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1,25-Dihydroxyvitamin D_3 Pretreatment Limits Prostaglandin Biosynthesis by Cytokine-Stimulated Adult Human Osteoblast-Like Cells

Philip E. Keeting,[1]* Chun Hong Li,[1] Diana L. Whipkey,[1] Ray Thweatt,[1] JiaQuan Xu,[1] Madhavi Murty,[1] J. David Blaha,[2] and Geoffrey M. Graeber[3]

[1]Departments of Biology, West Virginia University, Morgantown, West Virginia 26506
[2]Department of Orthopedics, West Virginia University School of Medicine, Morgantown, West Virginia 26506
[3]Department of Surgery, West Virginia University School of Medicine, Morgantown, West Virginia 26506

Abstract The steroid derivative 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a regulator of bone biology, and there is evidence that $1,25(OH)_2D_3$ modulates arachidonic acid metabolism in osteoblastic cell model systems and in bone organ cultures. In the present studies, $1,25(OH)_2D_3$ decreased prostaglandin (PG) biosynthesis by normal adult human osteoblast-like (hOB) cell cultures by about 30%. The decrease was observed under basal incubation conditions, or in specimens stimulated by transforming growth factor-β_1 (TGF-β) or by tumor necrosis factor-α (TNF). The inhibition of the TGF-β-stimulated PG production appeared to reflect a diminished efficiency of arachidonic acid conversion into PGs by the cells, while the efficiency of substrate utilization for PG biosynthesis was unaffected by $1,25(OH)_2D_3$ pretreatment in the unstimulated samples, or in samples stimulated with TNF or with TNF plus TGF-β. Free arachidonic acid levels were decreased following $1,25(OH)_2D_3$ pretreatment in the TNF stimulated samples. hOB cell phospholipase A_2 activity was measured in subcellular fractions, and this activity was decreased by 20–25% in the $1,25(OH)_2D_3$ pretreated samples. The addition of the selective inhibitor $AACOCF_3$ to the phospholipase A_2 assays provided evidence that it was the cytoplasmic isoform of the enzyme that was affected by the $1,25(OH)_2D_3$ pretreatment of the hOB cells. Thus, $1,25(OH)_2D_3$ regulation of hOB cell biology includes significant effects on arachidonic acid metabolism. In turn, this could influence the effects of other hormones and cytokines whose actions include the stimulated production of bioactive arachidonic acid metabolites. J. Cell. Biochem. 68:237–246, 1998.

Key words: transforming growth factor-β; tumor necrosis factor-α; phospholipase A_2; arachidonic acid; $AACOCF_3$

Prostglandins (PGs) are enzymatically oxygenated derivatives of free arachidonic acid that are well suited to serve as important, responsive regulators of bone biology [Smith, 1992]. We have reported [Xu et al., 1997] that the production of PGE_2 by cultured adult human osteoblast-like (hOB) cells increased over 20-fold following stimulation with a combination of transforming growth factor-β_1 (TGF-β) and tumor necrosis factor-α (TNF). PGs are capable of eliciting either anabolic or catabolic effects on bone [reviewed by Kawaguchi et al., 1995; Raisz et al., 1993; Raisz, 1995], suggesting their functional importance during bone remodeling or in the response of the tissue to trauma. Several cytokines mediate PG biosynthesis by hOB cells and other osteoblastic cell models. Some of these, including TNF, TGF-β, and interleukin-1β, are osteoblastic cell products and could act in an autocrine manner to provide a basis for a focal regulation of PG production [Gowen et al., 1990; Robey et al., 1987; Oursler et al., 1991; Keeting et al., 1991].

Osteoblasts are also subject to organismal and systemic factor effects that influence the biosynthesis of PGs. Local PG production is increased during adaptive bone remodeling [Rawlinson et al., 1991; Forwood, 1996]. Multiple hormones, such as parathyroid hormone and the various osteotropic steroids, including 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), target osteoblasts to condition bone cell functions,

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*Correspondence to: Philip E. Keeting, Department of Biology, P.O. Box 6057, West Virginia University, Morgantown, WV 26506-6057.

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and can affect the production of PGs [Raisz et al., 1993; Kawaguchi et al., 1995]. Klein-Nulend et al. [1991] reported that basal PG production by cultured mouse parietal bones was dose-dependently increased by a 48-h treatment with $1,25(OH)_2D_3$, and that this treatment increased the tissue's PGE_2 response to interleukin-1β or parathyroid hormone stimulation. Other studies reported no $1,25(OH)_2D_3$-dependent changes in PGE_2 production in fetal rat calvariae or in chicken calvariae-derived osteoblastic cells [Feyen et al., 1984; Raisz and Simmons, 1985]. MacDonald et al. [1984] reported that basal [^{14}C]PGE_2 production by a human osteoblast-like cell model was decreased by treatment with $1,25(OH)_2D_3$. Subsequently, we reported that hOB cell uptake of 1-[^{14}C]arachidonic acid into the glycerophospholipid pools of the cells was decreased by $1,25(OH)_2D_3$ treatment, as was the proportional incorporation of the radiolabeled fatty acid specifically into phosphatidylinositol [Cissel et al., 1995]. Those findings suggest that MacDonald's observation could reflect $1,25(OH)_2D_3$ effects on the specific activity of the mobilizable pool of radiolabeled arachidonic acid, rather than a direct regulation of PG biosynthesis by the hormone. The present studies were performed to test the efficiency of substrate mobilization and utilization by hOB cells, and their capacity for PGE_2 biosynthesis, following $1,25(OH)_2D_3$ pretreatment under basal and cytokine stimulated conditions.

METHODS AND MATERIALS

Patient Population

Trabecular bone explants were obtained from surgical waste generated from the femoral head during routine bone grafting procedures or, after gaining informed consent, from the manubrium of the sternum of patients undergoing elective thoracic surgery; these procedures were approved by the West Virginia University Internal Review Board. Patient records were evaluated, and explants were not taken from patients with diagnosed osteoporosis or from those presenting with endocrine disorders that affect bone. Femoral bone explants were obtained from 17 women, aged 27–78 (median age 59), and from 12 men, aged 39–72 (median age 56). Sternal bone samples were obtained from 2 men, aged 43 and 60, and from 2 women, aged 47 and 58. Owing to the limited sample sizes the data in the Results section do not distinguish between hOB cells derived from different bone compartments, or by the explant donor's age or sex.

hOB Cell Cultures

Trabecular bone explants were prepared and placed into culture according to the method developed by Robey and Termine [1985] as previously described [Cissel et al., 1996]. The essential elements of this method include stripping the explants in a 2-h collagenase (1 mg/ml; Gibco, Grand Island, NY) digestion, after which the explants are placed in a calcium-free, phenol red-free mixture (1:1) of DMEM:Ham's F12K (Biofluids, Rockville, MD) supplemented to 10% heat-inactivated fetal calf serum (FCS; Gibco). These culture methods yield nearly homogeneous cell cultures that display multiple aspects of the mature osteoblast phenotype [Borke et al., 1988; Cissel et al., 1996]. The hOB cell phenotype is stable through at least two passages [Keeting et al., 1991b; Marie, 1994]. All experiments described in this report were performed with hOB cells subcultured at the end of first or second passage.

Reagents

Recombinant human TNF and recombinant human TGF-β_1 were purchased from R&D Systems (Minneapolis, MN). Stock solutions of each were prepared as suggested by the supplier and stored at −20°C for no more than 3 months. Unlabeled PGs and a PGE_2-Monoclonal Enzyme Immunoassay Kit were obtained from Cayman Chemical Co. (Ann Arbor, MI). 1-[^{14}C]Arachidonic acid (55 mCi/mmol) and L-α-palmitoyl-2-[1-^{14}C]arachidonoyl- phosphatidylethanolamine (55 mCi/mmol) were purchased from New England Nuclear (Boston, MA). $AACOCF_3$, a trifluoromethylketone analog of arachidonic acid that selectively inhibits human cytosolic 85-kDa phospholipase A_2 [Riendieu et al., 1994], HELSS, reported to be a selective inhibitor of the calcium independent $cPLA_2$ [Lehman et al., 1993], $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$ were purchased from BIOMOL (Plymouth Meeting, PA).

hOB Cell Incubations

Thin-Layer Chromatography. Thin-layer chromatographic (TLC) studies of hOB cell arachidonic acid metabolism were performed as previously described [Xu et al., 1997]. Briefly, hOB cells were subcultured into 6-well plates at 250,000 cells/well in 1 mM calcium contain-

ing 10% FCS-supplemented culture medium, and 48 h later, the FCS content was reduced to 1% for an additional 48-h period. During this period, 10 nM $1,25(OH)_2D_3$ or ethanol, at a final concentration of 0.1% (v/v), was added. Over the final 24 h of this preincubation period, cells were exposed to 0.55 µCi 1-[^{14}C]arachidonic acid/well [Cissel et al., 1995]. The media were then aspirated, and the cell layers were washed to remove unincorporated [^{14}C]arachidonic acid. Experimental manipulations were initiated by the addition of 20 nM TNF, 40 pM TGF-β, both cytokines, or vehicle for 20 h in 10% FCS-supplemented culture medium. Some samples were pretreated with 50 µM ibuprofen for 30 min prior to cytokine stimulation to inhibit PG production. Following the incubation, the hOB cell-conditioned media were collected on ice, microfuged to remove cell debris, and stored at −80°C in siliconized glass tubes until analyzed by TLC using solvent system C of Nugteran and Hazelhof [1973]. Radiolabeled bands were located by autoradiography using BioMax X-ray film (Kodak, Rochester, NY), and the TLC plates were scraped and scintillation counted to determine the radioactivity associated with the various bands.

Enzyme Immunoassays. hOB cells were subcultured at 50,000 cells/well into 24-well plates and pretreated as described above, omitting the preradiolabeling of the cells with 1-[^{14}C]arachidonic acid. Cytokine treatments were performed as described, and PGE_2 was measured in diluted aliquots of the hOB cell conditioned media using an EIA according to the supplier's instructions (Cayman Chemical, Ann Arbor, MI). The detection limit was 60 pg/ml at 80% B/B_0.

Phospholipase A_2 Assay

Nearly confluent hOB cells in 3 175 cm^2 culture flasks per experimental group were grown in 1 mM Ca^{2+}-supplemented, 10% FCS containing medium for 48 h. FCS was reduced to 1% for 48 h in the presence or absence of 10 nM $1,25(OH)_2D_3$. The cells were released by trypsinization, pelleted, resuspended in 1.5 ml homogenization buffer (8% sucrose, 10 mM HEPES, 20 µg/ml leupeptin, pH 7.2), and homogenized in a Dounce homogenizer using 40 up and down strokes. The homogenates were centrifuged at 10,000*g* for 15 min at 4°C, and the resultant supernatants were centrifuged at 100,000*g* for 1 h at 4°C. The pellet was designated as the particulate fraction and the supernatant as the cytosolic fraction. Protein content in the fractions was measured using the BioRad assay (BioRad, Richmond, CA) as per the suppliers instructions with bovine serum albumin as a standard.

The artificial substrate L-α-palmitoyl-2-[^{14}C]arachidonoylphosphatidylethanolamine ([^{14}C]PE) was resuspended in 10 mM tris-HCl buffer (pH 7.5) using 15 low-energy 2-min pulses of a Sonic Dismembranator (Fisher Scientific). The mixture was cooled on ice between each sonication. Ten µl of the sonicated substrate suspension containing 60,000 cpm/10-µl aliquot was added to a reaction mixture composed of 20 µg protein from the cytosolic or particulate fractions, 2% (w/v) sucrose, 2.5 mM HEPES, 0.5 µg leupeptin, 20 µg/ml BSA, 1.0 mM $CaCl_2$, and 0.1 M Tris–HCl, pH 7.5, in a final volume of 100 µl [Paglin et al., 1993]. In some experiments, the subcellular fractions were preincubated with $AACOCF_3$ or with HELSS for 5 min at room temperature before the addition of substrate. The reaction mixture was incubated in a shaking water bath at 37°C for 120 min. (In preliminary experiments, it was observed that hydrolysis of the [^{14}C]PE by the hOB cell subcellular fractions remained linear for at least 240 min [data not shown].) The reaction was terminated by the addition of 400 µl of chloroform–methanol (1:2) and stored at −20°C until analysis.

The frozen samples (500 µl) were acidified with 210 µl of 4 N formic acid and extracted twice with 3 ml ethyl acetate with 0.5 ml H_2O added. The organic layers were pooled, dried under a stream of N_2 gas, the residues redissolved in 100 µl of chloroform–methanol (1:2), and an aliquot taken for scintillation counting. A volume of each sample equivalent to 20,000 cpm was then spotted onto Analtech silica gel G preadsorbent TLC plates and developed in the solvent system described above for the separation of PGs.

Authentic standards for arachidonic acid and PE were co-chromatographed on the plates to confirm the identities of the radiolabeled bands in the samples. The TLC plates were used to produce autoradiographs, the plates were then scraped, and radioactivity determined by scintillation counting. The data on [^{14}C]PE hydrolysis by the $1,25(OH)_2D_3$-pretreated samples are expressed as a percentage of the hydrolysis of the substrate measured in their respective controls.

Statistical Analyses

Data are presented as the mean ±SEM of n experiments using different hOB cell strains.

Data on PG production and arachidonic acid release (Tables I–V) were analyzed by analysis of variance (ANOVA) and by Tukey–Kramer comparison of all pairs, and the effects of the $1,25(OH)_2D_3$ pretreatment were compared with their respective vehicle-pretreated controls by the paired Student t-test. The effects of $1,25(OH)_2D_3$ on [^{14}C]PE hydrolysis by the cytosolic and particulate fractions of the hOB cells were analyzed by the Student's paired t-test after an outlier was eliminated by inspection. When the outlier was included in the analysis, the Wilcoxon rank-sum method was used due to the resultant abnormal distribution of the data. The effects of the $cPLA_2$ inhibitors were analyzed by ANOVA and the Tukey–Kramer comparison of all pairs.

RESULTS

[^{14}C]PG production by 48 hr $1,25(OH)_2D_3$-pretreated hOB cells was qualitatively similar to the production by ethanol (vehicle) pretreated control samples (Fig. 1). [^{14}C]PGE_2 was

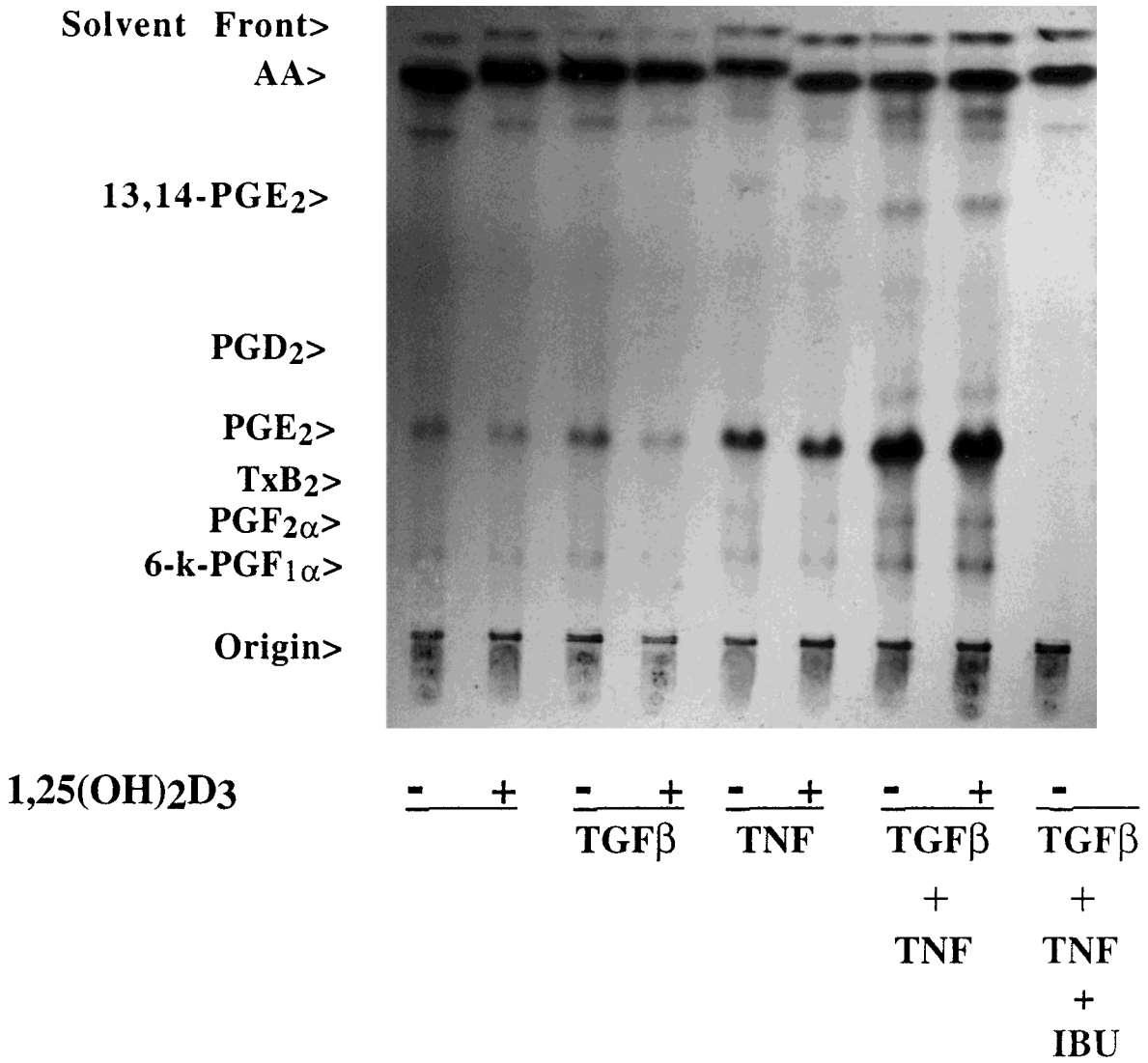

Fig. 1. PG biosynthesis by vehicle or $1,25(OH)_2D_3$ pretreated hOB cells. hOB cells derived from a 58 yr old man were pretreated for 48 hr with vehicle (0.1% ethanol) or with 10 nM $1,25(OH)_2D_2$. Over the final 24 hr of the pretreatment period, the cells were radiolabeled with 1-[^{14}C]-arachidonic acid. Cells were then stimulated with or without TGFβ, TNF, or both cytokines, as indicated beneath the lanes. Ibuprofen (IBU) was added to inhibit PG biosynthesis. The autoradiograph displayed is representative of the results from 9–10 independent trials of the various incubation conditions. The migration of authentic thromboxane B_2 (TxB_2) and PGD_2 in this system are shown, although bands at these positions were not seen in any of the hOB cell conditioned media extracts.

readily observed as the principal PG product of the hOB cells, irrespective of the pretreatment they had received, and this observation held under basal or cytokine-stimulated conditions. [^{14}C]-6-keto-$PGF_{1\alpha}$ and [^{14}C]$PGF_{2\alpha}$ could sometimes be visualized in the autoradiographs as relatively minor products of hOB cells stimulated by the combination of TGF-β plus TNF. No bands corresponding to PGD_2 or to thromboxane B_2 were evident in these experiments.

The TLC plates were scraped, and cpm for the PG bands, arachidonic acid, and the total cpm in each lane were measured. The efficiency of hOB cell conversion of free [^{14}C]arachidonic acid into [^{14}C]PGE_2, or into [^{14}C]-6-keto-$PGF_{1\alpha}$, was calculated as the ratio of (individual) PG cpm–arachidonic acid cpm (Table I). This approach to the data analysis was preferred to a direct comparison of PG cpm between the ethanol-pretreated and $1,25(OH)_2D_3$-pretreated specimens, since earlier work has determined that $1,25(OH)_2D_3$ alters arachidonic acid uptake and its distribution into the cellular glycerophospholipids [Cissel et al., 1995]. Thus, the pools of available radiolabeled arachidonic acid were not directly comparable between the experimental groups. The efficiency of [^{14}C]PGE_2 formation by both the ethanol-pretreated and the $1,25(OH)_2D_3$-pretreated samples was significantly increased ($P < 0.05$) by cytokine stimulation. In only the TGF-β-stimulated samples did the pretreatment with $1,25(OH)_2D_3$ result in a significant decrease ($P < 0.05$) in the ratio of [^{14}C]PGE_2 cpm–[^{14}C]arachidonic acid cpm, indicating a diminished efficiency of conversion of substrate into product by these cells. [^{14}C]-6-keto-$PGF_{1\alpha}$ was also scraped from these TLC plates, and again, only in the TGFβ-stimulated samples did the pretreatment with $1,25(OH)_2D_3$ decrease the ratio of product to substrate ($P < 0.05$). Table II shows the actual cpm data from which the analyses of PGE_2 formation were made. The availability of free [^{14}C]arachidonic acid was decreased by the pretreatment with $1,25(OH)_2D_3$ in the samples stimulated by TNF ($P < 0.05$); however, its efficiency in using the available substrate for PGE_2 formation was not diminished (Table I). The availability of

TABLE I. Efficiency of PG Formation by Ethanol or $1,25(OH)_2D_3$-Pretreated hOB Cells*

	PGE_2/AA (ratio)[a]		6-Keto-$PGF_{1\alpha}$/AA (ratio)[a]	
Stimulus (n)	Ethanol	$1,25(OH)_2D_3$	Ethanol	$1,25(OH)_2D_3$
Vehicle (9)	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
TGF-β (10)	0.25 ± 0.06[b]	0.15 ± 0.03[b,c]	0.07 ± 0.01	0.05 ± 0.01[c]
TNF (9)	0.26 ± 0.06[b]	0.25 ± 0.06[b]	0.07 ± 0.02	0.07 ± 0.01
TGF-β + TNF (10)	0.79 ± 0.16[b]	0.83 ± 0.13[b]	0.10 ± 0.03	0.11 ± 0.02[b]

*hOB cells were pretreated with ethanol or with 10 nM $1,25(OH)_2D_3$ for 48 h. Cells were radiolabeled with 0.55 μCi 1-[^{14}C]AA over the final 24 h of the preincubation period, the cell layers were washed and treated with vehicle, 40 pM TGF-β, 20 nM TNF, or with both cytokines for 20 h. Media were collected and extracted and products separated by thin-layer chromatography. Radiolabeled bands were scraped and scintilation counted.
[a]Data represent the mean ± SEM of n independent trials.
[b]Significantly different ($P < 0.05$) from vehicle-stimulated (ANOVA).
[c]Significantly different ($P < 0.05$) from the ethanol-pretreated samples (paired t-test).

TABLE II. Radiochemical Data on PGE_2 Production and Arachidonic Acid Availability From the Samples Analyzed in Table I*

	PGE_2 (cpm)[a]		AA (cpm)[a]		Lane total (cpm)	
Stimulus (n)	Ethanol	$1,25(OH)_2D_3$	Ethanol	$1,25(OH)_2D_3$	Ethanol	$1,25(OH)_2D_3$
Vehicle (9)	238 ± 53	190 ± 20	5,497 ± 1,568	4,374 ± 852	8,520 ± 1,788	7,114 ± 999
TGF-β (10)	880 ± 402	462 ± 109	3,495 ± 790	3,489 ± 765	7,443 ± 1,342	6,377 ± 877
TNF (9)	938 ± 211	648 ± 137	4,417 ± 973	2,518 ± 323[c]	8,446 ± 1,112	5,548 ± 515[c]
TGF-β + TNF (10)	3,124 ± 778[b]	2,391 ± 463[b]	3,429 ± 547	3,022 ± 448	10,726 ± 1,713	9,008 ± 1,275

*hOB cells were pretreated and incubated as described in Table I. Media were collected and extracted and products separated by thin-layer chromatography. Radiolabeled bands were scraped and scintilation counted.
[a]Data represent the mean ± SEM of n independent trials.
[b]Significantly different ($P < 0.05$) from vehicle-stimulated (ANOVA).
[c]Significantly different ($P < 0.05$) from the ethanol-pretreated samples (paired t-test).

free [^{14}C]arachidonic acid was not significantly lowered by the 1,25(OH)$_2$D$_3$-pretreatment in the other samples.

Quantitative studies measured the effect of the 48-h 1,25(OH)$_2$D$_3$ pretreatment on hOB cell PGE$_2$ production using an EIA (Table III). Basal release of PGE$_2$ was decreased by the pretreatment with 1,25(OH)$_2$D$_3$ ($P < 0.01$). Stimulation of the cells with TGF-β, TNF, or the combination of both cytokines increased PGE$_2$ release by the ethanol-pretreated and by the 1,25(OH)$_2$D$_3$-pretreated samples ($P < 0.05$). PGE$_2$ production in response to TGF-β stimulation ($P < 0.01$) or to TNF stimulation ($P < 0.05$) was decreased in the 1,25(OH)$_2$D$_3$ pretreated samples relative to production by their respective ethanol-pretreated controls. The 1,25(OH)$_2$D$_3$-dependent decrease in PGE$_2$ formation did not reach significance in the samples stimulated by the combination of TGFβ and TNF ($P = 0.08$). Generally, similar responses were obtained when the preincubation period with 1,25(OH)$_2$D$_3$ was extended to 96 h (Table IV). The effects of 1,25(OH)$_2$D$_3$ on PGE$_2$ production could not be duplicated by 24,25(OH)$_2$D$_3$, the inactive metabolite of vitamin D$_3$ (Table V).

PG biosynthesis requires the release of arachidonic acid from glycerophospholipid stores, usually via the actions of cytoplasmic PLA$_2$ (cPLA$_2$) [Burch and Axelrod, 1987]. The possibility that the 1,25(OH)$_2$D$_3$ pretreatment limited hOB cell cPLA$_2$ activity, thereby potentially limiting PG biosynthesis, was evaluated in radiochemical studies (Fig. 2A). The hydrolysis of [^{14}C]PE by the particulate fractions (79.3 ± 4.7% of control sample hydrolysis of [^{14}C]PE) and the cytosolic fractions (74.4 ± 6.7%) of 1,25(OH)$_2$D$_3$-pretreated hOB cells was decreased ($P < 0.005$; n = 5; paired t-test). In the data analysis presented, an outlier from each group was excluded; however, inclusion of the outlier in the analysis did not change the conclusion ($P <$

TABLE III. PGE$_2$ Biosynthetic Capacity of Ethanol- or 1,25(OH)$_2$D$_3$-Pretreated hOB Cells*

	PGE$_2$, ng/ml[a]	
Stimulus (n)	Ethanol	1,25(OH)$_2$D$_3$
Vehicle (14)	9.7 ± 1.7	6.4 ± 1.1[c]
TGFβ (14)	50.0 ± 9.9[b]	31.8 ± 8.0[b,c]
TNF (14)	89.9 ± 17.9[b]	69.3 ± 18.9[b,d]
TGF-β + TNF (12)	160.3 ± 22.9[b]	135.0 ± 19[b]

*hOB cells were pretreated with ethanol or with 10 nM 1,25(OH)$_2$D$_3$ for 48 h, and then stimulated with vehicle, 40 pM TGFβ, 20 nM TNF, or with both cytokines for 20 h. Media were collected and assayed for PGE$_2$ by EIA.
[a]Data represent the mean ± SEM of n independent trials.
[b]Significantly different ($P < 0.05$) from vehicle-stimulated (ANOVA).
[c]Significantly different ($P < 0.01$) from the ethanol-pretreated samples (paired t-test).
[d]Significantly different ($P < 0.05$) from the ethanol-pretreated samples (paired t-test).

TABLE IV. Effects of 96-h Pretreatment With Ethanol or 1,25(OH)$_2$D$_3$ on the PGE$_2$ Biosynthetic Capacity of hOB Cells*

	PGE$_2$, ng/ml[a]	
Stimulus	Ethanol	1,25(OH)$_2$D$_3$
Vehicle	14.7 ± 3.3	9.2 ± 2.5
TGF-β	69.1 ± 10.6[b]	42.9 ± 6.1[b,c]
TNF	80.1 ± 24.8[b]	69.5 ± 18[b]
TGF-β + TNF	242.8 ± 32.9[b]	147.6 ± 33.1[b,c]

*hOB cells were pretreated with ethanol or with 10 nM 1,25(OH)$_2$D$_3$ for 96 h, and then stimulated with vehicle, 40 pM TGF-β, 20 nM TNF, or with both cytokines for 20 h. Media were collected and assayed for PGE$_2$ by EIA.
[a]Data represent the mean ± SEM of four independent trials.
[b]Significantly different ($P < 0.05$) from vehicle-stimulated (ANOVA).
[c]Significantly different ($P < 0.05$) from the ethanol-pretreated samples (paired t-test).

TABLE V. 24,25(OH)$_2$D$_3$ Was Unable to Influence PGE$_2$ Biosynthesis by hOB Cells*

	PGE$_2$, ng/ml[a]		
Stimulus	Ethanol	1,25(OH)$_2$D$_3$	24,25(OH)$_2$D$_3$
Vehicle	15.2 ± 3.7	8.9 ± 2.5[c]	13.7 ± 3.8
TGF-β	44.7 ± 8.9[b]	27.1 ± 5.7[b,c]	41.7 ± 4.0[b]
TNF	99.4 ± 44.8[b]	69.5 ± 18[b,c]	91.4 ± 34.0[b]
TGF-β + TNF	217.0 ± 44.7[b]	191.6 ± 40.1[b]	241.3 ± 55.3[b]

*hOB cells were pretreated with ethanol, 10 nM 1,25(OH)$_2$D$_3$, or 10 nM 24,25(OH)$_2$D$_3$ for 48 h, and then stimulated with vehicle, 40 pM TGF-β, 20 nM TNF, or with both cytokines for 20 h. Media were collected and assayed for PGE$_2$ by EIA.
[a]Data represent the mean ± SEM of four independent trials.
[b]Significantly different ($P < 0.05$) from vehicle-stimulated (ANOVA).
[c]Significantly different ($P < 0.05$) from the ethanol-pretreated samples (paired t-test).

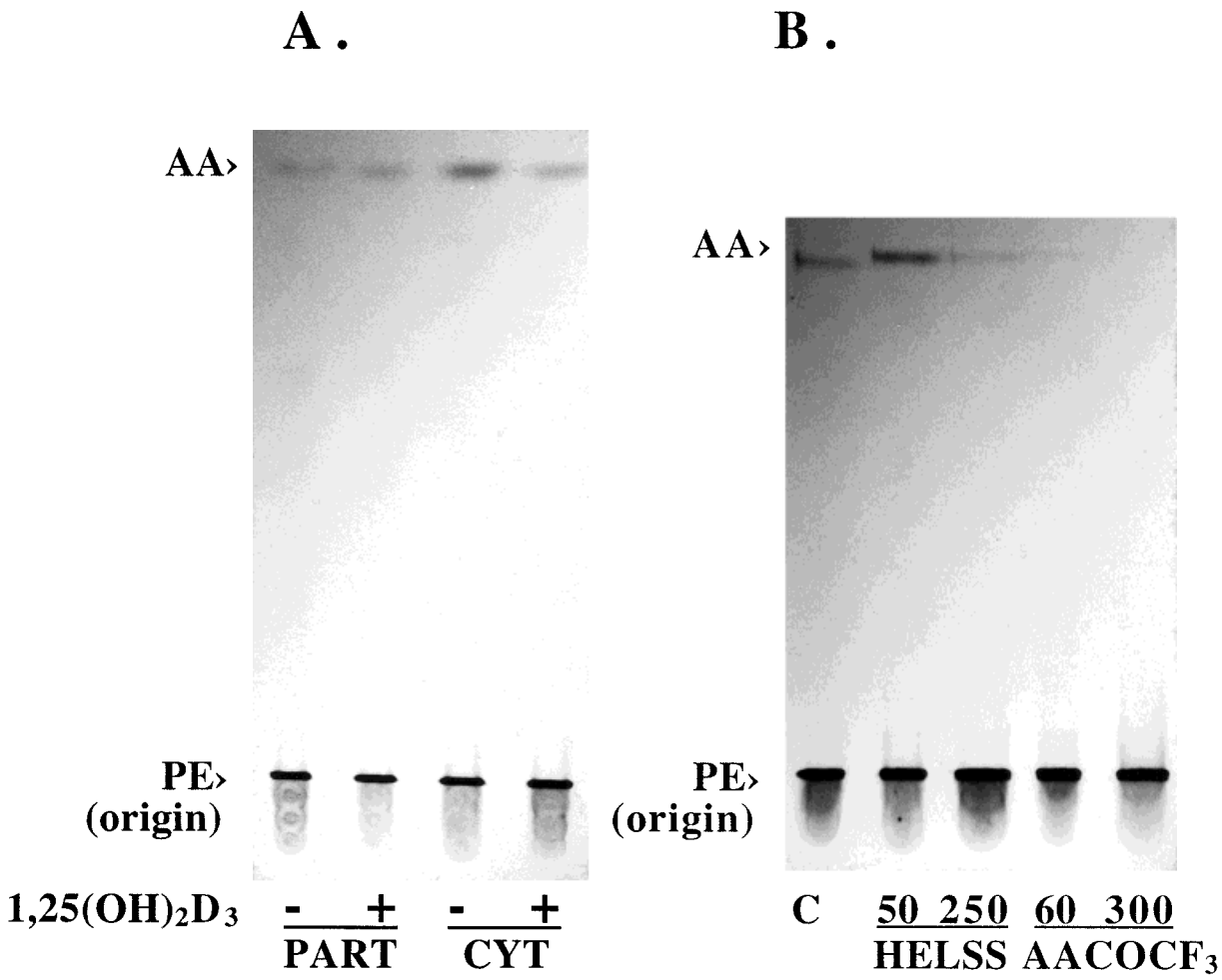

Fig. 2. Assays of hOB cell cPLA$_2$ activity. The cytosolic (CYT) and particulate (PART) fractions of vehicle or 1,25(OH)$_2$D$_3$ pretreated hOB cells from a 40 yr old man were prepared by differential centrifugation and used in 120 min incubations with [^{14}C]PE (**A**). The autoradiograph shown is representative of the results obtained in 5 of 6 independent trials. **B** displays the results of an incubation of [^{14}C]PE with the cytosolic fraction of an hOB cell preparation from cells grown from an explant from a 65 yr old man. HELSS treatment at 50 and 250 μM produced inconsistent effects on the cPLA$_2$ activity, while the treatments with 60 and 300 μM AACOCF$_3$ were routinely effective. These results were repeated in 5 independent trials; similar results were obtained in incubations using the particulate fraction of the hOB cells.

0.05, n = 6, Wilcoxon-rank-sum analysis). The effects of HELSS, a selective inhibitor of the calcium-independent isoform of PLA$_2$, were inconsistent in these studies (Fig. 2B). The addition of 300 μM AACOCF$_3$, a selective cPLA$_2$ inhibitor, to the incubation mixture decreased [^{14}C]PE hydrolysis by hOB cell fractions to 31.5 ± 6.1% (cytoplasmic) and to 37.5 ± 4.7% (particulate) of control sample hydrolysis ($P < 0.05$; n = 5). Placing the particulate fractions prepared from hOB cells into a boiling water bath for 10 min prior to the incubation with substrate reduced the enzyme activity to 34 ± 12% of the activity measured in paired control samples ($P < 0.05$; n = 3). Collectively, these data indicate that the mediation of hOB cell phospholipase A$_2$ activity by 1,25(OH)$_2$D$_3$ was targeted towards the calcium-dependent cytoplasmic isoform of the PLA$_2$ enzyme. The effects of 1,25(OH)$_2$D$_3$ pretreatment on [^{14}C]PE hydrolysis were not evaluated in cells stimulated with TNF or with TGF-β.

Table VI is presented as a comparative summary of the observed effects of 1,25(OH)$_2$D$_3$ pretreatment on hOB cell arachidonic acid metabolism from the present studies.

DISCUSSION

The effective regulation of PG biosynthesis within the bone compartment must be a critical

TABLE VI. Summary of the Effects of $1,25(OH)_2D_3$ Pretreatment on hOB Cell Arachidonic Acid Metabolism*

	Stimulus			
	None	TGF-β	TNF	TGF-β + TNF
Arachidonic acid release	—	—	↓	—
PGE_2 formation: efficiency	—	↓	—	—
PGE_2 formation: capacity	↓	↓	↓	—
PLA_2 activity	↓	ND	ND	ND

*Arrows, results that are significantly different from control samples, dashes indicate no change; ND, not determined.

feature of bone homeostasis. PGs elicit diverse anabolic and catabolic actions in bony tissue. Adaptive bone remodeling is dependent on PG production, and there is evidence that PG production is elevated by either an increase or decrease in mechanical force transmitted through bone [Raisz et al., 1993]. The inhibition of PG synthesis by nonsteroidal anti-inflammatory drugs (NSAIDs) interferes with bone repair during fracture healing or after bone grafting procedures [Allen et al., 1980; Engesaeter et al., 1992; Trancik et al., 1989]. Pre-osteoclast fusion and differentiation are subject to PG mediation, as is osteoclast activation [Akatsu et al., 1991]. Distinct structure–activity relationships for the different PGs have been demonstrated in various bone and bone cell model systems [Fall et al., 1994]. PGE_2 is a bone cell mitogen under most conditions, biphasically regulates collagen synthesis, increases regional bone remodeling when administered exogenously, and stimulates bone formation in vivo [Kawaguchi et al., 1995]. We have identified PGE_2, PGI_2 (as its stable metabolite, 6-keto-$PGF_{1\alpha}$), and $PGF_{2\alpha}$ as hOB cell products of arachidonic acid metabolism [Xu et al., 1997]. Using a variety of models, other investigators have uniformly reported PGE_2 synthesis, and variously the production of PGI_2, $PGF_{2\alpha}$, PGD_2, and thromboxane as well [Kawaguchi et al., 1995; Voelkel et al., 1980; Feyen et al., 1984].

PG biosynthesis is subject to regulation at several levels, including the release of arachidonic acid from the *sn*-2 position of cellular glycerophospholipids by the actions of phospholipases, the two-step conversion of the substrate into PGH_2 by the cyclooxygenase enzymes, and the utilization of PGH_2 as substrate by various isomerases, synthetases, or reductase enzymes to yield the specific PG products of the pathway in a given tissue [Smith, 1992]. In the present studies, the pretreatment of the hOB cells with $1,25(OH)_2D_3$ limited $cPLA_2$ activity by about 20–25%, a decrease roughly comparable to the approximately 30% decrease in PGE_2 synthesis measured in the cell conditioned media, under basal or stimulated conditions. Recent work by Lyons-Giordano et al. [1993] and by Hulkower et al. [1996] have demonstrated temporal and quantitative correlations between $cPLA_2$ activity and PG production in rabbit articular chondrocytes and in human rheumatoid synovial fibroblasts. Nevertheless, it would be premature to conclude that the decrease in hOB cell PGE_2 production is solely the result of $1,25(OH)_2D_3$ effects on hOB cell $cPLA_2$ activity. Berenbaum et al. [1996] found that the synergistic regulation of rabbit articular chondrocyte PGE_2 production by interleukin-1β and TNF did not involve $cPLA_2$ stimulation. In those studies, PGE_2 production increased 160-fold following stimulation, while measured $cPLA_2$ activity was unchanged from controls. Further complicating the mechanistic analyses of the effects of $1,25(OH)_2D_3$ are the data of Kawaguchi et al. [1996] who reported that $cPLA_2$ steady-state mRNA levels were increased in neonatal mouse parietal bone cultures treated with TNF. Marusic et al. [1991] suggested that TGF-β increased PGE_2 production in MC3T3 osteoblastic cells by increasing arachidonic acid release. Jackson et al. [1993] reported that TGF-β treatment elevated steady-state $cPLA_2$ mRNA levels in bovine pulmonary artery endothelial cells, but not in human embryo lung fibroblasts. Since TNF and TGF-β regulate $cPLA_2$ mRNA accumulation in some tissues, the potential interactions of $1,25-(OH)_2D_3$ and the cytokines will need to be independently described.

The conversion of arachidonic acid into bioactive products can shift as a function of tissue development and differentiation [Lysz et al., 1991]. $1,25(OH)_2D_3$ treatment shifts hOB cells toward a more differentiated phenotype [Borke et al., 1988; Cissel et al., 1996], as described by Rodan and Rodan [1984]. The radiochemical analyses of hOB cell arachidonic acid metabolism indicated that PGE_2 formation predominated in both the ethanol-pretreated and the $1,25(OH)_2D_3$-pretreated samples. Novel prod-

ucts, such as PGD_2 or thromboxane B_2, were not observed following the pretreatment, nor was the production of 6-keto-$PGF_{1\alpha}$ or $PGF_{2\alpha}$ accentuated. The efficiency of hOB cell conversion of 1-[^{14}C]arachidonic acid into [^{14}C]PGE_2 and into [^{14}C]-6-keto-$PGF_{1\alpha}$ was diminished by the $1,25(OH)_2D_3$-pretreatment in the samples stimulated by TGF-β. This effect of $1,25(OH)_2D_3$ was specific for the TGF-β-stimulated samples, and the efficiency of [^{14}C]PG formation was not altered by $1,25(OH)_2D_3$-pretreatment in unstimulated hOB cell samples, in the samples stimulated with TNF, or in the samples stimulated by the combination of TNF plus TGF-β. The EIA-based analyses of the PGE_2 biosynthetic capacity of the $1,25(OH)_2D_3$-pretreated samples revealed that total PGE_2 formation was decreased under basal conditions, as well as in the samples stimulated by either cytokine. Although PGE_2 production by hOB cells stimulated with both cytokines together was not significantly changed by a 48-hr pretreatment with $1,25(OH)_2D_3$, a 96-hr pretreatment did inhibit production (Table IV). The radiochemical data presented in Tables I and II suggest that distinct mechanisms can be used to regulate PG biosynthesis by the hOB cells. 1-[^{14}C]Arachidonic acid release was not affected by $1,25(OH)_2D_3$ pretreatment during the 20 hr incubation in samples stimulated by TGF-β, although the efficiency of PGE_2 formation was decreased by the pretreatment. In contrast, the availability of 1-[^{14}C]arachidonic acid was decreased in the $1,25(OH)_2D_3$-pretreated, TNF-stimulated samples while the efficiency of PG synthesis from the substrate was unaffected by $1,25(OH)_2D_3$. These data imply that $1,25(OH)_2D_3$ may affect the regulation of COX-2 expression by TGF-β [Xu et al., 1997], whereas the effects of $1,25(OH)_2D_3$ on basal or TNF-stimulated PGE_2 production might be accomplished through its mediation of $cPLA_2$ activity.

The complexities inherent in the regulation of hOB cell PG biosynthesis are entirely consistent with the pleiotropic effects of the PGs on bone biology. Various steroid hormones, locally produced and systemic polypeptide regulators of bone, mechanical loading, and trauma all exert influences on osteoblastic cell PG biosynthesis. In certain instances, the PGs appear to be involved in elevating the rate of bone resorption, in others, the pace of bone formation. The interaction of multiple regulators, perhaps acting on diverse components of the arachidonic acid cascade, presumably operating in a temporally and sequentially influenced manner, indicates the potential for a fine control of PG elaboration within the bone compartments.

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REFERENCES

Akatsu T, Takahashi N, Udagawa N, Imamura K, Yamaguchi A, Sato K, Nagata N, Suda T (1991): Role of prostaglandins in interleukin-1-induced bone resorption in mice in vitro. J Bone Miner Res 6:183–189.

Allen HL, Wase A, Bear WT (1980): Indomethacin and aspirin: effect on fracture repair in the rat. Acta Orthop Scand 51:595–600.

Berenbaum F, Jacques C, Thomas G, Corvol MT, Bereziat G, Masliah J (1996): Synergistic effect of interleukin-1β and tumor necrosis factor α on PGE_2 production by articular chondrocytes does not involve PLA_2 stimulation. Exp Cell Res 222:379–384.

Borke JL, Eriksen EF, Minami J, Keeting P, Mann KG, Penniston JT, Riggs BL, Kumar R (1988): Epitopes of the human erythrocyte Ca^{2+}-Mg^{2+} ATPase pump in human osteoblast-like cell plasma membranes. J Clin Endocrinol Metab 67:1299–1304.

Burch RM, Axelrod J (1987): Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: evidence for G protein regulation of phospholipase A_2. Proc Natl Acad Sci USA 84:6374–6378.

Cissel DS, Birkle DL, Whipkey DL, Blaha JD, Graeber GM, Keeting PE (1995): 1,25-Dihydroxyvitamin D_3 or dexamethasone modulate arachidonic acid uptake and distribution into glycerophospholipids by normal adult human osteoblast-like cells. J Cell Biochem 57:599–609.

Cissel DS, Murty M, Whipkey DL, Blaha JD, Graeber GM, Keeting PE (1996): Estrogen pretreatment increases arachidonic acid release by bradykinin stimulated normal human osteoblast-like cells. J Cell Biochem 60:260–270.

Dekel S, Lenthall G, Francis MJO (1981): Release of prostaglandins from bone and muscle after tibial fracture. J Bone Joint Surg 63B:185–189.

Engesaeter LB, Sudmann B, Sudmann E (1992) Fracture healing in rats inhibited by locally administered indomethacin. Acta Orthop Scand 63:330–333.

Fall PM, Breault DT, Raisz LG (1994): Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic clonal cell line, Py 1a: structure activity relations and signal transduction mechanisms. J Bone Miner Res 9:1935–1942.

Feyen JHM, Van der Wilt G, Moonen P, Di Bon A, Nijweide PJ (1984): Stimulation of arachidonic acid metabolism in primary cultures of osteoblast-like cells by hormones and drugs. Prostaglandins 28:769–782.

Forwood MR (1996): Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J Bone Miner Res 11:1688–1693.

Gowen M, Chapman K, Littlewood A, Hughes D, Evans D, Russell RGG (1990): Production of tumor necrosis factor by human osteoblasts is modulated by other cytokines, but not by osteotropic hormones. Endocrinology 126:1250–1255.

Hulkower KI, Coffey JW, Levin W, Anderson CM, Chen T, Hope WC, Bolin DR, Morgan DW (1993): Interleukin-1β induces cytosolic PLA_2 in parallel with prostaglandin E_2 in rheumatoid synovial fibroblasts. Agents Actions 9:C5–C7.

Jackson BA, Goldstein RH, Roy R, Cozzani M, Taylor L, Polgar P (1993): Effects of transforming growth factor β and interleukin-1β on expression of cyclooxygenase 1 and 2 and phospholipase A_2 mRNA in lung fibroblasts and endothelial cells in culture. Biochem Biophys Res Commun 197:1465–1474.

Kawaguchi H, Pilbeam CC, Harrison JR, Raisz LG (1995): The role of prostaglandins in the regulation of bone metabolism. Clin Orthop 313:36–46.

Kawaguchi H, Nemoto K, Raisz LG, Harrison JR, Voznesensky OS, Alander CB, Pilbeam CC (1996): Interleukin-4 inhibits prostaglandins G/H synthase-2 and cytosolic Phospholipase A_2 induction in neonatal mouse parietal bone cultures. J Bone Miner Res 11:358–366.

Keeting PE, Rifas L, Harris SA, Colvard DS, Spelsberg TC, Peck WA, Riggs BL (1991a): Evidence for interleukin-1β production by cultured normal human osteoblast-like cells. J Bone Miner Res 6:827–833.

Keeting PE, Scott RE, Colvard DS, Han IK, Spelsberg TC, Riggs BL (1991b): Lack of a direct effect of estrogen on proliferation and differentiation of normal human osteoblast-like cells. J Bone Miner Res 6:297–304.

Klein DC, Raisz LG (1970): Prostaglandins: stimulation of bone resorption in tissue culture. Endocrinology 86:1436–1440.

Klein-Nulend J, Pilbeam CC, Raisz LG (1991): Effect of 1,25-dihydroxyvitamin D_3 on prostaglandin E_2 production in cultured mouse parietal bones. J Bone Miner Res 6:1339–1344.

Lehman JJ, Brown KA, Ramanadham S, Turk J, Gross RW (1993): Arachidonic acid release from aortic smooth muscle cells induced by [Arg^8]vasopressin is largely mediated by calcium independent phospholipase A_2. J Biol Chem 268:20713–20716.

Lyons-Giordano B, Pratta MA, Galbraith W, Davis GL, Arner EC (1993): Interleukin-1 differentially modulates chondrocyte expression of cyclooxygenase-2 and phospholipase A_2. Exp Cell Res 206:58–62.

Lysz TW, Fu S-CJ, Brash A, Keeting PE, Chengren L, Shariff H, Wu Y (1991): Identification of 12(S)-hydroxyeicosatetraenoic acid in the young rat lens. Curr Eye Res 10:331–337.

MacDonald BR, Gallagher JA, Ahnfelt-Ronne I, Beresford JN, Gowen M, Russell RGG (1984): Effect of bovine parathyroid hormone and 1,25-dihydroxyvitamin D_3 on the production of prostaglandins by cells derived from human bone. FEBS Lett 169:49–52.

Marie PJ (1994): Perspectives—Human osteoblast-like cells: A potential tool to assess the etiology of pathologic bone formation. J Bone Miner Res 9:1847–1850.

Marusic A, Kalinowski JF, Harrison JR, Centrella M, Raisz LG, Lorenzo JA (1991): Effects of transforming growth factor-β and IL-1α on prostaglandin synthesis in serum deprived osteoblastic cells. J Immunol 146:2633–2638.

Nugteran DH, Hazelhof E (1973): Isolation and properties of intermediates in prostaglandin biosynthesis. Biochim Biophys Acta 326:448–461.

Oursler MJ, Cortese C, Keeting PE, Anderson M, Bonde S, Riggs BL, Spelsberg TC (1991): Modulation of transforming growth factor-beta production in normal human osteoblast-like cells by 17-[β]-estradiol and parathyroid hormone. Endocrinology 129:3313–3319.

Paglin S, Roy R, Polgar P (1993): Characterization of hormonally regulated and particulate-associated phospholipase A_2 from bovine endothelial cells. J Biol Chem 268: 11697–11702.

Raisz LG (1995): Physiologic and pathologic roles of prostaglandins and other eicosanoids in bone metabolism. J Nutr 125:2024S–2027S.

Raisz LG, Simmons HA (1985): Effects of parathyroid hormone and cortisol on prostaglandin production by neonatal rat calvaria in vitro. Endocr Rev 11:59–74.

Raisz LG, Pilbeam CC, Fall PM (1993): Prostaglandins: mechanisms of action and regulation of production in bone. Osteoporosis Int Suppl 1S136–S140.

Rawlinson SCF, El-Haj AJ, Mintor SL, Tavares IA, Bennett A, Lanyon LE (1991): Loading-related increases in prostaglandin production in cores of adult canine cancellous bone in vitro: A role for prostacyclin in adaptive bone remodeling? J Bone Miner Res 6:1345–1351.

Riendeau D, Guay J, Weech PK, Laliberte F, Yergey J, Li C, Desmarais S, Perrier H, Liu S, Nicoll-Griffith D, Street IP (1994): Arachidonoyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase A_2, blocks production of arachidonic acid and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. J Biol Chem 269:15619–15624.

Robey PG, Termine JD (1985): Human bone cells in vitro. Calcif Tissue Int 37:453–460.

Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB (1987): Osteoblasts synthesize and respond to TGFβ in vitro. J Cell Biol 105:457–463.

Rodan GA, Rodan SB (1984): Expression of the osteoblast phenotype. In Peck WA (ed) Bone and Mineral, Annual II. Excerpta Medica, Amsterdam, pp 244–286.

Smith W (1992): Prostanoid biosynthesis and mechanisms of action. Am J Physiol 263:F181–F191.

Trancik T, Mills W, Vinson N (1989): The effect of indomethacin, aspirin, and ibuprofen on bone ingrowth into a porous-coated implant. Clin Orthop 249:113–121.

Voelkel EF, Tashjian AH, Levine L (1980): Cyclooxygenase products of arachidonic acid metabolism by mouse bone in organ culture. Biochim Biophys Acta 620:418–428.

Xu J-Q, Cissel DS, Varghese S, Whipkey DL, Murty M, Blaha JD, Graeber GM, Keeting PE (1997): Cytokine treatment stimulates prostaglandin production by normal adult human osteoblast-like cells. J Cell Biochem 64:618–631.