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Prostaglandin (PG) E_2 generation by cultured canine synovial fibroblasts exposed to microcrystals containing calcium

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SUMMARY Immunoreactive prostaglandin (PG) E_2 was released into the ambient medium in a dose dependent fashion when either hydroxyapatite (HA) or calcium pyrophosphate dihydrate (CPPD) crystals were added to canine synovial fibroblasts in tissue culture. PGE_2 release peaked 6 to 9 hours after HA or CPPD crystals were added in the presence of serum but at 24 hours if they were added in the presence of lactalbumin hydrolysate. PGE_2 release correlated with crystal endocytosis estimated qualitatively by serial phase contrast microscopy and time lapse photography. As postulated previously by others for monosodium urate crystals, prostaglandin production by synovial cells may also be related to the pathogenesis of the destructive arthropathies associated with HA or CPPD crystals.

Key words: hydroxyapatite, calcium pyrophosphate.

Both acute inflammation and chronic destructive arthropathy have been described in association with basic calcium phosphate (BCP=hydroxyapatite, octacalcium phosphate, tricalcium phosphate) or calcium pyrophosphate dihydrate (CPPD) crystals.[1-5] Such tissue reactions resemble in some respects the acute inflammatory attack and chronic (tophaceous) destructive arthropathy associated with gout, the hallmark of which is the formation in tissues of monosodium urate (MSU) crystals. Each of these crystal species undergoes endocytosis by neutrophils and to a lesser extent by monocytes in acute inflammatory exudates.[1 3 6] Products of both the lipoxygenase and cyclo-oxygenase pathways of arachidonic acid metabolism have been identified when MSU crystals are exposed to these cells in vitro (reviewed by McCarty[7]), and products of both pathways have been identified when cultured human synovial fibroblasts[8] or human neutrophils[9] were exposed to MSU crystals. Cyclo-oxygenase products, mostly prostaglandin (PG)E_2 were elaborated in impressive quantities when MSU crystals were added to murine macrophages[10] or lapine synovial fibroblasts.[11 12] Microcrystalline, but not amorphous, calcium oxalate produced effects similar to that of MSU crystals when incubated with cultured lapine synovial fibroblasts.[13]

PGE_2, and to a lesser extent, PGF_α, generation by cultured human or canine synovial fibroblasts[14] and by lapine chondrocytes in primary culture[15] was augmented when cells were incubated with hydroxyapatite or CPPD crystals in concentrations greater than those actually found in synovial fluid from patients. As prostaglandins have been implicated as mediators in both acute inflammation[16] and in bone resorption,[17] they may relate to the pathogenesis of both the acute and chronic reactions to microcrystals.

The work reported here was performed with highly characterised synthetic hydroxyapatite (HA) or CPPD crystals in concentrations actually found in human joint fluid.

Materials and methods

General. All reagents were prepared in doubly deionised, glass distilled, charcoal filtered water.

Cultures. Canine synovial cells were obtained from anaesthetised animals by a modification of the

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method of Clarris *et al.*[18] Visually confluent second passage cultures from four mongrel dogs were used experimentally. There were about 1·5 to 2 × 10^6 cells/plate. Cells were grown in 100 mm plastic dishes (Scientific Products, McGaw Park, Ill.) containing 10 ml of Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% horse serum and 1% penicillin, streptomycin, and amphotericin B (all from Gibco, Grand Island, NY). At the end of each experiment the cells were washed three times with Hanks's balanced salt solution, released with 2 ml of 20 mM EDTA, and precipitated with an equal volume of 20% trichloroacetic acid. After dissolution of the centrifuged precipitate in 2 ml 0·1 N NaOH, the protein content was measured.[19]

Crystals. Hydroxyapatite crystals were a kind gift from M D Francis, PhD, the Proctor and Gamble Co., Cincinnati, Ohio. They had a molar Ca/P of 1·67 and showed only HA adsorption bands by Fourier transform infrared spectrophotometry.[20] Crystal aggregates were sieved to 10–20 μM with Endicott test sieves (London, England) and heated to 200°C for 2 h to assure sterility and to eliminate possible pyrogen contamination. Calcium pyrophosphate dihydrate crystals were synthesised, characterised, and sieved to 10–20 μM and heated as described previously.[21] Monosodium urate monohydrate crystals were prepared and characterised as described previously.[22] They were sonicated to 10–20 μM and then heated to 170°C for 2 h, which does not alter crystal structure.[23]

Prostaglandin (PG) E_2 assay. This was done by radioimmunoassay by the method of Dunn *et al.*[24] From 0·5 to 3 ml of conditioned medium was extracted for each determination to give the desired sensitivity. The higher values required dilution to give results that fell on the standard curve.

Experiments. Culture medium was replaced with DMEM alone, DMEM with 10% serum, or 0·2% lactalbumin hydrolysate (LA) with or without HA crystals, 100 μg/ml (mg/l) PGE_2 generation by synovial cells was determined. All cultures were examined at intervals up to 24 hours by phase-contrast microscopy. Several cultures containing crystals were examined continuously by time lapse photography with a microscope equipped with a camera (Nikon).

Samples of conditioned media were removed for PGE_2 assay at periodic intervals up to 24 hours, at which time the cells were released and protein content measured. Similar experiments were performed with MSU or CPPD crystals. Dose response was determined by addition of HA or CPPD crystals in concentrations varying from 0 to 500 μg/ml. Indomethacin was added to at least one plate containing crystals and serum in each experiment at final concentration of 10^{-6}M. PGE_2, 45 500 pg/ml (ng/l) and 2400 pg/ml (ng/l) were incubated in DMEM–10% horse serum that had been conditioned for 24 hours by incubation with synovial cells, and samples were removed for radioimmunoassay at hourly intervals for the first six hours and then at 24 hours.

Results

All data are expressed as pg of PGE_2 per mg of cell protein. Cell protein per plate varied from 350 to 900 μg. Except for occasional clumps, nearly all crystals appeared to have undergone endocytosis within 4 to 6 hours when serum was present. In the presence of DMEM alone or when 0·2% LA was substituted, intracellular crystals were noted in large numbers only at 24 hours. These observations were confirmed by time lapse photography.

PGE_2 concentration in the conditioned medium increased in all cultures (Table 1). The increased

Table 1 *PGE_2 (pg/mg cell protein) release from cultured canine synovial cells by hydroxyapatite (HA) crystals*

Time (h)	*DMEM*	*DMEM +HA**	*DMEM +HA†*	*DMEM 10% HS‡ mean±SEM (n=4)*	*DMEM 0·2% LA§*	*DMEM 10% HS +HA†*	*DMEM 0·2% LA+HA**	*DMEM 10% HS+HA+Ind¶*
0	0	0	0	114	0	120	0	
3	24	36	19	2480±588	159±77	5,734±1,166	155±67	
6	31	87	78	3659±1068	3565±1789	18,907±1,790	2,609±1,564	
9	124	471	714	4629±954	3424±1540	23,939±4,057	5,227±2,978	
24	821	30,285	88,109	2080±468	3238±1496	46,149±18,911	70,884±49,098	100±33

* HA=hydroxyapatite crystals, opsonised in 10% horse serum in DMEM washed 3 times; 100 μg/ml (mg/l) used throughout 10–20 μm aggregate size.
† HA=not opsonised.
‡ HS=horse serum.
§ LA=lactalbumin hydrolysate.
¶ Ind=indomethacin 10^{-6}M.

level in the presence of DMEM alone was just detectable; in the presence of 10% serum or 0·2% LA, PGE_2 levels increased after 3 to 6 hours but remained stable. HA crystals added with 10% serum produced progressively greater PGE_2 levels up to 24 hours; when incubated with DMEM alone or with 0·2% LA the PGE_2 rise was delayed but showed a very large rise at 24 hours, sometimes exceeding that noted with crystals in the presence of serum (Fig. 1). Repeated experiments with cells from different animals gave widely variable results in terms of absolute numbers, but the shapes of the curves representing the time course of PGE_2 release were nearly identical.

The time course of release of PGE_2 from canine synovial cells by triclinic CPPD crystals and by MSU crystals is shown in Table 2. In all cultures MSU crystals resulted in a massive and progressive release of PGE_2 into the ambient medium. CPPD behaved like HA crystals, producing an early (3–6 h) rise in the presence of serum. The PGE_2 concentrations at 9 and 24 hours were similar with opsonised crystals in DMEM alone or in DMEM with 10% serum.

The PGE_2 released into the culture medium 9 hours after addition of varying concentrations of HA or CPPD crystals is shown in Table 3. The levels increased in proportion to the concentration of CPPD crystals and to the concentration of HA crystals at 50, 100, and 500 μg/ml (mg/l). Concentrations of HA crystals of 10 μg/ml (mg/l) or less were not associated with PGE_2 generation greater than that found in 10% horse serum alone.

Immunoreactive PGE_2 levels decreased gradually over time when incubated in conditioned media. Only 71% and 48% of the higher concentration remained at 6 and 24 hours respectively. At the lower concentration 66% and 48% remained at these times (data not shown).

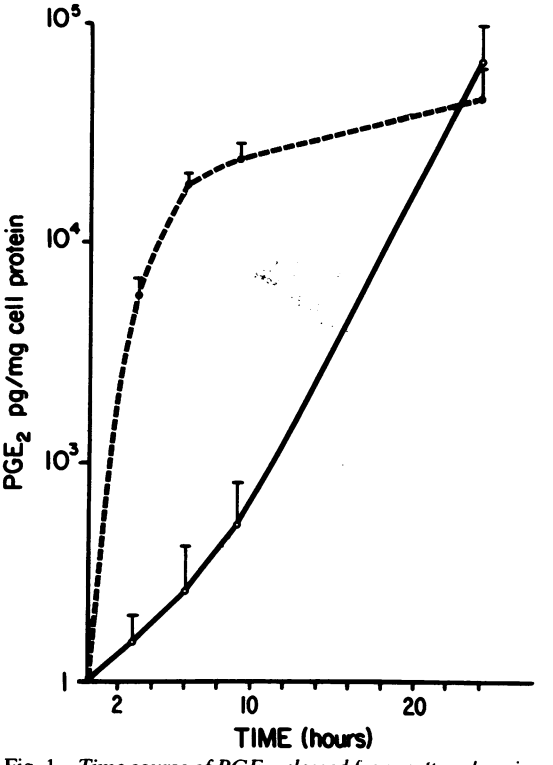

Fig. 1 *Time course of PGE_2 released from cultured canine synovial cells after addition of HA crystals (- - -) 100 μg/ml (mg/l) in DMEM+10% HS, or (——) in DMEM+0·2% lactalbumin hydrolysate. Data shown as mean±SEM, n=4.*

Table 2 *PGE_2 (pg/mg cell protein) released from cultured canine synovial cells by monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) crystals*

Time (h)	*MSU (500 μg/ml)*			*CPPD (100 μg/ml)*		
	DMEM[*]	*DMEM 0·2% LA*[†]	*DMEM 10% HS*[‡]	*DMEM OP*[§]	*DMEM 10% HS*	*DMEM 10% HS+Ind*[‖]
0	0	ND[¶]	ND	0	324	—
3	20,031	15,535	11,888	0	742	—
6	39,891	28,469	62,598	2,244	11,904	—
9	152,036	84,201	200,618	21,083	19,641	—
24	304,581	ND	ND	4,822	4,636	128

[*] DMEM=Dulbecco's modified Eagle's medium.
[†] LA=lactalbumin hydrolysate.
[‡] HS=horse serum.
[§] OP=opsonised crystals.
[‖] Ind=indomethacin 10^{-6}M.
[¶] ND=not measured.

Table 3 *PGE_2 released from cultured canine synovial cells by various concentrations of crystals after nine hours of incubation*

*HA** (μg/ml)	*PGE_2†* (pg/mg protein)	*CPPD‡* (μg/ml)	*PGE_2†* (pg/mg protein)	*PGE_2§* (pg/mg protein)
0	4,630	0	3,454	333
1	2,734	5	6,784	1,058
10	3,834	10	8,494	1,063
50	13,704	100	10,623	2,100
100	18,526	500	20,893	14,344
500	24,457			

* HA=hydroxyapatite 10–20 μm aggregate size.
† DMEM=10% horse serum.
‡ CPPD=calcium pyrophosphate dihydrate—10–20 μm.
§ DMEM=opsonised crystals only.
SI conversion: μg/ml=mg/l.

Discussion

The results of these experiments with canine cells confirm previous reports that monosodium urate crystals stimulate PGE_2 production by cultured human[8] or lapine[11–13] synovial fibroblasts. Although precise comparison is not possible because of the variability of response when cells from different animals are used, it seems evident that hydroxyapatite or calcium pyrophosphate dihydrate crystals are much less potent stimulators of PGE_2 production than are MSU crystals (Tables 1 and 2). PGE_2 levels in the ambient culture medium showed a definite lag period after addition of either HA or CPPD crystals in serum, with a marked increase between 3 and 6 hours and a further rise at 9 and/or 24 hours. PGE_2 release correlated with qualitative assessment of endocytosis estimated by serial phase contrast microscopy and time lapse photography, as noted previously with MSU crystals.[8] This release was found when synthetic HA crystals were used with properties similar to natural BCP crystals but only in concentrations of 50 μg/ml (mg/l) or greater, levels not generally found in patients joint fluid.[25] PGE_2 release by CPPD crystals was noted even at 5 μg/ml (mg/l), far less than the mean of 72 μg/ml (mg/l) found in chronic and 280 μg/ml (mg/l) found in acute effusions.[26]

Both endocytosis and PGE_2 generation occurred but were delayed when either opsonised or uncoated crystals were added to cultured cells in the absence of serum. Wigley *et al.* found that serum was required for PGE_2 generation from human synovial cells and that this was not accompanied by cell death as estimated by lactate dehydrogenase (LDH) release into the culture fluid.[8] Hasselbacher and colleagues found no need for serum when MSU crystals were added to murine macrophages or rabbit synovial fibroblasts, but LDH was released at 2 hours and this became massive after 20 hours.[10–13] We had previously reported no significant LDH release when either synovial cells[14] or lapine chondrocytes[15] were exposed to HA or CPPD crystals in the presence of serum.

MSU, calcium oxalate, BCP, or CPPD crystals are all capable of releasing both PGE_2 and collagenase from cultured cells. The latter event was originally demonstrated by Werb and Reynolds by incubating cultured lapine cells with latex beads[27] and the former by Fine *et al.*, who exposed human synovial fibroblasts and murine macrophages to MSU crystals.[28] The mechanism of prostaglandin generation by cells stimulated with any of these particulates remains to be determined.

It is possible that crystal-induced release of prostaglandins from synovial cells contributes to the destructive arthropathies which occur in some patients with BCP or CPPD crystal deposition diseases. Determinations of the types and amounts of eicosinoids in synovial fluid from patients with these conditions may shed further light on this possibility.

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