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Estrogen Pretreatment Increases Arachidonic Acid Release by Bradykinin Stimulated Normal Human Osteoblast-Like Cells

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Abstract Eicosanoids are multifunctional autocrine/paracrine regulators of bone that are enzymatically derived from arachidonic acid (AA). The rate-limiting step in the eicosanoid biosynthetic pathways may be the release of AA from membrane glycerophospholipids by activated phospholipases. Free AA can serve as the substrate for cyclooxygenase(s) or lipoxygenases that catalyze the commitive steps in eicosanoid synthesis; alternatively, free AA may be used in reacylation processes, resulting in its reincorporation into cellular lipids. The hormones 17β-estradiol (17β-E_2), dexamethasone (a synthetic glucocorticoid), and 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) have been identified as regulators of AA metabolism, at various levels, in several tissues including bone. The possibility that these osteotropic steroids modulate the availability of free AA in bone cells was studied in the human osteoblast-like (hOB) cell model system. Following a 48-h steroid pretreatment, bradykinin or the calcium ionophore A23187 were used as agonists to stimulate hOB cell release of AA. The principal findings from these investigations were that (1) 17β-E_2 pretreatment potentiated the appearance of free AA following bradykinin stimulation of the cells but, did not alter their response to A23187 stimulation; (2) dexamethasone pretreatment limited bradykinin-induced increases in free AA levels but did not alter cell response to A23187 stimulation; (3) hOB cells derived from different trabecular bone compartments (manubrium of the sternum, femoral head) differed quantitatively in their responses to bradykinin stimulation of AA release; and (4) 1,25$(OH)_2D_3$ did not effect AA release stimulated by either agonist. The ability of the steroids to modulate AA release by hOB cells suggests that these hormones may indirectly mediate bone cell responses to other osteotropic hormones that act through eicosanoid-dependent processes.

Key words: osteoporosis, dexamethasone, glucocorticoids, prostaglandins, phospholipase

In healthy adults the rates of bone resorption and bone formation are in an approximate dynamic equilibrium and bone mass is maintained [Parfitt, 1982; Riggs and Melton, 1986]. The biochemical coordination of these processes is complex and multifactorial and involves both systemic and locally produced factors. The systemic regulators of bone include estrogens, glucocorticoids, and 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$). Whereas appropriate concentrations of these hormones support bone homeostasis, inadequate or excessive levels can result in pathogenesis [Raisz, 1988; Riggs and Melton, 1986; Villareal et al., 1991]. A principal target of the steroid hormones in bone appears to be the osteoblast cell population since the receptors for these hormones have been identified in cells of the osteoblast lineage studied in vitro [Eriksen et al., 1988; Subrumaniam et al., 1992; Narabitz et al., 1983].

Among the locally produced regulators of bone are the prostaglandins, and perhaps, hydroxyeicosatetraenoic fatty acids (HETEs) and the leukotrienes [Raisz, 1988; Zhang and Dziak, 1993]. These oxygenated metabolites of arachidonic acid (AA), collectively termed eicosanoids, are the bioactive products of enzymatic pathways initiated by either prostaglandin endoperoxide G synthetase (cyclooxygenase) or lipoxygenase enzymes [Smith, 1989]. Each of these enzymes utilizes free AA as substrate. The one resorp-

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tion promoting activities of bradykinin, parathormone, interleukin-1 (IL-1), and several other peptidyl regulators can be blocked, or diminished, by inhibiting prostaglandin synthesis [Lerner et al., 1987; Pilbeam et al., 1989; Akatsu et al., 1991].

In unstimulated cells free AA is maintained at a low concentration, thereby limiting basal rates of eicosanoid production [Irvine, 1982]. Most cellular AA is esterified at the *sn*-2 position of membrane glycerophospholipids, with lesser amounts found in the neutral lipids. Cellular phospholipases may be activated following occupancy of plasma membrane receptors, or after other stimuli such as calcium ionophores, mechanical manipulation, or ischemia [Smith, 1989; Flower and Blackwell, 1976; Karmazyn, 1986]. Depending on the type of stimulus and the involved tissue, AA can be released from the glycerophospholipids by the hydrolytic actions of phospholipase A_2 or phospholipase C in concert with diglyceride lipase [Whatley et al., 1990]. Substrate specificities vary between the different phospholipase classes, and each class is composed of multiple isozymes [Exton, 1994; Cockcroft and Thomas, 1992]. The relative contribution of each AA release pathway towards eicosanoid synthesis is unknown [Abdel-Latif, 1986]. Following its stimulated release, the majority of the free AA is rapidly reincorporated into cellular lipids by highly efficient acylating systems, while the remaining fraction can be used for eicosanoid synthesis [Balsinde et al., 1992].

Estrogens, glucocorticoids, and $1,25(OH)_2D_3$ modulate aspects of AA metabolism in various tissues, including bone [Klein-Nulend et al., 1991; Pilbeam et al., 1989; Schwartz et al., 1992; Cissel et al., 1995]. The possibility that these hormones modulate AA release by stimulated human osteoblast-like (hOB) cells in primary culture was tested in the present study. The bone resorption promoting nonapeptide bradykinin was chosen as a representative physiological stimulator of AA release [Lerner et al., 1987]. The calcium ionophore A23187 was employed as a non-receptor-dependent agonist that should maximally elevate the activities of Ca^{2+}-dependent cellular phospholipases to stimulate AA release [Reed and Lardy, 1972; Whatley et al., 1990; Clark et al., 1991].

MATERIALS AND METHODS

Patient Populations

Trabecular bone explants were obtained from the manubrium of the sternum of patients undergoing elective thoracic surgery after obtaining informed consent, or from surgical waste generated from the femoral head during routine bone grafting procedures. All procedures were approved by the West Virginia University Internal Review Board. Patient records were evaluated for all potential explant donors and explants were not taken from patients with diagnosed osteoporosis or from patients presenting with endocrine disorders that effect bone. Six of the femur explant donors were diagnosed with osteoarthritis. The responses noted for the resultant hOB cell strains were not different from the other femur-derived strains tested and the data reported do not distinguish between these groups. The arachidonic acid studies were conducted using cells established from sternal explants taken from 5 women aged 46–73 (median age of 64), and from 21 men aged 20–79 (median age of 62). Femoral explants used in these studies were from 9 women aged 39–79 (median age of 67), and from 8 men aged 39–68 (median age of 55). No correlation existed between donor age and the release of [^{3}H]arachidonic acid following bradykinin stimulation. [^{3}H]AA release by bradykinin stimulated femur-derived hOB cell strains was slightly higher in males than in females ($P < 0.05$; unpaired t-test), but with the limited number of samples tested (8 males, 9 females) the importance of the difference is difficult to evaluate. Release by sternum-derived male hOB cell strains was not higher than the release by the female strains. Donor sex did not influence hOB cell responses to the steroid hormone pretreatments for strains derived from either bone compartment; the data within the Results section are reported without regard to donor sex or age. The demographics of the patient populations contributing sternal and femoral explants used in alkaline phosphatase and osteocalcin assays was approximately similar to those described above which were used in the arachidonic acid analyses.

hOB Cell Cultures

Trabecular bone explants obtained from the sternum or the femur were treated and cultured identically. hOB cell cultures were established by the method of Robey and Termine [1985] with modification. Following the removal of any

adherent tissue, the explants were washed and minced by scalpel into small fragments (2–4 mm in diameter). The fragments were digested in Dulbecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD) containing 1 mg/ml crude bacterial collagenase (Gibco, Grand Island, NY) for 2 h in a shaking water bath at 37°C. The collagenase-treated fragments were placed in culture in a calcium-free phenol red-free medium consisting of DMEM:Ham's F12K (1:1; Biofluids) supplemented to 10% heat inactivated fetal calf serum (FCS; Gibco), 1 mM glutamine, and PSN antibiotic/antimycotic (Sigma, St. Louis, MO). hOB cell outgrowth typically required 4–5 weeks to approach confluence, whereupon the cultures were passaged once at a 1:2 split. These methods have been found to yield nearly homogeneous (95%+) cell cultures [Borke et al., 1988]. Previous reports indicate that the hOB cell phenotype remains stable in culture through at least two passages [Eriksen et al., 1988; Keeting et al., 1991a,b; Marie, 1994]. All experiments with arachidonic acid were performed using cells subcultured from nearly confluent first passage cultures seeded into 24-well plates at a density of 20,000 cells/well in media supplemented to 1 mM calcium and 10% FCS. Forty-eight h later, the media were replaced, maintaining calcium at 1 mM but reducing serum to 1% FCS, with steroid or vehicle added. Unless otherwise noted, all steroid pretreatments were for 48 h.

Reagent Preparation

Stock solutions of 17β-estradiol (17β-E_2), 17α-E_2, the synthetic glucocorticoid dexamethasone (all Sigma), and 1,25$(OH)_2D_3$ (Biomol Research, Plymouth Meeting, PA) were prepared in ethanol and stored at −20°C until used. The final concentration of ethanol in all samples was 0.1%. These hormones were used at concentrations previously found effective in hOB cell studies, specifically 10 nM 17β-E_2 [Oursler et al., 1991], 50 nM dexamethasone [Subramaniam et al., 1992], or 1 nM 1,25$(OH)_2D_3$ [Cissel et al., 1995]. These hormones doses over a 48 hour incubation have no effect on hOB cell viability or proliferation [data not shown; Keeting et al., 1991b].

A stock solution of bradykinin (Bachem, Torrance, CA) was prepared in dilute HCl, and a stock solution of the calcium ionophore A23187 (Sigma) was prepared in dimethylsulfoxide (DMSO; Sigma). Each was stored at −20°C until used. Based on the results from preliminary dose-response experiments (data not shown), bradykinin was used at 100 nM and A23187 at 1 μM to maximally stimulate [^{3}H]AA release by hOB cells. The final concentrations of the HCl or DMSO vehicles applied were 10 μM or 0.1% (v/v), respectively.

hOB Cell Protein Assays

Cell alkaline phosphatase activity was determined by colorimetrically measuring the hydrolysis of p-nitrophenyl phosphate using a commercial assay kit (Sigma). hOB cells were subcultured into 96-well microtitre plates at 5,000 cells/well. Following a 48-h recovery period, 8 wells each were treated with 10 nM 1,25$(OH)_2D_3$, 50 nM dexamethasone, both steroids, or with vehicle (0.1% ethanol), in media supplemented to 1% FCS. Sample incubations were for a duration of 72 h with a media change after 48 h. Five of the wells per treatment were used to measure alkaline phosphatase activity. Three wells per treatment were used in determinations of cell density which were made with the Cell Titre^{96} assay performed according to suppliers' instructions (Promega, Madison, WI). Data are expressed as nmol product/minute and were normalized across all experiments according to the cell density determinations.

Osteocalcin measurements were made using the Novocalcin ELISA kit from Metra Biosystems, Inc. (Mountain View, CA). hOB cells were subcultured into 24 well plates at a density of 50,000 cells/well. Following a 48-h recovery period, samples were treated with 10 nM 1,25$(OH)_2D_3$, a combination of 1,25$(OH)_2D_3$ and 50 nM dexamethasone, or with vehicle (ethanol) in 1% FCS-supplemented media. Cell-free incubation blanks were run in parallel to account for assayable osteocalcin contributed by the FCS. Media were changed at 48 h, and samples were incubated for an additional 24 h. These media were harvested and stored at −80°C until assayed. Measurements were made in duplicate samples for each treatment, the osteocalcin level in the cell-free blanks subtracted, and data are reported as pg osteocalcin/ml/24 h.

[^{3}H]Arachidonic Acid Incorporation into hOB Cell Lipids

[^{3}H]Arachidonic acid (100 Ci/mmol; New England Nuclear, Boston, MA) was prepared in 0.25% (wt/v) fatty acid free bovine serum albumin (BSA; Calbiochem, La Jolla, CA) in DMEM. hOB cells were prelabeled for the final 18–24

hours of the steroid treatment period with 0.15 μCi [^{3}H]AA, washed thoroughly to remove unincorporated radiolabel, and agonist (bradykinin, A23187), or respective vehicles, applied immediately. Under these conditions, hOB cells from either bone source incorporated approximately 80,000 cpm during the prelabeling period, of which approximately 78% was incorporated into glycerophospholipids.

In some experiments, vehicle or 17β-E_2-pretreated samples were not prelabeled, but instead were exposed to 0.1 μCi [1-^{14}C]AA (55 mCi/mmol; New England Nuclear) for a 60 minute period to compare the acylating capacities of the cells. After the 60-min incubation, media were aspirated, the cell layers washed twice with 0.1% fatty acid free BSA in DMEM, and lysed into a 2% SDS buffer. Cellular lipids were extracted twice [Bligh and Dyer, 1959] and separated by thin layer chromatography as previously described [Cissel et al., 1995]. Radioactivity in the glycerophospholipid and neutral lipid bands was measured by solid state radioactivity scanning using a radioanalytic imaging system (AMBIS, San Diego, CA).

[^{3}H]Arachidonic Acid Release by Stimulated hOB Cells

Immediately following the hOB cell prelabeling period, bradykinin at 100 nM, A23187 at 1 μM, or their respective vehicles were applied in serum free DMEM containing 0.1% fatty acid free BSA and 1 mM calcium. Following a 60-min incubation at 37°C, the conditioned media were collected and centrifuged to remove cell debris. An aliquot of each supernatant was removed and scintillation counted to determine [^{3}H]AA release by the hOB cell samples. Baseline release of [^{3}H]AA was determined in agonist-free, paired samples for all pretreatment groups in all experiments. The release of radiolabel in response to bradykinin or A23187 stimulation was then calculated as the percentage increase over the baseline release measured in the vehicle-treated paired samples. This allowed the release data to be normalized across multiple experiments, and controlled for the potentially confounding effects of steroid-induced changes on the uptake and distribution of AA into glycerphospholipids by the hOB cells [Cissel et al., 1995].

The possibility that radiolabel uptake, even between identically pretreated paired specimens, might vary sufficiently to compromise the accuracy of our measurements of [^{3}H]AA release was a concern. Therefore, in many experiments, following conditioned media collection (as above) the remaining cell layers were lysed with 2% sodium dodecyl sulfate, and radioactivity in the lysates determined. In these experiments, [^{3}H]AA release was also calculated as the percentage of total radioactivity respectively present in the paired specimens (cpm released into the medium × 100/cpm in lysate + cpm in the medium).

Statistical Analyses

Data are presented as the mean ±SEM of N experiments using different hOB cell strains. When appropriate, the data were analyzed by the paired Student's t-test. When population means were compared the t-test for independent means was used. Regression analysis was performed by the least-squares method to evaluate whether donor age influenced the observed responses.

RESULTS

Cell alkaline phosphatase activity and osteocalcin release (Table I) responded to steroid hormone treatment in a manner consistent with that of the mature osteoblast phenotype [Rodan and Rodan, 1984; Borke et al., 1988]. hOB cell alkaline phosphatase activity was elevated by 72-h treatment with 1,25$(OH)_2D_3$, dexamethasone, or in samples treated simultaneously with both hormones ($P < 0.05$ vs. control). hOB cell release of osteocalcin was undetectable in vehicle treated specimens, whereas following 72-h incubation with 1,25$(OH)_2D_3$ osteocalcin release was significantly increased ($P < 0.05$). This 1,25$(OH)_2D_3$-dependent increase in osteocalcin release was blocked by the addition of dexamethasone. As shown in Table I, the responses of the hOB cell cultures derived from the sternum or from the femur were qualitatively and quantitatively similar.

Bradykinin and A23187 proved to be effective agonists, and each stimulated [^{3}H]AA release by the hOB cells derived from either bone compartment over a 60-min incubation (Table II). The measured stimulation in [^{3}H]AA release was essentially the same whether release was determined as the increase in free [^{3}H]AA in the media above basal release, or if release was calculated as a percentage of the total radioactivity present in the different cell cultures. The hOB cell strains derived from the femur were somewhat more responsive to bradykinin stimu-

TABLE I. hOB Cell Protein Assays

Treatment	Alkaline phosphatase activity[a,b] (nmol/min)[c]		Osteocalcin release (pg/ml/24 hr)[c]	
	Sternum	Femur	Sternum	Femur
Vehicle	20.0 ± 4.8	18.0 ± 3.3	0	0.1 ± 0.1
$1,25(OH)_2D_3$	31.9 ± 6.2*	34.5 ± 5.9*	7.1 ± 2.1*	6.6 ± 1.4*
Dexamethasone	24.6 ± 4.8	27.3 ± 4.9*	ND	ND
$1,25(OH)_2D_3$ + dexamethasone	51.6 ± 10.1*	50.9 ± 7.6*	0.6 ± 0.3	0.4 ± 0.2

[a]hOB cells were treated with the indicated hormones for 72-h period prior to assay.
[b]The alkaline phosphatase activity has been normalized across experiments to reflect the measured cell density for each sample.
[c]Data represent the mean ± SEM, N = 8–9 for each condition.
*Significantly different from vehicle treated ($P < 0.05$). ND, not determined.

TABLE II. Bradykinin and A23187 Stimulate [^{3}H]Arachidonic Acid Release by hOB Cells

hOB cell source	Agonist[a]	N	[^{3}H]Arachidonic acid release[b] Method 1		[^{3}H]Arachidonic acid release[c] Method 2	
			CPM[d]	% increase over baseline	% of total cell CPM	% increase over baseline
Femur	HCl (control)	16	3,240 ± 364	—	4.1 ± 0.3	—
	Bradykinin	16	5,996 ± 675	89 ± 13*,†	7.6 ± 0.7	87 ± 12*,†
	DMSO (control)	6	3,034 ± 519	—	3.7 ± 0.5	—
	A23187	6	8,002 ± 1,117	174 ± 32*	10.0 ± 0.9	184 ± 27*
Sternum	HCl (control)	19	3,338 ± 218	—	4.3 ± 0.3	—
	Bradykinin	19	5,039 ± 388	52 ± 9*	6.6 ± 0.5	55 ± 9*
	DMSO (control)	8	3,934 ± 731	—	ND	ND
	A23187	8	7,734 ± 1,455	104 ± 19*	ND	ND

[a]hOB cells derived from the femur and the sternum bone compartments were incubated in 1% FCS media for 48 h and radiolabeled with 0.15 μCi of [^{3}H]AA over the last 24 h. hOB cells were washed and stimulated with the indicated agonist for 1 h; [^{3}H]AA release was determined as described in Materials and Methods.
[b]Values determined from [^{3}H]AA released into the cell culture media.
[c][^{3}H]AA released into the cell culture media is represented as a percentage of total cell cpm.
[d]Data represent the mean ± SEM.
*Significantly different ($P < 0.01$) from control.
†Mean responses of the hOB cell populations to bradykinin stimulation were significantly different ($P < 0.05$). ND = not determined.

lation than were the strains derived from the sternal explants ($P < 0.05$), indicating that hormone stimulated AA deacylation–reacylation processes exhibit bone compartment specific characteristics. The increase in [^{3}H]AA release stimulated by A23187 treatment approximately doubled the response of the cells elicited by bradykinin treatment ($P < 0.01$). These experiments did not confirm a significantly greater release by the femur derived hOB cell strains than by the sternum derived strains following A23187 stimulation of [^{3}H]AA release by the cells ($P = 0.10$).

The possibility that 17β-E_2 pretreatment might modulate agonist induced release of [^{3}H]AA was evaluated in studies using hOB cell strains derived from the sternum (Table III). The bradykinin stimulated release of [^{3}H]AA was significantly potentiated by a 24-h (N = 8; $P < 0.01$) or a 48 hour (N = 24; $P = 0.01$) pretreatment of the cells with 17β-E_2. A 48-h pretreatment of the cells with 10 nM 17α-E_2 did not influence the response of the cells to bradykinin stimulation ([^{3}H]AA release by vehicle pretreated: 53 ± 9% increase over basal release; 17α-E_2 pretreated: 52 ± 10% over basal; N = 9) indicating the stereospecific nature of the estradiol effect on the hOB cells. The increase in bradykinin stimulated [^{3}H]AA release in 17β-E_2 pretreated samples was not a pharmacological effect of the steroid on hOB cell AA deacylation–reacylation processes. That possibility was tested in experiments in which 17β-E_2 and bradykinin were added concurrently to samples for a 60-min incubation. Bradykinin stimulated [^{3}H]AA release was not effected by the concurrent addi-

TABLE III. Time Course Effect of 17β-estradiol Pretreatment on [^{3}H]Arachidonic Acid Release by Stimulated hOB Cells

Pretreatment time (h)	Bradykinin[a]				A23187[a]			
		%increase over baseline				%increase over baseline		
	N	Vehicle[b]	17β-Estradiol	*P*	N	Vehicle[b]	17 β-Estradiol	*P*
0	8	44 ± 14	51 ± 15	.627		ND	ND	
24	8	28 ± 12	52 ± 10	.003		ND	ND	
48	24	51 ± 8	70 ± 10	.010	8	104 ± 20	100 ± 19	.692

[a]hOB cells derived from the sternum bone compartment were preincubated with 17β-estradiol or ethanol (vehicle) for the indicated time and radiolabeled with 0.15 μCi of [^{3}H]AA over the last 24 h. hOB cells were washed and stimulated with bradykinin or A23187 for 1 h, and [^{3}H]AA release was determined as described in Materials and Methods.
[b]Data represent the mean ± SEM.

tion of the steroid (bradykinin only: 44 ± 14% over basal; bradykinin plus 17β-E_2: 51 ± 15; N = 8; p = 0.63). A23187 stimulated release of [^{3}H]AA by the hOB cells (Table III) was not sensitive to pretreatment with 17β-E_2 (N = 8).

The effect of a 48 hour pretreatment with 17β-E_2 on [^{3}H]AA release was then tested in experiments using hOB cell strains derived from the femur. Once again, 48-h pretreatment with 17β-E_2 increased the release of [^{3}H]AA by bradykinin stimulated hOB cells. In 18 experiments, bradykinin stimulated [^{3}H]AA release increased from 84 ± 12% over basal in the vehicle pretreated samples to 107 ± 16% over basal following the estrogen pretreatment ($P < 0.05$). 17β-E_2 pretreatment had no effect on the release of [^{3}H]AA elicited by A23187 stimulation of the femur derived hOB cell strains (vehicle pretreated: 174 ± 32% over basal; 17β-E_2 pretreated: 168 ± 22% over basal; N = 6). Thus, the specific potentiation of bradykinin-stimulated [^{3}H]AA release by 17β-E_2 pretreatment was not dependent on the bone compartment of strain origin.

The appearance of [^{3}H]AA in the media of stimulated hOB cells is not a direct measure of the release of the radiolabeled fatty acid by activated phospholipases. The increased [^{3}H]AA levels found in the media of 17β-E_2-pretreated hOB cells following stimulation could have reflected a diminished capacity of the acylating pathways to reincorporate released AA into cellular lipids. This potential complication was evaluated in femur-derived hOB cell strains pretreated with 17β-E_2 or vehicle but that were not preradiolabeled. Following the 48 hour pretreatment with steroid or vehicle, hOB cell uptake of exogenous [1-^{14}C]AA into cellular neutral lipids and glycerophospholipids over a 60-min incubation was measured. No differences between vehicle and the paired 17β-E_2 pretreated samples were observed (uptake of [^{3}H]AA by 17β-E_2-pretreated hOB cells: 101 ± 4% of vehicle-pretreated controls; N = 4).

Figure 1 summarizes the observed effects of a 48-h 1,25$(OH)_2D_3$, dexamethasone, or 17β-E_2 pretreatment of hOB cells on bradykinin or A23187-stimulated [^{3}H]AA release. The ratio of release by steroid pretreated agonist stimulated to vehicle pretreated agonist-stimulated samples are shown. The effects of the steroids on agonist induced [^{3}H]AA release were qualitatively similar for hOB cells derived from the sternum or from the femur. Pretreatment with 1,25$(OH)_2D_3$ had no effect on bradykinin stimulated [^{3}H]AA release. hOB cell responses to dexamethasone and, as described above, 17β-E_2 were evident. In samples stimulated with bradykinin, cells that had been pretreated with dexamethsone exhibited a significantly diminished response compared with their vehicle-pretreated controls (dexamethasone-pretreated/vehicle ratios: sternum, 0.14 ±0.16, N = 6, $P < 0.05$; femur, 0.22 ± 0.08, N = 5, $P = 0.01$). In these studies, none of the steroids significantly effected the release of [^{3}H]AA elicited by A23187 treatment.

DISCUSSION

The eicosanoids are pleiotropic local modulators of bone biology produced in response to a variety of stimuli [Klein and Raisz, 1970; Hakeda et al., 1987; Norrdin et al., 1990; Collins and Chambers, 1991]. The synthesis of the eicosanoids begins with the release of AA from cellular glycerophospholipid stores [Irvine, 1982]. The availability of free AA in unstimulated cells is not sufficient to support more than minimal amounts of eicosanoid synthesis. The activation of specific phospholipase A_2 isozymes that selectively hydrolyze AA from the sn-2 position of

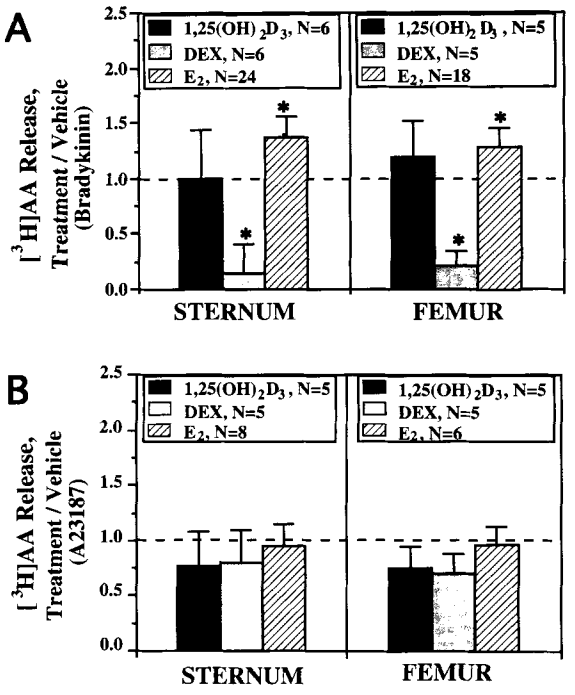

Fig. 1. Effect of a 48-h steroid pretreatment on [^{3}H]AA release in bradykinin (**A**) or A23187 (**B**) stimulated hOB cells. Cells derived from the sternum or the femur bone compartments were incubated with 1 nM 1,25(OH)$_2$D$_3$ (black), 50 nM dexamethasone (DEX; gray), 10 nM 17β-estradiol (E_2; cross-hatched), or ethanol vehicle for 48 h and radiolabeled with 0.15 μCi of [^{3}H]AA over the final 24 h. hOB cells were washed twice to remove unincorporated radiolabel and stimulated with the indicated agonist for 1 h, and [^{3}H]AA release determined as described in the Materials and Methods section. Data are expressed as Treatment/Vehicle ratios (mean + SEM) to normalize for differences between experiments. *$P < 0.05$ vs. vehicle.

glycerophospholipids is an important component of many signal transduction pathways [Whatley, 1987; Glaser et al., 1993; Exton, 1994]. Alternatively, or in some cases in addition, the activation of phospholipase C isozymes provides substrate for diglyceride lipase, which in turn releases AA [Whatley, 1987; Cockcroft and Thomas, 1992].

The recruitment of phospholipases in receptor-dependent signal transduction processes occurs variously by specific G protein activation, by phosphorylation of dissociable phospholipase subunits, or by secondary effects commonly mediated through Ca^{2+}, cAMP, or protein kinase regulated pathways [Burch and Axelrod, 1987; Carter et al., 1989; Ullrich and Schlessinger, 1990; Yanaga et al., 1991; Piomelli and Greengard, 1991; Ljunggren et al., 1993]. Bradykinin receptor activation is reportedly coupled to both phospholipase A_2 and to phospholipase C by G proteins in osteoblastic cells, and elsewhere [Yanaga et al., 1991; Burch and Axelrod, 1987]. Secondary effects of bradykinin receptor activation may include elevated cytosolic free Ca^{2+} concentration, protein kinase C activation, and the generation of cAMP. Whether bradykinin directly stimulates cAMP formation in osteoblastic cell systems, or if cAMP increases are mediated indirectly through elevated prostaglandin production and the autocrine activation of adenylyl cyclase is unclear and may differ according to species of osteoblastic cell origin [Ljunggren et al., 1991; Tatakis et al., 1992]. Each of these direct and secondary consequences of bradykinin receptor activation can regulate phospholipase activity. The multiple, and frequently overlapping, signal transduction pathways potentially involved may make it difficult to elucidate the mechanism(s) through which 17β-E_2 pretreatment potentiates bradykinin stimulated release of [^{3}H]AA by hOB cells. Further complicating these determinations are the possibilities that estrogen might increase hOB cell bradykinin receptor levels, or even alter the bradykinin isoreceptor composition expressed in these cells [Ljunggren et al., 1991; Tatakis et al., 1992; Field et al., 1992].

The present studies of agonist induced [^{3}H]AA release by steroid pretreated hOB cells do provide some clues upon which future evaluations may be based. First, the nonpharmacologic basis of 17β-E_2 effects (shown by the coincubation of the steroid with bradykinin) and the stereospecificity of the response (17α-E_2 was ineffective) suggest a gene regulatory requirement for the estrogen effect. Second, A23187 stimulated release of [^{3}H]AA was not sensitive to 17β-E_2 pretreatment. This observation decreases the likelihood that Ca^{2+}-dependent processes, such as calmodulin-dependent protein kinase activation, or even increased expression of Ca^{2+}-activated phospholipases lie at the root of the estrogen-dependent increase in [^{3}H]AA release. Third, estrogen pretreatment had no apparent effect on hOB cell acylation processes studied over a 60-min incubation of the cells with exogenous [1-^{14}C]AA. An additional indication that 17β-E_2 pretreatment did not inhibit AA reacylation is gleaned from the A23187 experiments, which showed no differences between release by steroid or vehicle pretreated samples. Reacylation of AA may be sensitive to protein kinase C modulation. In platelets [Fuse et al., 1989] and in neutrophils [Yamazaki and Tai, 1990] arachi-

donoyl CoA synthetase and arachidonoyl CoA lysophosphatide acyltransferase were inhibited by protein kinase C activation. Those reports, and the present findings, are tentatively interpreted to indicate that estrogen dependent effects on protein kinase C are not fundamental to the potentiation of [^{3}H]AA release by 17β-E_2 pretreatment observed in the present bradykinin experiments.

We have previously reported that 48-h pretreatment of hOB cells with 17β-E_2 had no effect on the uptake of radiolabeled AA, nor on its distribution into glycerophospholipids [Cissel et al., 1995]. Thus, the enhanced release of [^{3}H]AA by bradykinin stimulated, 17β-E_2-pretreated hOB cells was not a secondary event to alterations in the radiolabeled AA pool available for bradykinin activated phospholipases. Furthermore, the present data were derived from release evaluations made on pairwise matched vehicle versus agonist-treated samples. This analytical approach was necessary for the evaluation of regulation by dexamethasone, since dexamethasone pretreatment did alter radiolabel uptake and distribution by hOB cells in those earlier studies.

A preliminary report from Walker et al. [1994] indicated that prostaglandin E_2 production by interleukin-1 stimulated MG-63 human osteosarcoma cells was increased by 17β-E_2 pretreatment. Since the rate-limiting step in prostaglandin synthesis may be the release of AA, the concordance between bradykinin and interleukin-1 activation of AA-release processes might provide additional focus for further analysis of 17β-E_2 regulation. For example, those findings suggest limiting the consideration of estrogen effects at the level of the bradykinin receptor. Concordance between bradykinin and interleukin-1 activation may be found at the phospholipase and, perhaps, the G protein levels [Konieczkowski and Sedor, 1993].

Phospholipase A_2 enzymes can be classified into two general categories based on molecular weight [Clark et al., 1991; Exton, 1994; Glaser et al., 1993]. High-molecular-weight phospholipase A_2 isozymes are considered cytosolic enzymes, while the lower-molecular-weight phospholipase A_2 isozymes usually represent secreted forms of the enzyme. Secreted phospholipase A_2 has been identified as a proinflammatory component in the exudate in rheumatoid and osteoarthritis [Pruzanski et al., 1984]; its synthesis and release into the exudate are regulated by various inflammatory cytokines [Konieczkowski and Sedor, 1993; Pfeilschifter et al., 1993]. Thus, 17β-E_2 potentiation of bradykinin-stimulated [^{3}H]AA release might involve mediation at the level of the cytosolic isozymes, the secreted isozymes, or both categories of the phospholipase A_2 enzyme family. If a secreted phospholipase A_2 isoform is important in producing the 17β-E_2 effect, the source of the [^{3}H]arachidonoyl-containing glycerophospholipids hydrolyzed by the phospholipase A_2 might include osteoblast-like cell-derived matrix vesicle membranes [Termine, 1993; Schwartz et al., 1992]. AA released by vesicle membrane hydrolysis could provide substrate for osteoblastic cell eicosanoid synthesis since transcellular eicosanoid metabolism is a recognized phenomenon [Marcus, 1988; Pfeilschifter et al., 1993].

The benefits of glucocorticoid-based antiinflammatory therapy can be blunted by subsequent osteopenia. This pathological response may be secondary to an inhibited absorption of dietary calcium [LoCasio, 1984; Villareal et al., 1991]. Maturation of the osteoblastic phenotype from precursor cells may require exposure to glucocorticoids [Cheng et al., 1994]. However, the hOB cell model system used in the present studies typically reflects a relatively mature osteoblast phenotype [Keeting et al., 1992; Borke et al., 1988]. In these studies, hOB cell pretreatment with a pharmacological dose of dexamethasone elicited a profound decrease in bradykinin-stimulated [^{3}H]AA release. The antiinflammatory effects of glucocorticoid therapy are attributed, in part, to their capacity to inhibit eicosanoid synthesis in vivo [Masferrer et al., 1992]. Flower and Blackwell [1979] and Hirata et al. [1980] determined that an inhibition of phospholipase-dependent AA release was the probable mechanism by which glucocorticoids inhibited eicosanoid synthesis. The molecular basis of that inhibition of AA release is unclear, but may include decreased phospholipase A_2 synthesis [Hoeck et al., 1993]. The inability of dexamethasone to inhibit A23187 induced [^{3}H]AA release by hOB cells is suggestive that bradykinin receptor activation recruits a limited subset of cellular phospholipases that is sensitive to dexamethasone modulation.

$1,25(OH)_2D_3$ was without effect in the present experiments. Previous reports on the effects of $1,25(OH)_2D_3$ on mineralizing tissue phospholipase A_2 activity generally agree with this obser-

vation. Differential effects of $1,25(OH)_2D_3$ on rat costochondral cells were reported [Schwartz and Boyan, 1988], but in the human MG-63 osteosarcoma cell line, $1,25(OH)_2D_3$ treatment did not alter phospholipase A_2 activity when tested on an artificial phosphatidylethanolamine substrate [Schwartz et al., 1992]. That finding can now be extended, since in the present studies, each of the major glycerophospholipid classes become radiolabeled during the preincubation period [Cissel et al., 1995].

In light of the similarities found between sternum- and femur-derived hOB cell alkaline phosphatase activity and osteocalcin release, the quantitatively greater release of [^{3}H]AA by bradykinin-stimulated femur-derived hOB cells compared with the sternum derived strains is of interest. We speculate that this difference may reflect the distinctive functional roles of the two bone compartments in vivo. Bone mass is adjusted and maintained locally due, in part, to load-bearing forces. Work in various in vitro model systems indicates that prostaglandins have important roles in the mediation of adaptive bone remodeling [Lanyon, 1992; Rawlinson et al., 1993]. The femur is subjected to far greater load-bearing forces than is the sternum. An increased capacity for AA release for subsequent eicosanoid synthesis may be an integral component of the remodeling requirements of the femur.

Whether the distinct effects of dexamethasone and estrogen on hOB cell AA release might be related to their contrasting effects on bone homeostasis warrants consideration. Following osteoclastic cell excavation of bone, the resorption pit must be refilled by new bone formation through the actions of the osteoblast cell population to maintain bone mass. Prostaglandin regulation of osteoblast lineage cells include promitogenic actions and the promotion of increased cell differentiation [Hakeda et al., 1987; Norrdin et al., 1990], responses entirely consistent with localized increases in bone formation. Antiinflammatory agents which interfere with eicosanoid synthesis are avoided by clinicians in the early treatment of fractures, or immediately after bone-grafting procedures, because the steroidal and nonsteroid anti-inflammatory drugs (NSAIDs) can compromise bone healing [Ro et al., 1976; Dekel et al., 1981]. Physiologic bone remodeling may also be dependent on an attenuated, or extremely localized, inflammatory response at the level of the bone remodeling unit. Estrogen may help maintain the responsive capacities of osteoblasts to proinflammatory stimuli, while dexamethasone might limit these capacities, each with resultant consequences on the efficacy of new bone formation.

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