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1,25-Dihydroxyvitamin D_3 or Dexamethasone Modulate Arachidonic Acid Uptake and Distribution Into Glycerophospholipids by Normal Adult Human Osteoblast-Like Cells

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Abstract The effects of treatment with the osteotropic steroids 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 17β-estradiol, or dexamethasone on [1-^{14}C]arachidonic acid (AA) uptake and distribution into glycerophospholipid classes by normal adult human osteoblast-like (hOB) cells were investigated. Total uptake of [1-^{14}C]AA was decreased in cells treated with dexamethasone when assayed after a 24-, 48-, or 96-h exposure to the hormone. Specific radiolabel incorporation into phosphatidylcholine was reduced by a 48-h treatment with dexamethasone with a concurrent increase in the radiolabeling of phosphatidylethanolamine. However, these changes were transient, and by 96 h of dexamethasone treatment the distribution of the radiolabeled fatty acid had reequilibrated to resemble the pattern found for vehicle treated samples. Total uptake of [1-^{14}C]AA was diminished by 96-h treatment with $1,25(OH)_2D_3$ (79 ± 3% of control, $P < 0.01$); at that time point, a significant decrease in the proportional radiolabeling of the phosphatidylinositol pool was identified (92 ± 2% of control, $P < 0.05$). The $1,25(OH)_2D_3$-dependent decrease in total uptake and in phosphatidylinositol incorporation of [1-^{14}C]AA were found to be hormone dose dependent. Treatment with $24,25(OH)_2D_3$ was without effect on either total [1-^{14}C]AA uptake or the specific [1-^{14}C]AA radiolabeling of the phosphatidylinositol pool. $1,25(OH)_2D_3$ treatment decreased hOB cell uptake of [1-^{14}C]oleic acid and decreased its proportional incorporation into the phosphatidylinositol pool. Gas chromatographic analyses revealed no $1,25(OH)_2D_3$-dependent effects on total phosphatidylinositol lipid mass or on the mole percent of arachidonic acid within the phosphatidylinositol pool, leaving the mechanism of the effects of the secosteroid on hOB cell AA metabolism unexplained. 17β-Estradiol had no effects on the parameters of AA metabolism measured. As a consequence of their modulation of arachidonic acid uptake and its distribution into hOB cellular phospholipids, steroids might alter the biological effects of other hormones whose actions include the stimulated production of bioactive AA metabolites, such as prostaglandins or the various lipoxygenase products.

Key words: 17β-estradiol, phosphatidylinositol, gas chromatography, fatty acid metabolism

Free arachidonic acid (AA) is the principal precursor of the prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs), a group of oxygenated bioactive lipids collectively referred to as the eicosanoids [Smith, 1989]. Typically, AA is found esterified in the *sn*-2 position of the cellular glycerophospholipids [Irvine, 1982]. In its esterified form, the fatty acid is not a useful substrate for the enzyme systems that produce the eicosanoids [MacDonald and Sprecher, 1991]. Following appropriate stimulus, specific cellular phospholipases are activated and hydrolyze the arachidonoyl ester linkage releasing free AA [Axelrod et al., 1988]. Various phospholipases show specificity for different phospholipid classes [Cockcroft and Thomas, 1992; Exton, 1994]. The appearence of free AA is believed to be the rate-limiting step for eicosanoid biosynthesis [Irvine, 1982; MacDonald and Sprecher, 1991].

Following the seminal work of Raisz and associates [Klein and Raisz, 1970], numerous bone and bone cell studies have confirmed that the

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prostaglandins have important bioregulatory roles in bone physiology and can contribute to the pathology of bone diseases [Hakeda et al., 1987; Norrdin et al., 1990; Collins and Chambers, 1991]. Several of the peptidyl bone resorption-promoting hormones and cytokines depend, at least in part, on their capacity to stimulate prostaglandin synthesis for their activity [Lerner et al., 1987; Akatsu et al., 1991]. Curiously, although bone resorption is an osteoclast function, the receptors for many of these resorption-promoting stimulators are found in the osteoblast and are not expressed in the osteoclast [Kahn and Partridge, 1987]. Thus, in addition to their bone-forming functions, emerging work indicates that osteoblasts are key regulators of the osteoclast population with mediating activities in osteoclast recruitment, differentiation, and activation [McSheehy and Chambers, 1986; Kahn and Partridge, 1987; Suda et al., 1992]. A part of this paracrine regulation of the osteoclast cell population is accomplished by prostaglandins, since various nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandin synthesis interfere with osteoblast mediation of osteoclast functions [Akatsu et al., 1991]. Recently, evidence has been presented that lipoxygenase products can activate the bone resorption process [Meghji et al., 1988; Gallwitz et al., 1993]. Although it is unknown whether human osteoblasts possess lipoxygenase activity, those studies have served to heighten interest in AA metabolism in cells of the osteoblast lineage.

The steroid hormones, including 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$) [Lamberg-Allardt, 1991], 17β-estradiol (17β-E_2) [Lindsay, 1987], and glucocorticoids [Villareal et al., 1991], are all systemic regulators of bone homeostasis and pathology. These hormones have all been identified as regulators, at various levels, of AA metabolism in some target tissues [Feyen and Raisz, 1987; Duval and Freyss-Beguin, 1992; Swain et al., 1992]. If steroid treatment modulates AA metabolism in osteoblasts, then the biological effects of other hormones whose actions include the stimulation of eicosanoid biosynthesis within bone may be indirectly sensitive to steroid treatment. Accordingly, the effects of these steroids on the uptake of AA into specific glycerophospholipid classes were investigated in normal human osteoblast-like (hOB) cells derived from trabecular bone explants taken from adult donors.

MATERIALS AND METHODS

Patient Population

Trabecular bone explants were obtained after obtaining informed consent from patients without evidence of metabolic bone disease, or diabetes, undergoing elective thoracic surgery. The procedures used have been approved by the WVU-IRB. The patient population sampled included nine women, aged 46–86 years, with a median age of 63, and 23 men, aged 20–75, with a median age of 63. No evidence for donor age- or sex-linked effects on steroid hormone regulation of AA metabolism was identified in these studies.

hOB Cell Cultures

Normal adult human trabecular bone was obtained from the manubrium of the sternum of the patients. Human osteoblast-like (hOB) cells were established in culture according to the procedure of Robey and Termine [1985] with modifications. After the removal of any adherent fibrous tissue, the explants were washed and minced by scalpel into small fragments (2–4 mm in diameter). These small fragments were digested with 1 mg/ml crude bacterial collagenase (Gibco, Grand Island, NY) in Dulbecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD) for 2 h at 37°C in a shaking water bath. After the collagenase digestion, the bone fragments were placed in a calcium-free phenol red-free medium consisting of DMEM:Ham's F12K (1:1; Biofluids) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 1 mM glutamine (Sigma, St. Louis, MO), 0.5 mg/ml penicillin, 0.5 mg/ml streptomycin sulfate, and 1 mg/ml neomycin sulfate. hOB cell growth in primary culture typically required 4–6 weeks to approach confluence, and the cultures were passaged once at a 1:2 split. Previous reports indicate that the hOB phenotype remains stable in culture through at least two passages [Erickson et al., 1988; Keeting et al., 1991a,b]. All experiments were performed using hOB cells subcultured from near-confluent first passage cells seeded into 24-well plates at a density of 20,000 cells per well in 10% fetal calf serum (FCS) containing medium supplemented to 1 mM calcium. After seeding, the cells were incubated for 48 h in this media to attach and recover from trypsinization before initiating the experimental manipulations. All experiments

were conducted in medium supplemented to 1% FCS and 1 mM calcium.

Preparation of Steroids

Stock solutions of 17β-E_2 (Sigma), dexamethasone (a synthetic glucocorticoid; Sigma), 1,25[OH]$_2D_3$ (BIOMOL Research Lab., Plymouth Meeting, PA) and 24,25-dihydroxyvitamin D_3 (24,25[OH]$_2D_3$; BIOMOL) were prepared in ethanol and stored at −20°C until use. The final concentration of ethanol in the experimental samples was 0.1%.

Hormones were used at concentrations previously found effective in hOB cell studies, specifically, 1 nM 1,25$(OH)_2D_3$ [Keeting et al., 1991b], 50 nM dexamethasone [Subramaniam et al., 1992], and 10 nM 17β-E_2 [Oursler et al., 1991], unless otherwise noted. These dosages did not effect hOB cell viability or proliferation [data not shown; and Keeting et al., 1991b].

Incorporation of Radiolabeled Fatty Acids Into Glycerophospholipids

[1-^{14}C]Arachidonic acid ([1-^{14}C]AA, 55 mCi/mmol; New England Nuclear, Boston, MA) was prepared in 0.25% (w/v) fatty acid-free bovine serum albumin (BSA) in DMEM. hOB cell cultures were radiolabeled for the final 18–24 h prior to the termination of all incubations with 0.1 μCi [1-^{14}C]AA. In some experiments, hOB cell cultures were radiolabeled with 0.1 μCi [1-^{14}C]oleic acid (55 mCi/mmol; New England Nuclear) under identical conditions.

Thin-Layer Chromatographic Analysis of [1-^{14}C]AA-Radiolabeled Glycerophospholipids

After radiolabeling, media were aspirated and the hOB cell layers washed once with a 0.1% (w/v) BSA solution to remove remaining unincorporated [1-^{14}C]AA. A lysis buffer (2% sodium dodecyl sulfate [SDS], 2 mM EDTA, 20 mM $NaHCO_3$, pH 7.4) was applied to the washed cell layers for 1 min; lysates were harvested into borosilicate glass tubes on ice and stored at −20°C until assayed. Cellular lipids were extracted twice according to the method of Bligh and Dyer [1959], and the organic layers were pooled. An aliquot of the extract was scintillation counted (Fisher Scintiverse BD, Fisher Scientific, Pittsburgh, PA; Beckman Instruments, Irvine, CA), to measure total [1-^{14}C]AA uptake by each hOB sample. The remaining extract was dried under a stream of nitrogen gas, reconstituted in 30 μl of $CHCl_3$:MeOH (1:2), and spotted onto Analtech (Newark, DE) silica gel G preadsorbant thin-layer chromatography (TLC) plates. TLC plates were developed in a chloroform–ethanol–triethylamine–H_2O (30:40:30:8) solvent system. Authentic phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylserine (PS) standards (Sigma) were co-chromatographed in adjacent lanes and visualized by staining with I_2 vapor. Following autoradiography, migration distances (R_f values) of radiolabeled bands were compared to the authentic glycerophospholipid standards to identify sample glycerophospholipids. Radioactivity in the bands was measured by solid state radioactivity scanning using a radioanalytic imaging system (AMBIS, San Diego, CA). The percentage of total [1-^{14}C]AA distributed into each glycerophospholipid class was calculated by the formula

$$\%\text{ of total [1-}^{14}\text{C]AA cpm} = \frac{\text{cpm per individual phospholipid band} \times 100}{\text{total cpm in sample lane.}}$$

When [1-^{14}C]oleic acid was used instead of [1-^{14}C]AA to radiolabel cells, thin layer chromatographic analyses of [1-^{14}C]oleic acid radiolabeled glycerophosopholipids were performed identically to the procedure described above.

Gas Chromatographic Analysis of Fatty Acids

Following incubation with 1,25$(OH)_2D_3$ or vehicle for 96 h, hOB cell layers were lysed into 0.2% SDS. Each experimental sample used 300,000–700,000 cells. hOB cellular glycerophospholipids were extracted and separated on Analtech TLC plates as described above but, since the [1-^{14}C]AA radiolabeling step was omitted in these experiments, the separated glycerophospholipids were visualized by spraying the plates with 1% dichlorofluorescein (in methanol), followed by exposure to UV light. Sample bands corresponding to authentic PC, PI, PE, and PS standards were scraped into screwcap glass tubes and subjected to methanolysis in BF_3:methanol [Morrison and Smith, 1964]. The fatty acid methyl esters were analyzed on a Perkin-Elmer capillary gas–liquid chromatograph with dual columns and flame ionization detection, using a DB-23 (cyanopropyl polysiloxane, 30-m × 0.25-mm ID) column (Supelco, Bellefonte, PA), operated in a temperature gradient mode from 70°C to 220°C. Fatty acid methyl esters were identi-

fied by comparison of retention times to authenic standards and quantified by an internal standard method using heptadecanoate methyl ester (17:0). An equal volume of the 0.2% SDS solution was extracted, co-chromatographed in lanes adjacent to the sample extracts, and subjected to gas chromatography to serve as blanks in each experiment. Data are expressed as pmol lipid/500,000 cells to correct for differences in cell number between experiments. Phospholipid mass is expressed as a percentage of total phospholipid (PC, PE, PI, and PS) mass to control for differences in sample loading onto the TLC plates.

Statistical Analysis

Data are presented as the mean ±SEM of n experiments using different hOB cell strains. When appropriate, the data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's test to compare a set of treatment group means against the mean of its control (vehicle treated). Gas chromatographic data were analyzed by the paired Student's *t*-test.

RESULTS

Total [1-^{14}C]AA uptake by hOB cells was evaluated at 24, 48, and 96 h following the initiation of steroid treatment. At each measured interval, dexamethasone-treated cells demonstrated a significantly diminished capacity for [1-^{14}C]AA uptake (24 h: 86 ± 3% of control, n = 7, $P < 0.01$; 48 h: 86 ± 4%, n = 6, $P < 0.01$; 96 h: 90 ± 4%, n = 9, $P < 0.05$). hOB cells treated with $1,25(OH)_2D_3$ exhibited no change in total [1-^{14}C]AA uptake following a 24-h (102 ± 2% of control, n = 8) or a 48-h (93 ± 4%, n = 9) treatment, whereas in samples treated for 96 h, uptake was decreased (79 ± 3%, n = 13, $P < 0.01$). This response was specific for the active metabolite of vitamin D_3, since $24,25(OH)_2D_3$ treatment of the hOB cells for 96 h elicited no such change (97 ± 4%, n = 7). 17β-Estradiol treatment did not alter [1-^{14}C]AA uptake by the hOB cells at any of the time intervals (24 h: 106 ± 2%, n = 8; 48 h: 101 ± 2%, n = 8; 96 h: 104 ± 5%, n = 10).

The major glycerophospholipid classes were a primary site for the incorporation of the radiolabeled arachidonic acid (Table I). Steroid hormone effects on the proportional radiolabeling of the major glycerophospholipid classes were evaluated. Figure 1 illustrates a representative TLC of the separation of extracted hOB cell lipids, demonstrating the resolution of the TLC method employed in these studies. Phosphatidylcholine incorporation of [1-^{14}C]AA decreased to 92 ± 2% of control (n = 6, $P < 0.01$) following a 48-h treatment with dexamethasone with a concurrent increase of incorporation into phosphatidylethanolamine (117 ± 8%, n = 6, $P < 0.01$). These changes were transient, and by 96 h of treatment with dexamethasone, the pattern of [1-^{14}C]AA distribution into specific glycerophospholipids resembled the pattern seen in control samples (Table II). In addition to dexamethasone, Table II also illustrates the effects of a 96-h treatment with $1,25(OH)_2D_3$ or 17β-E_2 on the incorporation of the [1-^{14}C]AA into the major glycerophospholipid classes. No evidence of 17β-E_2-dependent changes in specific phospholipid labeling was found at the 96-h time point (or after shorter exposures of 24 or 48 h; data not shown). The proportional incorporation of [1-^{14}C]AA into phosphatidylinositol was inhibited in samples treated with $1,25(OH)_2D_3$ for 96 h to 92 ± 2% of control (n = 12, $P < 0.05$). This effect was time dependent and no change in the incorporation of [1-^{14}C]AA into phosphatidylinositol was seen in hOB cells treated with $1,25(OH)_2D_3$ for 24 h (95 ± 2, n = 8) or for 48 h (101 ± 5, n = 8). The response observed at 96 h was specific for the active metabolite of vitamin D_3, since $24,25(OH)_2D_3$ treatment for 96 h did not alter [1-^{14}C]AA radiolabeling of phosphatidylinositol (98 ± 2%, n = 6).

In order to confirm the observed responses of the hOB cells to $1,25(OH)_2D_3$ treatment, dose–

Table I. Incorporation of [^{14}C]Arachidonic Acid and [^{14}C]Oleic Acid Into the Lipid Fraction of hOB Cells

Lipid fraction	% Distribution[a]	
	[^{14}C]arachidonic acid[b]	[^{14}C]oleic acid[b]
NL	10.6 ± 0.7	11.2 ± 0.9
PE	20.8 ± 0.4	10.4 ± 0.2
PI	12.0 ± 0.5	5.6 ± 0.4
PS	5.3 ± 0.4	3.5 ± 0.2
PC	39.8 ± 0.5	59.1 ± 0.9

[a]hOB cells were labeled with 0.1 μCi [^{14}C]arachidonic acid or 0.1 μCi [^{14}C]oleic acid for 18–24 h. Cellular lipids were extracted and separated by thin-layer chromatography, and the proportional distribution of radiolabeled fatty acid was determined as described under Materials and Methods. [^{14}C]Arachidonic acid labeled cell cultures averaged a total uptake of 65,000 ± 5,163 cpm (n = 10). [^{14}C]oleic acid labeled cell cultures averaged a total uptake of 58,343 ± 12,702 cpm (n = 6).

[b]Data represent the mean ±SEM.

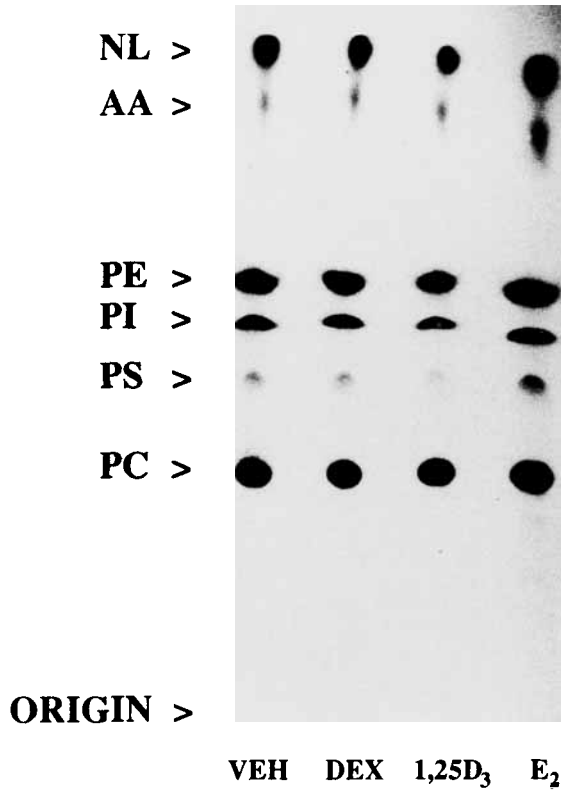

Fig. 1. Autoradiograph of radiolabeled lipids from steroid treated hOB cells. Cells were incubated with ethanol vehicle (VEH), dexamethasone (DEX), 1,25-dihydroxyvitamin D_3 ($1,25D_3$), or 17β-estradiol (E_2), for 96 h and labeled over the last 24 h with 0.1 μCi [1-^{14}C]AA. Cell lipids were extracted and separated by thin-layer chromatography as described under Materials and Methods. PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; AA, arachidonic acid; NL, neutral lipids.

response studies were performed over a 96-h incubation period (Fig. 2). Both the decrease in total [1-^{14}C]AA uptake ($r = 0.98$, $P < 0.01$, $n = 4$) and the altered profile of glycerophospholipid radiolabeling ($r = 0.91$, $P < 0.01$, $n = 4$) were dose dependent over the dosage range 0.001–30 nM $1,25(OH)_2D_3$.

Attempts to uncover the mechanism by which $1,25(OH)_2D_3$ treatment had specifically decreased the incorporation of [1-^{14}C]AA into phosphatidylinositol were made using gas chromatography to analyze cellular glycerophospholipid amounts and arachidonic acid content. Treatment of hOB cells with the hormone for 96 h had no effect on the total phosphatidylinositol lipid mass (vehicle: 9.7 ± 0.9%; $1,25(OH)_2D_3$ treated: 10.7 ± 1.0%, n = 4, NS) or on the total lipid mass of the other major glycerophospholipid classes (n = 4, NS; Table III). The mole

Table II. Effect of Steroids on the Proportional Incorporation of [^{14}C]Arachidonic Acid Into hOB Cell Glycerophospholipids

Pretreatment[a]	n	% of control[b] PE	PI	PS	PC
$1,25(OH)_2D_3$	12	94 ± 2	92 ± 2*	107 ± 5	101 ± 1
17β-Estradiol	9	101 ± 3	98 ± 3	110 ± 6	98 ± 2
Dexamethasone	8	103 ± 2	96 ± 3	114 ± 9	98 ± 1

[a]hOB cells were incubated with the indicated steroid or ethanol vehicle (control) for 96 h and radiolabeled with 0.1 μCi of [^{14}C]AA over the last 24 h of incubation. Cellular lipids were extracted and separated by thin-layer chromatography, and the proportional distribution of [^{14}C]AA was determined as described in materials and methods. Data are expressed as a percentage of vehicle-treated cells.
[b]Data represent the mean ±SEM.
*Significantly ($P < 0.05$) different from control.

percent of arachidonic acid found in phosphatidylinositol was not different between vehicle treated control samples and samples receiving $1,25(OH)_2D_3$ (vehicle: 22.0 ± 1.8%; $1,25(OH)_2D_3$: 21.8 ± 2.4%, n = 4, NS). In fact, as indicated in Table IV, the mole percent of arachidonic acid in each of the major glycerophospholipids was unchanged after treatment with $1,25(OH)_2D_3$. Further analysis of the effects of $1,25(OH)_2D_3$ on phosphatidylinositol lipid mass indicated that the hormone had no discernible effect on the fatty acid composition of the glycerophospholipid (n = 4, NS; Table V).

On a mole percent basis, arachidonic acid (20:4), followed by oleic acid (18:1), was found to be the major unsaturated fatty acids of phosphatidylinositol in both vehicle and $1,25(OH)_2D_3$-treated cells (Table V). In some experiments [1-^{14}C]oleic acid was used instead of [1-^{14}C]AA to radiolabel cells. The apparent differences in the proportional distribution of [1-^{14}C]AA versus [1-^{14}C]oleic acid in the glycerophospholipids of control cells suggests that hOB cells have specific acylating systems that distinguish between arachidonic acid and oleic acid (Table I). Even so, a 96-h $1,25(OH)_2D_3$ treatment altered [1-^{14}C]oleic acid incorporation and distribution a manner similar to that observed in the [1-^{14}C]AA-labeled samples. $1,25(OH)_2D_3$ treatment decreased total [1-^{14}C]oleic acid uptake to 84 ± 4% of control ($n = 7$, $P < 0.01$) and decreased the proportional incorporation of [1-^{14}C]oleic acid into phosphatidylinositol to 86 ± 3% of control cells ($n = 6$, $P < 0.01$).

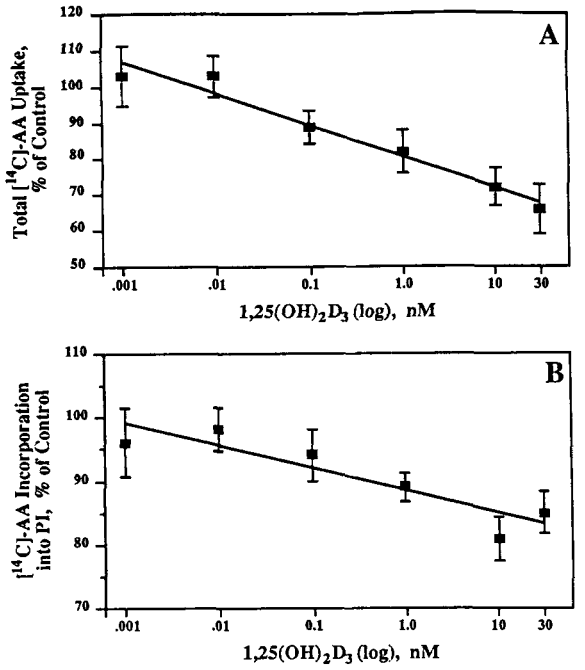

Fig. 2. Dose–response relationship for the effect of 1,25-$(OH)_2D_3$ on total [1-^{14}C]AA uptake (A) and the proportional [1-^{14}C]AA labeling of the phosphatidylinositol pool (B). hOB cells were incubated with the indicated concentrations of 1,25-$(OH)_2D_3$ for 96 h and labeled with 0.1 μCi of [1-^{14}C]AA during the last 24 h of incubation. Total [1-^{14}C]AA uptake and the proportional incorporational of [1-^{14}C]AA into phosphatidylinositol was determined as described under Materials and Methods. Regression analysis was performed for data from A ($r = 0.98$; $P < 0.01$; $n = 4$) and B ($r = 0.91$; $P = 0.01$; $n = 4$). Data (mean ± SEM) are expressed as the percentage of vehicle-treated hOB cells.

DISCUSSION

Eicosanoids are synthesized upon need and act locally. Owing to their instability and/or rapid catabolism after release, plasma eicosanoid concentrations are low, indicating that systemic function is unlikely [Granstrom and Kumlin, 1987; Smith, 1989]. Since eicosanoids are not stored, their release at a site represents de novo synthesis from precursors [Smith, 1989]. Arachidonic acid is stored, predominantly, in cellular phospholipids with lesser amounts of the fatty acid found in the neutral lipids [Snyder et al., 1992]. Neither the cyclooxygenases nor the lipoxygenases use arachidonic acid for eicosanoid biosynthesis until the fatty acid is released from its *sn*-2 position within the cellular glycerophospholipids [MacDonald and Sprecher, 1991]. Cellular phospholipases may be activated following agonist occupancy of plasma membrane receptors or after other stimuli, such as mechanical manipulation or ischemia [Karmazyn, 1986; Smith, 1989]. Various phospholipases participating in signal transduction pathways show substrate selectivity, preferentially acting on arachidonoyl containing specific phospholipid classes such as phosphatidylcholine [Exton, 1994; Schutze et al., 1991] or phosphatidylinositol [Cockcroft and Thomas, 1992]. Most released arachidonic acid is rapidly reacylated into phospholipids [Balside et al., 1992]. The remaining fraction of the released arachidonic acid is available for metabolism by the eicosanoid synthesizing enzymes.

The activation of phospholipase C is an important component of many signal transduction systems. Phospholipase C cleavage of the phosphoinositol head group from the glyceryl moiety of phosphatidylinositol(4,5)-bisphosphate yields two products capable of mediating intracellular biochemistries. The initiation of Ca^{2+} release/influx by inositol 1,4,5-trisphosphate is amply documented [Abdel-Latif, 1986; Berridge and Irvine, 1989], as is the role of 1,2-diacylglycerol as a protein kinase C activator [Berridge, 1984; Hug and Sarre, 1993]. Some studies indicate that the alkyl side chains of the fatty acyl groups of 1,2-diacylglycerol can be critical determinants of protein kinase C activity [Bell and Sargent, 1987; Kerr et al., 1987]. Furthermore, free arachidonate may also serve to modulate intracellular signal transduction during phosphoinositide metabolism and studies indicate that arachidonic acid itself can stimulate protein kinase C activity [McPhail et al., 1984; Hug and Sarre, 1993]. Recently, free arachidonic acid was found to modify inositol polyphosphate-mediated calcium mobilization in pancreatic cells [Maruyama, 1993]. It is also now recognized that free polyunsaturated fatty acids, without further metabolism, can be important cellular modulators of steroid hormone [Nunez, 1993] and peptide factor signal transmission [Sumida et al., 1993]. Thus, the altered characteristics of arachidonic acid uptake and distribution into specific glycerophospholipids in hOB cells treated with $1,25(OH)_2D_3$ may modify signal transduction and intercellular signaling pathways at several points.

Although $1,25(OH)_2D_3$ treatment decreased total uptake of [1-^{14}C]AA into the hOB cells, the proportionate incorporation of the radiolabeled fatty acid into the glycerophospholipids was unchanged with the notable exception of phosphatidylinositide labeling. The present investiga-

Table III. Phospholipid Composition of hOB Cells

	Vehicle[b]		$1,25(OH)_2D_3$[b]	
	pmol lipid[a]		pmol lipid	
Phospholipid	500,000 cells	% total phospholipid	500,000 cells	% total phospholipid
PE	1,805 ± 227	16.8 ± 1.8	1,744 ± 212	20.3 ± 1.0
PI	1,043 ± 126	9.7 ± 0.9	902 ± 77	10.7 ± 1.0
PS	1,589 ± 229	14.8 ± 2.0	1,149 ± 234	12.8 ± 1.0
PC	6,257 ± 205	58.7 ± 2.4	4,906 ± 718	56.2 ± 1.2

[a]hOB cells were incubated with vehicle or 1 nM $1,25\text{-}(OH)_2D_3$ for 96 h. Gas chromatographic analyses were performed as described under Materials and Methods. Phospholipid mass is expressed as a percentage of total phospholipid (PE, PI, PS, and PC) mass to correct for differences in sample loading onto the thin-layer chromatography plates.
[b]Data represent the mean ±SEM; n = 4.

Table IV. Arachidonic Acid (20:4) Content in hOB Cell Phospholipids

	Vehicle[b]		$1,25(OH)_2D_3$[b]	
Phos-	pmol 20:4[a]		pmol 20:4	
pho-	500,000	Mole	500,000	Mole
lipid	cells	%	cells	%
PE	730 ± 157	19.9 ± 2.6	793 ± 266	21.2 ± 5.9
PI	449 ± 43	22.0 ± 1.8	398 ± 69	21.8 ± 2.4
PS	129 ± 51	3.6 ± 1.2	76 ± 33	2.9 ± 1.0
PC	765 ± 143	6.1 ± 1.1	758 ± 211	7.3 ± 1.2

[a]hOB cells were incubated with vehicle or 1 nM $1,25\text{-}(OH)_2D_3$ for 96 h. Gas chromatographic analyses were performed as described under Materials and Methods.
[b]Data represent the mean ±SEM; n = 4.

Table V. Fatty Acid Composition of Phosphatidylinositol in hOB Cells

	Vehicle[b]		$1,25(OH)_2D_3$[b]	
	pmol/PI[a]		pmol/PI	
Fatty	500,000		500,000	
acid	cells	Mole %	cells	Mole %
14:0	<50	<1.0	<50	<1.0
16:0	237 ± 107	10.3 ± 3.7	150 ± 72	8.0 ± 3.9
18:0	935 ± 86	45.9 ± 4.0	839 ± 110	46.6 ± 4.3
20:0	<50	<1.0	<50	<1.0
22:0	<50	<1.0	<50	<1.0
18:1	260 ± 34	12.5 ± 0.7	188 ± 20	10.7 ± 1.6
18:2	50 ± 27	2.3 ± 1.0	60 ± 26	3.4 ± 1.4
18:3	<50	<1.0	<50	<1.0
20:3	59 ± 6	2.9 ± 0.4	51 ± 7	2.9 ± 0.5
20:4	449 ± 43	22.0 ± 1.8	398 ± 69	21.8 ± 2.4
22:1	<50	<1.0	<50	<1.0
22:4	<50	<1.0	<50	<1.0
22:6	<50	<1.0	<50	<1.0

[a]hOB cells were incubated with vehicle or 1 nM $1,25\text{-}(OH)_2D_3$ for 96 h. Gas chromatographic analyses were performed as described under Materials and Methods.
[b]Data represent the mean ±SEM; n = 4.

tions failed to determine the level at which the modification in phosphatidylinositide acylation–deacylation pathways had occurred in $1,25(OH)_2D_3$ treated hOB cells. The possibilities examined were that either hOB cell phosphatidylinositol lipid mass was decreased by the treatment or that the mole percent of arachidonic acid within the phosphatidylinositides had declined from control levels. Our analysis was unable to provide experimental evidence to support either of these potential mechanisms. Although in vivo and in vitro feeding experiments have demonstrated that arachidonic acid can be replaced within the glycerophospholipids by excess amounts of other long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (20:5) [Galli et al., 1971; Philbrick et al., 1987; Bourre et al., 1993], the present studies were not designed to examine arachidonic acid replacement in the hOB cells. In general, long-chain polyunsaturated fatty acids are metabolized differently than are shorter-chain, less unsaturated fatty acids within cells [Voss et al., 1991]. Arachidonic acid is expected to have a low rate of β-oxidation, which combined with the highly efficient reacylation processes for it, operate to preserve this important fatty acid for its specialized functions. Vossen's work with human endothelial cells cultured in media with varied fatty acid compositions led the investigators to propose a homeostatic control of arachidonic acid levels in phosphatidylinositides that may maintain essential signal transduction processes [Vossen et al., 1993]. The elevated levels of docosahexaenoic acid (22:6) and eicosapentaenoic acid (20:5) in platelet membranes from patients 18–24 weeks removed from dietary supplements with these fatty acids illustrates the conservative metabolism of the long-chain polyunsaturated fatty acids [Galli et al., 1993]. Longer-term studies of $1,25(OH)_2D_3$-treated hOB cells might reveal a diminished arachidonic acid content in the phosphatidylinositides, as the pre-

sent data suggest would be the ultimate outcome of treatment.

The sequential actions of arachidonoyl CoA synthetase and arachidonoyl CoA lysophosphatide acyltransferase esterify arachidonic acid in the *sn*-2 position of the glycerophospholipids [Irvine, 1982; Snyder et al., 1992]. Modulation of arachidonic acid incorporation between glycerophospholipid classes has been linked to alterations in arachidonoyl CoA lysophosphatidylcholine acyltransferase activity in $1,25(OH)_2D_3$-treated duodenal cells [O'Doherty, 1978] and to the stimulation of CoA-independent transacylase activity in platelets [Breton and Colard, 1991]. Protein kinase C activation stimulated the CoA-independent transacylase of platelets [Breton and Colard, 1991], and arachidonoyl CoA synthetase and arachidonoyl CoA lysophosphatide acyltransferase were inhibited by activated protein kinase C in platelets [Fuse et al., 1989] and in neutrophils [Yamazaki and Tai, 1990]. The inhibition of the arachidonoyl CoA-synthetase and acyltransferase enzymes were linked with the inhibition of total uptake of radiolabeled arachidonic acid in both systems. It remains to be determined if similar changes can be identified in the $1,25(OH)_2D_3$-treated hOB cells and if any such changes are the consequence of an altered protein kinase C activity.

Our findings indicated that $1,25(OH)_2D_3$ treatment decreased the proportional incorporation of either [1-^{14}C]AA or [1-^{14}C]oleic acid into phosphatidylinositol. Acylating systems in various cell types are reported to display a high degree of substrate specificity towards arachidonic acid [Fuse et al., 1989; MacDonald and Sprecher, 1991]. However, the possibility that $1,25(OH)_2D_3$ treatment altered an acylation pathway still merits consideration. Various unsaturated acyl-CoA's can be esterified within 1-acyl-glycerophosphatides when the acyl-CoA's are tested individually as substrates for acyltransferase activity in isolated microsomes [Inoue et al., 1984; MacDonald and Sprecher, 1991]. However, specificity for arachidonoyl CoA became apparent only when mixtures containing equal molar concentrations of arachidonoyl- and oleoyl CoA were used in the assay system [Inoue et al., 1984; MacDonald and Sprecher, 1991]. O'Doherty [1978] reported that lysophosphatidylcholine acyltransferase activity was higher with [^{14}C]arachidonoyl CoA as the supplied substrate then when [^{14}C]oleoyl CoA was used. Nevertheless, $1,25(OH)_2D_3$ treatment of duodenal cells did alter acyltransferase activity towards both of the substrates [O'Doherty, 1978].

An alternative explanation for the observed decrease in the proportional incorporation of [1-^{14}C]AA or [1-^{14}C]oleic acid into phosphatidylinositol is that $1,25(OH)_2D_3$ treatment decreased phosphatidylinositol turnover. The stimulation of the rapid formation of inositol phosphates by agonist activation of phospholipase C was reported to be inhibited by $1,25(OH)_2D_3$ pretreatment of MC3T3-E1 cells, a rodent osteoblastic model system [Tokuda et al., 1993]. The enzymatic pathways utilized for synthesis and degradation of second messenger molecules generated during the stimulation of the phosphatidylinositol cycle have been characterized [Abdel-Latif, 1986]. However, the regulation of constitutive phosphatidylinositol turnover is poorly understood.

MacDonald et al. [1984] reported that $1,25(OH)_2D_3$-treated hOB cells released less radiolabeled arachidonic acid subsequent to parathyroid hormone challenge than did untreated control cells. MacDonald et al. [1984] did not report whether the pretreatment of the hOB cells with $1,25(OH)_2D_3$ had decreased their capacity to introduce the radiolabel into the cellular glycerophospholipids or whether the distribution of the radiolabel within the various glycerophospholipids had been altered. The present findings may account for the decreased release that was reported. MacDonald and colleagues [1984] also reported a decreased basal release of prostaglandin by $1,25(OH)_2D_3$-treated hOB cell cultures. Similar findings were reported by Feyen et al. [1984] in studies using a chick calvaria culture system. Changes in total arachidonic acid content in cellular glycerophospholipids have been correlated with altered prostaglandin synthetic capacities in several systems [Vossen et al., 1991; Kawashima et al., 1994].

An intriguing question is whether the observed effects of steroids on arachidonic acid metabolism are linked to osteoblastic cell differentiation. Based on evidence obtained from various osteoblastic model systems, $1,25(OH)_2D_3$ and dexamethasone are considered differentation promoting hormones. The reported effects of those hormones include the stimulation of gene expression and protein synthesis of bone matrix components [Kasugai et al., 1991; Owen et al.,

1991; Subramaniam et al., 1992] and the modulation of responsiveness to osteotropic hormones [Ikeda et al., 1990; Suarez and Silve, 1992]. Studies concerning the interactions between $1,25(OH)_2D_3$ and dexamethasone indicate that the two hormones have overlapping but distinct effects on hOB differentiation [Subramaniam et al., 1992]. The effects of 17β-E_2 on osteoblastic cell differentation are controversial. Studies using normal hOB cells suggest that 17β-E_2 treatment does not effect the differentation or proliferation of those cells [Keeting et al., 1991b]. However, results obtained from neonatal [Ernst et al., 1988] and transformed [Ernst et al., 1989] rodent osteoblastic cell model systems indicates that 17β-E_2 stimulated proliferation and increased the gene expression of various bone matrix components.

While a growing body of work has indicated that steroid hormones modulate cellular glycerophospholipid composition and fatty acid distribution, little has been done using osteoblastic cell model systems. Haining and co-workers [1988] found that treatment of an hOB cell model system with $1,25(OH)_2D_3$ for a 24-h period increased the rate of [^{3}H]serine incorporation into phosphatidylserine. A similar effect on [^{3}H]serine incorporation was reported from a 48-h $1,25(OH)_2D_3$ treatment of UMR 106 cells, a rodent osteoblast-like cell line [Matsumoto et al., 1985]. The increased rate of [^{3}H]serine incorporation in UMR 106 cells was accompanied by increased phosphatidylserine phospholipid content, as well as a decreased phosphatidylethanolamine mass [Matsumoto et al., 1985]. Our studies indicated that 96-h treatment of a hOB model system with $1,25(OH)_2D_3$ altered arachidonic acid metabolism without effecting phospholipid mass. Haining et al. [1988] also reported that 2-h stimulation of human osteoblast-like cells with the secosteroid nearly doubled the uptake of [^{3}H]inositol into phosphatidylinositol. The latter change probably reflects nongenomic effects since reports of rapid inositol phosphate formation following $1,25(OH)_2D_3$ stimulation have been made for several osteoblastic cell model systems [Civitelli et al., 1990; Grosse et al., 1993; Sorensen and Baron, 1993]. Treatment of neonatal mouse osteoblastic cells for 7 days with dexamethasone increased radiolabeled arachidonic acid incorporation into phosphatidylcholine and diminished its incorporation into phosphatidylinositol [Suarez and Silve, 1992]. Those findings may illustrate distinctions between steroid regulation of neonatal mouse and adult human osteoblastic cell models, or perhaps time-dependent effects not seen in our studies with dexamethasone that extended only to 96 h of treatment.

The prostaglandins and lipoxygenase products are pleiotropic regulators of bone biology. Among the effects described for various eicosanoids are mediation of osteoblastic cell proliferation and differentiation [Hakeda et al., 1987], the fusion [Chenu et al., 1990] and differentiation [Collins and Chambers, 1991] of osteoclastic cell precursors, as well as the mediation of osteoclast recruitment [Shinar and Rodan, 1990], activation [Akatsu et al., 1991; Gallwitz et al., 1993], and, perhaps, inactivation [Chambers and Ali, 1983]. Through modulation of arachidonic acid uptake and distribution into glycerophospholipids, osteotropic steroid hormones may indirectly influence the biological effects of the peptidyl regulators of bone which act through the stimulation of arachidonic acid release and eicosanoid production.

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