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

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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 periarthrithis, 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 mitogenes [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 prostanoids. 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 prostanoid 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 the purposes of this study, signal transduction inhibitors (Merck Biosciences, Nottingham, UK) were added 45 min prior to BCP crystal stimulation with BCP crystals at the following concentrations: 100 nM UO124 (a negative control for UO126), 20 \mu M SB202474 (a c-Jun N-terminal kinase (JNK) pathway inhibitor) and 10 \mu M bisindolylmaleimide I (a PKC inhibitor), with 0.1% dimethylsulfoxide (DMSO) as a vehicle control. Recruitment of concentrations and timing of administration of inhibitors was informed by prior experiments, cytotoxicity assays, the available literature and the manufacturers’ instructions.

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 1000 g 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 carbonate/di-oxyrate, 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 24 h. Fresh 0.5% FBS-containing media was then added and the cells treated with BCP crystals were left untreated. RNA or cell lysates were harvested at the time 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 \mu M U0126 [an inhibitor of the extracellular regulated kinase 1/2 (ERK1/2) pathway], 20 \mu M U0124 (a negative control for U0126), 20 \mu M SB203580 (a p38 mitogen-activated kinase pathway inhibitor), 20 \mu M SB202474 (a negative control for SB203580), 20 \mu M SP600125 [a c-jun N-terminal kinase (JNK) pathway inhibitor] and 10 \mu M bisindolylmaleimide I (a PKC inhibitor), with 0.1% dimethylsulfoxide (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.

Western blot

Samples containing equal protein concentrations were electrophoresed on 10% SDS-PAGE and proteins were transferred to nitrocellulose membranes over 2 h. Membranes were blocked in 3% BSA. The membranes were then incubated for 3 h with 1 : 200 dilution of primary anti-COX-1 antibody (SPI-Bio, Montigny-le-Bretonneaux, 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 32 h. 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 \mu M) and COX-2 (SC-236; 1 \mu M) and aspirin (200 \mu M). Synthesis of prostanoids [PGF_2 alpha, prostacyclin (measured as 6-keto-PGF_1 alpha), PGF_2 beta and thromboxane A_2 (measured as thromboxane B_2)] 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

PGD_2 alpha, PGJ_2 and 15d-PGJ_2 were measured by a HPLC/tandem mass spectrometry technique. Briefly, OASFs were pre-treated with 50 \mu M arachidonic acid prior to incubation with or without BCP crystals for 24 h. Control media containing 50 \mu M arachidonic acid (without cells) was also incubated at 37°C for 24 h. Cell supernatant/control media of about 5 ml was spiked with either 2.5 ng of 18:0C_18:2, PGJ_2, 5 ng of 14:1-15d-PGJ_2 or 4d-PGD_2 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].

**BCP crystals enhance synthesis of PGE$_2$ and prostacyclin, but not other major prostanoids**

Figure 2 indicates that BCP caused an increase in PGE$_2$ 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]. PGE$_{2\alpha}$ and thromboxane A$_2$ (TXA$_2$; measured as its stable metabolite TXB$_2$) 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, PGD$_2$ 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 PGE$_2$ 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 PGE$_2$ production (Fig. 3B). Therefore, the early and late peaks in PGE$_2$ 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. 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.
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The second rise in \([Ca^{2+}]\) may be prevented by cycloheximide, 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 NH4Cl or without cycloheximide (CHX) at the doses indicated, COX-1 mRNA expression was measured by real-time PCR, normalized to 18S values and then expressed as fold change over the value obtained for the sample treated with BCP crystals alone. Therefore, the samples treated with BCP crystals alone assumed a value of 1 (mean ± s.e.m., n = 3, **P < 0.01 vs samples treated with BCP crystals alone).

Inhibitory effect of cycloheximide, along with the fact that BCP stimulation is consistent with its independence from the later 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 NH4Cl or baflomycin 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, baflomycin 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-1β, 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-1ra. However, as IL-1 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 in up-regulation of COX by BCP crystals in OASF.

This report is the first to examine the production of PGs other than PGE2 and prostacyclin in BCP crystal-stimulated OASF. PGE2 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 PGF2α production by hydroxyapatite crystals in animal models [12, 45], no such induction was found in BCP crystal-stimulated human OASF. Roles for 15d-PGJ2 in the propagation and resolution of inflammation have been proposed. A number of studies have suggested that exogenous 15d-PGJ2 may exert biological effects [44]. However, in BCP crystal-stimulated OASF, despite marked up-regulation of COX-1 and COX-2, and increased production of other PGs, no 15d-PGJ2 was detected. This observation is consistent with the findings of Bell-Parikh et al. [49] that 15d-PGJ2 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 pathogenesis also requires further investigation. The precise role of PGE2 and other PGs in OA pathophysiology is incompletely understood. 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.

In conclusion, our findings indicate that BCP crystals enhance PG production in OASF through induction of COX-1 and COX-2 signal transduction pathways are required for BCP crystal-induced COX-1 up-regulation. These data provide further evidence implicating BCP crystals in the pathogenesis of OA and emphasizing their up-regulation. These data provide further evidence implicating pathways are required for BCP crystal-induced COX-1 PG production in OASF through induction of both COX-1 and COX-2. 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.

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