: cyclooxygenase; PG, prostaglandin; PPAR: peroxisome proliferator-activated receptor.

Radiolabeled arachidonic acid metabolites in the samples were separated by RP-HPLC using a 4.6 × 250 mm Hypersil ODS (C_{18}) column with a 5 μm pore size (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was a mixture of acetonitrile : water : acetic acid (33:67:0.1 v/v) adjusted to pH 3.0 with ammonium hydroxide. A flow rate of 1 ml/min was used for 50 min. The column effluent was monitored at 192 nm (26) with a ProStar 310 UV/Visible detector connected to a ProStar 230 Solvent Delivery System (Varian Inc., Walnut Creek, CA, USA). Results were determined as a correlation between absorbance and retention times. The HPLC fractions were collected every 60 s and radioactivity determined using a liquid scintillation counter (Beckman Coulter, Inc.). 6-Keto-PGF$_{1\alpha}$ (used as the standard for the stable metabolite of PGI$_2$), PGD$_2$, PGE$_2$ (Sigma-Aldrich, St Louis, MO, USA), Δ^{12}PGJ$_2$ and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (Cayman Chemical, Ann Arbor, MI, USA) were individually diluted in acetonitrile to a concentration of 0.1 μg/ml. Each prostaglandin was detected at 192 nm. The chromatograms of the prostaglandin standards were evaluated as absorbance units (AU) and retention time in minutes.

Statistical analysis

Each value reported is the mean ± standard error of the mean (SEM) of triplicate independent samples. Repeated measures analysis of variance (ANOVA) was used to determine time-dependent differences in gene expression. When significant group differences were present, ANOVA was used to assess statistical significance between control and experimental groups at various time points. Differences were considered significant if $p < 0.05$.

Results

Effect of mechanical loading on the prostaglandin D$_2$ pathway

The effect of stretching on the production of prostaglandins and the PGD$_2$ pathway (Fig. 1) was examined by analyzing the ^{14}C arachidonic acid metabolites released into the media of stretched osteoblasts. The levels of radiolabeled PGD$_2$ and its metabolites, Δ^{12}PGJ$_2$ and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$, as well as PGE$_2$ and PGI$_2$ were determined by RP-HPLC using a liquid scintillation counter. As shown in Fig. 2(A), elution times were optimized using authentic standards and absorbance was detected at 192 nm. The 6-keto-PGF$_{1\alpha}$ and PGD$_2$ standards eluted at 7 and 16 min, respectively. In addition, PGE$_2$, 15-deoxy- $\Delta^{12,14}$-PGJ$_2$ and Δ^{12}PGJ$_2$ eluted at 18, 20 and 26 min, respectively. In control, unstretched labeled osteoblasts, a radioactive peak eluted at 7 min, which is thought to correspond to 6-keto-PGF$_{1\alpha}$ (Fig. 2B). No other peak was detected.

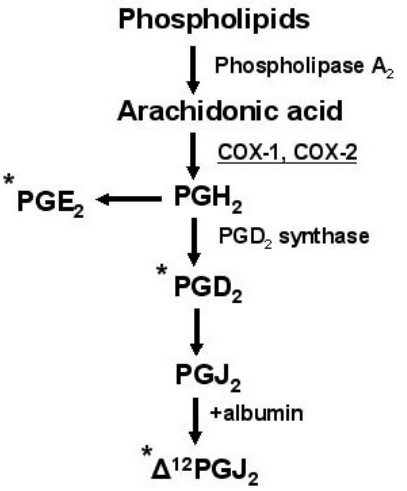

Fig. 1. Schematic of the putative prostaglandin D$_2$ (PGD$_2$) pathway induced by mechanical load in osteoblasts. Phospholipase A$_2$ reacts with membrane phospholipids to yield arachidonic acid. Arachidonic acid is converted to the prostanoid precursor PGH$_2$ via cyclooxygenases (COX-1, COX-2). Next, PGH$_2$ is converted to PGE$_2$ or PGD$_2$ by specific synthases (PGE$_2$ synthase, PGD$_2$ synthase) (29). PGD$_2$ spontaneously dehydrates in aqueous solution to yield PGJ$_2$. In the presence of albumin serum or plasma, Δ^{12}PGJ$_2$ is the ultimate metabolite of PGD$_2$. Enzymes found transcriptionally induced in this report are underlined. Prostaglandin metabolites found induced in the media of stretched cells are denoted with an asterisk (*).

However, in the stretched experimental group (Fig. 2C), radioactive peaks at 7, 16, 18 and 26 min of elution were identified, corresponding to 6-keto-PGF$_{1\alpha}$, PGD$_2$, PGE$_2$ and Δ^{12}PGJ$_2$, respectively. An unknown peak at 44 min of elution time was also detected. No radioactive peak corresponding to 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ was detected.

The unstretched and stretched cells demonstrated a basal secretion of PGI$_2$ as measured by the stable metabolite 6-keto-PGF$_{1\alpha}$ (Figs 2B and C). Mechanical stretching at a level of 1% resulted in a significant increase in PGE$_2$, PGD$_2$ and its metabolite Δ^{12}PGJ$_2$; however, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ was not detected.

Transcriptional levels of enzymes of the prostaglandin D$_2$ pathway are induced by stretching

One possible mechanism for increased levels of prostaglandins in the media is through induction of enzymes from the PGD$_2$ pathway. Levels of mRNA for COX-1, COX-2 and PGD$_2$ synthase were measured in stretched vs. unstretched cells by RQ RT–PCR using 18S rRNA as an internal quantitative standard (Fig. 3). As shown in Fig. 3 (left), unstretched control cells exhibited basal constitutive expression of COX-1 in lane 1 (baseline, before stretching) throughout 24 h. Levels of COX-2 and PGD$_2$ synthase mRNA were undetectable at all time points. However in stretched cells (Fig. 3, right), mRNAs were induced for all three components, COX-1, COX-2 and PGD$_2$ synthase.

We further determined the relative amount of induction of the former candidate genes. Figure 4(A–C) shows that mRNA levels for COX-1 were increased modestly (e.g. 50% increase at 4 h). In contrast, between 5 min to 24 h of stretching, COX-2 and PGD$_2$ synthase increased significantly (from approximately 4 to 7-fold and from 3.5 to 8.5-fold, respectively).

These results suggested that the induction of mRNA expression of the major components of the PGD$_2$ pathway was associated with the increased levels of PGD$_2$ and Δ^{12}PGJ$_2$ that were observed in the media following stretching.

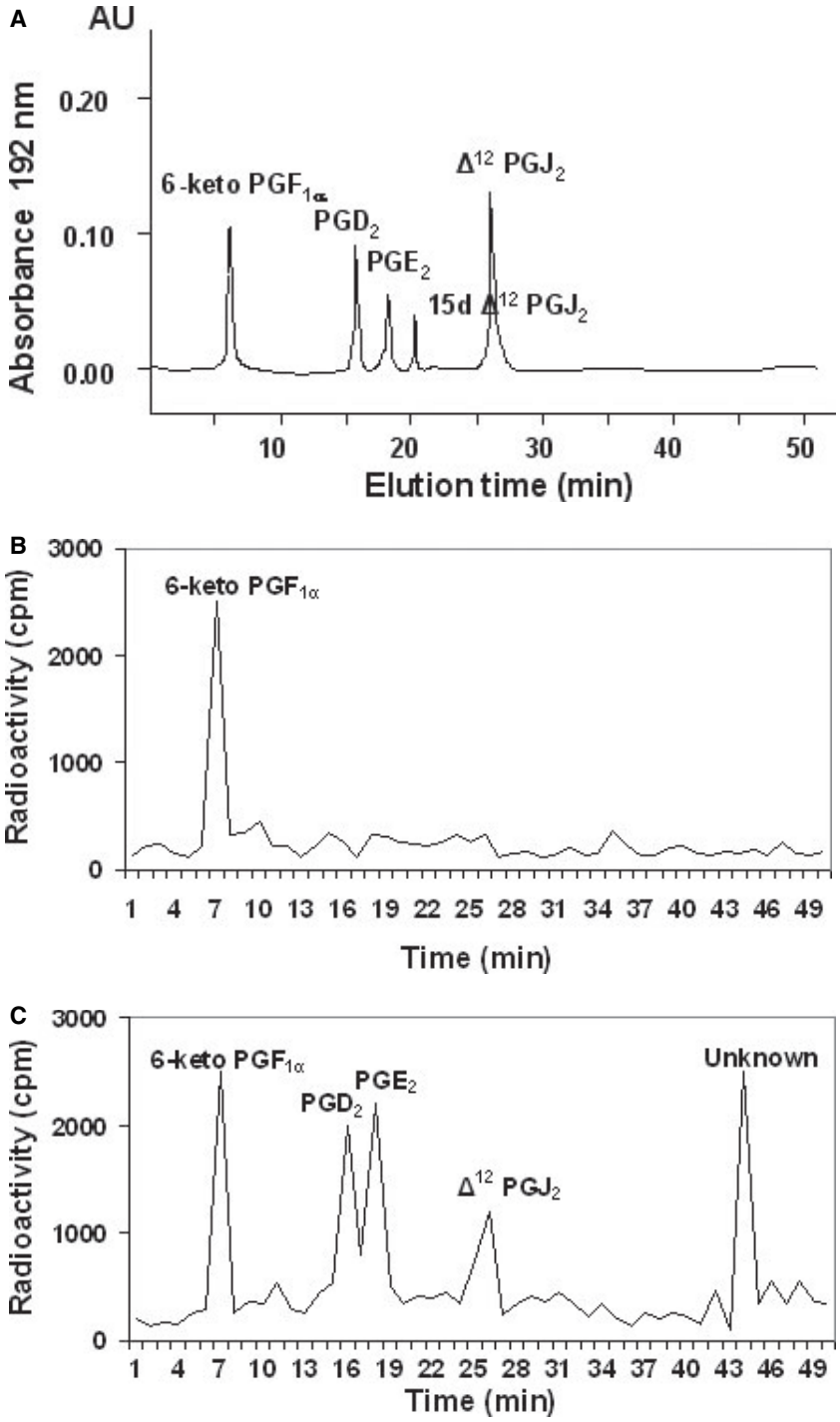

Fig. 2. Reverse-phase high performance liquid chromatography chromatogram of major arachidonic acid metabolites. (A) Chromatogram of 6-keto-prostaglandin (PG)$F_{1\alpha}$, PGD_2, PGE_2, 15-deoxy-Δ12-PGJ2 (15d-$\Delta^{12}PGJ_2$) and $\Delta^{12}PGJ_2$ standards. Absorbance at 192 nm is shown on the vertical axis. Retention time is shown on the horizontal axis. $PGF_{1\alpha}$, PGD_2, PGE_2, 15d-$\Delta^{12}PGJ_2$ and $\Delta^{12}PGJ_2$ eluted at 7, 16, 18, 20 and 26 min, respectively. Labeled arachidonic acid metabolites found in the media of unstretched control osteoblasts (B) and stretched osteoblasts (C) are shown. Counts per min (cpm) are shown on the vertical axis and elution time is shown on the horizontal axis.

Nuclear receptor for prostaglandin J_2

After finding that stretching induced the PGD_2 pathway, we investigated to see whether mRNA of the receptors for $\Delta^{12}PGJ_2$ (PPARγ-1 and PPARγ-2) was also up-regulated. As shown in Fig. 3 (left), unstretched control osteoblasts did not show detectable levels of PPARγ-1 and PPARγ-2 mRNA. However, in stretched cells (Fig. 3, right), PPARγ-1 mRNA was induced, but not PPARγ-2 mRNA. The relative amount of PPARγ-1 induction was also determined. Data in Fig. 4(D) show that from 5 min to 24 h of stretching, mRNA levels for PPARγ-1 increased significantly (between approximately 1.5 and 3-fold). No PPARγ-2 mRNA was detected at any time point in stretched or unstretched osteoblasts. Amplification of a positive control indicated that the PPARγ-2 primers were functioning properly (Fig. 3).

Effect of cellular stretching on bone nodule formation

We investigated the effect of cellular stretching on bone nodule formation. Osteoblast-like MC3T3-E1 cells were mechanically stretched at a level of 1% and bone nodule formation was determined by microscopic examination (Fig. 5). We observed a 3.5-fold increase in the number of cell nestings (from 15 to 52) in stretched osteoblasts compared to the unstretched controls, $p < 0.05$ (Table 2). There were no bone nodules seen in control unstretched cells and 62 were noted in stretched cultures. Stretching at the level of 1% elongation had little effect on cell viability (above 90%); however, cells stretched at a level greater than 1% decreased cell viability to less than 50% (data not shown). Thus, we found that stretching cells at a level of 1% elongation significantly induced bone nodule formation.

Discussion

In this study, we investigated the effect of mechanical stretching of osteoblasts on arachidonic acid metabolism.

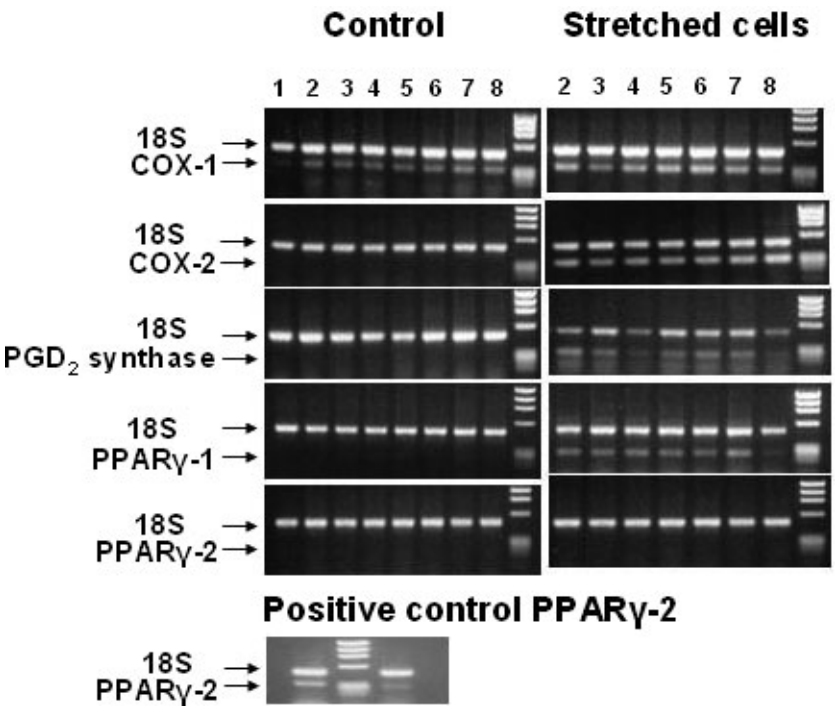

Fig. 3. Relative quantitative reverse transcriptase–polymerase chain reaction of the mRNA of cyclooxygenase (COX)-1, COX-2, prostaglandin (PG)D_2 synthase, peroxisome proliferator-activated receptor (PPAR)γ-1 and PPARγ-2. Left shows unstretched control osteoblasts. Right shows stretched osteblasts. Top (lanes 2–8) shows the time of stretching for 5, 15, and 30 min and 1, 4, 16 and 24 h, respectively. Lane 1 is the baseline control before the stretching regimen. The positive control for PPARγ-2 is shown at the bottom. Control 18S rRNA standards amplified in the same reaction are also shown.

Furthermore, we examined whether stretching enhanced transcriptional expression of the key enzymes and receptors associated with the PGD_2 pathway.

The hypothesis was tested that when mechanical load induces small strains applied to whole bone (within the physiological range of about 0.1% to 0.5%), these strains are amplified to 1% or larger at the level of the membrane of the osteocytes (27). Biaxial stretching was used to mimic this physiological load that is critical for normal maintenance of bone mass. A 1% level of mechanical load retained cell viability and resulted in bone nodule formation in the presence of β-glycerophosphate, which indicates an activation of anabolic bone signals. Higher loads greater than 1% did not induce PGD_2 synthase or PPARγ-1 expression and they decreased cell viability (data not shown). Based upon the results of this study, we present an updated model of the PGD_2 activation pathway, which is induced by cell stretching in osteoblasts (Fig. 1).

Beyond confirming previous reports that stretched osteoblasts secrete PGE_2 and PGI_2, we provide new evidence that mechanical loading also induces both PGD_2 synthase mRNA expression (Fig. 3) as well as increased PGD_2 release (Fig. 2). These results suggest that the increased arachidonic acid metabolites observed from stretched cells were associated with transcriptional activation of individual enzymes (COX-1, COX-2, PGD_2 synthase) of the PGD_2 pathway.

Several studies have shown that $\Delta^{12}PGJ_2$ stimulates osteogenesis and osteoblastic responses (including the stimulation of alkaline phosphatase activity and calcification), the induction of type I collagen production, and the increase in Ca^{2+} influx into cells (10, 14, 28). Thus, $\Delta^{12}PGJ_2$ may modulate osteogenesis through induction of the synthesis and secretion of multiple bone proteins and cellular processes related to osteoid formation and mineralization. This study is the first report of an induction of PGD_2 and $\Delta^{12}PGJ_2$ in osteoblasts subjected to mechanical strain *in vitro*. Considering that PGD_2 is a major arachidonic acid metabolite in bone marrow (15), $\Delta^{12}PGJ_2$ may be physiologically involved in the modulation of osteogenesis induced by mechanical loading.

Because COX-1 and/or COX-2 enzymes are crucial in producing PGH_2, which serves as the substrate for PGD_2, PGI_2 and PGE_2 synthases (29), it was essential to detect and measure them in these experiments. It is well accepted that COX-1 is expressed constitutively in most cells and tissues, including bone (30), and that COX-2 is induced in osteoblasts following mechanical loading (31). However, the understanding of the roles of COX-1 and COX-2 in prostaglandin biosynthesis is changing. There is some evidence that COX-1 and COX-2 play equal roles in PGD_2 biosynthesis (32, 33), yet other evidence suggests that PGD_2 biosynthesis is predominantly mediated by COX-1 (34). In the present study, we found that mechanical loading on osteoblastic cells robustly induced COX-2 mRNA expression, which supports previous findings (31), and mildly induces COX-1 mRNA expression as well. As COX inhibition would be expected to decrease $\Delta^{12}PGJ_2$ synthesis, the inhibition of bone formation by non-steroidal anti-inflammatory drugs (NSAIDs) (35–37) may in part be attributable to a net decrease in the synthesis of this anabolic cyclopentenone class of prostanoids. Further studies are needed to clarify the effects of COX inhibitors on induction of the PGD_2 pathway in stretched osteoblasts.

It is notable, however, that stretching is an anabolic signal that induces significant shunting of arachidonic acid metabolism through the PGD_2 synthase pathway. When osteoblasts were stretched, approximately equivalent proponents of arachidonic acid were metabolized by PGD_2 synthase as by PGE_2 synthase (Fig. 2). These results suggest that the PGD_2 pathway is a major arachidonic acid pathway in stretch-stimulated osteoblasts.

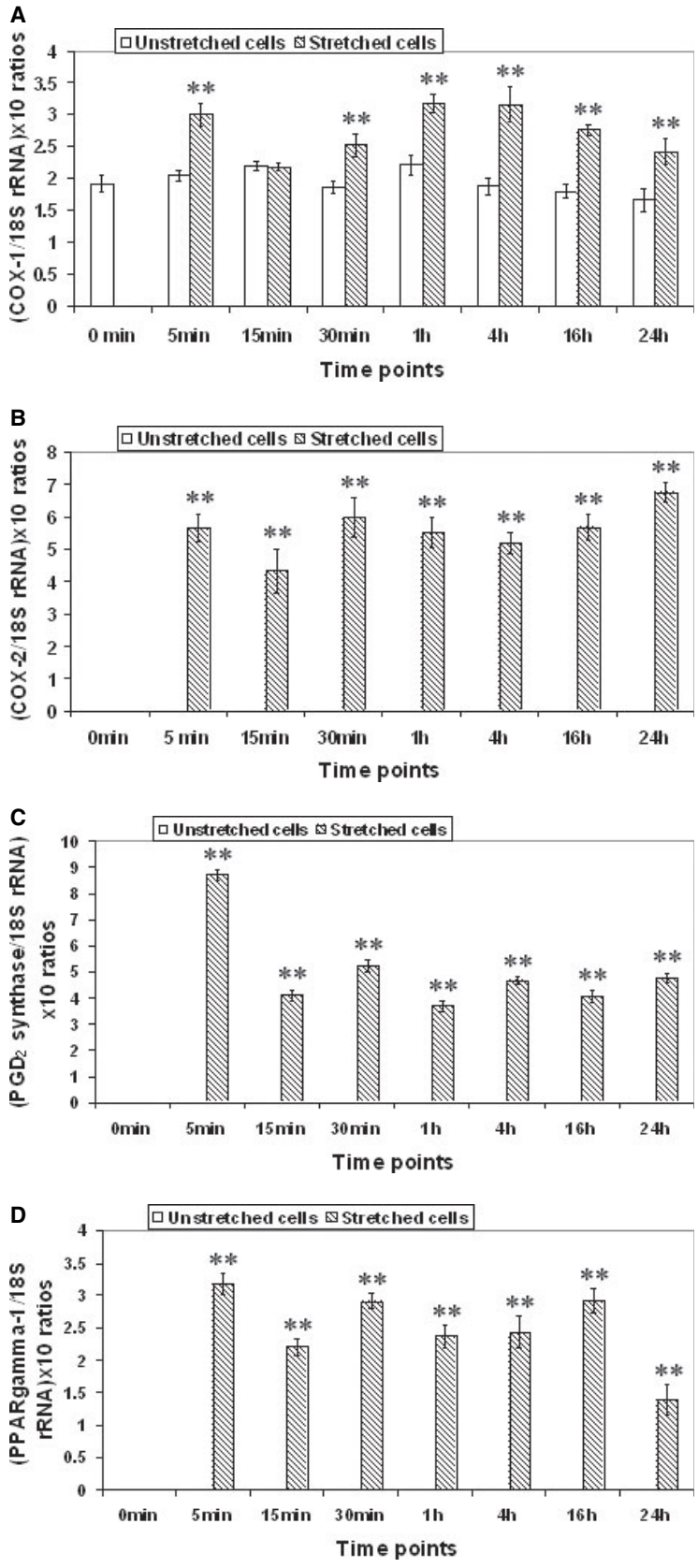

Fig. 4. Relative levels of expression of mRNA for cyclooxygenase (COX)-1 (A), COX-2 (B), prostaglandin (PG)D_2 synthase (C), and peroxisome proliferator-activated receptor (PPAR)γ-1 (D) detected by relative quantitative reverse transcriptase–polymerase chain reaction. Vertical axis shows the ratios of mRNA to 18S rRNA (multiplied by a factor 10 for representation). Horizontal axis shows the total time of stretching. Open bars are representative of unstretched control osteoblasts and hatched bars of stretched osteoblasts. Error bars represent standard error of the mean (SEM) from three independent experiments. Asterisks (*) denote a significant difference between the control and the experimental groups ($p < 0.05$).

In stretched osteoblasts, there was no detectable production of 15-deoxy-$\Delta^{12,14}$-PGJ_2, a metabolite identified as a product of the albumin-catalyzed transformation of PGD_2 *in vitro* (11, 38). The 15-deoxy-$\Delta^{12,14}$-PGJ_2 substrate was recently found to be a high affinity ligand for the PPARγ receptor and to serve as a transcriptional factor that promotes adipocyte differentiation (17, 18). Thus, results of the present study suggest that mechanical loading on bone cells should not promote adipogenesis via 15-deoxy-$\Delta^{12,14}$-PGJ_2. Results of RT–PCR experiments revealed that the levels of PPARγ-1 increased significantly through the period of stretching. As $\Delta^{12}PGJ_2$ also binds to PPARγ, the possibility that $\Delta^{12}PGJ_2$ may play a role in ostogenesis via an interaction of $\Delta^{12}PGJ_2$ with PPARγ cannot be excluded. This possibility is supported by the observation that stretching results in increased $\Delta^{12}PGJ_2$ and its ligand PPARγ.

There are three forms of PPARγ (PPARγ-1, PPARγ-2 and PPARγ-3), which are expressed in different tissues (39). In this investigation, only PPARγ-1 was expressed in osteoblasts as a result of mechanical stress. This suggests that PPARγ-1 may play an important role in osteogenesis induced by mechanical stress. Even though there is strong evidence that $\Delta^{12}PGJ_2$ is a ligand for PPARγ (17, 18), the potential role of this pathway in osteoblasts and the potential effects on

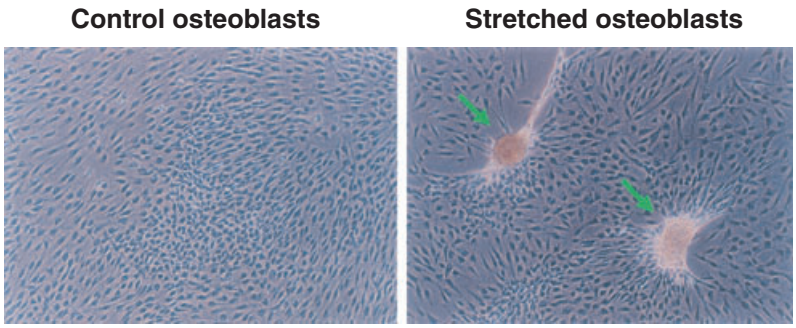

Fig. 5. Comparison of bone nodule formation in control unstretched osteoblasts and stretched osteoblasts. Cell monolayers were examined 2 days after the stretching procedure and the number of bone nodules quantitated. Unstretched control cells (left) showed no bone nodule formation, whereas stretched cells (right) showed formation of bone nodules (green arrow).

Table 2. The number of cell nestings and bone nodules per 10 cm^2 in stretched and unstretched osteoblasts

	Cell nesting Mean ± SEM	Bone nodule Mean ± SEM
Control unstretched cells	15.6 ± 1.5	0
Stretched cells	52.0 ± 1.0*	62.6 ± 2.0*

SEM: standard error of the mean.
*Significant difference between control and experimental groups at $p < 0.05$.

osteogenesis are not known and need further investigation.

We further show that cell stretching caused dramatic changes in osteoblastic phenotype evident by an increase in formation of cell nestings and a significant induction of bone nodule formation, a late stage event in osteogenesis (Fig. 5). These findings further extend previous studies indicating an important role for physical mechanotransduction in bone growth and development (6). These results also support a model for osteogenesis via mechanotransduction that results in the early stages of prostaglandin release, the induction of various cytokines and growth factors (7–9, 31, 40) and an increase in cell nestings and bone nodule formation. However, the direct link between $\Delta^{12}PGJ_2$ induction and its possible role as an anabolic signaling agent in bone nodule formation require further investigation.

In conclusion, we determined that physiological mechanical loading of osteoblasts by physiological stretching resulted in the activation of the COX-1, COX-2 and PGD_2 synthase cascade and led to the release of PGD_2 and its $\Delta^{12}PGJ_2$ metabolite. Stretching also induced PPARγ-1, which is a natural ligand for $\Delta^{12}PGJ_2$. These findings advance the knowledge of the biomechanical transduction pathways that initially involve the activation of COXs and suggest a possibility of interaction between $\Delta^{12}PGJ_2$ and PPARγ-1. These findings also suggest that PGD_2 biosynthesis induced in bone cells by mechanical stress may be one of the major mechanotransduction pathways in osteogenesis. Further studies are needed to clarify the role of this pathway in relation to osteogenesis.

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