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Arachidonic Acid Metabolism by Adult Human Osteoblast-like Cells Exhibits Sexually Dimorphic Characteristics

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Abstract The eicosanoids, including prostaglandin E_2 (PGE_2) and other bioactive arachidonic acid metabolites, are important local mediators of bone remodeling. Presumably, the limited or excessive synthesis of the eicosanoids could compromise bone homeostasis. We have noted that the stimulated release of arachidonic acid by adult male donor derived human osteoblast-like (hOB) cells exceeded the stimulated release measured for female-derived hOB cells by 1.5-fold. Assays of PGE_2 biosynthesis by cytokine-stimulated hOB cells also demonstrated a sex-linked difference, such that male hOB cell PGE_2 production exceeded female cell production by 1.6–2.2-fold. The calcium-dependent cytoplasmic phospholipase A_2 activity in subcellular fractions prepared from hOB cell homogenates was higher in both the cytosolic (1.6-fold) and particulate (1.5-fold) fractions from the male cells than in those prepared from female hOB cells, suggesting a molecular basis for the observed sexually dimorphic characteristics related to arachidonic acid metabolism by hOB cells. The relatively limited capacity of the female cells may limit needed intracellular and intercellular signaling during bone remodeling, thereby contributing to the development of bone pathology. J. Cell. Biochem. 71:74–81, 1998.

Key words: prostaglandin; phospholipase A_2; age; tumor necrosis factor-α; transforming growth factor-β_1

As early as 1970, Klein and Raisz reported that prostaglandin E_2 (PGE_2) stimulated bone resorption. Since that seminal work, numerous bone and bone cell studies have confirmed that PGE_2 and other members of the prostaglandin family have important bioregulatory roles in bone physiology and that PGE_2 may contribute to the pathology of bone diseases [reviewed by Raisz et al., 1993; Raisz, 1995; Kawaguchi et al., 1995]. Several of the peptidyl bone resorption-promoting hormones and cytokines depend at least in part on their capacity to stimulate bone cell prostaglandin biosynthesis. In some species, the released PGE_2 acts to increase the replication and differentiation of osteoclast precursors, thereby promoting increased bone resorption. On the whole, PGE_2 has been regarded as an agent that stimulates increased bone resorption.

An expanded role for prostaglandin mediation of bone biology has been increasingly recognized over more recent years. The replication and differentiation of osteoblast precursors was enhanced by PGE_2 treatment in in vitro studies, suggesting a possible anabolic function of the eicosanoid [Hakeda et al., 1986]. Support for that possibility was gained in studies of whole animals treated systemically with pharmacologic doses of exogenous PGE_2, which demonstrated that the treatment elicited increased bone formation [Jee et al., 1985; Kimmel et al., 1994]. Such treatments acted both to stimulate new bone formation and to limit bone resorption. PGE_2 treatment of ovariectomized rats opposed the onset of osteopenia by promoting the formation of new woven bone trabeculae in marrow cavity spaces, by increasing bone forma-

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tion at existing surfaces, and by slowing bone resorption [Jee et al., 1990; Mori et al., 1992; Welsh et al., 1993]. Additional evidence that PGE_2 can promote bone formation comes from analyses of the effects of mechanical stresses placed on bone, which elicit adaptive bone modeling and remodeling responses. These stresses induced bone formation and increased local prostaglandin formation [Somjen et al., 1980; Pead and Lanyon, 1989]. The inhibition of prostaglandin biosynthesis in those studies markedly decreased the bone formation response of the stressed animals. It has also been observed that systemic treatment of infants with PGE_1 to stimulate the closure of a patent ductus arteriosus caused the deposition of bone at periosteal surfaces [Ueno et al., 1980]. Thus, the prostaglandins are pleiotropic regulators of bone and can serve as anabolic or catabolic agents, depending on the specifics of a given set of biological conditions.

Free arachidonic acid is the most common precursor of the prostaglandins. Typically, arachidonic acid is found esterified in the *sn*-2 position of the cellular glycerophospholipids [Irvine, 1982]. In its esterified form, arachidonic acid is not a useful substrate for the eicosanoid generating enzymes; therefore, the release of the fatty acid must precede the formation of the eicosanoids [Smith, 1989]. In unstimulated cells, free arachidonic acid is maintained at a low concentration, thereby serving to limit basal eicosanoid production. In hormone-, cytokine-, or tumor promoter-stimulated cells, the release of arachidonic acid for eicosanoid production is elevated, probably as the responsibility of the cytoplasmic (85-kDa) isoform of phospholipase A_2 ($cPLA_2$), an enzyme that displays selectivity for the hydrolysis of arachidonic acid from the glycerophospholipids [Dennis, 1994; Roberts, 1996]. Various pathways of extant $cPLA_2$ activation have been described, such as a calcium-induced translocation of the enzyme from the cytosolic cell compartment to the cellular membranes, G-protein mediation, protein kinase activation, and the phosphorylation of the enzyme [Clark et al., 1991; Dennis, 1994; Roberts, 1996]. The regulation of $cPLA_2$ is also achieved by modulation of its expression, and increases in $cPLA_2$ activity and expression have been linked to the differentiation of various cells [Bomalaski et al., 1988; Gao and Serreto, 1990; Peters-Golden and Feyss, 1994]. However, other phospholipases can contribute to the free arachidonic acid pool needed for eicosanoid production. The secreted (14-kDa) isoform of PLA_2 ($sPLA_2$) releases arachidonic acid for eicosanoid synthesis in some cells stimulated with proinflammatory cytokines [Konieczkowski and Sedor, 1993]. The phospholipase C (PLC)-initiated pathway, acting in concert with diacylglyceride lipase, can also supply free arachidonic acid [Neufeld and Majerus, 1983; Balsinde et al., 1991]. Sources other than the glycerophospholipids may in some instances serve as a source of mobilizable arachidonic acid [Habenicht et al., 1990; Wellnor et al., 1991], and transcellular arachidonic acid metabolism has been described [Parker et al., 1989; Smith et al., 1991; Smith and DeWitt, 1996]. PGE_2 is enzymatically formed from free arachidonic acid by the sequential actions of cyclooxygenase (also called prostaglandin endoperoxide H synthase-1 or -2) and the $PGH_2 \rightarrow PGE_2$ isomerase.

In a previous study, we described certain characteristics of arachidonic acid release in primary cultures of adult human osteoblast-like (hOB) cells that had been stimulated with the proinflammatory nonapeptide bradykinin [Cissel et al., 1996]. In that report, it was noted that bradykinin-stimulated hOB cells derived from male femoral bone explant donors released more arachidonic acid than did stimulated hOB cells derived from female explant donors. (Those data are herein presented for the first time.) This distinction suggested that arachidonic acid metabolism by the hOB cells may exhibit additional sexually dimorphic characteristics. This possibility was evaluated in the present studies in which PGE_2 production and PLA_2 activity are compared between male and female hOB cell strains

METHODS AND MATERIALS

Patient Population

Trabecular bone explants were from surgical waste generated from the femoral head during bone grafting procedures. Because only surgical waste was harvested and patient confidentiality is protected, a waiver for the procedure was approved by the West Virginia University Internal Review Board. Explants were not taken from individuals diagnosed with osteoporosis or from patients presenting with endocrine disorders that affect bone. The femoral bone ex-

plants used in these studies were obtained from 31 women, aged 32–77 years (median age = 66 years), and from 30 men, aged 39–85 years (median age = 60 years).

hOB Cell Cultures

Trabecular bone explants were prepared and placed into culture in a low-calcium, phenol red-free medium according to the method of Robey and Termine [1985], with modifications as previously described [Cissel et al., 1996]. These methods produce nearly homogeneous cell cultures that exhibit multiple aspects of the mature osteoblast phenotype [Borke et al., 1988; Cissel et al., 1996]. The hOB cell phenotype remains stable in culture for at least two passages [Marie, 1994]. All experiments described in this report were performed by using cultures at the end of first or second passage.

Reagents

Human OB cell culture medium consisted of a 1:1 mix of calcium-free, phenol red-free Dulbecco's Modified Eagle Medium (DMEM):Ham's F12 K (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 mM glutamine, and penicillin-streptomycin-neomycin (Gibco, Grand Island, NY). [^{3}H]Arachidonic acid ([^{3}H]AA; 100 Ci/mmol) and L-α-palmitoyl-2-[1-^{14}C]arachidonoyl-phosphatidylethanolamine (55 mCi/mmol) were purchased from New England Nuclear (Boston, MA). PGE_2-Monoclonal Enzyme Immunoassay kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). Recombinant human tumor necrosis factor-α (TNF) and recombinant human transforming growth factor-β_1 (TGFβ) were purchased from R&D Systems (Minneapolis, MN). Concentrated stock solutions of each cytokine were prepared according to the suppliers' instructions and stored at −20°C for no more than 3 months. A stock solution of bradykinin (Bachem, Inc., Torrence, CA) was prepared in 0.1 N HCl and stored at −20°C until used. Silica gel G preadsorbent thin layer chromatography (TLC) plates were obtained from Analtech (Newark, DE), and Biomax X-ray film was obtained from Fisher Chemical (Pittsburgh, PA). The Bio-Rad protein assay (Bio-Rad, Richmond, CA) was used to measure protein concentrations, with bovine serum albumin used as a standard. Other chemicals and reagents employed were of the highest grade available.

hOB Cell Incubations

[^{3}H]Arachidonic acid release by bradykinin stimulated cells. The assay for hOB cell release of [^{3}H]AA by bradykinin-stimulated hOB cells was fully described in the original publication of that work [Cissel et al., 1996]. Briefly, hOB cells were subcultured at 20,000 cells/well and preradiolabeled with 0.15 μCi [^{3}H]AA for 18–24 h. The media were aspirated, the cell layers were washed thoroughly, and the cells were immediately stimulated with 100 nM bradykinin or vehicle in serum-free DMEM containing 0.1% fatty-acid-free bovine serum albumin and 1 mM calcium for 60 min. The media were collected and the release of [^{3}H]AA determined by scintillation counting. The data for the bradykinin-stimulated samples were expressed as the percentage increase in counts per minute (cpm) in the media above the basal (i.e., unstimulated) cpm measured in their respective vehicle-stimulated controls.

Enzyme immunoassays (EIA). Nearly confluent first passage hOB cells were subcultured into 24-well plates at a density of 50,000 cells/well and pretreated as previously described [Xu et al., 1997]. Following attachment, specimens were incubated for 48 h in a 1% FCS-supplemented medium with 1 mM calcium added. This medium was then replaced with 10% FCS-supplemented medium, with calcium maintained at 1 mM. The specimens were stimulated with 40 pM TGFβ, 20 nM TNF, both cytokines, or with vehicle for 20 h, and the conditioned media were harvested and stored frozen at −20°C. PGE_2 was measured in diluted aliquots of the conditioned media by using an EIA according to the supplier's instructions (Cayman Chemical). The detection limit was 60 pg/ml at 80% B/B_o. The cyclooxygenase inhibitor ibuprofen (50 μM) completely blocked PGE_2 production by the hOB cells (data not shown).

Phospholipase A_2 Assay

Nearly confluent second passage hOB cells in three 175-cm^2 culture flasks per experimental group were grown in 1 mM calcium-supplemented, 10% FCS-containing media for 48 h. The FCS concentration was reduced to 1% for the next 48 h, after which the cells were released by trypsinization. As previously described [Keeting et al., 1998], the cells were

pelleted, resuspended, and homogenized, and particulate and cytosolic fractions (post-100,000*g* pellet and supernatant, respectively) of the homogenates were prepared for the PLA_2 assay. Twenty micrograms of protein from each subcellular fraction were incubated with 60,000 cpm of sonicated suspensions of L-α-palmitoyl-2-[1-^{14}C]arachidonoyl-phosphatidylethanolamine ([^{14}C]PE) added for 60 min at 37°C in a shaking water bath according to the method of Paglin et al. [1993], with modification [Keeting et al., 1998]. The addition of chloroform:methanol (2:1) terminated the incubations. The samples were extracted, dried, and redissolved, and volumes containing 20,000 cpm were spotted onto TLC plates. These were developed in solvent system "C" of Nugteran and Hazelhof [1973] and used to expose X-ray film. The radioactivity associated with [^{14}C]PE and with free [^{14}C]arachidonic acid was measured by scintillation counting after scraping the plates.

Statistical Analyses

The release of [^{3}H]AA by bradykinin-stimulated male and female hOB cells was compared by using Student's t-test. The comparisons of PGE_2 production by male and female hOB cells were made by the Wilcoxon rank-sum test because the data were not normally distributed. The relationship between PGE_2 production and bone donor age was evaluated for each sex by using regression analysis. PLA_2 activity comparisons between the subcellular fractions assayed and between the bone donor sexes were made by analysis of variance. In all experiments, N represents the number of different hOB cell strains tested.

RESULTS

[^{3}H]Arachidonic acid release by hOB cells was stimulated by bradykinin treatment, and hOB cell strains derived from male bone explant donors were quantitatively more responsive to stimulation than were the hOB cell strains derived from female donors (Fig. 1). Bradykinin-stimulated [^{3}H]AA release by the male strains was increased to 106 ± 18% above basal (vehicle stimulated) release, whereas the stimulated release by the female strains increased to only 72 ± 14% above basal release (N = 9 and 8, respectively; $P < 0.05$). The possibility that the difference in stimulated AA release between the sexes may be predictive of differences in their capacities for the biosynthesis of PGE_2 was evaluated in male (N = 12–13) and female (N = 10–12) hOB cells (Fig. 2). In unstimulated specimens, PGE_2 production did not differ by donor sex. In all the cytokine-stimulated specimens, PGE_2 formation by the

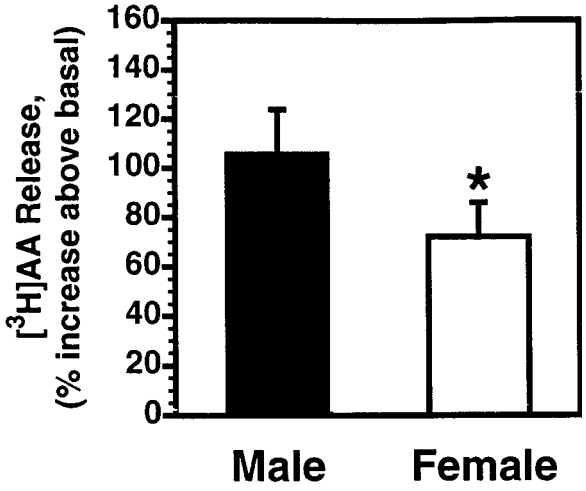

Fig. 1. [^{3}H]Arachidonic acid ([^{3}H]AA) release by bradykinin-stimulated male and female hOB cells. Human osteoblast-like (hOB) cells were subcultured at 20,000 cells/well and preradiolabeled with [^{3}H]AA. Samples were stimulated with 100 nM bradykinin for 1 h, media were collected, and the release of the radiolabeled fatty acid was determined as the percentage increase above basal [^{3}H]AA release by unstimulated paired controls. *$P < 0.05$ (Student's t-test).

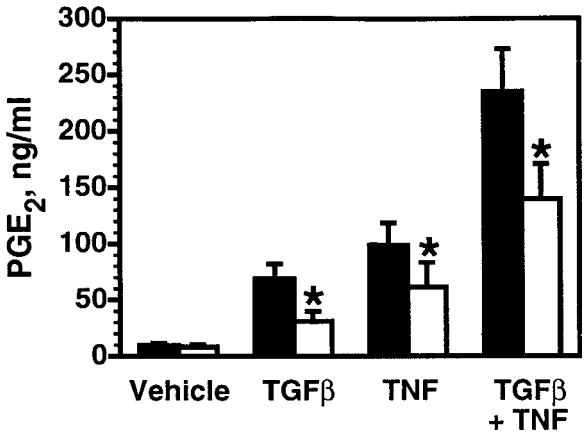

Fig. 2. Prostaglandin E_2 (PGE_2) production by male and female human osteoblast-like (hOB) cells. hOB cells were subcultured at 50,000 cells/well and subsequently stimulated with vehicle (dilute HCl), 40 pM recombinant human transforming growth factor-β_1 (TGFβ), 20 nM recombinant human tumor necrosis factor-α (TNF), or TGFβ and TNF for 24 h. Media were harvested, and diluted aliquots were assayed for PGE_2 by using an EIA. Data shown represent the mean ± SEM of 10–13 independent trials using different hOB cell strains. PGE_2 formation was analyzed by the Wilcoxon rank-sum test because the data were not normally distributed. *$P < 0.03$ for the male/female pairs.

male hOB cell strains exceeded the PGE_2 production by the female cell strains ($P < 0.03$ for each stimulated pair), specifically by 2.2-fold in the TGFβ-stimulated specimens, by 1.6-fold in the TNF-stimulated specimens, and by 1.7-fold in the samples stimulated with both cytokines.

These studies were not specifically designed to evaluate the effects of donor age on hOB cell PGE_2 biosynthesis, and the number of specimens examined per decade was limited and variable. Nevertheless, the PGE_2 production by the male and female hOB cells stimulated with the combination of TGFβ and TNF is shown in Figure 3 to illustrate the heterogeneity of the responses of the individual hOB cell strains and to demonstrate the ultimate clarity of the observed differences in PGE_2 production between the sexes. The age of the explant donors did not correlate with PGE_2 biosynthesis by the derived hOB cell cultures in these studies (males: $r^2 = 0.009$, NS; females: $r^2 = 0.015$, NS). Donor age also did not correlate with PGE_2 production in the specimens stimulated with vehicle, TGFβ, or TNF in these studies (data not shown).

Arachidonic acid release for prostaglandin production in hormone- and cytokine-stimulated cells is typically the responsibility of the cytoplasmic isoform of PLA_2 [Roberts, 1996]. After differential centrifugation of male (N = 12–13) and female (N = 13) hOB cell homogenates, $cPLA_2$ activity in the resultant particulate and cytosolic subcellular fractions was assayed by measuring the hydrolysis of [^{14}C]AA from the artificial $cPLA_2$ substrate [^{14}C]PE (Fig. 4). Cytoplasmic PLA_2 activity in the male hOB cells exceeded the activity measured in the female hOB cells by 1.5-fold in the particulate subcellular fraction and by 1.6-fold in the cytosolic fraction ($P < 0.01$). The use of $cPLA_2$ isoform-selective inhibitors has previously provided evidence that the assay for hOB cell $cPLA_2$ activity measures the activity associated with the calcium-dependent isoform of the enzyme [Keeting et al., 1998].

DISCUSSION

Bone loss is always the result of an imbalance between the rates of bone resorption and bone formation, such that resorption exceeds formation. The slow phase of bone loss during aging that affects both men and women is associated with decreased bone formation. Postmenopausal women also suffer an accelerated

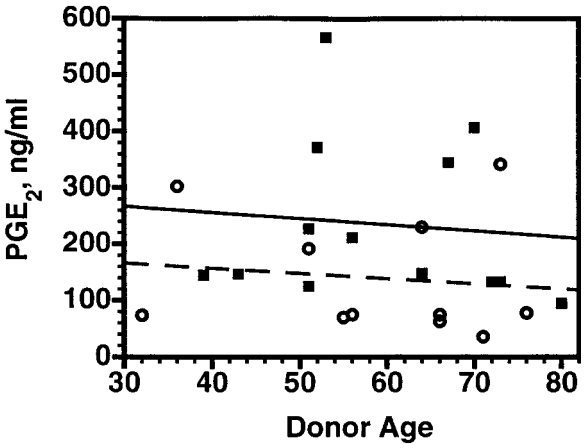

Fig. 3. Human osteoblast-like (hOB) cell donor age did not correlate with stimulated prostaglandin E_2 (PGE_2) biosynthesis. The production of PGE_2 by the hOB cells stimulated with recombinant human transforming growth factor-β_1 (TGFβ) plus recombinant human tumor necrosis factor-α (TNF) (see Fig. 2) are plotted on the basis of donor age and sex (male hOB cells, open squares; female hOB cells, filled circles). Analysis for an effect of age was made by regression analysis, yielding r^2 values of 0.009 (NS) and 0.015 (NS) for male (solid line) and female (dashed line) strains, respectively. Similar results (not shown) were obtained for samples stimulated with vehicle, TGFβ, or TNF.

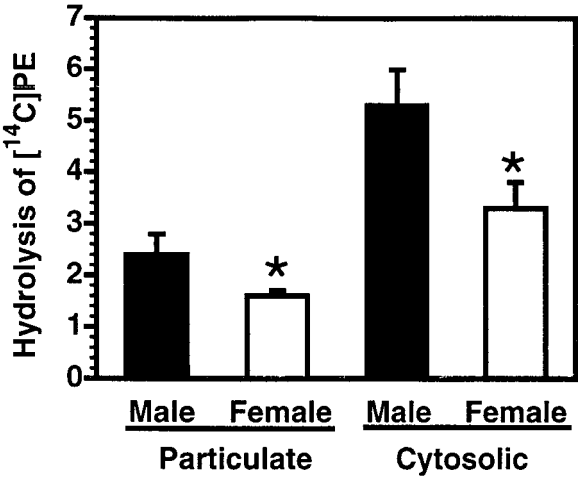

Fig. 4. Phospholipase A_2 activity in male and female human osteoblast-like (hOB) cell subcellular fractions. The hydrolysis of L-α-palmitoyl-2-[1-^{14}C]arachidonoyl-phosphatidylethanolamine ([^{14}C]PE) sonicated suspensions by 20 μg protein from the particulate (100,000*g* pellet) and cytosolic (100,000*g* supernatant) fractions of homogenized hOB cells was measured after a 60-min incubation at 37°C in a shaking water bath. Counts per minute associated with free [^{14}C]arachidonic acid and unmetabolized [^{14}C]PE were measured by scintillation counting, and the results shown represent the mean ± SEM percentage hydrolysis of substrate in 12–13 trials using different hOB cell strains. *$P < 0.01$ vs. percentage hydrolysis by the corresponding fraction in males (analysis of variance).

rate of bone resorption that is inadequately compensated for by an increased rate of bone formation [Lips et al., 1978; Riggs and Melton, 1986; Parfitt, 1987].

Physiological, or so-called scheduled, bone remodeling occurs continuously at discrete foci scattered throughout the skeleton [Parfitt, 1987]. In contrast, a localized burst of intensive remodeling activity is called for during the healing of a bone fracture or during the adaptive bone modeling and remodeling that occurs in response to load-bearing stresses placed on the skeleton [Hulth, 1989; Lanyon, 1993]. The biochemical regulation of these different categories of bone remodeling is not thoroughly defined [Raisz, 1988]. Although distinctions in regulation must exist under the different physiological conditions during which remodeling occurs, producing at least differences in scale and intensity, certain similarities in regulation are highly probable. In all cases, osteoclasts are recruited and activated to resorb bone. Subsequently, new osteoid is produced at the resorption site and later mineralized; both activities comprise a part of osteoblast functions. It is inadequacies in the bone formation phase during the physiological remodeling process that can result in osteoporosis [Riggs and Melton, 1986]. Intriguingly, the remodeling that occurs during fracture healing or in response to load-bearing stresses effectively demonstrates a reserve capacity for bone formation that is evident even in aged individuals and in postmenopausal women. This reserve capacity is also demonstrated by the results of the clinical trials of sodium fluoride as an anti-osteoporosis agent, in which the experimental group actually increased their bone mass during the treatment period [Riggs et al., 1994].

If PGE_2 is an important anabolic regulator of bone, as has been indicated in several recent studies, then the diminished capacity for PGE_2 biosynthesis by the female hOB cells may be a contributing factor to the bone loss that occurs after menopause. In the absence of the increased bone formation demands imposed on the skeleton by the increased rate of resorption that accompanies the menopause, the limited capacity for PGE_2 biosynthesis may not compromise the maintenance of bone mass in women. However, as the remodeling demands are increased, the osteoblast and preosteoblast cell populations may be unable to respond adequately in the absence of sufficiently elevated PGE_2 production. The inadequate response may simply be the result of an insufficient expansion of the preosteoblast population [Parfitt, 1990] because PGE_2 is promitogenic for these cells [Hakeda et al., 1986]. However, because of the pleiotropic effects of PGE_2 in bone, the consequences of a limited production of PGE_2 may also affect other involved cell populations.

An additional point for consideration is that PGE_2 is not the only osteotropic arachidonic acid metabolite produced by hOB cells [Xu et al., 1997]. PGI_2 and $PGF_{2\alpha}$ are also hOB cell products. Whereas the release of these products is quantitatively less than is the release of PGE_2, and PGI_2 and $PGF_{2\alpha}$ formation are less responsive to stimulation by various cytokine treatments, their potential importance as remodeling regulators cannot be overlooked [Kawaguchi et al., 1995]. Whether or not female hOB cell production of PGI_2 and $PGF_{2\alpha}$ is less than that of the male cells has not yet been evaluated, but this seems a likely outcome in light of the analyses of PLA_2 activity.

We previously reported that hOB cell PGE_2 production is increased in a greater than additive manner by treatment with multiple cytokines [Xu et al., 1997]. In that work, we found that PGE_2 production by specimens maximally stimulated with the combination of TGFβ and TNF could be further elevated by the addition of interleukin-1β. It is reasonable to expect that in vivo the elaboration of cytokines by osteoblasts within the remodeling site microenvironment might be supplemented, or complemented, by cytokine release by resident and recruited immune cells, particularly during the inflammatory phase of bone healing [Raisz, 1988; Hulth, 1989]. This could elicit an increased formation of PGE_2, which then reaches a level that provides for the required increase in bone formation at that site. Age and the menopause could also be expected to exert an influence on the microenvironment of the bone cells, with the potential to affect prostaglandin production [Riancho et al., 1994; Manolagas and Jilka, 1995]. The accretion of bone during adaptive bone remodeling may also depend on differential production of the proinflammatory cytokines, which, collectively, elevate PGE_2 formation to useful levels.

Sexually dimorphic characteristics related to arachidonic acid metabolism are not unique to hOB cells. Both thromboxane (Tx) and PGI_2 are products of the cyclooxygenase pathway, and

each of these is a vasoactive substance that may have a role in vascular disease [Moncada et al., 1976; Needleman et al., 1979]. In light of the increased risk for atherosclerosis and downstream organ ischemia in males, the possibility of sex-linked differences in vascular tissue and platelet prostaglandin generation has been studied. The production of the potent vasoconstrictor TxA_2 (measured as TxB_2) by male rat, rabbit, and human platelets exceeded the TxA_2 production by the corresponding female platelets [Kelton et al., 1982; Morikowa et al., 1986; Pinto et al., 1990]. Male human umbilical endothelial cells released more of the potent vasodilator PGI_2 than did similar female cells [Batres and Dupont, 1987]. Homogenates of male rat aorta produced 30% more PGI_2 than did such homogenates from female animals [Lennon and Poyser, 1987]. However, others have not found differences in prostanoid production on the basis of sex in related studies [Buchanon et al., 1983; Maugeri et al., 1989].

The release of [^{3}H]AA by bradykinin-stimulated male hOB cells exceeded the release measured in female specimens. PGE_2 production by cytokine-stimulated male hOB cells exceeded the stimulated production by female hOB cells. Subcellular fractions prepared from male and female hOB cell strains displayed quantitative differences in $cPLA_2$ activity, suggesting a molecular basis for the results obtained in the whole cell studies. These sexually dimorphic characteristics of hOB cell arachidonic acid metabolism may have consequences on the maintenance of bone mass in humans.

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