Transcriptional Induction of Prostaglandin G/H Synthase-2 by Basic Fibroblast Growth Factor

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Abstract

In serum-free mouse osteoblastic MC3T3-E1 cells, basic fibroblastic growth factor (bFGF) induced mRNA and protein for prostaglandin G/H synthase-2 (PGHS-2), the major enzyme in arachidonic acid (AA) conversion to prostaglandins. mRNA accumulation peaked at 1 h with bFGF 1 nM. In cells stably transfected with a 371-bp PGHS-2 promoter-luciferase reporter, bFGF stimulated luciferase activity, which peaked at 2–3 h with bFGF 1–10 nM. In the presence of exogenous AA, bFGF stimulated PGE_2 production, which paralleled luciferase activity. In serum-free neonatal mouse calvarial cultures, bFGF stimulated PGE_2 production in the absence of exogenous AA. bFGF stimulated PGHS-2 mRNA accumulation, which peaked at 2–4 h and then decreased; there were later mRNA elevations at 48 and 96 h that were inhibited by indomethacin. In both MC3T3-E1 cells and neonatal calvariae, bFGF produced smaller and slower increases in PGHS-1 mRNA levels than for PGHS-2. bFGF stimulated bone resorption in mouse calvariae with a maximal increase of 80% at 1 nM. Stimulation was partially inhibited by nonsteroidal anti-inflammatory drugs. We conclude that bFGF rapidly stimulates PGE_2 production in osteoblasts, largely through transcriptional regulation of PGHS-2, and that prostaglandins mediate some of bFGF's effects on bone resorption. (*J. Clin. Invest.* 1995. 96:923–930.) Key words: osteoblasts • bone resorption • prostaglandins • indomethacin • luciferase

Introduction

Basic fibroblast growth factor (bFGF),[1] a member of the family of heparin-binding growth factors, exerts potent mitogenic activity on a variety of cells of mesodermal and ectodermal origin (1–4). Bone cells produce bFGF at 10-fold higher levels than they produce acidic fibroblast growth factor (FGF) another heparin-binding factor that exhibits ~ 55% sequence identity to bFGF (5, 6). Recently, we found that bFGF mRNA and protein are expressed in mouse osteoblastic MC3T3-E1 cells (7). bFGF is stored in the extracellular matrix in association with heparin sulfate and is activated by proteases or heparin-like molecules (8–10). bFGF could act as an autocrine/paracrine factor for bone cells. bFGF modulates bone formation through the regulation of proliferation and differentiation of cells of osteoblastic lineage in vitro (4). We have also reported that bFGF stimulates bone resorption in cultured fetal rat long bones (11).

Among prostaglandins (PGs), PGE_2 is the major product of osteoblasts (12, 13) and is a potent bone resorber in vitro (12, 14, 15). PGE_2 also has mitogenic effects on bone cells (16, 17). Both exogenous bFGF (18–20) and PGE_2 (21–24) have been shown to stimulate new bone formation in vivo. In some cell lines, including fibroblasts and synovial cells, bFGF has been shown to stimulate PG production (25–27). Therefore, it is possible that some of the effects of bFGF on bone metabolism might be mediated by PG synthesis.

The major enzyme regulating the conversion of arachidonic acid (AA) to PGs is prostaglandin G/H synthase (PGHS), also called cyclooxygenase, which oxidizes AA to PGG_2 and reduces PGE_2 to PGH_2. Two forms of PGHS, constitutive PGHS (PGHS-1) and inducible PGHS (PGHS-2), have been identified. PGHS-1 mRNA has been identified in many ovine, murine, and human tissues (28), while the PGHS-2 mRNA was first found as an immediate-early gene from murine fibroblasts and chick embryo fibroblasts (29–31). These two enzymes have 60% homology in nucleic acid and amino acid sequence, although they are products of separate genes (28). Both are glycoproteins whose molecular weight is 70–74 kD. PGHS-1 is constitutively expressed, while the recently identified PGHS-2 is likely to be more important in regulating prostaglandin production by extracellular ligands. Both PGHS-1 and PGHS-2 are expressed in osteoblastic cells. PGHS-2 is the main enzyme regulating the production of PGE_2 in response to hormones and cytokines in MC3T3-E1 cell cultures and neonatal mouse calvarial cultures (32–34).

The present study was undertaken to examine the effects of bFGF on PG production and the role of PGs in the response to bFGF in bone. Mouse osteoblastic MC3T3-E1 cells were used to investigate the cellular mechanisms by which bFGF regulates PG synthesis, and neonatal mouse calvarial cultures were used to examine the involvement of PGs in bFGF-stimulated bone resorption.

Methods

Materials. The PGHS-2 promoter-luciferase fusion genes containing either 963 or 371 bp of PGHS-2 5′-flanking sequence in pXp-2 vector

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1. *Abbreviations used in this paper:* APC, aphidicolin; bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSAID, nonsteroidal anti-inflammatory drug; PGHS, prostaglandin G/H synthase.

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(P2-Luc963 or P2-Luc371, respectively) have been described previously (35). Human recombinant bFGF was provided by California Biotechnology (Mountain View, CA). Murine PGHS-1 cDNA was the gift of Drs. David DeWitt and William Smith (Michigan State University, East Lansing, MI). Murine PGHS-2 cDNA was obtained from Oxford Biomedical Research Inc. (Oxford, MI). Polyclonal rabbit anti-murine PGHS-2 antiserum was obtained from Cayman Chemical Co. (Ann Arbor, MI). Murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified by PCR using a mouse GAPDH control amplimer set from Clontech (Palo Alto, CA). PGE_2 antibody was purchased from Dr. Lawrence Levine (Waltham, MA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Stable DNA transfection. The PGHS-2 promoter-luciferase fusion constructs contain either 963 or 371 bp of the 5′ flanking sequence immediately proximal to the transcription start site and 70 bp of downstream untranslated DNA (35). Promoterless luciferase vector was made by cutting P2-Luc371 with BamHI and BglII, followed by ligation with T4 ligase (GIBCO BRL, Gaithersburg, MD).

PGHS-2 promoter-reporter constructs were purified by CsCl banding and cotransfected with pSV_2-neo into cultured MC3T3-E1 cells using Lipofectamine (GIBCO BRL). For transfection, MC3T3-E1 cells were plated in 6 well dishes (Costar Corp., Cambridge, MA) at 1×10^5 cells/well and grown to 50–80% confluency (18–24 h) in DME without phenol red (Sigma) containing 10% heat-inactivated FCS (GIBCO BRL), penicillin (100 U/ml), and streptomycin (50 μg/ml). Cells were rinsed twice with serum-free medium and incubated with 1 μg of promoter-reporter DNA, 0.067 μg of pSV_2-neo DNA, and 8 μl of Lipofectamine reagent in 1 ml of serum-free medium without antibiotics. After 5 h of incubation, a second ml of medium with 20% FCS was added; 19 h later the medium was replaced with fresh complete medium. After 48 h, cells were split 1:10 into 100-mm dishes and placed under selection with 400 μg/ml of G418 for 2 w. Stable colonies (> 200) were pooled to randomize effects of variation in integration site. Cells were grown in culture medium containing 200 μg/ml G418.

Cell culture. MC3T3-E1 cells were plated in 6-well dishes (Costar Corp.) at a density of 50,000 cells/cm^2 unless stated otherwise and grown for 6–7 d in DME containing 10% heat-inactivated FCS and antibiotics. They were precultured for another 24 h in serum-free DME with antibiotics, 1 mg/ml BSA (RIA grade; Sigma Chemical Co.), and 100 μg/ml phosphoascorbic acid (L-ascorbic acid phosphate magnesium salt; Wako Pure Chemical Industries, Osaka, Japan) before treatment. Cycloheximide was added 45 min before addition of bFGF. Control cultures were treated with appropriate vehicles. For test materials dissolved in ethanol, the final concentration of ethanol was 0.1%.

PGHS-2 promoter activity. Luciferase activity was measured in soluble cell extracts prepared with a luciferase detection kit (Promega, Madison, WI) using an automatic injection luminometer (Analytical Luminescence Lab. Inc., San Diego, CA). Activity was normalized to total proteins measured with a BCA protein assay kit (Pierce, Rockford, IL). For each experiment, six wells were analyzed per treatment group, and data were expressed as means±SEM.

Neonatal mouse calvarial culture. Parietal bones were excised from 7-d-old CD-1 mice (Charles River Laboratories, Wilmington, MA). Bones were precultured for 24 h in a chemically defined medium, BGJb (1 mM proline, 3 mM phosphate, 0.4 mM L-glutamine, 100 μg/ml L-ascorbic acid phosphate, and 1 mg/ml BSA) (GIBCO BRL). They were cultured in the experimental medium in 24 well dishes (Costar Corp.) on a rocking platform.

Bone resorption assay. Bone resorption was measured as the release of previously incorporated ^{45}Ca from neonatal mouse calvariae. Timed pregnant mice were injected with 0.05 mCi ^{45}Ca on the 16th day of gestation, and parietal bones were excised from 7-d-old neonatal mice. Bones were precultured in BGJb medium for 16–24 h and then cultured with experimental agents for 96 h, with a medium change after 48 h. Indomethacin or aphidicolin was added 2 h before addition of bFGF. ^{45}Ca in the medium and in TCA extracts of bone was determined by liquid scintillation counting. ^{45}Ca release in the medium was expressed as the percentage of total counts in the medium and bone extracts. Data from multiple experiments were pooled after individual values were normalized to the mean control for each experiment.

Steady state mRNA analysis. Three wells of cells in 6-well dishes or 6–8 half calvariae were pooled for RNA extraction using the method of Chomczynski and Sacchi (36). Briefly, cells or calvariae were homogenized in 4 M guanidinium thiocyanate, extracted with phenol/chloroform-isoamyl alcohol (24:1), and RNA precipitated with isopropanol and washed with 80% ethanol. After quantitation at 260 nm, 20 μg of total RNA was run on a 1% agarose–2.2 M formaldehyde gel and transferred to a nylon membrane (Genescreen; New England Nuclear Research, Boston, MA) by positive pressure and fixed to the membrane by ultraviolet irradiation. After 3 h of prehybridization in a 50% formamide solution at 42°C, filters were hybridized overnight in a similar solution in rotating cylinders at 42°C with a random primer [^{32}P]dCTP (New England Nuclear Research)–labeled cDNA probe for PGHS-1, PGHS-2, or GAPDH. Filters were washed in a 1 × SSC, 1% SDS solution, 0.1 × SSC, at room temperature. Then the filters were washed three times at 65°C with 0.1% SDS. The filters were exposed to XAR-5 film (Eastman Kodak, Rochester, NY) at − 70°C. Signals were quantitated by densitometry (Bio-Rad Laboratories, Richmond, CA), and optical densities for PGHS-1 and PGHS-2 were normalized to the corresponding values for GAPDH.

Western blot analysis. MC3T3-E1 cells were plated in 100-mm dishes (Costar Corp.) at a density of 5,000 cells/cm^2 and grown to confluence in DME containing 10% serum. Cells were serum deprived for 24 h and treated for 2, 6, and 24 h with or without bFGF (10 nM). Cells were then washed with PBS, harvested by centrifugation, and extracted with 0.5% Tween 20 in a 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM phenylmethyl-sulfonyl fluoride, 1 mM EDTA, and 1 mM *N*-ethylmaleimide at 4°C for 30 min. This mixture was centrifuged at 14,000 *g* for 30 min. The supernatant was dialyzed against *N*-ethylmaleimide without Tween 20 for 16 h, and an aliquot was mixed with DEAE cellulose (200-μl bed volume/mg protein) preequilibrated with the potassium phosphate buffer containing 0.05% Tween 20. DEAE cellulose was precipitated by centrifugation, and protein in the supernatant was measured by the BCA protein assay kit (Pierce). 25 μg of protein per treatment group was run on an SDS–polyacrylamide gel (10%) and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with 1% nonfat dry milk at 4°C for 16 h to block nonspecific binding and then treated with a 1:2,000 dilution of polyclonal rabbit anti–PGHS-2 antiserum or nonimmune rabbit serum. Immunoreactive bands were stained using a Western exposure chemiluminescent detection kit (Clontech) according to the manufacturer's instructions.

Immunocytochemistry. MC3T3-E1 cells were plated on glass coverslips at a density of 5,000 cells/cm^2 and grown in DME with 10% FCS. When at least 80% confluent, cells were serum deprived for 24 h and then cultured for 6 or 24 h with or without bFGF (10 nM). Cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. Cells were rinsed twice for 10 min with PBS. Cells were permeabilized with 0.1% Triton X-100 for 20 min on ice (37). After a 20-min incubation with 0.1% gelatin in PBS, a 1:40 dilution of the rabbit anti–murine PGHS-2 antiserum or rabbit nonimmune serum was added to cells for 2 h at room temperature. A 1:200 dilution of rhodamine-conjugated anti–rabbit IgG was added to the cells for 1 h at room temperature. To prevent quenching of the fluorescence, 2.5% n-propyl gallate in 1:1 PBS:glycerol was added. The cells were photographed with a fluorescence microscope (Optiphot; Nikon Inc., Melville, NY).

PGE_2 assays. Medium was removed from cultured cells or bones, and PGE_2 accumulation was measured by radioimmunoassay as described previously (38). In some instances indicated in the text, AA (10 μM) was added to MC3T3-E1 cell culture to provide substrate for PGE_2 production.

Statistical analysis. Means of groups were compared using ANOVA, and significance of differences was determined by post-hoc testing using Bonferroni's method.

Results

PGHS-2 mRNA was undetectable in unstimulated MC3T3-E1 cells, but was rapidly induced by bFGF (10 nM). mRNA levels

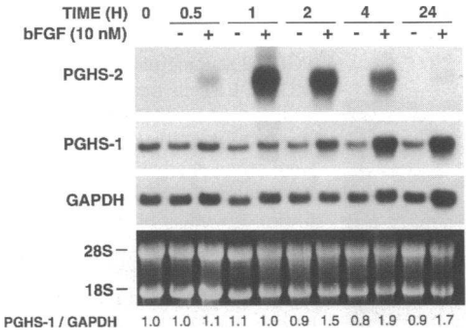


Figure 1. Time course of the effect of bFGF on steady state mRNA levels of PGHS-2 and PGHS-1 in MC3T3-E1 cells. MC3T3-E1 cells were serum deprived for 24 h and cultured with or without bFGF (10 nM) for the time indicated. Total RNA was extracted and 20 μg of RNA was fractionated on a 1% agarose gel, transferred to nylon filters, and hybridized to PGHS-2, PGHS-2, and GAPDH. The numbers on the bottom are the treated/control ratios of the intensity of PGHS-1 normalized to that of GAPDH measured by densitometry.

were maximal at 1 h, remained elevated for at least 4 h, and then decreased over the next 24 h (Fig. 1). Fig. 2 shows the dose response of the effect of bFGF on PGHS-2 mRNA at 1 h of treatment in cells initially plated at different densities (5,000 and 50,000 cells/cm^2). PGHS-2 mRNA was induced with 10^{-10} M bFGF, and accumulation was maximal at 10^{-9} M. These effects were independent of the plating density. PGHS-1 mRNA was expressed constitutively and was not altered by 1 h of treatment with bFGF (Figs. 1 and 2). Stimulation of PGHS-1 by bFGF only appeared after 2 h and was less than twofold when normalized to GAPDH mRNA. Cycloheximide (3 μg/ml), a protein synthesis inhibitor, induced PGHS-2 mRNA and potentiated the effect of bFGF (Fig. 3), indicating that new protein synthesis is not essential for the stimulatory effect of bFGF.

PGHS-2 protein levels in MC3T3-E1 cells were measured by Western blotting (Fig. 4). Immunoreactive protein was induced by bFGF (10 nM) at 2 h and was still present at 24 h. Multiple bands were present at 24 h, perhaps due to deglycosylation during culture (39). The localization of PGHS-2 protein was examined by immunocytochemistry at 6 and 24 h of culture (Fig. 5). Immunofluorescence microscopy demonstrated an increase in cytoplasmic PGHS-2 levels at 6 h with bFGF (10 nM). Staining was still present at 24 h. The nuclear staining

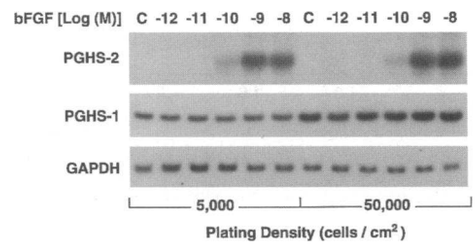


Figure 2. Dose response of the effect of bFGF on steady state mRNA levels of PGHS-2 and PGHS-1 in MC3T3-E1 cells at 1 h of culture. Cells were plated at 5,000 or 50,000 cells/cm^2 and cultured for 6 d. Cells were serum deprived for 24 h and cultured for 1 h with various concentrations of bFGF. 20 μg of RNA was hybridized to PGHS-2, PGHS-1, and GAPDH.

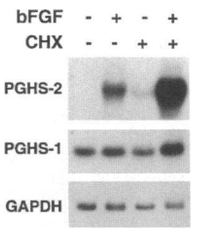


Figure 3. Effect of cycloheximide (CHX) on steady state mRNA levels of PGHS-2 and PGHS-1 in the presence and absence of bFGF in MC3T3-E1 cells. Cells were precultured for 24 h and cultured for 1 h with bFGF (10 nM) in the presence and absence of cycloheximide (3 μg/ml). Cycloheximide was pulsed into the culture 45 min before bFGF treatment. 20 μg of RNA was hybridized to PGHS-2, PGHS-1, and GAPDH.

seen in control cultures was also seen with preimmune serum (data not shown), indicating that this was nonspecific.

Medium PGE_2 was not detectable (< 0.1 nM) in serum-free cell cultures, despite the constitutive expression of PGHS-1 mRNA and the induction of PGHS-2 by bFGF. However, if cells were given exogenous AA (10 μM), the substrate for both PGHS-1 and PGHS-2, during the last 10 min of culture, PGE_2 production was increased by bFGF (10 nM) treatment as early as 2 h (Table I).

To examine the transcriptional regulation of PGHS-2 by bFGF, PGHS-2 promoter–luciferase fusion genes containing 963 or 371 bp of 5′ flanking sequence (P2-Luc 963 or P2-Luc 371, respectively) were stably transfected into MC3T3-E1 cells. Basic FGF (10 nM) stimulated luciferase activity in P2-Luc 963 and P2-Luc 371 to a similar extent at 3 h (treated/control ratios for luciferase activity were 4.7±0.3 and 4.9±0.1, respectively), suggesting that a bFGF response element is likely to reside within the proximal 371 bp. Fig. 6 shows the time course of the effect of bFGF on luciferase activity in cells transfected with P2-Luc 371. Significant stimulation was observed at 1 h, reached a maximum at 2–3 h, and decreased to the control level at 24 h.

Luciferase activity of P2–Luc 371 transfected cells and promoterless-Luc transfected cells were measured with or without bFGF at 3 h of culture at different plating densities of 5,000 and 50,000 cells/cm^2 (Fig. 7). Cells were cultured continuously in the presence of arachidonic acid (10 μM), and medium PGE_2 levels were also measured at 3 h. There were similar dose-dependent responses for both luciferase activity and PGE_2 lev-

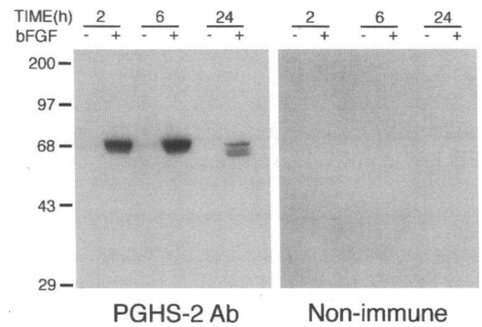


Figure 4. Time course of the effect of bFGF on immunoreactive PGHS-2 protein in MC3T3-E1 cells. Cells were serum deprived for 24 h and then treated for 2, 6, and 24 h with or without bFGF (10 nM). Proteins were extracted as shown in Methods, and 25 μg of protein was loaded on a 10% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with 1% nonfat dry milk to block nonspecific binding and then with polyclonal rabbit anti–murine PGHS-2 antiserum or nonimmune rabbit serum (both 1:2,000 dilutions). Immunoreactive bands were stained using alkaline phosphatase immunostaining and detected by chemiluminescence.

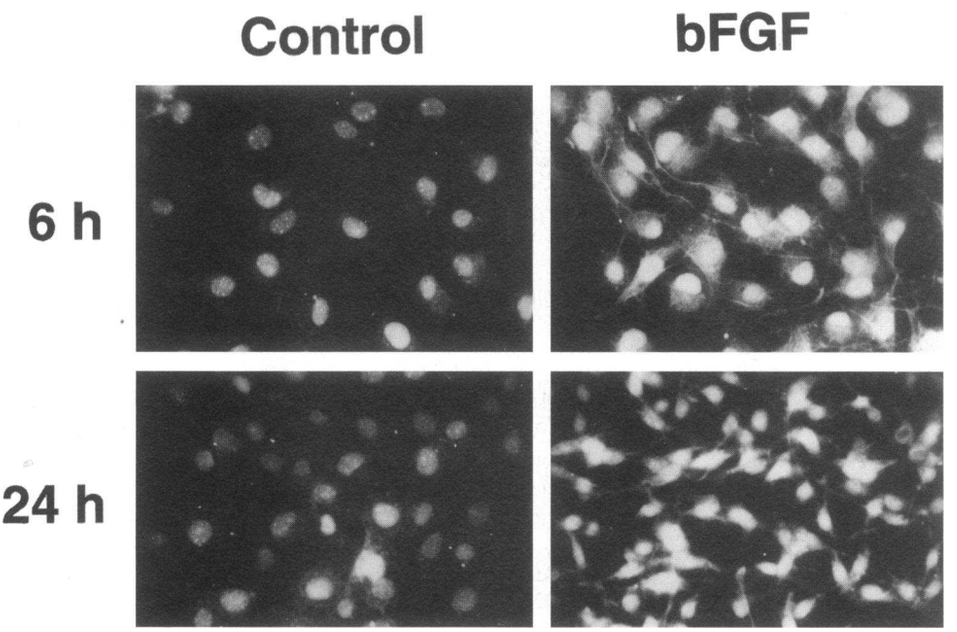


Figure 5. Immunofluorescence microscopy of PGHS-2 staining in MC3T3-E1 cells treated with or without bFGF for 6 or 24 h. MC3T3-E1 cells were serum-deprived for 24 h, cultured for 6 or 24 h with or without bFGF (10 nM), and then fixed, permeabilized, and incubated with polyclonal rabbit anti-murine PGHS-2 antiserum or nonimmune rabbit serum and then treated with rhodamine-conjugated anti-rabbit IgG. An increase in PGHS-2 staining is apparent in the cytoplasm of the cells. The nuclear staining which is seen in control cultures was also observed in nonimmune cells × 200 (data not shown).

els. There was an increase in PGE_2 production with bFGF (10 nM) of 10-fold or more and an increase in PGHS-2 promoter activity of sevenfold or more. The similarities in these responses, along with the twofold or less increase in PGHS-1 mRNA levels at 3 h (Fig. 1), suggest that the stimulation of PGE_2 production was largely dependent on transcriptional regulation of PGHS-2. Plating density did not influence bFGF's effect on either PGE_2 production or PGHS-2 promoter activity. Cells transfected with the promoterless luciferase construct showed minimal luciferase activity, but produced PGE_2 levels similar to the P2–Luc 371 transfected cells.

The neonatal mouse calvarial culture system can be used to examine and compare physiological responses of bone cells (such as cellular proliferation), bone formation (measured by collagen synthesis), and bone resorption (measured by ^{45}Ca release from prelabeled calvariae). Because bFGF and PGE_2 have similar effects on some responses, we used the calvarial culture system to determine if PGE_2 mediated these responses to bFGF. 7-d-old neonatal mouse calvariae were cultured in serum-free media. PGHS-2 mRNA was not detectable in freshly dissected calvariae but was induced in control cultures during the first 24 h in culture, as previously reported (33). The spontaneously induced PGHS-2 mRNA levels in control cultures decreased after 24 h of culture, eventually becoming undetectable. Hence, the calvariae were given a 24 h preculture in control media to allow the spontaneous induction of PGHS-2 mRNA to decrease so as not to obscure induction by bFGF. After preculture, calvariae were placed in culture with or without bFGF and cultured for another 96 h, with a medium change after 48 h (Figs. 8 and 9). At the beginning of treatment, control cultures still had elevated levels of PGHS-2 mRNA (Figs. 8 *A* and 9). bFGF stimulated PGHS-2 mRNA levels with a biphasic time course. Levels peaked at 2–4 h, decreased to undetectable at 24 h, and then increased again at 48 h. After a change to fresh

Table I. PGE_2 Production by Serum-free MC3T3-E1 Cells Treated for 2 h with bFGF (10 nM) and for the Last 10 min of Culture with Arachidonic Acid (AA; 10 μM) or Vehicle (Ethanol)

Treatment	PGE_2 (nM)
Control	< 0.1
bFGF	< 0.1
Control + AA	6.2±3.6
bFGF + AA	16.0±3.6*

Values are means±SEM; $n = 3$. * Significantly different from control + AA; $P < 0.05$.

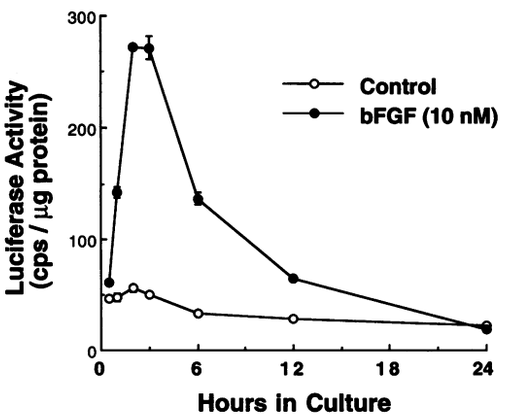


Figure 6. Time course of the effect of bFGF on PGHS-2 promoter activity of MC3T3-E1 cells stably transfected with the 371-bp PGHS-2 promoter-luciferase fusion construct (P2-Luc 371). Cells were stably transfected with a PGHS-2 promoter-luciferase fusion construct containing 371 bp of the 5′ flanking sequence and 70 bp of downstream untranslated DNA, and selected with G418 as described in Methods. Transfected cells were serum deprived for 24 h and treated with or without bFGF (10 nM) for the time indicated. Assay for luciferase activity was done on soluble cell extracts and normalized to total proteins. Symbols indicate means and the vertical bar; SEM for 3–6 wells.

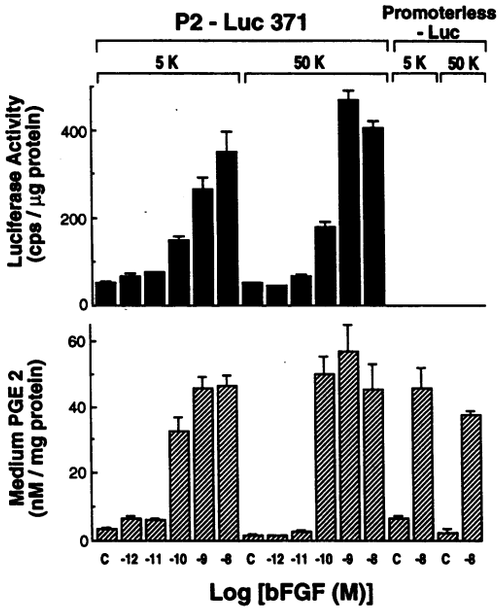


Figure 7. Dose response of the effect of bFGF at 3 h on PGHS-2 promoter activity and medium PGE_2 levels in MC3T3-E1 cell culture stably transfected with P2-Luc 371 or a promoterless luciferase. Cells were plated at 5,000 or 50,000 cells/cm^2 and cultured for 6 d. Cells were serum deprived for 24 h and cultured for 3 h with various concentrations of bFGF. The assay for luciferase activity was performed on soluble cell extracts and normalized to total proteins. Medium PGE_2 was measured by radioimmunoassay in the presence of AA (10 μM).

media with bFGF, PGHS-2 mRNA levels were again elevated at 96 h. PGHS-1 mRNA levels were also stimulated by bFGF but only after 4 h, and this increase was maintained. In contrast with MC3T3-E1 cell cultures, measurable PGE_2 accumulated in the medium without the addition of arachidonic acid. A dose–response study showed that at 4 h, the induction of PGHS-2 and PGHS-2 mRNA and PGE_2 production were maximum at bFGF 1 nM (Fig. 8 *B*).

In addition to the 4.2-kb PGHS-2 band, a smaller (2.5 kb)

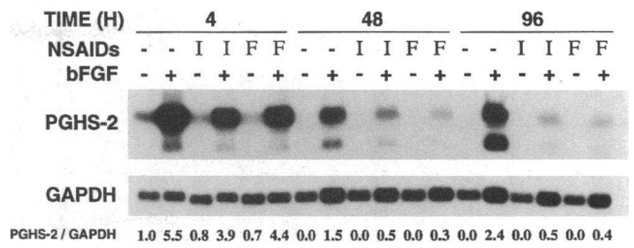
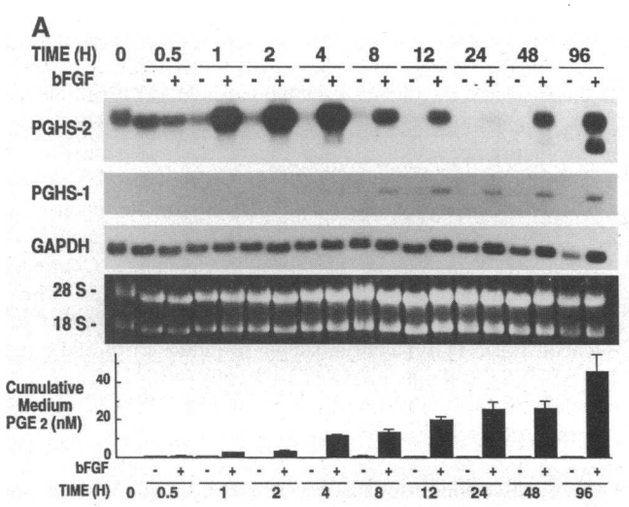


Figure 9. Effects of two structurally unrelated NSAIDs, indomethacin and flurbiprofen, on bFGF-stimulated PGHS-2 mRNA levels in cultured neonatal mouse calvariae at 4, 48, and 96 h. Calvariae were precultured for 24 h and cultured with or without bFGF (1 nM) for the time indicated. 1 μM indomethacin (I) and 1 μM flurbiprofen (F) were added 2 h before adding bFGF. Total RNA was extracted, and 20 μg of RNA was fractionated on a 1% agarose gel, transferred to nylon filters, and hybridized to PGHS-2 and GAPDH.

mRNA was present in RNA extracted from calvariae treated for 48 h with bFGF. This band increased at 96 h. The smaller band probably reflects a different site of polyadenylation of PGHS-2 mRNA as reported previously for murine PGHS-2 (40).

Because we have previously found that endogenous prostaglandins can stimulate PGHS-2 mRNA in both MC3T3-E1 cells (32, 41) and mouse calvarial cultures (33), we examined the effects of two structurally unrelated nonsteroid anti-inflammatory drugs (NSAIDs) indomethacin and flubiprofen (both at 1 μM) on the induction of PGHS-2 in mouse calvariae cultures by bFGF (Fig. 9). At 4 h the induction was only slightly inhibited. At 48 and 96 h, both NSAIDs inhibited the bFGF induction of PGHS-2 mRNA accumulation by about 80%, suggesting that the recurrence of PGHS-2 induction at later time points was probably due to autoamplification by the production of prostaglandins.

Both bFGF and PGE_2 can have potent effects on osteoblastic proliferation and synthesis of collagen. We have previously reported that bFGF inhibited collagen synthesis in MC3T3-E1 cells but that this inhibition was not PG dependent (42). In the present study, we examined the effect of bFGF (10 nM) on



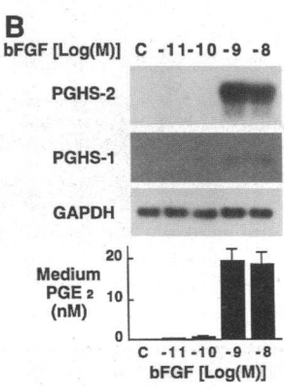


Figure 8. (*A*) Time course of the effect of bFGF on steady state mRNA levels of PGHS-2 and PGHS-1 in cultured neonatal mouse calvariae. 7-d-old neonatal mouse calvariae were precultured for 24 h and cultured with or without bFGF (1 nM) for the time indicated. Time 0 is time of bFGF addition. Total RNA was extracted and 20 μg of RNA was fractionated on a 1% agarose gel, transferred to nylon filters, and hybridized to the probes for PGHS-2, PGHS-1, and GAPDH. (*B*) Dose response of the effect of bFGF on steady state mRNA levels of PGHS-2 and PGHS-1 in cultured neonatal mouse calvariae at 4 h of culture. Calvariae were precultured for 24 h and then treated for 4 h with various concentrations of bFGF. 20 μg of RNA was hybridized to PGHS-2, PGHS-1, and GAPDH.

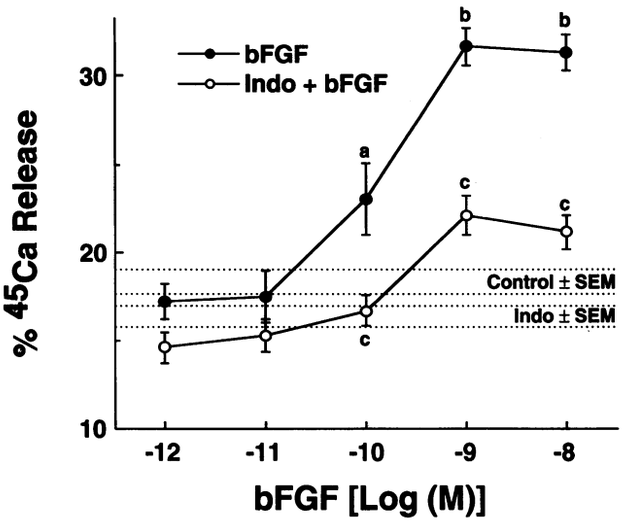


Figure 10. Dose response of the effect of bFGF on ^{45}Ca release from prelabeled neonatal mouse calvariae in the presence and absence of indomethacin at 96 h of culture. ^{45}Ca prelabeled calvariae were precultured for 16–24 h and then cultured with experimental agents for 96 h with a medium change after 48 h. Treatment with indomethacin (1 μM) was begun 2 h before the addition of bFGF (1 nM). ^{45}Ca in medium and TCA extracts of bone was determined by liquid scintillation counting and the cumulative percentage of ^{45}Ca release was calculated. Data from multiple experiments were pooled after calculating individual values as a percentage of the mean control for each experiment. Similar results were seen at 48 h of culture, although bFGF stimulation was less than that at 96 h. *a*, significant effect of bFGF: $P < 0.05$. *b*, $P < 0.01$. *c*, significant effect of indomethacin: $P < 0.01$.

[^{3}H]thymidine (TdR) incorporation into MC3T3-E1 cells and cultured mouse calvariae in the presence and absence of indomethacin (1 μM). bFGF stimulated [^{3}H]TdR incorporation in both cell and organ culture (4.6±0.5-fold and 3.8±0.2-fold, respectively) at 24 h, and this stimulation was not inhibited by indomethacin in either system (4.7±0.5-fold and 3.8±0.2-fold, respectively).

Both bFGF and PGE_2 are potent stimulators of bone resorption. To examine the possible role of PGE_2 in bFGF-stimulated resorption, we measured ^{45}Ca release from prelabeled neonatal mouse calvariae at 48 and 96 h of culture. Medium PGE_2 accumulation was also measured at these time points, in the absence of exogenous arachidonic acid. bFGF increased the release of ^{45}Ca dose dependently, with a maximal increase of 80% at 1 nM after 96 h of culture (Fig. 10 and Table II). Indomethacin (1 μM) blocked PGE_2 production, abrogated the effect of bFGF 0.1 nM, and decreased the effects of higher concentrations of bFGF, suggesting that bFGF effects on bone resorption are partially dependent on PG production. Similar results were seen at 48 h of culture (data not shown).

To examine the role of the mitogenic effect of bFGF in bone resorption, we added 30 μM aphidicolin (APC), an inhibitor of DNA synthesis, to the neonatal mouse calvarial cultures. At 48 and 96 h, APC decreased [^{3}H]TdR incorporation into calvariae by 92–96% (data not shown). bFGF still increased bone resorption in the presence of APC (Table II). However, APC alone increased PGE_2 production and bone resorption; both effects were blocked by indomethacin (Table II). Therefore, we added both APC and indomethacin and found that the combination abrogated the effects of bFGF on bone resorption.

Table II. Effects of bFGF (10^{-9} M) on ^{45}Ca Release from Prelabeled Neonatal Mouse Calvariae and Medium PGE_2 Level in the Presence and Absence of Indomethacin (Indo: 10^{-6} M) and/or Aphidicolin (APC; 3×10^{-5} M)

Treatment	*n*	^{45}Ca release (% of control) 48 h	96 h	Medium PGE_2 (nM)
Control	32	100±4	100±4	0.7±0.1
bFGF	32	155±5 (55%)*§	180±5 (80%)*§	36.6±6.7*§
Indo	21	85±4‡	92±4	N.D.
Indo + bFGF	20	110±6 (29%)§	124±6 (36%)*§	N.D.
APC	24	131±5*	142±7*	7.3±1.6*
APC + bFGF	24	146±4 (12%)*	187±5 (32%)*§	143.0±23.3*§
Indo + APC	19	95±7	102±6	N.D.
Indo + APC + bFGF	19	105±4 (11%)	105±4 (3%)	N.D.

Values are the mean±SEM for *n* bone cultures. Data for ^{45}Ca release were normalized to 100 for those of the control. The mean control values of the percentage of ^{45}Ca release were 11.9±0.4% for 48 h and 17.8±0.7% for 96 h of culture. The data in parentheses indicate the percentage of stimulation of ^{45}Ca release produced by bFGF. Medium PGE_2 was measured at 2 d of culture. * Significant difference from control; $P < 0.01$. ‡ $P < 0.05$. § Significant effect of bFGF; $P < 0.01$. N.D., not detectable (< 0.1 nM).

Discussion

This study has shown that bFGF is a potent stimulator of PGHS-2 mRNA and protein accumulation in osteoblastic MC3T3-E1 cells. In these cells, PGHS-2 mRNA was not detectable under control conditions. In contrast, PGHS-1 mRNA was constitutively expressed. Basic FGF transiently induced PGHS-2 mRNA in MC3T3-E1 cells, with levels peaking at 1 h and undetectable at 24 h. This induction was not blocked by cycloheximide. The induction of PGHS-2 mRNA was accompanied by induction of PGHS-2 protein, which was still detectable at 24 h. In MC3T3-E1 cells stably transfected with a 371-bp PGHS-2 promoter-luciferase reporter construct, bFGF also rapidly and transiently stimulated luciferase activity. We conclude that bFGF regulates PGHS-2 expression in large part through a transcriptional mechanism. Despite the induced expression of PGHS-2, PGE_2 production was not measurable (< 0.1 nM) up to 24 h in these cultures unless arachidonic acid was given as substrate, suggesting that substrate release may be a limiting factor for PG production in these cells under these conditions. In the presence of exogenous arachidonic acid, bFGF-stimulated PGE_2 production paralleled the stimulation of PGHS-2 promoter-luciferase activity, indicating a major dependence on transcriptional regulation of PGHS-2.

The 5′ flanking region of the PGHS-2 promoter containing the 371-bp proximal to the transcription start site appears to contain one or more major bFGF response elements. Computer search of this region has identified an AP-1–like site, which may bind the Fos-Jun dimer and two NF-IL6 consensus sequences. Basic FGF has been shown to induce c-fos and c-jun and NF-IL6 transcripts in MC3T3-E1 cells (43, 44). However, maintaining the bFGF induction of pPGHS-2 mRNA in the presence of cycloheximide suggests that the major effect of bFGF is to directly activate one or more transcription factors. Other putative consensus sequences identified within this region

include an SP-1 site, an ATF/CRE region, and an E-box or helix-loop-helix protein-binding element. The ATF/CRE site has been recently shown to mediate *v-src* induction of the murine PGHS-2 promoter (45).

Basic FGF also rapidly stimulated PGHS-2 mRNA levels in neonatal mouse calvarial cultures. Basic FGF increased medium PGE_2 levels in these cultures, which were measurable in the absence of exogenous substrate, and stimulation of PGE_2 production paralleled the stimulation of PGHS-2 mRNA expression. The mitogenic effects of bFGF in MC3T3-E1 cells and the inhibitory effects of bFGF on collagen synthesis in MC3T3-E1 cells (42) have been shown to be independent of PG production. However, in the present study, the inhibition of PG production by NSAIDs reduced bFGF-stimulated resorption in neonatal mouse calvariae, indicating that the resorptive effect of bFGF was mediated in part by PGs. Although an inhibitor of DNA synthesis alone did not block PG-mediated resorption, inhibition of both DNA synthesis and PG production completely blocked bFGF-stimulated resorption. Hence, we propose that bFGF has a direct effect on resorption through stimulation of proliferation of osteoclast precursors and an indirect effect mediated by PGs. We have shown by histological examination that bFGF increases the number of osteoclast-like cells in cultures of 21-d fetal rat calvariae (46). In a previous study in cultured fetal rat long bones, we found that bFGF-stimulated bone resorption was independent of PGs (11). Many stimulators of resorption show different degrees of dependence on endogenous prostaglandin production in different model systems, and even in the same model systems. In general, endogenous prostaglandin production is more likely to play a role in resorptive responses in neonatal mouse calvariae than in fetal rat long bones. Similar observations have been made for thyroid hormone (47) and interleukin 1 (48, 49). These differences may reflect the fact that the two culture systems contain a different population of osteoclast precursor cells or differences in other cell populations, particularly since the neonatal mouse calvariae contain some hematopoetic marrow, while fetal rat long bones do not.

Although the induction of PGHS-2 mRNA by bFGF in calvarial cultures had decayed by 24 h, there was a rebound induction at 48 and 96 h. This rebound could be inhibited by treatment with NSAIDs and hence was probably due largely to autoamplification by endogenous PGs. Autoamplification, perhaps in conjunction with the smaller, slower, but more sustained increases in PGHS-1 expression, may play an important role in maintaining physiologic responses mediated by PGs, such as bFGF-stimulated resorption, which are initiated by transient inductions of PGHS-2 expression.

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