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Fluid flow induces COX-2 expression in MC3T3-E1 osteoblasts via a PKA signaling pathway☆

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Abstract

Mechanical loading of bone generates fluid flow within the mineralized matrix which can exert fluid shear stress (FSS) at cell membranes. FSS induces new transcription of cyclooxygenase-2 (COX-2) in MC3T3-E1 osteoblasts, with peak effects at 4–5 h. Using MC3T3-E1 cells stably transfected with the COX-2 promoter fused to a luciferase reporter, we examined involvement of the protein kinase A (PKA) and protein kinase C (PKC) signaling pathways in the peak COX-2 mRNA and luciferase responses to FSS (10 dyn/cm^2). Neither inhibition nor down-regulation of the PKC pathway affected the FSS stimulation of COX-2 mRNA or luciferase activity. In contrast, inhibitors of the PKA pathway, used at doses which inhibited forskolin-stimulated luciferase activity by 70–80%, reduced FSS-stimulated COX-2 mRNA expression and luciferase activity by 50–80%. Hence, peak FSS induction of COX-2 expression in MC3T3-E1 osteoblastic cells is largely dependent on the PKA signaling pathway.

Keywords: Prostaglandins; Signal transduction; Cyclooxygenase; Mechanical loading; Bone

Mechanical loading of bone is essential for maintaining bone mass and integrity. Prostaglandins may mediate the anabolic effects of mechanical loading on bone [1–3]. The limiting enzyme in the conversion of membrane-released arachidonic acid to prostaglandins is cyclooxygenase (COX). There are two isoforms of COX: COX-1, which is constitutively expressed, and COX-2, which is inducible. COX-2 is highly expressed in osteoblasts and the prostaglandin response to most important regulators of bone metabolism, including mechanical loading, is dependent on the activity of COX-2 [4,5]. Fluid shear stress (FSS), which is thought to transduce the effects of mechanical loading on bone to the osteoblasts and osteocytes within the mineralized matrix, has been shown to stimulate COX-2 expression and prostaglandin production in osteoblastic cells [6–9].

We previously found that FSS induced COX-2 mRNA expression in the immortalized murine osteoblastic cell line, MC3T3-E1 cells, with peak effects at 4–5 h, and that this induction was mediated predominantly by the ERK signaling pathway [10]. Both the PKA and PKC pathways have been implicated as being upstream of the FSS activation of ERK [11,12]. Studies have reported that the PKC pathway was involved in the FSS induction of prostaglandin production in osteoblasts [13] and in osteocytes [14]. In this study, we examined the roles of the PKA and PKC pathways in the peak FSS induction of COX-2 mRNA expression and luciferase activity in MC3T3-E1 cells stably transfected with 371 bp of the COX-2 promoter fused to a luciferase reporter. After documenting the effects of inhibitors on known PKC and PKA agonists of COX-2, we used these inhibitors at the most specific and/or ef-

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ficacious doses. We found that inhibitors of the PKA, but not the PKC, pathway inhibited the FSS induction of COX-2 mRNA expression and promoter activity in MC3T3-E1 cells.

Materials and methods

Materials. Murine COX-2 cDNA was the gift of Harvey Herschman (UCLA, Los Angeles, CA) and has been previously described [15,16]. cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH) was obtained by PCR using a control amplifier set from Clontech (Palto Alto, CA). GF109203X, myristoylated protein kinase inhibitor 14–22 (PKI), and H-89 were purchased from Biomol (Plymouth Meeting, PA). Phorbol myristate acetate (PMA) and other chemicals were purchased from Sigma (St. Louis, MO).

Fluid flow chamber. The parallel plate fluid flow chamber was designed by Epstein [10] and Peterson [17] and has been previously described. This chamber generated a uniform flow field in which magnitude and direction of the velocity vector were constant under steady flow conditions. The flow medium was Dulbecco's modified Eagle's medium without phenol red (DMEM, Sigma) containing 0.05% heat-inactivated fetal calf serum (FCS, Gibco BRL, Grand Island, NY). The flow medium was maintained at 37 °C. A gassing system saturated the medium with 5% CO_2.

Cell culture. Stable transfections of MC3T3-E1 cells, the gift of Dr. Y. Hakeda (Meikai University School of Dentistry, Sakado, Saitama, Japan), with DNA constructs containing −371/+70 bp of the COX-2 promoter fused to a luciferase reporter (Pluc371) were performed as previously described [10]. For experiments, cells were plated on type I collagen (rat tail collagen; Collaborative Biomedical Products, Bedford, MA)-coated glass slides at 5000/cm^2 in phenol red-free DMEM with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (50 μg/ml) and grown to near confluence over 4–6 d at 37 °C in a humidified atmosphere of 5% CO_2 in air. Slides were coated according to manufacturer's instructions with 5 μg/cm^2 collagen. Concentration of the vehicle for inhibitors (dimethyl sulfoxide for H-89 and GF109203X and ethanol for indomethacin) was 0.1% or below in both treated and control cultures. For inhibitor experiments, cells were pretreated with vehicle or inhibitor for 30 min.

Luciferase assay. Luciferase activity was measured in soluble cell extracts prepared with a kit from Promega (Madison, WI) using an automatic injection luminometer (Berthold Lumat, Wallac, Gaithersburg, MD). Activity in counts per second (cps) was normalized to total protein measured with a BCA Protein Assay Kit (Pierce, Rockford, IL). For each experiment, 3–4 glass slides plated of cells that were subjected to FSS simultaneously were analyzed per treatment group.

Extraction of RNA and Northern blot analysis. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH) following manufacturer's instructions. Ten to twenty μg of total RNA was run on a 1% agarose–2.2 M formaldehyde gel, transferred to a nylon membrane by capillary pressure, and fixed to the membrane by UV 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 the same temperature with a random [^{32}P]dCTP-labeled cDNA probe. Filters were washed once in a 1× SSC, 1% SDS solution at room temperature, once in 0.1× SSC, 0.1% SDS solution at 65 °C, and three times in the latter solution at room temperature. After washing, the filters were exposed to Kodak XAR-5 film at −70 °C. Bands were scanned into the computer and the band density was quantitated by NIH Image 1.61 software (free software available from NIH).

Statistics. Means of groups were compared by ANOVA and significance of differences was determined by post hoc testing using Bonferroni's method (SigmaStat software, Jandel Scientific, San Rafael, CA).

Results

Involvement of the PKC pathway in the FSS induction of COX-2 expression

We have previously shown that the PKC agonist, phorbol myristate acetate (PMA), and the PKA agonist, forskolin, induce luciferase activity in MC3T3-E1 cells stably transfected with 371 bp COX-2 promoter fused to a luciferase reporter (Pluc371), with peak effects at about 3 h in stationary culture [18,19]. We used the luciferase responses to these agonists as a convenient assay for determining the lowest effective dose of inhibitor with the highest specificity under our experimental conditions. GF109203X has been shown to be an inhibitor of the PKC pathway in human platelets and Swiss 3T3 fibroblasts [20]. We compared the effects of GF109203X, from 1 to 5 μg/ml, on the luciferase responses to forskolin (10 μM) and PMA (1 μM) (Fig. 1). GF109203X at 1 μg/ml inhibited PMA-stimulated luciferase activity by 90% without any effect on forskolin-stimulated luciferase activity (Fig. 1). In a second similar experiment, we found that GF109203 at 1.25 μg/ml inhibited PMA-stimulated luciferase activity by 84% without any effect on forskolin-stimulated luciferase activity (data not shown).

GF109203X at 1.25 μg/ml was the dose used for subsequent FSS experiments. We have previously shown that FSS coordinately induces COX-2 mRNA and stimulates luciferase activity in MC3T3-E1 cells stably transfected with Pluc371, with peak effects on both at 4–5 h [10]. GF109203X did not inhibit the FSS (10 dyn/cm^2) induction of COX-2 mRNA at 4 h in MC3T3-E1 cells in the experiment shown in Fig. 2 or in a second independent experiment (data not shown). FSS (10 dyn/

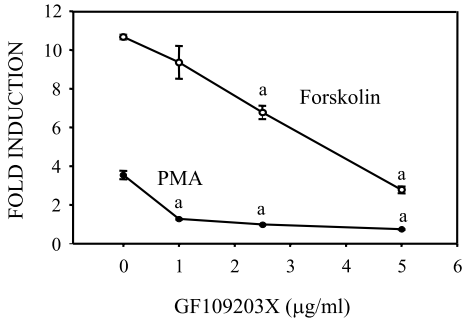

Fig. 1. Comparison of the inhibitory effects of the PKC inhibitor, GF109203X, on luciferase activity in MC3T3-E1 cells (stably transfected with Pluc 371) treated for 3 h with phorbol myristate acetate (PMA, 1 μM), a PKC agonist, or with forskolin (10 μM), a PKA agonist. Luciferase activity is expressed as treated/control ratios. Each point is the mean and SEM for $n = 3$ slides of cells. (a) Significant effect of GF109203X compared to control, $P < 0.01$.

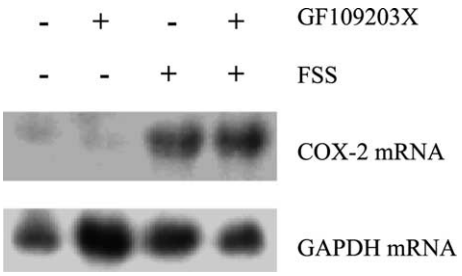

Fig. 2. Effect of GF109203X on the FSS induction of COX-2 mRNA in MC3T3-E1 cells. Cells were subjected to FSS, with and without GF109203X (1.25 μg/ml), for 4 h. Northern blot analysis was done for COX-2 mRNA and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

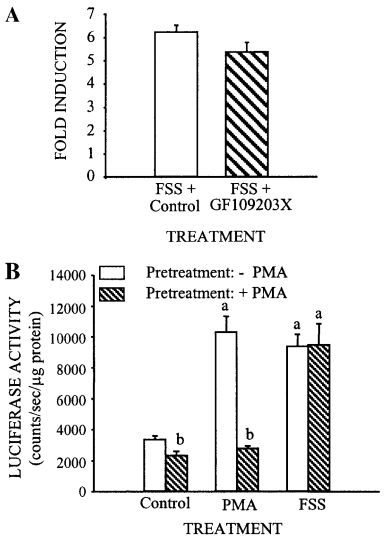

Fig. 3. Effects of inhibiting or down-regulating the PKC pathway on the FSS induction of luciferase activity in MC3T3-E1 cells stably transfected with Pluc371. Luciferase activity, expressed as treated/control ratios, is the mean ± SEM for $n = 4$ slides of cells. (A) Cells were subjected to FSS for 4.5 h without inhibitor (Control) or with GF109203X (1.25 μg/ml). (B) Cells were pretreated for 24 h with vehicle or with phorbol myristate acetate (PMA, 1 μM) and then subjected to PMA (1 μM) or vehicle (Control) in stationary culture for 3 h or to FSS (10 dyn/cm^2) for 4.5 h. (a) Significant effect of FSS, $P < 0.01$. (b) Significant effect of pretreatment, $P < 0.01$.

cm^2) applied for 4.5 h stimulated luciferase activity 6.2-fold and this stimulation was not significantly reduced by GF1090203X (Fig. 3A).

As an additional means of testing the involvement of the PKC pathway, we down-regulated the PKC pathway by pretreating cells with PMA (1 μM) for 24 h before treatment either with PMA (1 μM) for 3 h or with FSS (10 dyn/cm^2) for 4.5 h. Pretreatment with PMA blocked the ability of PMA to stimulate luciferase activity (Fig. 3B). FSS stimulated luciferase activity 7.6-fold and this stimulation was not reduced by PMA pretreatment. This experiment was repeated two more times with similar results (data not shown).

Involvement of the PKA pathway in the FSS induction of COX-2 expression

H-89 and PKI have been used as specific inhibitors of the PKA pathway [21,22]. To determine the most effective dose of these inhibitors under our experimental conditions, we performed similar dose–response experiments as described above in MC3T3-E1 cells stably transfected with Pluc371 treated with PMA and forskolin. PKI at 1 μM had maximal inhibitory effects of 65% on forskolin-stimulated luciferase activity and did not significantly inhibit PMA-induced luciferase activity (Fig. 4A). H-89 was most effective against forskolin at 30 μM, causing an 80% reduction of stimulated luciferase activity (Fig. 4B). However, at this concentration, H-89 inhibited PMA-induced luciferase activity by 60%. There was no dose of H-89 that selectively inhibited the PKA pathway. This experiment was repeated with similar results (data not shown). Since we found no involvement of the PKC pathway in the FSS induction of COX-2 and since H-89 at 30 μM was the most effective inhibitor of forskolin-induced luciferase, we performed the FSS experiments with H-89 at 30 μM.

In MC3T3-E1 cells, PKI (1 μM) and H-89 (30 μM) inhibited the FSS (10 dyn/cm^2, 4 h) induction of COX-2

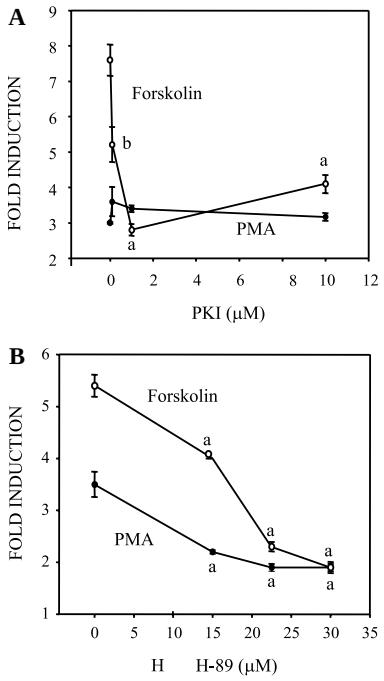

Fig. 4. Comparison of the inhibitory effects of PKA pathway inhibitors on luciferase activity in MC3T3-E1 cells (stably transfected with Pluc 371) treated for 3 h with phorbol myristate acetate (PMA, 1 μM), a PKC agonist, or with forskolin (10 μM), a PKA agonist. Effects of PKI (A) and H-89 (B) on PMA and forskolin-induced luciferase activity. Luciferase activity is expressed as treated/control ratios. Each point is the mean and SEM for $n = 4$ slides of cells. (a) Significant effect of inhibitor compared to control, $P < 0.01$. (b) Significant effect of inhibitor compared to control, $P < 0.05$.

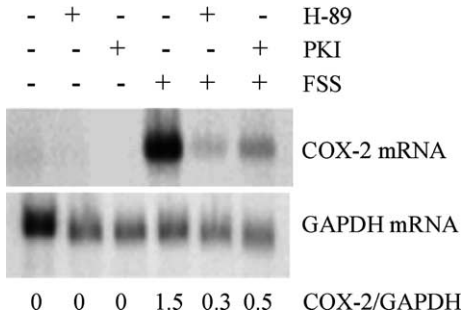

Fig. 5. Effect of PKA inhibitors on the FSS induction of COX-2 mRNA in MC3T3-E1 cells. Cells were subjected to FSS (10 dyn/cm^2) for 4 h, with and without PKI or H-89. Northern blot analysis was done for COX-2 and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

mRNA on Northern blot analysis by 67% and 80%, respectively (Fig. 5). Inhibition was calculated after normalization of COX-2 mRNA levels to the corresponding GAPDH mRNA levels. In a similar experiment, H-89 (30 μM) inhibited FSS induction of COX-2 mRNA expression on Northern blot analysis by 62% at 4 h (data not shown).

In MC3T3-E1 cells stably transfected with Pluc371, 4.5 h of FSS (10 dyn/cm^2) stimulated luciferase activity 9.7-fold and this stimulation was reduced 50% by PKI (Fig. 6A). In a second experiment, the FSS stimulation of luciferase activity was decreased by 66% by PKI (data not shown). In a similar experiment, FSS stimulated a 5.7-fold increase in luciferase activity, which was inhibited by H-89 at 4.5 h by 66% (Fig. 6B).

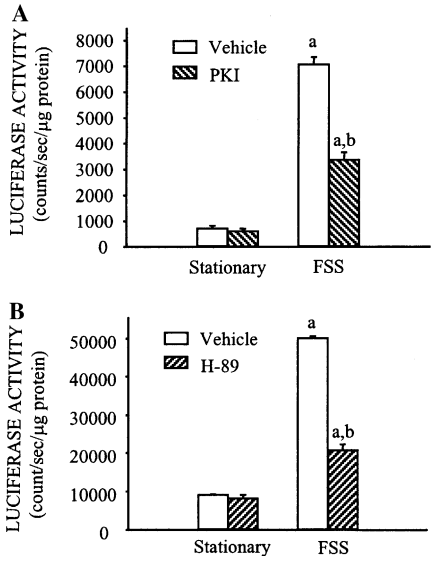

Fig. 6. Effect of PKA inhibitors on the FSS induction of luciferase activity in MC3T3-E1 cells stably transfected with Pluc371. Cells were subjected to FSS (10 dyn/cm^2) for 4.5 h, with and without (A) PKI (1 μM) or (B) H-89 (30 μM). Each bar is the mean and SEM for $n = 4$ slides of cells. (a) Significant effect of FSS, $P < 0.01$. (b) Significant effect of PKI or H-89, $P < 0.01$.

Discussion

We did not find any effect of inhibiting the PKC pathway on the FSS induction of COX-2 mRNA expression or luciferase activity at 4–4.5 h in MC3T3-E1 cells, despite previous studies suggesting involvement of the PKC pathway [13,14]. In one study that reported involvement of the PKC pathway in the FSS induction of prostaglandin in osteoblastic cells, the inhibitor H-7 was used [13]. However, H-7 is a non-specific kinase inhibitor [23] and has the ability to inhibit both the PKA and PKC signaling pathways approximately equally [24]. In another study, in which it was reported that the PKC pathway was involved in FSS stimulated prostaglandin production in osteocytes, prostaglandin levels were measured after 10 min of flow [14]. We found that the induction of COX-2 mRNA and protein was first detected after about 30 and 60 min, respectively, of flow in MC3T3-E1 cells [10]. It is likely therefore that the FSS stimulated prostaglandin production at 10 min was due to the action of constitutively expressed COX-1 on arachidonic acid that was released from membranes and not on the FSS induction of COX-2 expression.

Inhibitors of the PKA pathway inhibited the FSS induction of COX-2 mRNA and luciferase activity in MC3T3-E1 osteoblastic cells at 4–4.5 h by 50–80%. The forskolin induction of luciferase expression in these cells, which presumably occurs predominantly via the PKA pathway, was inhibited 70–80% by the same concentrations of inhibitors. We conclude therefore that FSS induction of COX-2 expression is dependent in large part on a PKA signaling pathway. Although it has previously been reported that FSS can increase cAMP levels in osteoblastic cells, this is the first report, that we are aware of, of the involvement of the PKA signaling pathway in the FSS induction of COX-2 expression or prostaglandin production in osteoblastic cells.

It seems likely that FSS causes an initial rapid increase in prostaglandin levels by providing substrate for the constitutively expressed COX-1 enzyme [9]. The FSS-induced increase in cAMP levels occurring 30 s after initiation of flow in osteoblastic cells was reported to be blocked by an inhibitor of prostaglandin production, ibuprofen, a nonselective inhibitor of COX activity [25]. In addition, prostaglandins themselves can induce COX-2 mRNA expression in osteoblastic cells via both PKA and PKC pathways [26]. Hence, prostaglandins produced either by COX-1 within minutes of FSS application or by the induction of COX-2 within 30 min of FSS application could activate the PKA signaling pathway and lead to the peak COX-2 induction seen at 4–5 h of FSS. However, we did not find any effect of indomethacin, a nonselective irreversible COX inhibitor, on the FSS induction of luciferase activity in MC3T3-E1 cells stably transfected with Pluc371 cells at 4.5 h, indicating that the FSS induction of COX-2 expression under our

experimental conditions was independent of prostaglandin production (data not shown).

We have previously shown that the FSS peak induction of COX-2 mRNA at 4–5 h in MC3T3-E1 cells and primary calvarial osteoblasts was largely dependent on an ERK signaling pathway [10]. The ERK pathway is tightly regulated by and cross-communicates with the PKA signaling pathway. cAMP may inhibit the ERK pathway, at least in part, by blocking the binding of Raf-1 to Ras [27]. On the other hand, cAMP can induce the activation of ERK through a PKA mediated activation of the Ras-related small G protein Rap1. The activated Rap1 is both a selective activator of B-Raf and an inhibitor of Raf-1 [28]. Thus, in cells with little or no B-Raf, cAMP inhibits the ERK pathway. The expression of B-Raf has not been reported in osteoblastic cells. Our preliminary data suggest that inhibition of the PKA pathway does not inhibit the induction of ERK phosphorylation seen at 5 min of FSS (data not shown). In support of this observation, a number of studies have found that cAMP activators inhibit ERK activation [29–31] in osteoblastic cells. However, in a recent study it was shown that strain-induced ERK activation in the ROS 17/2.8 osteoblastic cell line was dependent on a PKA pathway [11].

The −371/+70 bp region of the COX-2 promoter contains a cAMP response element (CRE) and an activator protein-1 (AP-1) binding site. A recent study has shown that these sites play crucial roles in FSS-induced COX-2 expression in osteoblasts [6]. Members of the CRE/ATF family (CREB, CREM, and ATF) preferentially bind to the CRE site, while members of the Fos and Jun family of proteins preferentially bind to the AP-1 site. However, interactions and heterodimerization between the different families are common. Activation of the AP-1 complex is thought to be mediated by the ERK signaling pathway [32], while activation of CREB is thought to be mediated by the PKA signaling pathway [33] in osteoblastic cells. Our studies suggest that the peak COX-2 response to FSS in MC3T3-E1 osteoblastic cells requires both PKA and ERK signaling pathways.

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