Mechanism of basic calcium phosphate crystal-stimulated cyclo-oxygenase-1 up-regulation in osteoarthritic synovial fibroblasts

E. S. Molloy1, M. P. Morgan1, G. A. Doherty2, B. McDonnell1, M. Hilliard2, J. O’Byrne3, D. J. Fitzgerald2 and G. M. McCarthy1,2,3,4

Objectives. Basic calcium phosphate (BCP) crystals have been implicated in the pathogenesis of OA and stimulate cyclo-oxygenase (COX) expression and PGE2 production. This study aimed to elucidate the mechanism of COX-1 up-regulation by BCP crystals and to characterize the PGs produced in OA synovial fibroblasts (OASFs) in response to BCP crystals.

Methods. OASFs were stimulated with BCP crystals in vitro. mRNA expression was measured by real-time PCR, PG production by EIA and protein production by western blot.

Results. Maximal (19-fold) up-regulation of COX-1 mRNA occurred 32 h after stimulation with BCP crystals; increased COX-1 protein production was also seen. At 32 h post-stimulation with BCP crystals, PGE2 (and prostacyclin) production was COX-1 dependent. In contrast, maximal (17-fold) up-regulation of COX-2, with corresponding COX-2-dependent PG production, occurred 4 h after BCP crystal stimulation. There was no appreciable increased production of other PGs such as PGF2α, thromboxane A2 or cyclopentanone PGs including 15d-PGJ2. Inhibition of protein kinase C (PKC) and extracellular regulated kinase 1/2 (ERK1/2) signal transduction pathways blocked BCP crystal-induced COX-1 mRNA expression. Bafilomycin A1, an inhibitor of intra-lysosomal BCP crystal dissolution, diminished BCP crystal-induced COX-1 mRNA expression.

Conclusions. These findings indicate that BCP crystals can augment PG production in OASF through induction of COX-1 and COX-2. Intra-lysosomal BCP crystal dissolution and activity of the PKC and ERK1/2 signal transduction pathways are required for BCP crystal-induced COX-1 up-regulation. These data add to the evidence suggesting that the constitutive COX-1/inducible COX-2 concept is an over-simplification and suggest that non-selective COX inhibition may be preferable to COX-2 selective inhibition in BCP crystal-associated OA.

Key words: Basic calcium phosphate crystals, Osteoarthritis, Cyclo-oxygenase-1, Prostaglandins, Synovial fibroblasts.

Introduction

Basic calcium phosphate (BCP) crystals are predominantly composed of partially carbonate-substituted hydroxyapatite, but also include octacalcium phosphate and tricalcium phosphate. They are associated with a number of rheumatic syndromes including acute calcific periarthritis, calcific tendinitis, Milwaukee shoulder syndrome and OA as well as non-rheumatic syndromes such as breast cancer and atherosclerosis [1, 2]. The prevalence of BCP crystals in synovial fluid from patients with knee OA is between 30% and 60% [3–6], in contrast to other destructive arthropathies such as RA where they are rarely found. Ample data support the role of BCP crystals in cartilage degeneration as their presence correlates strongly with radiographic severity of OA [6, 7] and larger joint effusions are seen in affected knee joints when compared with joint fluid from OA knees without crystals [8]. In vitro properties of BCP crystals have been observed which could contribute to OA pathogenesis. These include induction of mitogenesis [9, 10], and stimulation of MMPs [10–14]. BCP crystals also up-regulate nitric oxide production [15] as well as expression of TNF-α and IL-1β [15–17], both of which have been implicated in the pathogenesis of OA [18].

Cyclo-oxygenase (COX) catalyses the conversion of arachidonic acid to PGH2, the first committed step in the biosynthesis of prostanooids. NSAIDs inhibit cyclo-oxygenase activity and PG production and, thus partially ameliorate some aspects of the inflammatory state [19]. Two main COX isoforms, each encoded by distinct genes, have been described in mammalian cells. COX-2, is only expressed in a limited number of cells at baseline, but can be induced by various pro-inflammatory agents, and is thought to be the predominant COX isoform involved in the inflammatory response [20]. In contrast, the COX-1 isoform is thought to be expressed constitutively in most tissues. It is generally regarded as a ‘housekeeping gene’ that supports the levels of prostanooid biosynthesis required for maintaining organ and tissue homeostasis [20]. However, recent studies have shown that COX-1 is overexpressed in certain cancers [21–23] and can be induced by certain stimuli including oestrogen [24], phorbol ester [25, 26], pro-inflammatory cytokines [27, 28] and stem cell factor plus dexamethasone [29]. While the nuclear transcription factors involved in COX-1 up-regulation have been examined [24, 25], the signal transduction pathways involved in COX-1 up-regulation have not yet been defined.

It has been known for some time that BCP crystals cause an increase in PGE2 production [12, 30, 31]. We have recently shown, in the human foreskin fibroblast (HFF) cell line, that BCP crystal stimulation leads to a significant 23-fold up-regulation of COX-2 and to a slight, but significant, 1.75-fold increase in COX-1 mRNA expression. Increased COX-2 protein production was found by western blotting [17]. The up-regulation of COX-2 mRNA expression was diminished by inhibition of the protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) signal transduction pathways [17]. Although the HFF model has long been used in the investigation of the in vitro effects of BCP crystals...
and was considered comparable with synovial fibroblasts (SFs), biological responses differ between HFF and OASFs. Of particular relevance to this study, we have noted a markedly increased potential for COX-1 induction by BCP crystals in OASF, as compared with HFF. Because of these differences and as OASFs are more relevant from a pathophysiological viewpoint, OASFs were used in this study. The primary aim of this study was to elucidate the mechanism of BCP crystal-stimulated COX-1 up-regulation; a secondary aim was to define more fully the range of PGs produced by OASF in response to BCP crystal stimulation.

Methods

BCP crystal preparation

BCP crystals were synthesized by alkaline hydrolysis of brushite, as previously described [32]. Mineral prepared by this method has a calcium/phosphate molar ratio of 1.59 and contains partially carbonate-substituted hydroxyapatite as determined by Fourier transform infrared spectroscopy. The crystals were weighed into vials and rendered pyrogen-free by heating at 200°C for 90 min. The sterile crystals were resuspended by brief sonication in dulbecco’s modified Eagle’s medium (DMEM) prior to use. For purposes of this study, signal transduction inhibitors (Merck) implicated in cellular responses to BCP crystals [33]. For the BCP crystal preparation

Cell culture

Synovial tissue was obtained from four primary OA patients at the time of joint replacement surgery. Informed consent was obtained from all patients. Ethical approval was granted by the National Orthopaedic Hospital Ethics Committee. The synovial tissue was minced and enzymatically digested by 1.5 mg/ml collagenase in DMEM for 90 min at 37°C. Then 0.5% trypsin in DMEM was added for a further 30 min incubation. Cells were centrifuged at 1000g for 5 min and washed with phosphate-buffered saline/DMEM four times. Cells were resuspended, grown and maintained in DMEM containing 10% foetal bovine serum (FBS), 1% penicillin/streptomycin/fungizone and 1% sodium bicarbonate, in a humidified incubator at 37°C with 5% carbon-dioxide/95% air.

Prior to each experiment, cells were rendered quiescent by incubation in media containing 0.5% FBS for 24h. Fresh 0.5% FBS-containing media was then added and the cells treated with BCP crystals are left untreated. RNA or cell lysates were harvested at the times indicated. All cultures used were fifth or earlier passage cells. All experiments were repeated at least three times.

Inhibitor studies

A variety of signal transduction pathways have previously been implicated in cellular responses to BCP crystals [33]. For the purposes of this study, signal transduction inhibitors (Merck Biosciences, Nottingham, UK) were added 45 min prior to stimulation of cells at the following concentrations: 100 nM Wortmannin (a PI3K inhibitor), 20 μM UO126 [an inhibitor of the extracellular regulated kinase 1/2 (ERK1/2) pathway], 20 μM UO124 (a negative control for UO126), 20 μM SB203580 (a p38 mitogen-activated kinase pathway inhibitor), 20 μM SB202474 (a negative control for SB203580), 20 μM SP600125 [a c-jun N-terminal kinase (JNK) pathway inhibitor] and 10 μM bisindolylmaleimide I (a PKC inhibitor), with 0.1% dimethylsulphoxide (DMSO) as a vehicle control.

Studies to date have indicated that intra-lysosomal dissolution of BCP crystals is required for some, but not all, cellular effects of BCP crystals [34, 35]. In this study, inhibition of intra-lysosomal BCP crystal dissolution was achieved by pre-incubating OASF for 30 min with bafilomycin A1, a vacuolar-type proton pump inhibitor.

Cycloheximide, an inhibitor of protein synthesis, was added to OASF 45 min prior to BCP crystal stimulation. Inhibition of mRNA expression by cycloheximide indicates that new protein synthesis is required for the up-regulation of that mRNA transcript, thereby potentially implicating an intermediate factor in the up-regulation of that mRNA transcript.

Previous data has shown that BCP crystals up-regulate IL-1 in human fibroblasts [17]. The potential role of IL-1 in mediating BCP crystal-stimulated COX-1 (and COX-2) up-regulation was examined using recombinant human IL-1 receptor antagonist (IL-1ra; R&D Systems, Abingdon, UK). This was added 60 min prior to BCP crystal stimulation.

Choice of concentrations and timing of administration of inhibitors was informed by prior experiments, cytotoxicity assays, the available literature and the manufacturers’ instructions.

Western blot

Samples containing equal protein concentrations were electrophoresed on 10% SDS-PAGE and proteins were transferred to nitrocellulose membranes over 2h. Membranes were blocked in 3% BSA. The membranes were then incubated for 3h with 1:200 dilution of primary anti-COX-1 antibody (SPI-Bio, Montigny-le-Bretonneux, France). Secondary peroxidase-conjugated anti-mouse immunoglobulin was used at a dilution of 1/25000. Immunoreactive bands were detected using enhanced chemiluminescence reagents ECL-plus (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RNA extraction, reverse transcription and real-time PCR

RNA was isolated and reverse transcribed as previously described [17]. Real-time PCR was carried out using an ABI 7500 Sequence Detector (Applied Biosystems, Cheshire, UK). Real-time PCR methods and COX-1 and COX-2 primers and probe sequences were as previously described [17].

Prostanoid immunoassays

Prostanoid assays were carried out as previously described [17], in accordance with the manufacturer’s instructions. Cells were stimulated with BCP crystals or left untreated and incubated for in between 4 and 32h. The relative contributions of COX-1 and COX-2 to BCP crystal-stimulated PG production were assessed using specific inhibitors of COX-1 (SC-560; 1 μM) and COX-2 (SC-236; 1 μM) and aspirin (200 μM). Synthesis of prostanoids [PGE2, prostacyclin (measured as 6-keto-PGF1α), PGF2α and thromboxane A2 (measured as thromboxane B2)] was measured using commercially available PG immunoassays (R&D Systems). Choice of concentrations and timing of administration of inhibitors was informed by prior experiments, available literature and the manufacturer’s instructions, with the aim of preserving COX isoenzyme selectivity. Assays were carried out in duplicate at least 3 times.

Measurement of cyclopentanone PGs

PGD2, PGJ2 and 15d-PGJ2 were measured by a HPLC/tandem mass spectrometry technique. Briefly, OASFs were pre-treated with 50 μM arachidonic acid prior to incubation with or without BCP crystals for 24h. Control media containing 50 μM arachidonic acid (without cells) was also incubated at 37°C for 24h. Cell supernatant/control media of about 5ml was spiked with either 2.5 ng of 18O2- or 12PGJ2, 5 ng of d4-15d-PGJ2 or d4-PGD2 and solid-phase extraction performed followed by analysis using a triple quadruple mass spectrometer (Sciex API 3000, Perkin Elmer, Surrey, England).
Mechanism of COX-1 up-regulation by BCP crystals

Results

BCP crystals induce COX-1 mRNA and protein expression in OASF

A significant (17-fold) up-regulation of COX-2 mRNA expression in response to BCP crystal stimulation, comparable with that previously seen in HFF [17], was seen in OASF (Fig. 1A). Significant up-regulation of COX-1 mRNA expression in OASF was demonstrated by real-time PCR (Fig. 1B), with maximal (19-fold) expression seen at 32 h. Increased COX-1 protein was also observed following treatment with BCP crystals (Fig. 1C). This degree of induction of COX-1 expression in response to BCP crystal stimulation was much greater than the 1.75-fold induction previously seen in HFF [17].

Fig. 1. Effect of BCP crystals on COX-2 (A) and COX-1 (B) mRNA expression and COX-1 protein production (C) in OASF. Confluent, quiescent cultures of OASF cells were treated with BCP crystals (18 µg/cm²) or left untreated. RNA was collected at the times indicated. Transcript levels of COX-1 and COX-2 were determined by real-time PCR. All samples were assayed in duplicate. Data for each sample was normalized to 18S expression levels, and expressed as mean ± S.E.M. Relative to untreated control cells (data not shown). Data shown as the mean fold increase ± S.E.M., n = 3. (*P < 0.05 vs untreated control at each time point). For western blotting (C), confluent, quiescent cultures of OASFs were stimulated with BCP crystals (18 µg/cm²) or left untreated (U). After 32 h cell lysates were collected. Western blotting was performed using a monoclonal antibody to COX-1. Equal protein concentrations, as determined by Bradford assay, were loaded onto 10% SDS–PAGE gels. Blot shown is representative of three experiments.

BCP crystals enhance synthesis of PGE₂ and prostacyclin, but not other major prostanoids

Figure 2 indicates that BCP caused an increase in PGE₂ levels peaking at 4 h with a later lower peak at 32 h. A similar temporal pattern was previously reported for prostacyclin production in response to BCP crystal stimulation [36]. PGE₂ and thromboxane A₂ (TXA₂; measured as its stable metabolite TXB₂) were detected in small amounts by EIA but were unchanged with addition of BCP crystals (data not shown). As measured by HPLC and tandem mass spectrometry, PGD₂ was found in small quantities, but was not increased in response to BCP crystal stimulation; J-series PGs were not detected (data not shown). At 4 h, the increase in PGE₂ was inhibited by addition of aspirin or the COX-2 selective inhibitor SC-236 (Fig. 3A). After 32 h, SC236 was no longer a significant inhibitor, but aspirin and the COX-1 selective inhibitor SC-560 both significantly inhibited PGE₂ production (Fig. 3B). Therefore, the early and late peaks in PGE₂ production were inhibited by COX-2 and COX-1 selective inhibition, respectively. This is logical, given that these peaks also temporally coincided with the maximal up-regulation of COX-2 and COX-1 mRNA expressions, respectively.

Intra-lysosomal BCP crystal dissolution is necessary for BCP crystal-induced COX-1 but not COX-2 mRNA expression

Pre-treatment of OASF with bafilomycin A1 10 nM significantly inhibited BCP crystal-induced COX-1 mRNA expression as assessed by real-time PCR (Fig. 4A). However, COX-2 mRNA expression was not altered by bafilomycin A1 treatment (Fig. 4B). These results indicate that intra-lysosomal crystal dissolution is required for BCP crystal-induced up-regulation of COX-1, but not COX-2.

New protein synthesis is required for BCP crystal-induced COX-1 but not COX-2 mRNA expression

Cycloheximide dose-dependently inhibited BCP crystal-induced COX-1 mRNA expression (Fig. 5A). In contrast, pre-treatment of OASF with cycloheximide 10 µM significantly enhanced BCP crystal-induced COX-2 mRNA expression (Fig. 5B). This super-induction of COX-2 mRNA expression with cycloheximide
treatment was also seen in OASF not treated with BCP crystals (Fig. 5B); this effect has been previously described and is attributable to stabilization of the inherently unstable COX-2 transcript [37, 38].

**Effect of signal transduction inhibitors on BCP crystal-induced COX-1 mRNA expression**

Inhibitors of some of the signal transduction pathways previously reported to be involved in BCP crystal-induced signalling were used to elucidate the molecular mechanism of COX-1 induction by BCP crystals [15, 16, 33]. The effect on COX-1 mRNA was examined by quantitative real-time RT-PCR. UO216, the ERK1/2 pathway inhibitor, significantly inhibited BCP crystal-induced COX-1 mRNA expression, whereas its inactive control compound UO124 had no significant effect (Fig. 6A). In contrast, inhibition of the p38 MAP kinase pathway with SB203580 was not found to have a significant effect on BCP crystal-induced COX-1 mRNA expression levels relative to SB202474, the control compound (Fig. 6A). Bisindolylmaleimide I, the PKC inhibitor, abrogated the BCP crystal-induced COX-1 mRNA (Fig. 6B). Wortmannin, the PI3K inhibitor, had no significant effect and SP600125, the JNK pathway inhibitor, had only a partial inhibitory effect (Fig. 6B). There was no significant inhibitory effect of any of the inhibitors used on COX-1 expression in OASF in the absence of BCP crystals (data not shown).

**Role of IL-1β in up-regulation of COX by BCP crystals**

IL-1β has been demonstrated to up-regulate COX-2 [39] and BCP crystals enhance IL-1β expression in human fibroblasts [17]. However, using IL-1ra at doses of up to 250 ng/ml we were unable to substantially alter COX-1 or COX-2 mRNA expression (results not shown).

**Discussion**

COX-1 has traditionally been viewed as a constitutively expressed gene. However, evidence is emerging that COX-1 is inducible in some cell lines under certain conditions [24-29] and may modulate inflammatory pathways [22, 40]. In this study, COX-1 up-regulation in OASF treated with BCP crystals was strikingly greater than that previously observed in a non-diseased HFF [17]. This induction of COX-1 contributed significantly to the PG produced in response to BCP crystals. The signal transduction pathways involved in COX-1 up-regulation have not previously been examined. In this study, inhibitors of the ERK1/2 pathway and the highly selective cell-permeable PKC inhibitor, bisindolylmaleimide I, abrogated BCP crystal-induced COX-1 mRNA expression. Consistent with this, PKC translocation from the cytoplasm to the membrane is also stimulated by BCP crystals [41]. Interestingly, activation of ERK1/2 by PKC has previously been implicated in BCP crystal-induced MMP-1 and -3 production.
up-regulation [42]. PI3K inhibition was previously shown to inhibit BCP crystal-induced COX-2 mRNA expression [17], the specific PI3K inhibitor, Wortmannin did not, however, significantly diminish BCP crystal-induced COX-1 mRNA expression. COX-2 is generally believed to be the predominant COX isoform involved in the inflammatory process and this study has confirmed that COX-2 is up-regulated in OASF in response to BCP crystal treatment.

The cellular effects of BCP crystals rely upon a bimodal increase in intracellular calcium ([Ca\(^{2+}\)]) [43]. BCP crystals induce a rapid 10-fold increase in [Ca\(^{2+}\)], within seconds, derived principally from extracellular calcium, which returns to baseline within 8 min. A second increment in [Ca\(^{2+}\)], begins at 60 min and continues to increase up to at least 3 h after stimulation. Increasing lysosomal pH by pre-treatment with NH\(_4\)Cl or bafilomycin A1 prevents crystal dissolution and thereby abolishes the second rise in [Ca\(^{2+}\)], but does not affect the transient early peak [43]. Although crystal endocytosis is necessary for BCP crystals to induce MMP synthesis, intra-lysosomal crystal dissolution is not, whereas both events are required to obtain the maximal mitogenic response to BCP crystals [34, 35]. In this study, bafilomycin A1 inhibited BCP crystal-induced COX-1 but not COX-2 mRNA up-regulation. The early induction of COX-2 mRNA by BCP crystals (~7-fold induction seen 1 h post-stimulation) is consistent with its independence from the later event of intra-lysosomal BCP crystal dissolution.

The requirement for new protein synthesis, as evidenced by the inhibitory effect of cycloheximide, along with the fact that BCP crystal up-regulation of COX-1 is a late phenomenon, raised the possibility that BCP crystal induction of COX-1 occurs through an intermediate factor(s). We investigated whether one such putative factor could be IL-1\(\beta\), which is induced in human fibroblasts by BCP crystals [17]. In this study, BCP crystal-induced COX-1 and COX-2 up-regulation was not significantly influenced by the addition of IL-1\(\beta\). However, as IL-1\(\beta\) needs only to occupy 10% of its cell surface receptors to exert its biological effects [44], these results do not definitively exclude a role for IL-1\(\beta\) in up-regulation of COX by BCP crystals in OASF.

This report is the first to examine the production of PGs other than PGE\(_2\) and prostacyclin in BCP crystal-stimulated OASF. PGE\(_2\) and prostacyclin [36] were the only PGs produced in appreciable quantities in OASF in response to BCP crystal stimulation. Despite the previous reports of augmented PGF\(_{2\alpha}\) production by hydroxyapatite crystals in animal models [12, 45], no such induction was found in BCP crystal-stimulated human OASF. Roles for 15d-PGJ\(_2\) in the propagation and resolution of inflammation have been proposed. A number of studies have suggested that exogenous 15d-PGJ\(_2\) may exert biological effects in arthritic tissues in vitro [46–48]. However, in BCP crystal-stimulated OASF, despite marked up-regulation of COX-1 and COX-2, and increased production of other PGs, no 15d-PGJ\(_2\) was detected. This observation is consistent with the findings of Belli-Parikh et al. [49] that 15d-PGJ\(_2\) could not be identified in synovial fluid from patients with OA or RA.
These data add to the accumulating body of evidence that demonstrates that while COX-1 is generally constitutively expressed, it can be induced by certain stimuli and may influence the inflammatory process. This implies that the dichotomous paradigm of a ‘constitutive COX-1’ and ‘inducible COX-2’ is an over-simplification. The clinical relevance of these findings requires further investigation. It could be argued that the therapeutic equivalence of traditional NSAIDs and COX-2 selective inhibitors in OA suggests a limited role for COX-1 in OA pathogenesis. However, such trials to date have looked at short-term measures of symptomatic improvement, without looking at structural changes within the affected joints. It is possible that reducing PG levels below a certain threshold effectively diminishes PG-mediated nociceptive signalling, while having a lesser impact on other deleterious effects of PGs. In addition, no study of NSAIDs in OA has ascertained the presence or absence of intra-articular BCP crystals in the study population. Therefore, based on our data, it is conceivable that intra-articular BCP crystal deposition in OA predicts a greater response to non-selective NSAIDs than to selective COX-2 inhibitors. This hypothesis clearly requires further investigation.

The precise role of PGE2 and other PGs in OA pathophysiology is incompletely understood. However, it seems unlikely that inhibition of PG synthesis is uniformly beneficial in OA. The contribution of PGs to inflammation and nociception is presumably the basis of the symptomatic benefit derived by OA patients taking NSAIDs. Conflicting results have been reported regarding the potential effects of NSAIDs on cartilage integrity [50–57], although this may reflect differential effects of COX selectivity [58]. Other factors that may influence the net effect of PG on OA pathogenesis include the modulation of expression of terminal PG synthases and PG receptors. Future studies that unravel the intricacies of the effects of PGs in OA pathophysiology may permit identification of therapeutic strategies that preferentially target the deleterious effects of PGs over their beneficial actions.

In conclusion, our findings indicate that BCP crystals enhance PG production in OASF through induction of both COX isoenzymes. Intra-lysosomal crystal dissolution, new protein production in OASF through induction of both COX terminal PG synthases and PG receptors. Future studies that unravel the intricacies of the effects of PGs in OA pathophysiology may permit identification of therapeutic strategies that preferentially target the deleterious effects of PGs over their beneficial actions.

References
8. Carroll GJ, Stuart RA, Armstrong JA, Breidahl PD, Laing BA. Hydroxyapatite crystals are a frequent finding in osteoarthritic synovial fluid, but are not related to increased concentrations of keratan sulfate or interleukin 1 beta. J Rheumatol 1991;18:861–6.

Acknowledgements
Funding: This study has been funded by Health Research Board (ESM); Wellcome Trust; PRTLI Cycle III.

Disclosure statement: The authors have declared no conflicts of interest.

References
Mechanism of COX-1 up-regulation by BCP crystals

971


51 Ding C. Do NSAIDs affect the progression of osteoarthritis? Inflammation 2002;26:139–42.


