Published in final edited form as:

Calcif Tissue Int. 2003 December ; 73(6): 565–574. doi:10.1007/s00223-002-0023-z.

Estrogen Potentiates the Combined Effects of Transforming Growth Factor- β and Tumor Necrosis Factor- α on Adult Human Osteoblast-like Cell Prostaglandin E₂ Biosynthesis

F. J. Secreto¹, A. Grover¹, M. Pacurari¹, M. B. Rice¹, M. Kantorow¹, A. P. Bidwai¹, J. D. Blaha², and P. E. Keeting¹

P. E. Keeting: pkeeting@mail.wvu.edu

¹ Department of Biology, West Virginia University, Morgantown, West Virginia 26506-6057, USA

² Department of Orthopedics, West Virginia University School of Medicine, Morgantown, West Virginia 26506, USA

Abstract

Reports that estrogen treatment modulates arachidonic acid metabolism by bone and bone cells are found in the literature. However, conflicting indications of the relationship that exists between estrogen and arachidonic acid metabolism emerge from the analysis of those reports. The present studies were undertaken to determine if estrogen effected the production of prostaglandins (PG) in human osteoblast-like (hOB) cell cultures derived from adults, under basal or cytokine-stimulated conditions. A 48-hour estrogen pretreatment did not modify hOB cell PG biosynthesis on a qualitative basis, and PGE₂ formation predominated under all tested conditions. Estrogen pretreatment did lead to increased PGE₂ production in specimens stimulated conjointly with transforming growth factor- β_1 and tumor necrosis factor- α ($p < 0.001$). No changes in PGE₂ production were observed in estrogen pretreated specimens stimulated singly with either tested cytokine, nor in samples in which either TGF β or TNF was replaced by interleukin-1 β . Anti-estrogen (ICI 164,384) inclusion prevented the estrogen-dependent increase in PGE₂ production in the TGF β plus TNF-stimulated samples. These results suggest that an estrogen effect on bone cell prostaglandin biosynthesis may be most evident and significant under conditions in which the cells are exposed to multiple osteotropic cytokines, a condition that applies during the bone remodeling process.

Keywords

Cyclooxygenase; Cytokine; Remodeling; Phospholipase; Anti-estrogen

The mechanism(s) through which estrogen modulates bone biology remains incompletely defined despite numerous studies over several decades [1,2,3]. Effects of the sex steroid on bone may not be attributable to a single, dramatic response to treatment that is easily demonstrated; the bone-sparing effects of estrogen may, instead, reflect a montage of several modest changes that cumulatively act to maintain bone mass. It has been reported that estrogen may influence the cellular composition of bone by appropriately altering the apoptosis characteristics of osteoblasts and osteoclasts [4,5,6]. The release of osteotropic cytokines may be sensitive to estrogen modulation, with osteoblastic cell biosynthesis of interleukin-1 (IL-1), interleukin-6, osteoprotegerin, and transforming growth factor- β (TGF β), among others, cited as changing following estrogen administration [7,8,9,10,11].

The present report examines the effects of estrogen on the elaboration of prostaglandin E₂ (PGE₂) by adult human osteoblast-like (hOB) cell cultures variously stimulated by TGF β , tumor necrosis factor- α (TNF), or IL-1 β , cytokines that do increase prostaglandin biosynthesis by hOB cells [12]. Each of these cytokines are products of osteoblastic cells that mediate bone biology in an autocrine/paracrine manner [13,14,15]. Immunocytochemical work on human bone sections by Dodds et al. [16] provides evidence that osteoblast expression of TGF β , TNF, and IL-1 β is linked with the reformation phase of bone remodeling. Since PGE₂ acts as an anabolic regulator of bone that under some conditions stimulates catabolic responses, the modulation of PGE₂ production might comprise one of the aspects of estrogen's regulation of bone biology [17].

Materials and Methods

Patient Population

Cancellous bone explants were obtained as surgical waste generated from the femoral head during routine bone-grafting procedures. The harvest of this waste material following informed consent was approved by the West Virginia University Internal Review Board. Patient records were evaluated, and explants were not taken from patients with diagnosed osteoporosis or from patients presenting with endocrine disorders known to affect bone. Femoral explants were obtained from 47 women, aged 23–85 (median age 67), and from 37 men, aged 19–87 (median age 63).

hOB Cell Cultures

Cancellous bone explants were prepared and placed in culture according to the method of Robey and Termine [18], as previously described by Cissel et al. [19]. The essential elements of this method include stripping the explants in a 2-hour collagenase (1 mg/ml) (Gibco, Grand Island, NY) digestion after which the explants are placed in a calcium-free, phenol red-free mixture of DMEM:Ham's F12K (1:1) (Biofluids, Rockville, MD) supplemented by 10% heat-inactivated fetal calf serum (FCS; Gibco). These culture methods yield nearly homogeneous cell cultures that display multiple aspects of the mature osteoblast phenotype [19,20]. The hOB cell phenotype is stable through at least 4 passages [21]. All experiments described in this report used hOB cells subcultured at the end of first or second passage.

Reagents

Recombinant human tumor necrosis factor- α (TNF), recombinant human transforming growth factor- β ₁ (TGF β), and recombinant interleukin-1 β (IL-1 β) were purchased from R&D Systems (Minneapolis, MN). Stock solutions of each were prepared according to the supplier's instructions and stored at –20°C for no more than 4 months. Unlabeled PGs and PGE₂-Monoclonal Enzyme Immunoassay (EIA) Kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). 1[¹⁴C]Arachidonic acid (55 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Preadsorbent Silica Gel G thin layer chromatography (TLC) plates were from Analtech (Newark, DE), and Biomax-MR X-ray film was from Fisher Chemical (Pittsburgh, PA). An affinity purified goat polyclonal antibody to human cyclooxygenase-2 and an affinity purified goat polyclonal antibody to human cytosolic phospholipase A₂- α (Group IVA) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). A rabbit anti-goat alkaline phosphatase-conjugated affinity purified antibody was purchased from Jackson Immunos (West Grove, PA). RT-PCR One-Step kits and TOPO TA kits were obtained from Invitrogen (Carlsbad, CA), and Sequenase kits from Promega (Madison, WI). Other chemicals and reagents used were of the highest quality available.

Oligonucleotide primers recognizing human cyclooxygenase-1 (COX-1), human cyclooxygenase-2 (COX-2), and human cytosolic phospholipase A₂- α (cPLA₂- α) were

designed using the primer algorithm of the DNA Star software package (DNASTAR Inc., Madison, WI), and crossed one intron/exon boundary. The primers were compared against the known sequences using the BLAST algorithm and GENBANK sequence data (NCBI, Frederick, MD). Oligonucleotide primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPdH) were purchased from Invitrogen. Amplification specificity was assured by sequencing the subcloned gel purified PCR products from our experiments. The COX-2 sequence was determined in our laboratory using an Applied Biosystems 377 automated DNA sequencer, and the COX-1, cPLA₂- α , and GAPdH sequences were determined externally (Univ. California-Davis Sequencing Laboratory). RT-PCR products of 450 base pairs (COX-1), 450 bp (COX-2), 332 bp (cPLA₂- α), and 600 bp (GAPdH) were obtained from hOB cell RNA preparations. The oligonucleotide primers used were as follows: COX-1, forward 5'-CTCATAGGGGAGACCA TCAAG-3' and reverse 5'-CCTTCTCTCCTACGAGCTC CTG-3'; and COX-2, forward 5'-TGTGCCTGATGATTGC CCGACTCC-3' and reverse 5'-TGTTGTGTTCCCGCAGCCAGATTG-3'; and cPLA₂- α , forward 5'-CCAGCACATTAT AGTGGAGCAC-3' and reverse 5'-CCCACCTTCATAGA AGATACAG-3'.

hOB Cell Incubations

For Radiochemical Analyses

hOB cells were subcultured into 24-well plates at 50,000 cells/well in medium supplemented to 1 mM calcium and 10% (v/v) FCS (see flow chart below). The calcium concentration was thereafter maintained at 1 mM.

Forty-eight hours after subculturing, media were replaced and the FCS was reduced to 1% for an additional 48 hours, with or without supplementation to 10^{-8} M 17 β -estradiol (17 β -E₂). This 48-hour pretreatment regimen has previously been used in hOB cell studies to elicit estrogen-dependent regulation of arachidonic acid metabolism [19]. We elected not to charcoal-strip the FCS. At the serum concentrations used, based on the supplier's analysis the FCS contributed 17 β -E₂ was, at most, 10^{-13} M, while the K_d of the estrogen receptor is roughly 10^{-10} M [22]. Charcoal-stripping of serum is not specific for 17 β -E₂ and whatever steroids, small peptides, and other low molecular weight factors are present are also removed, a complication that we prefer to avoid. In some experiments, the anti-estrogen ICI 164,384 at 10^{-8} M was added during this preincubation period. Over the final 24 hours of the preincubation, cells were exposed to 0.55 μ Ci 1-[¹⁴C]arachidonic acid/well, a treatment that radiolabels each of the major glycerophospholipids of the hOB cells [23]. Following the preincubation period, the media were aspirated, and the cell layers washed twice with 0.1% BSA in phosphate-buffered saline to remove unincorporated 1-[¹⁴C]arachidonic acid. Media supplemented to 10% FCS were applied to the cells, and the experimental manipulations were initiated 30 minutes later by the addition of 20 nM TNF, 40 pM TGF β , both cytokines, or vehicle for 24 hours. In some experiments, IL-1 β at 300 nM was used in lieu of either TGF β or TNF. These cytokine concentrations elicit their maximal effects on hOB cell PGE₂ production [12]. During the 30 minutes preceding the cytokine additions, some specimens were pretreated with 50 μ M ibuprofen to inhibit prostaglandin production. After the 24-hour cytokine stimulation, the hOB cell conditioned media were collected on ice, microfuged to remove cell debris, and stored at -80°C in siliconized glass tubes until analyzed using thin layer chromatography (TLC).

The effect of the estrogen pretreatment on the total release of radioactivity by stimulated hOB cells was evaluated in several experiments in which the cells were radiolabeled with 1-[¹⁴C]arachidonic acid as described above. Following the media change at 48 hours, cytokines were added. Aliquots of the media were taken at 1, 6, and 24 hours of stimulation and scintillation counted. Some specimens were stimulated with 1 μ M A23187, a calcium ionophore. The

calcium influx elicited by the A23187 activates calcium-regulated phospholipases and elicits the translocation of the cPLA₂ from the cytosol to the intracellular membranes, and served as a positive control providing a basis for comparison with the cytokine-stimulated samples [24].

For Enzyme Immunoassay (EIA)

hOB cells were subcultured at 50,000 cells/well into 24-well plates and pretreated as described above, omitting the preradiolabeling of the cells with 1-[¹⁴C]arachidonic acid. Following incubation with or without the added cytokines for 24 hours, the conditioned media were harvested and stored at -80°C until assayed.

Thin Layer Chromatography and Autoradiography

hOB cell-conditioned media were acidified with 4N formic acid and extracted twice with 3 volumes each of ethyl acetate. In preliminary studies, ethyl acetate extraction was found to recover $72 \pm 5\%$ ($n = 4$) of added [³H]PGE₂ from hOB cell incubation media. The extracts were dried under a stream of nitrogen and the residues were redissolved into chloroform:methanol (2:1) and spotted onto Silica Gel G preadsorbent TLC plates. The plates were developed in the water-saturated organic phase of ethyl acetate:iso-octane:acetic acid:water (55:25:10:50) and used to expose Biomax-MR X-ray film for 10–14 days. Authentic PGs and arachidonic acid were co-chromatographed and visualized by iodine vapor staining to establish their R_f's in this system. Following autoradiography, the R_f values of the radiolabeled bands were compared to the R_f's of the authentic standards to identify the prostaglandin products of the incubation. The radiolabeled bands were scraped from the plates and radioactivity was determined by scintillation counting.

Immunochemical Assay for PGE₂

PGE₂ was measured in diluted aliquots of hOB cell-conditioned media using EIA kits according to the supplier's (Cayman Chemical) instructions. The detection limit was 60 pg/ml at 80% B/B₀.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Northern blot analyses of mRNA levels under multiple incubation conditions was not a viable option for these studies since the yield of cells from a single cell strain is limited. hOB cells were subcultured at a density of 150,000 cells/well in 12-well plates in 10% FCS medium supplemented with 1 mM calcium. After 48 hours, media were changed to 1% FCS with 1 mM calcium for 48 hours with either 10⁻⁸ M 17 β-E₂ or vehicle (0.1% ethanol) added. In samples stimulated with TGFβ plus TNF, the cytokines were added to the media 6 hours before harvesting the cells for RNA preparations. Time course studies had demonstrated that COX-2 mRNA and cPLA₂-α mRNA levels were maximal following a 6-hour stimulation with these cytokines (data not shown). Total RNA was isolated using the TRIZOL Reagent (Invitrogen), and DNAase treated. RNA yields were estimated by absorbance at 260 nM, and the reverse transcriptions and amplifications were performed starting with 50 ng of total RNA per sample, using the RT-PCR One-Step kits according to the manufacturer's recommendations. Thirty-three cycles of amplification at 61°C were performed when COX-1 was analyzed, 25 cycles for COX-2 at 55°C, 30 cycles for cPLA₂-α at 55°C, and GAPdH at 55°C, leaving each product in its linear range. Hybridization intensities in these experiments were determined using the BioRad Fluor-STM MultiImager gel documentation system with their software. All data were expressed as a percentage of concurrently amplified human GAPdH control bands.

Western Blots for Cyclooxygenase-2 and Cytosolic Phospholipase A₂-α

hOB cells at 300,000 cells/well were subcultured into 6-well plates in 10% FCS supplemented media with 1 mM calcium for 48 hours. Media were replaced with 1% FCS, calcium-supplemented media with 10^{-8} M 17β -E₂ or vehicle added for 48 hours. Following this, in samples treated with TGFβ plus TNF, the cytokines were added for 12 hours in 10% FCS, calcium-supplemented media. Cells were released from the wells by a brief trypsin digest, pelleted, and resuspended in 300 μl of a homogenization buffer consisting of 10 mM HEPES, 8% sucrose, and 1 μl/ml of Sigma's Protease Inhibitor Cocktail, at pH 7.2. The samples were sonicated on ice at low power for three 10–15 second bursts, and cooled on ice between each sonication for 30 seconds. Cell debris was cleared by microcentrifugation, and the supernatants were ultracentrifuged at $100,000 \times G$ for 60 minutes at 4°C. The resultant pellets, designated microsomes, were resuspended in buffer and protein measured using the Bradford assay. Protein (20 μg per sample) was separated on 10% SDS PAGE gels, and the separated proteins were transferred to nitrocellulose membranes. The blots were stained with Ponceau-S, washed for 30 minutes in Tris-Buffered saline, 0.1% Tween-20 (pH = 7.5; TBST), and blocked for 2 hours at room temperature with 5% normal rabbit serum in TBST. Blots were incubated with a goat affinity-purified antibody to human COX-2 (diluted 1:200), or to a goat affinity-purified antibody to human cPLA₂-α (1:200) overnight at 4°C, washed 3 times, and exposed to a rabbit anti-goat alkaline phosphatase-conjugated affinity-purified secondary antibody (1:5,000) for 2 hours, washed extensively, and developed for several minutes in a buffered Nitro Blue Tetrazolium chloride solution. Development was stopped with 0.5 M EDTA, and the bands were analyzed using the BioRad gel documentation system. Some specimens were incubated with COX-2 antiserum preabsorbed with a 100-fold excess of COX-2 blocking peptide (Santa Cruz) which confirmed the specificity of the antiserum (data not shown). A similar control was run for the cPLA₂-α Westerns, again confirming antibody specificity (data not shown).

Statistical Analyses

Data for PGE₂ production as measured by EIA are presented as “Fold-increase” to represent the response of the cells to treatment compared with basal production. Absolute PGE₂ production varied widely among cell strains, thus a normalization method was required to permit meaningful comparisons to be made. For example, basal PGE₂ production by the male hOB cell strains ranged from a low of 1.7 ng/ml to a high of 22.1 ng/ml, with an average value of 10.2 ± 1.4 ng/ml for the 17 strains assayed, as indicated in Figure 1.

Data are presented as the mean $\pm$ SEM of N experiments using different hOB cell strains. The effects of estrogen pretreatment on PGE₂ production and the effects of ICI 164,384 were analyzed by the paired *t*-test. Estrogen's effects on the release of radioactivity by stimulated hOB cells were analyzed by ANOVA and Dunnett's Method for Comparisons with a Control. In the RT-PCR studies, individual band intensities were first normalized to the sample's respective GAPdH band intensity. These values were then expressed as a percentage of control sample value, and treatment effects were analyzed with a blocked ANOVA model. A blocked ANOVA model was used to analyze the Western blot data.

Results

The release of PGE₂ by vehicle (0.1% ethanol) or 17β -E₂ pretreated hOB cells was measured by EIA under basal conditions, or following a 24-hour stimulation by cytokines (Fig. 1A, B). Because arachidonic acid metabolism by female hOB cells and male hOB cells exhibits sexually dimorphic characteristics [25], the responses of the cells of each sex were assessed independently in this experiment. The application of TGFβ, TNF, or both cytokines simultaneously significantly stimulated hOB cell PGE₂ production ($p \leq 0.01$), agreeing with previous observations [12]. Pretreatment with 17β -E₂ did not alter PGE₂ release under basal

conditions, nor in specimens stimulated singly with either TGF or with TNF. However, the 17 β -E₂ pretreatment potentiated the responses of samples stimulated conjointly with both TGF β plus TNF, an effect seen in both sexes (females: $p \leq 0.005$, $n = 15$; males: $p \leq 0.01$, $n = 17$). The percentage potentiation by the 17 β -E₂ pretreatment was similar for the female and male hOB cell cultures (females: $127 \pm 8\%$ of the ethanol pretreated TGF plus TNF-stimulated samples; males: $123 \pm 7\%$). On the basis of this similarity of response to 17 β -E₂ pretreatment, the results of the other experiments presented in this report do not distinguish findings as to the bone cell donor's sex. The potentiating effect of the 17 β -E₂ pretreatment on TGF β plus TNF-stimulated PGE₂ production was abolished when the anti-estrogen ICI 164, 384 was coincubated with the 17 β -E₂ (Fig. 2).

The cytokine specificity of the 17 β -E₂-dependent response was evaluated in a subset of the samples represented in Figure 1, wherein IL-1 β was used as a stimulatory cytokine in lieu of either TGF β or of TNF. As indicated in Figure 3, the fold-stimulation of PGE₂ production observed was the same in both the ethanol pretreated and 17 β -E₂ pretreated specimens stimulated singly with IL-1 β , or stimulated conjointly with IL-1 β plus TGF or with IL-1 β plus TNF ($n = 7$ for each condition). Thus, IL-1 β could not replace either TGF β or TNF in terms of the effectiveness of 17 β -E₂ on PGE₂ biosynthesis.

The possibility that the 17 β -E₂ pretreatment acted by increasing the availability of free arachidonic acid for conversion into PGE₂ via the cyclooxygenase pathway was considered. hOB cells were preradiolabeled with 1-[¹⁴C]arachidonic acid. Previous studies have demonstrated that 17 β -E₂ pretreatment does not alter the uptake of radiolabeled arachidonic acid by hOB cells, nor its distribution into the principal glycerophospholipid pools [21]. The preradiolabeled cells were stimulated concurrently with TGF β plus TNF ($n = 8-9$), or with the calcium ionophore A23187 as a positive control ($n = 6-10$), for up to 24 hours. No changes in the release of radioactivity into the media compared with controls were evident following 1, 6, or 24 hours of cytokine stimulation (data not shown). Estrogen pretreatment did not alter this outcome. A23187 stimulation elicited a significant increase in radioactivity release at each time point, reaching a maximum of $141 \pm 12\%$ of the release by unstimulated cells at 6 hours (A23187 vs unstimulated: $p \leq 0.05$, at each time point, Dunnett's Test). Samples pretreated with 17 β -E₂ before the A23187 addition produced similar results.

Since 17 β -E₂ could not be demonstrated to alter substrate availability, the possibility that an increased cyclooxygenase activity might account for the sex steroid's potentiation of PGE₂ biosynthesis was examined. Estrogen or vehicle pretreated, 1-[¹⁴C]arachidonic acid preradiolabeled cells were stimulated individually with TGF β , TNF, or with both cytokines concurrently. Following a 24-hour incubation, media were extracted, products were separated by thin layer chromatography, and used to expose x-ray film (Fig. 4). The product profiles displayed were not different between the ethanol and 17 β -E₂ pretreated groups, irrespective of stimulation, suggesting that estrogen-dependent changes did not include the induced production of additional cyclooxygenase products such as thromboxane or PGD₂. The prostanoid nature of the observed bands was confirmed by ibuprofen treatment of some specimens that eliminated the formation of the products (data not shown). Furthermore, there was no indication that the 17 β -E₂ pretreatment altered the metabolism of PGE₂ into 13, 14-dihydro-15-keto-PGE₂ via the 15-prostaglandin dehydrogenase and keto-prostaglandin- Δ^{13} -reductase pathway, nor that PGE₂ was converted into PGF_{2 α} by a 9-ketoreductase activity. The identified bands corresponding to PGE₂ and to 6-keto-PGF_{1 α} (the stable breakdown product of PGI₂) were scraped and scintillation counted. The remaining material of each lane was scraped from the TLC plates and scintillation counted as well, omitting the glycerophospholipids which remained at the origin in this developing system. The conversion of radiolabeled substrate into [¹⁴C] PGE₂ and [¹⁴C]6-keto-PGF_{1 α} was then calculated as the ratio of the cpm in each band to the total cpm of that lane (Table 1). These data are a

representation of the substrate utilization by the hOB cells over this 24-hour period, thereby providing a rough index of hOB cell cyclooxygenase activity. The 17β -E₂ pretreatment did not alter the net conversion of substrate into products under either basal or cytokine-stimulated conditions, suggesting that the 17β -E₂ pretreatment left cyclooxygenase levels unchanged. The effects of the cytokine treatments on increasing the cell's capacity for prostaglandin biosynthesis are obvious, and significant ($p \leq 0.05$ for all cytokine-stimulated samples vs. control).

Further evidence that 17β -E₂ pretreatment did not potentiate cytokine-stimulated PGE₂ biosynthesis by increasing the hOB cell complement of the COX-2 enzyme was obtained in RT-PCR-based studies. RNA prepared from ethanol or 17β -E₂ pretreated samples, stimulated with or without TGF plus TNF, were tested. Figure 5 shows the outcome of one such experiment, where COX-1, COX-2, and GAPdH were probed and band intensities were measured. Comparisons between all treatments and their respective ethanol pretreated, unstimulated controls were made as the ratios of the individual COX bands absorbances to that of the corresponding GAPdH bands (Fig. 5B). The TGF plus TNF treatment elevated the steady-state level of COX-2 mRNA by greater than 17-fold ($p \leq 0.001$; $n = 15$), but this increase was insensitive to the 17β -E₂ pretreatment ($p = 0.73$; $n = 14$). Supporting the RT-PCR data on COX-2 expression were the results seen in Western blots where the increased COX-2 protein levels elicited by the cytokines TGF and TNF were not affected by 17β -E₂ pretreatment of the cells ($p = 0.73$; $n = 7$) (Figs. 6A, B).

Although COX-2 is recognized to be the inducible isoform of the cyclooxygenase enzyme, in some tissues COX-1 expression has been found to be sensitive to estrogen treatment. Accordingly, RT-PCR analyses of COX-1 mRNA levels were also made in these studies (Fig. 5). TGF plus TNF stimulation did not significantly alter COX-1 levels ($n = 7$). This outcome was the same in paired 17β -E₂ pretreated specimens.

As described above, an increased release of radioactivity was not observed in cytokine-stimulated hOB cells, irrespective of 17β -E₂ pretreatment. The possibility that the rapid reacylation of released 1-[¹⁴C]arachidonic acid could have obscured any cytokine and/or 17β -E₂-dependent effects was considered. RT-PCR analyses of cPLA₂-α mRNA demonstrated a significant 3.0 ± 0.4 -fold increase ($p \leq 0.001$ vs. control; $n = 12$) in the steady-state level following a 6 hour TGF plus TNF stimulation (Fig. 7A, B). This cytokine-stimulated increase was insensitive to pretreatment of the cells with 17β -E₂, and the steady-state level of cPLA₂-α mRNA in these specimens demonstrated a 2.5 ± 0.4 -fold increase compared with controls ($p \leq 0.001$; $n = 12$). Estrogen alone had no effect on the cPLA₂-α mRNA level. A Western blot of cPLA₂-α protein is illustrated in Figure 8A, and the summary data in Figure 8B. In 5 tested hOB cell strains stimulated with TGF plus TNF for 12 hours, cPLA₂-α protein was not significantly increased compared to their respective unstimulated controls (1.4 ± 0.3 -fold of control; $p = 0.28$; $n = 5$). However, in those 5 cell strains, samples pretreated with 17β -E₂ prior to the 12 hour application of the cytokines, cPLA₂-α protein was significantly elevated compared with their controls (1.7 ± 0.3 -fold of control; $p \leq 0.05$; $n = 5$).

Discussion

Estrogen's actions in bone has been linked with effects on arachidonic acid metabolism [17]. Calvariae cultures from ovariectomized rats released about twice the amount of PGE₂ than did cultures prepared from intact animals [26]. The *in vivo* administration of estrogen to these ovariectomized animals blocked the increased release of PGE₂ by the resultant calvarial cultures, while estrogen's *in vitro* administration was ineffective. Estrogen administration to cultured neonatal mouse calvariae stimulated with parathyroid hormone opposed prostaglandin release [27]. The non-steroidal antiinflammatory drug (NSAID) naproxen prevented trabecular

bone loss in ovariectomized rats [28]. NSAID actions typically include the inhibition of prostaglandin biosynthesis. Since numerous reports can be found asserting that PGE₂ stimulates bone resorption in mouse and rat bone cultures [17,29], collectively these results suggest that estrogen may oppose bone loss by limiting prostaglandin biosynthesis within the bone compartment.

A different view emerges from other studies. Work by Jee et al. [30,31] and Kimmel et al. [32] shows that exogenous PGE₂ can be an anabolic agent for rat cancellous and cortical bone, and further that it prevents ovariectomy-induced cancellous bone loss [31,33,34]. Others report that the administration of the NSAID ibuprofen blunts the protective effect of estrogen against cancellous bone loss in ovariectomized rats, and it entirely blocks the effects of the estrogen analog tamoxifen on those animals [35], while indomethacin opposed high-dose estrogen-induced osteogenesis in the mouse [36]. These data suggest that prostaglandin biosynthesis plays a role in the bone-sparing effects of estrogen. It should be noted that NSAID effects on peroxisome proliferator-activated receptors (PPAR) α and γ , and on NF κ B-mediated responses have also been reported [37,38,39].

The present work supports the proposition that a positive modulation of prostaglandin biosynthesis may play a role in the bone-sparing effects of estrogen. In contrast to the broadly held view that estrogen opposes osteoblastic cell PGE₂ biosynthesis, as noted in recent reviews by Riggs [1], Spelsberg et al. [3], and Raisz [29,40], based on the work of Feyen and Raisz [26] and Pilbeam et al. [27] with rat and mouse models, no evidence that estrogen might limit human osteoblastic cell prostaglandin biosynthesis was found under the varied conditions tested. Those reports leading to a different conclusion regarding estrogen, prostaglandins, and bone remodeling may reflect distinctions among adult and fetal/neonatal cell models, bone compartment effects, species effects, the complications inherent in working at the organ rather than the cellular level, as well as the highly specific conditions that were required to demonstrate a positive regulation of PGE₂ biosynthesis in the present study. hOB cells are a challenging model system. There are inherent limitations in cell numbers available which restricts the scope of some investigations. The heterogeneity of the donor population is typically reflected in the data generated. Responses are often quite variable from cell strain to cell strain, forcing cautious interpretation of results, and imposing a burden on the investigator with respect to the number of experiments required for accurate and convincing statistical analysis. Nevertheless, the value of conducting studies in untransformed, adult human cell strains must also be recognized. The complications of studying cell physiology in transformed cell lines derived from a single aberrant cell, and the limitations regarding trans-species comparisons and conclusions should not be minimized.

Prostaglandin biosynthesis is a multistep process [41]. Esterified arachidonic acid is released from glycerophospholipids by activated phospholipase(s). Cyclooxygenase activity is rate-limited by free arachidonic acid availability, supplied through PLA₂ hydrolysis of glycerophospholipids at the *sn*-2 position [41]. The contributions of specific PLA₂'s to prostaglandin biosynthesis is complex and controversial [42], but it is clear that cPLA₂ is of fundamental importance [43]. The free arachidonic acid can then be used as substrate by the cyclooxygenase enzymes to form the unstable cyclic endoperoxide intermediate PGH₂. Various PGH₂-utilizing enzymes, typically expressed in a tissue-specific manner, then convert PGH₂ into one of the bioactive prostanoids. The present study was unable to define which of these steps was influenced by estrogen pretreatment that led to enhanced PGE₂ production.

The formation of radiolabeled arachidonic acid metabolites was increased in cytokine-stimulated specimens, irrespective of estrogen pretreatment (Fig. 4), although an overall increase in total radioactivity release in those experiments could not be demonstrated. The sensitivity of the assay methods may have been inadequate to detect a difference that might

have been solely the result of increased prostaglandin release. A23187 stimulation of the preradiolabeled hOB cells did elicit the release of radioactivity. An A23187-dependent increase in intracellular calcium increases cellular calcium-regulated phospholipase activity [24]. The lack of an estrogen effect on A23187-stimulated cells suggests that estrogen did not alter other metabolic pathways that could have modified levels of arachidonic acid in the media, such as the reacylation reactions that reincorporate free arachidonic acid into cellular lipids [44,45]. This agrees with previous results from this laboratory showing that estrogen does not influence arachidonic acid uptake and insertion into hOB cell lipids [22].

Cyclooxygenase activity was unaffected by the estrogen pretreatment as judged by the conversion of substrate into product (Table 1). In those experiments, 1-[¹⁴C]arachidonic acid conversion into 1-[¹⁴C]PGE₂ did vary according to the stimulation of the cells with TGFβ, TNF, or with both used in combination, results in accord with previously published work [12,46]. However, estrogen pretreatment did not further modify the cytokine-induced increases in product formation from substrate. These radiochemical analyses also provided evidence that the metabolism of 1-[¹⁴C]PGH₂, the direct product of cyclooxygenase, was insensitive to estrogen pretreatment since the array of prostaglandin products formed was not different from the array produced by control samples. The RT-PCR studies and Western blot data provide additional evidence that estrogen's potentiation of PGE₂ production by the hOB cells was not a consequence of effects on COX-2 expression. The steady-state level of COX-1 mRNA was unaffected by the administration of estrogen as well. In these findings, the hOB cells are distinguished from various cell models of human and animal origin that exhibited increased expression of COX-1 or COX-2 following estrogen exposure [47,48,49].

The site of estrogen modulation of hOB cell PGE₂ biosynthesis was not determined by the present studies. The observation that PGE₂ production was elevated in estrogen-pretreated samples stimulated by TGFβ and TNF is firmly established, as is the observation that 17β-E₂ treatment did not limit hOB cell PGE₂ biosynthesis. Experiments using 32 hOB cell strains (17 male and 15 female) indicated a consistent, although modest response of the cells to the estrogen pretreatment in terms of the potentiation of PGE₂ biosynthesis, a response that was independent of the sex of the bone donor. The methods used to evaluate arachidonic acid release and the efficiency of substrate utilization may have lacked the sensitivity required to identify whatever specific modest changes may be induced by estrogen. It has proven a difficult task to link prostaglandin biosynthesis with the activity of a specific phospholipase [41], and these studies did not provide direct evidence that the estrogen treatment altered substrate availability. The modest effect of estrogen pretreatment on cPLA₂-α protein levels in the cytokine-stimulated specimens does suggest a possible mechanism that could contribute to the sex steroid's potentiation of PGE₂ biosynthesis. cPLA₂-α does co-localize with COX-1 and COX-2 to the perinuclear membranes [24,41]. An elevated free arachidonic acid concentration within the perinuclear membrane microenvironment would support an increased prostaglandin biosynthesis. The reacylation of the released arachidonic acid may be rapid enough to prevent the release of the fatty acid from the cell. The reacylation processes would not effect the release of PGE₂. Thus, changes in PGE₂ production could be measured, while changes in arachidonic acid release might escape detection. It is difficult to envision an effect of estrogen leading to increased PGE₂ production that would not be directed towards the various key components of the arachidonic acid cascade, as discussed above.

Whole animal studies and clinical evidence demonstrates that estrogen's regulation of bone remodeling is critical for the maintenance of a healthy bone mass [50,51,52]. However, the responses of bone and bone cells thus far attributed to estrogen treatment are largely unremarkable and of limited magnitude [1,2,3,4,51]. Among the responses reported, it has been found that the proliferation of hOB cells was unaffected, or minimally affected, by estrogen, as were several tested aspects of hOB cell differentiation [53,54]. TGFβ mRNA and protein

were elevated in estrogen-treated hOB cell cultures [55], but this elevation did not elicit TGF β -typic responses in the cells. Interleukin-6 (IL-6) production was diminished in estrogen-treated, stimulated mouse stromal osteoblastic cells [56]. Stimulated IL-6 mRNA and protein production was decreased in estrogen-treated fetal human osteoblast-like cells (hFOB) overexpressing the estrogen receptor [57]. The release of [³H]arachidonic acid by bradykinin-stimulated hOB cells increased following estrogen pretreatment [19]. The present report indicates that PGE₂ production is increased in estrogen pretreated hOB cells conjointly stimulated with TGF β and TNF. The magnitude of the estrogen-induced changes in each of these parameters typically ranged from approximately 30–60% when compared with control samples.

Whether any of the responses noted can independently account for the bone-sparing effects of estrogen, or if the effect results from the collective consequences of multiple modest changes, as recently postulated by Riggs [1], is uncertain. The array of the reported effects of estrogen on osteoblastic cells almost uniformly involve the modulation of cellular signaling pathways. The present characterization of estrogen's modulation of hOB cell PGE₂ production fits into this general categorization. It is of interest that the consequences of estrogen pretreatment on PGE₂ production were not apparent in specimens stimulated singly with TGF β or with TNF. In our view, this suggests that the effects of estrogen on PGE₂ biosynthesis may be of particular importance during the most active phases of bone remodeling, when the elaboration of cytokines within the bone compartment increases and becomes more complex [16,40]. The present studies demonstrate that estrogen could increase PGE₂ formation by cytokine-activated resident osteoblasts, thereby elevating the local concentration of this anabolic mediator specifically at the remodeling site.

Acknowledgments

This work was supported in part by grants AR41769 and AG16656 from the National Institutes of Health, United States Public Health Service.

References

1. Riggs BL. The mechanism of estrogen regulation of bone resorption. *J Clin Invest* 2000;106:1203–1204. [PubMed: 11086020]
2. Manolagas SC, Kousteni S, Jilka RL. Sex steroids and bone. *Recent Prog Horm Res* 2002;57:385–409. [PubMed: 12017554]
3. Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. *Molec Endocrinol* 1999;13:819–828. [PubMed: 10379881]
4. Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 2000;21:115–137. [PubMed: 10782361]
5. Hughes DE, Dai A, Tiffée JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF- β . *Nat Med* 1996;2:1132–1136. [PubMed: 8837613]
6. Gohel A, McCarthy M-B, Gronowicz G. Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts *in vivo* and *in vitro*. *Endocrinology* 1999;140:5339–5347. [PubMed: 10537165]
7. Pivrotto LA, Cissel DS, Keeting PE. Sex hormones mediate interleukin-1 β production by human osteoblastic HOBIT cells. *Mol Cell Endocrinol* 1995;111:67–74. [PubMed: 7649354]
8. Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC. Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science* 1992;257:88–91. [PubMed: 1621100]
9. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology* 1999;140:4367–4370. [PubMed: 10465311]

10. Oursler MJ, Cortese C, Keeting PE, Anderson M, Bonde S, Riggs BL, Spelsberg TC. Modulation of transforming growth factor beta production in normal human osteoblast-like cells by 17[β]-estradiol and parathyroid hormone. *Endocrinology* 1991;129:3313–3320. [PubMed: 1954907]
11. Oursler MJ. Estrogen regulation of gene expression in osteoblasts and osteoclasts. *Clin Rev Eukaryot Gene Expr* 1998;8:125–140.
12. Xu JQ, Cissel DS, Varghese S, Whipkey DL, Blaha JD, Graeber GM, Keeting PE. Cytokine regulation of adult human osteoblast-like cell prostaglandin biosynthesis. *J Cell Biochem* 1997;64:618–631. [PubMed: 9093911]
13. Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB. Osteoblasts synthesize and respond to TGF β *in vitro*. *J Cell Biol* 1987;105:457–463. [PubMed: 3475276]
14. Gowen M, Chapman K, Littlewood A, Hughes D, Evans D, Russell RGG. Production of tumor necrosis factor by human osteoblasts is modulated by other cytokines, but not by osteotropic hormones. *Endocrinology* 1990;126:1250–1255. [PubMed: 2404745]
15. Keeting PE, Rifas L, Harris SA, Colvard DS, Spelsberg TC, Peck WA, Riggs BL. Evidence for interleukin-1 β production by cultured normal human osteoblast-like cells. *J Bone Miner Res* 1991;6:827–833. [PubMed: 1785373]
16. Dodds RA, Merry K, Littlewood A, Gowen M. Expression of mRNA for IL1 beta, IL6 and TGF beta 1 in developing human bone and cartilage. *Histochem Cytochem* 1994;42:733–744.
17. Kawaguchi H, Pilbeam CC, Harrison JR, Raisz LG. The role of prostaglandins in the regulation of bone metabolism. *Clin Orthop Rel Res* 1995;313:36–46.
18. Robey PG, Termine JD. Human bone cells in vitro. *Calcif Tissue Int* 1985;37:453–460. [PubMed: 2998572]
19. Cissel DS, Murty M, Whipkey DL, Blaha JD, Graeber GM, Keeting PE. Estrogen pretreatment increases arachidonic acid release by bradykinin-stimulated normal human osteoblast-like cells. *J Cell Biochem* 1996;60:260–270. [PubMed: 8655636]
20. Borke JL, Eriksen EF, Minami J, Keeting P, Mann KG, Penniston JT, Riggs BL, Kumar R. Epitopes of the human erythrocyte Ca²⁺-Mg²⁺ ATPase pump in human osteoblast-like cell plasma membranes. *J Clin Endocrinol Metab* 1988;67:1299–1304. [PubMed: 2461388]
21. Marie PJ. Human osteoblast-like cells: a potential tool to assess the etiology of pathologic bone formation. *J Bone Miner Res* 1994;9:1847–1850. [PubMed: 7872048]
22. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucl Acids Res* 2001;29:2905–2919. [PubMed: 11452016]
23. Cissel DS, Birkle DL, Whipkey DL, Blaha JD, Graeber GM, Keeting PE. 1,25-Dihydroxyvitamin D₃ or dexamethasone modulate arachidonic acid uptake and distribution into glycerophospholipids by normal adult human osteoblast-like cells. *J Cell Biochem* 1995;57:599–609. [PubMed: 7615644]
24. Clark JD, Lin L, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N, Knopf JL. A novel arachidonic acid-selective cytosolic phospholipase A₂ contains a Ca²⁺-dependent translocation domain with homology to PKC and GAP. *Cell* 1991;65:1043–1051. [PubMed: 1904318]
25. Keeting PE, Li CH, Murty M, Xu J, Cissel DS, Whipkey DL, Graeber GM, Blaha JD. Arachidonic acid metabolism by adult human osteoblast-like cells exhibits sexually dimorphic characteristics. *J Cell Biochem* 1998;71:74–81. [PubMed: 9736456]
26. Feyen JHM, Raisz LG. Prostaglandin production from sham-operated and ovariectomized rats: effects of 17 β -estradiol *in vivo*. *Endocrinology* 1987;121:819–821. [PubMed: 3474144]
27. Pilbeam CC, Klein-Nulend J, Raisz LG. Inhibition by 17 β -estradiol of PTH-stimulated resorption and prostaglandin production in cultured neonatal mouse calvariae. *Biochem Biophys Res Commun* 1989;163:1319–1324. [PubMed: 2783137]
28. Lane N, Coble T, Kimmel DB. Effects of naproxen on cancellous bone in ovariectomized rats. *J Bone Miner Res* 1990;5:1029–1035. [PubMed: 2080715]
29. Raisz LG. Physiologic and pathologic roles of prostaglandins and other eicosanoids in bone metabolism. *J Nutr* 1995;125:2024S–2027S. [PubMed: 7602387]
30. Jee WSS, Ueno K, Deng YP, Woodbury DM. The effects of prostaglandin E₂ in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. *Calcif Tissue Int* 1985;37:148–157. [PubMed: 3924371]

31. Jee WSS, Mori S, Li XJ, Chan S. Prostaglandin E₂ enhances cortical bone mass and activates intracortical bone remodeling in intact and ovariectomized female rats. *Bone* 1990;11:253–266. [PubMed: 2242291]
32. Kimmel DB, Slovik DM, Lane NE. Current and investigational approaches for reversing established osteoporosis. *Rheum Dis Clin North America* 1994;20:735–758.
33. Mori S, Jee WSS, Li XJ. Production of new trabecular bone in osteopenic ovariectomized rats by prostaglandin E₂. *Calcif Tissue Int* 1992;50:80–87. [PubMed: 1739875]
34. Welch RD, Johnston CE, Waldron MJ, Poteet B. Intraosseous infusion of PGE₂ in the caprine tibia. *J Orthop Res* 1993;11:110–121. [PubMed: 8423513]
35. Sibonga JD, Bell NH, Turner RT. Evidence that ibuprofen antagonizes selective actions of estrogen and tamoxifen on rat bone. *J Bone Miner Res* 1998;13:863–870. [PubMed: 9610751]
36. Samuels A, Perry MJ, Tobias JH. High-dose estrogen-induced osteogenesis in the mouse is partially suppressed by indomethacin. *Bone* 1999;25:675–680. [PubMed: 10593412]
37. Kopp E, Ghosh S. Inhibition of NK κ B by sodium salicylate and aspirin. *Science* 1994;265:956–959. [PubMed: 8052854]
38. Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors are activated by indomethacin and other non-steroidal antiinflammatory drugs. *J Biol Chem* 1997;272:3406–3410. [PubMed: 9013583]
39. Paik JH, Ju JH, Lee JY, Boudreau MD, Hwang DH. Two opposing effects of non-steroidal antiinflammatory drugs on the expression of the inducible cyclooxygenase. *J Biol Chem* 2000;275:28173–28179. [PubMed: 10866999]
40. Raisz LG. Physiology and pathophysiology of bone remodeling. *Clin Chem* 1999;45:1353–1358. [PubMed: 10430818]
41. Smith WL, DeWitt DL. Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* 1996;62:167–215. [PubMed: 8781269]
42. Murakami M, Kambe T, Shimbara S, Kudo I. Functional coupling between various phospholipase A₂'s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J Biol Chem* 1999;274:3103–3115. [PubMed: 9915849]
43. Fujishima H, Sanchez Mejia RO, Bingham CO III, Lam BK, Sapirstein A, Bonventre JV, Austen KF, Arm JP. Cytosolic phospholipase A₂ is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone-derived mast cells. *Proc Natl Acad Sci* 1999;96:4803–4807. [PubMed: 10220374]
44. Breton M, Colyard O. Protein kinase C promotes arachidonate mobilization through enhancement of Co-A-independent transacylase activity in platelets. *Biochem J* 1991;280:93–98. [PubMed: 1741761]
45. O'Doherty PJA. 1,25-Dihydroxyvitamin D₃ increases the activity of the intestinal phosphatidylcholine deacylation-reacylation cycle. *Lipids* 1978;14:75–77.
46. Keeting PE, Li CH, Whipkey DL, Thweatt R, Xu JQ, Murty M, Blaha JD, Graeber GM. 1,25-Dihydroxyvitamin D₃ pretreatment limits prostaglandin biosynthesis by cytokine-stimulated adult human osteoblast-like cells. *J Cell Biochem* 1998;68:237–246. [PubMed: 9443079]
47. Jun SS, Chen Z, Pace MC, Shaul PW. Estrogen upregulates cyclooxygenase-1 gene expression in ovine fetal pulmonary artery endothelium. *J Clin Invest* 1998;102:176–183. [PubMed: 9649571]
48. Sato T, Michizu H, Hashizume K, Ito A. Hormonal regulation of PGE₂ and COX-2 production in rabbit uterine cervical fibroblasts. *J Appl Physiol* 2001;90:1227–1231. [PubMed: 11247918]
49. Akarasereenont P, Techatrasak K, Thaworn A, Chotewuttakorn S. The induction of cyclooxygenase-2 by 17 β -estradiol in endothelial cells is mediated through protein kinase C. *Inflamm Res* 2000;49:460–465. [PubMed: 11071120]
50. Turner RT, Evans GL, Wakley GK. Mechanism of action of estrogen on cancellous bone balance in tibiae of ovariectomized growing rats: inhibition of indices of formation and resorption. *J Bone Miner Res* 1993;8:359–365. [PubMed: 8456590]
51. Turner RT, Riggs BL, Spelsberg TC. Skeletal effects of estrogen. *Endocr Rev* 1994;15:275–300. [PubMed: 8076582]
52. Lindsay R. The menopause: sex steroids and osteoporosis. *Clin Obstet Gynecol* 1987;30:847–858. [PubMed: 3319317]

53. Keeting PE, Scott RE, Colvard DS, Han IK, Spelsberg TC, Riggs BL. Lack of a direct effect of estrogen on proliferation and differentiation of normal human osteoblast-like cells. *J Bone Miner Res* 1991;6:827–833. [PubMed: 1785373]
54. Robinson JA, Harris SA, Riggs BL, Spelsberg TC. Estrogen regulation of human osteoblastic cell proliferation and differentiation. *Endocrinology* 1997;138:2919–2927. [PubMed: 9202236]
55. Oursler MJ, Cortese C, Keeting PE, Anderson M, Bonde S, Riggs BL, Spelsberg TC. Modulation of TGF β production in normal human osteoblast-like cells by 17 β -estradiol and parathyroid hormone. *Endocrinology* 1991;129:3313–3320. [PubMed: 1954907]
56. Girasole G, Jilka RL, Passeri G, Howell S, Boder G, Williams DS, Manolagas SC. 17 β -Estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts *in vitro*: a potential mechanism for the antiosteoporotic effect of estrogens. *J Clin Invest* 1992;89:883–891. [PubMed: 1541679]
57. Kassem M, Harris SA, Spelsberg TC, Riggs BL. Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. *J Bone Miner Res* 1996;11:193–199. [PubMed: 8822343]

Figure 1.

PGE₂ biosynthesis was measured in female donor (A) and male donor (B) hOB cell strains by EIA. Specimens were pretreated with 17 β -E₂ or vehicle (0.1% ethanol) and stimulated with cytokines, singly or in combination, as indicated for 24 hours. Data are represented as fold increase in production compared with basal release of PGE₂. Basal PGE₂ biosynthesis by the female strains was 7.5 ± 0.8 ng/ml and 10.2 ± 1.4 ng/ml for the male strains. Data for the specimens stimulated by either TGF β or TNF represent an N of 6 for the female strains and 8 for the males, and data for the samples stimulated by TGF β plus TNF conjointly represent an N of 15 for the females and 17 for the males. * $p \leq 0.01$ vs. ethanol pretreated, TGF β plus TNF stimulated.

Figure 2.

hOB cells were pretreated with vehicle (0.1% ethanol), the anti-estrogen ICI 164,384, 17 β -E₂, or with both ICI 164,384 and 17 β -E₂ for 48 hours. The cells were then stimulated with the combination of TGF β plus TNF for 24 hours, media were harvested, and PGE₂ was assayed by EIA. The PGE₂ production data from 13 cell strains tested were expressed as the fold-increase from basal production, and comparisons using the paired *t*-test are shown in the figure. The inclusion of the anti-estrogen ICI 164,384 blocked the potentiation of PGE₂ biosynthesis by 17 β -E₂. **p* $\leq$ 0.05 vs. vehicle.

Figure 3. hOB cells were pretreated with vehicle or 17 β -E₂ and then stimulated with cytokines as indicated beneath the bars, and PGE₂ biosynthesis was measured by EIA. The 17 β -E₂ pretreatment was without effect on PGE₂ biosynthesis.

Figure 4. hOB cells were pretreated with or without 17 β -E₂, radiolabeled with 1-[¹⁴C]arachidonic acid, and stimulated with or without cytokines, as indicated. Twenty-four-hour cell-conditioned media were collected, extracted, products separated by thin layer chromatography, and the TLC plates used to expose x-ray film. Shown above are the results from a representative experiment (of 13 independent trials) with cells derived from a 61-year-old man. Authentic PGs were co-chromatographed in this system in order to identify the hOB cell products. See also Table 1.

Figure 5. RT-PCR-based experiments tested whether the mRNA levels of COX-1 or COX-2 were sensitive to 17β-E₂ pretreatment. **(A)** A representative experiment using an hOB cell strain from an 80-year-old female. Specimens were pretreated with or without 17β-E₂ for 48 hours, and then stimulated with or without TGFβ plus TNF for 6 hours. **(B)** The left panel represents the relative levels of COX-1 (N = 9) and COX-2 (right) mRNA (N = 15), after correcting for GAPDH levels in the various samples. No significant effects of 17β-E₂ pretreatment were identified, **p* ≤ 0.001 vs. Control.

Figure 6.

COX-2 protein expression was assessed in Western blots using microsomes prepared from hOB cells pretreated and stimulated as indicated. **(A)** The results of an experiment using microsomes prepared from an 84-year-old female shows that COX-2 protein expression was induced by cytokine stimulation. **(B)** Figure represents the summary data of the Western blots performed using 7 different hOB cell strains. The 17β -E₂ pretreatment did not alter COX-2 protein levels in the absence of added cytokines ($p = 0.50$ vs. Control), or when cytokines were added ($p = 0.73$). * $p \leq 0.05$ vs. Control.

Figure 7. RT-PCR based experiments tested whether the mRNA level of cPLA₂-α was sensitive to treatments with 17β-E₂ and cytokines. **(A)** A representative experiment using an hOB cell strain from a 68-year-old male. Specimens were pretreated with or without 17β-E₂ for 48 hours, and then stimulated with or without TGFβ plus TNF for 6 hours. **(B)** The relative level of cPLA₂-α mRNA (N = 12), after correcting for GAPdH levels in the various samples. No significant effects of 17β-E₂ pretreatment were identified, **p* ≤ 0.001 vs. Control.

Figure 8. cPLA₂-α protein expression was assessed in Western blots using microsomes prepared from hOB cells pretreated and stimulated as indicated. **(A)** The results of an experiment using microsomes prepared from a 72-year-old male. **(B)** The summary data of the Western blots performed using 5 different hOB cell strains. The addition of the cytokines without pretreating the cells with 17β-E₂ did not significantly change cPLA₂-α protein levels. The 17β-E₂ pretreatment alone did not alter cPLA₂-α protein levels, but protein was elevated in specimens pretreated with 17β-E₂ and then stimulated with the cytokines. **p* ≤ 0.5 vs. Control.

Table 1

Effects of vehicle or 17 β -estradiol (E₂) pretreatment on hOB cell conversion of 1-[¹⁴C]arachidonic acid into prostaglandins^a

Treatment	PGE ₂ , % ^b	6-keto-PGF _{1α} , %
Control	2.8 $\pm$ 0.3	1.9 $\pm$ 0.2
Control + E ₂	2.4 $\pm$ 0.4	1.5 $\pm$ 0.3
TGF β	10.3 $\pm$ 2.3	2.8 $\pm$ 0.2
TGF β + E ₂	9.1 $\pm$ 1.6	2.6 $\pm$ 0.3
TNF	10.9 $\pm$ 2.3	2.7 $\pm$ 0.4
TNF + E ₂	9.6 $\pm$ 1.5	2.5 $\pm$ 0.4
TGF β + TNF	27.6 $\pm$ 2.4	2.7 $\pm$ 0.4
TGF β + TNF + E ₂	28.6 $\pm$ 2.9	2.7 $\pm$ 0.4

^a hOB cells were incubated for 48 hours with vehicle (0.1% ethanol) or with 17 β -estradiol (E₂), and then stimulated with the cytokines, as indicated, for 24 hours. The conditioned media were collected and individual prostaglandins were separated by TLC.

^b Data are calculated as the % cpm of individual prostaglandin bands vs. the total cpm of the scraped and scintillation-counted TLC lane. Each of the cytokine treatments stimulated product (PGE₂, 6-keto-PGF_{1 α}) formation, irrespective of 17 β -E₂ pretreatment ($p \leq 0.05$)