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Synovial fluid pyrophosphate and nucleoside triphosphate pyrophosphatase: comparison between normal and diseased and between inflamed and non-inflamed joints

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Abstract

Deposition of intra-articular calcium pyrophosphate is associated with both aging and arthropathy; increased concentrations of free pyrophosphate (PPi) may contribute to such deposition. Free pyrophosphate and nucleoside triphosphate pyrophosphatase (NTPase) were estimated in synovial fluids from 50 subjects with normal knees and from 44 patients with rheumatoid arthritis, 61 with pyrophosphate arthropathy, and 59 with osteoarthritis. For arthropathic knees clinically assessed inflammation was classified as active or inactive using a summated score of six clinical features.

The order of PPi (μmol/l) and NTPase (μmol PPi/30 min/mg protein) was pyrophosphate arthropathy> osteoarthritis> rheumatoid arthritis (median PPi, NTPase respectively: for pyrophosphate arthropathy 15·9, 0·45; for osteoarthritis 9·3, 0·25; for rheumatoid arthritis 4·4, 0·18), with significant differences between all groups. In pyrophosphate arthropathy both PPi (μmol/l) and NTPase (μmol PPi/30 min/mg protein) were higher than normal (15·9, 0·45 *v* 8·6, 0·2 respectively), but findings in osteoarthritis did not differ from normal. The inflammatory state of the knee had a distinct but variable effect on synovial fluid findings in rheumatoid arthritis and pyrophosphate arthropathy, but not in osteoarthritis. There was no correlation of either PPi or NTPase with age, or between PPi and NTPase in any group.

This study provides in vivo data for synovial fluid PPi and NTPase. It suggests that factors other than PPi need to be considered in a study of crystal associated arthropathy. Clinical inflammation, as well as diagnosis, is important in synovial fluid studies.

Although intra-articular deposition of crystalline calcium pyrophosphate dihydrate (CPPD) most commonly occurs as an identical, age related phenomenon,[1] such deposition has been associated with 'pyrophosphate arthropathy', a subset of osteoarthritis characterised by typical clinical and radiographic features.[2 3] The relation between crystals, inflammation, and arthropathy, however, remains uncertain.[1–3] The two principal hypotheses suggest that CPPD crystals either are (*a*) primary pathogenic particles or (*b*) are formed as a secondary event to joint insult, reflecting processes that accompany the articular response that is recognised as 'osteoarthritis'.[1]

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Many factors may affect crystal deposition and possibly, intra-articular inorganic pyrophosphate (PPi) concentration is important. Previous studies have estimated PPi in various arthropathies and reported raised concentrations in osteoarthritis synovial fluid. A possible source of excess PPi is through hydrolysis of nucleoside triphosphates spilled by metabolically active, dividing or damaged cells. This might be facilitated by activity of the chondrocyte ectoenzyme nucleoside triphosphate pyrophosphatase (NTPase), which is found 'free' in synovial fluid from osteoarthritic joints.[4–16] Estimation of PPi and NTPase in biological systems is technically difficult, however[16–21]; various methods have been used, with conflicting results.[22–27] Furthermore, sample numbers in most studies have been limited, and there is a paucity of data for normal joints.[5]

We used a modification of the sensitive and specific assay for PPi developed by McGuire *et al*[6] to estimate PPi and NTPase in knee synovial fluid from well defined patient groups and from normal controls. For abnormal knees the degree of clinical inflammation at the time of aspiration was assessed by a summated score using six variables[28]; comparison between inflamed and non-inflamed knees, as well as between diagnostic categories, was therefore made.

Subjects and methods

Approval for aspiration of normal knees (by means of the medial infrapatellar approach) was obtained from the local ethical committee.

NORMAL SUBJECTS AND PATIENTS

Normal volunteers (29 female, 21 male; median age 44, interquartile range 26–63 years) gave informed consent and had no symptoms or clinical signs of knee disease; radiographs were obtained in those aged >50 years to exclude occult osteoarthritis or other abnormality. All patients with rheumatoid arthritis fulfilled American Rheumatism Association criteria for classic or definite disease (29 female, 15 male; median age 63, interquartile range 54–69 years). Patients with knee osteoarthritis (29 female, 30 male; median age 70, interquartile range 63–75 years) had symptomatic gonarthrosis with weightbearing knee radiographs showing cartilage loss plus subchondral sclerosis, osteophyte, or both; none had radiographic chondrocalcinosis, synovial fluid CPPD crystals, or evidence of other primary joint disease. Patients

with chronic pyrophosphate arthropathy (40 female, 21 male; median age 77, interquartile range 72–83 years) had persistent (>3 months) symptomatic gonarthrosis with synovial fluid CPPD crystals and radiographic features of osteoarthritis (with or without chondrocalcinosis).

ASSESSMENT OF CLINICAL INFLAMMATION

For patients with rheumatoid arthritis, osteoarthritis, and pyrophosphate arthropathy each knee was designated as either 'active' or 'inactive' according to clinically assessed inflammation, using a summated score (0–6) of six features: duration of early morning and inactivity stiffness, warmth, joint line tenderness, effusion, and synovial thickening.[28] Scores of 4–6 were regarded as active, 0–2 as inactive; knees with intermediate scores were not included in this study.

SAMPLE HANDLING

All fluids were taken into sterile plastic containers and processed as soon as possible (within two hours). Crystal identification was by characteristic morphology and birefringence using compensated polarised light microscopy of unspun synovial fluid. Samples were centrifuged at 2500 *g* for 15 minutes to remove cells and crystals; supernatants were stored at −20°C and assayed within three months.

ASSAY OF INORGANIC PYROPHOSPHATE

Inorganic pyrophosphate was estimated by a modification of the radiometric assay developed by McGuire *et al.*[16] Briefly, the sample PPi was allowed to react with added tritiated uridine diphosphogluconate, and the product, glucose-1-phosphate, was converted to a labelled, stable, recoverable product—tritiated 6-phosphogluconate (each sample assayed in duplicate[29–32]).

The PPi assay mixture contained 57 mM TRIS acetate; 5·2 mM magnesium acetate; 4 μM nicotinamide-adenine dinucleotide phosphate; 18·6 μM glucose-1,6-diphosphate; 7·5 μM uridine diphosphogluconate; 0·4 U/l glucose-6-phosphate dehydrogenase; 0·2 U/l phosphoglucomutase; 0·136 U/l uridine diphosphoglucose pyrophosphorylase; and tritiated uridine diphosphogluconate (specific activity 110–370 GBq/mmol). A 100 μl aliquot of this mixture was incubated with 40 μl of either sample or standard PPi (0·05–0·8 μmol/l).

The incubation reaction was performed in triplicate for 75 minutes at 37°C, then terminated by cooling on ice. A 250 μl aliquot of activated charcoal solution (deionised water) was added to separate any unreacted substrate from labelled 6-phosphogluconate. After mixing, samples were centrifuged for 15 minutes at 2500 *g* at 4°C. Supernatant (200 μl) was counted for radioactivity in scintillation fluid.

ASSAY OF NTPase ACTIVITY

NTPase activity is expressed as μmol PPi (per mg total protein) generated over 30 minutes in the presence of added ATP.

Aliquots (200 μl) of synovial fluid were incubated at 37°C in the presence of 120 μM ATP and tracer ^{32}P PPI to determine any breakdown of PPi during subsequent incubation and extraction procedures.

The incubation mixture contained 5·4 mM KCl; 0·8 mM $MgSO_4$; 1·8 mM $CaCl_2$; 0·118 mM NaCl; 1 mM NaH_2PO_4; 5·56 mM glucose; and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid). The mixture was held for 30 minutes in a shaking water bath and then incubation was terminated by cooling on ice and by addition of 0·1 ml trichloroacetic acid (50% w/v). After centrifugation at 2500 *g* for 15 minutes at 4°C trichloroacetic acid was removed by the addition of one volume of tri-n-octylamine dissolved in three volumes of 1,1,2-trichlorofluoroethane. This mixture draws the trichloroacetic acid into the lower organic layer. The resulting 500 μl aqueous extract was neutralised by addition of 100 μl 0·1 M TRIS acetate/8 mM magnesium acetate.

To measure any hydrolysis of PPi to orthophosphate during incubation and extraction the following recovery procedure was carried out on each sample. Orthophosphate was removed by mixing 100 μl of each sample with an equal volume of ammonium molybdate (5% w/v) in 2·7 M HCl. The resulting phosphomolybdate complex was recovered by extraction into isobutanol/light petroleum (4:1 v/v). The remaining ^{32}P PPi was then counted in the aqueous phase in 0·1 M HCl. These reactions were performed in duplicate. Total synovial fluid protein was estimated by the Biuret method.

ANALYSIS

Differences between continuously variable data were tested by the Wilcoxon rank sum test, and correlation by Spearman's method.

Results

Table 1 shows the numbers of synovial fluids examined (one per subject) with subject characteristics. The three groups were divided into those with active and inactive joints. Median PPi and NTPase data are shown graphically in the figure. Table 2 provides an analysis of all the data. The principal findings were:

1 The order of PPi concentrations and NTPase activities was pyrophosphate arthropathy> osteoarthritis> rheumatoid arthritis, with highly significant differences between all groups ($p<0·001$).

2 In pyrophosphate arthropathy both PPi and NTPase were higher than in normal controls. In rheumatoid arthritis PPi concentrations were lower than in controls, though NTPase activities were the same. Results for osteoarthritis, however, were the same as those for normal controls.

3 The inflammatory state of the knee at the time of aspiration had little effect on synovial fluid findings in osteoarthritis. In pyrophosphate arthropathy, however, PPi concentrations were

Table 1 Median (interquartile range) of inorganic pyrophosphate and NTPase by disease category and clinical activity

	n	Age	M:F	Inorganic pyrophosphate (μmol/l)	NTPase (μmol PPi/30 min/mg protein)
Osteoarthritis					
Active	34			10·2 (4·7–17·2)	0·29 (0·14–0·39)
Inactive	25			6·8 (4·0–10·7)	0·24 (0·16–0·48)
Total	59	70 (63–75)	1:0·97	9·3 (5·7–15·8)	0·25 (0·14–0·42)
Pyrophosphate arthropathy					
Active	32			12·8 (9·3–15·9)	0·45 (0·24–0·64)
Inactive	29			25·0 (16·6–36·8)	0·41 (0·31–0·73)
Total	61	77 (72–83)	1:1·91	15·9 (10·5–28·8)	0·45 (0·29–0·68)
Rheumatoid arthritis					
Active	20			5·7 (4·5–8·9)	0·13 (0·07–0·19)
Inactive	24			1·7 (0·9–4·8)	0·27 (0·17–0·47)
Total	44	63 (54–69)	1:1·90	4·4 (1·5–8·3)	0·18 (0·10–0·29)
Normal	50	44 (26–63)	1:1·38	8·6 (6·4–10·7)	0·2 (0·08–0·42)

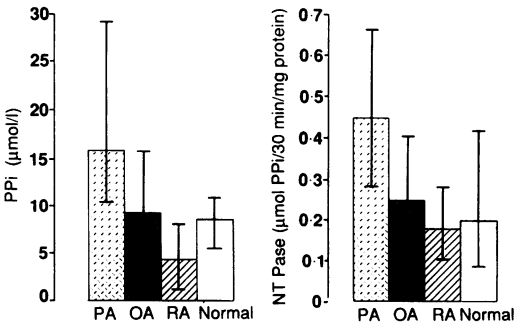
higher in inactive than in active knees, but there were no differences in NTPase. In rheumatoid arthritis, by contrast, PPi concentrations were higher in active joints, paradoxically accompanied by lower NTPase activity.

4 Neither PPi nor NTPase correlated with age in any group.

5 There was no correlation between the concentration of PPi and NTPase activity in any group.

Discussion

Several studies have examined PPi metabolism in patients with evidence of CPPD crystal deposition. Urine[7 32] and subsequently plasma[33 34] concentrations were reported to be the same as for normal controls. With development of more sensitive assays PPi concentration was estimated in synovial fluid and found to be greater than in matched plasma, the increase being reported to correlate with radiographic deterioration.[8] Smaller increases of synovial fluid PPi are also reported in osteoarthritis and in joints containing 'basic calcium phosphate' but not CPPD crystals[35]; the presence of consistently higher concentrations in synovial fluid than in matched plasma suggests local production within the joint. Synovial fluid NTPase activity has also been found to be increased when PPi is increased, suggesting that this enzyme is the major source of local PPi production.[1]



Median and interquartile range of inorganic pyrophosphate (PPi) and nucleoside triphosphate pyrophosphatase (NTPase) activity by diagnostic group.

Table 2 Significant findings for intergroup and intragroup comparisons

Between group comparisons	
PPi	PA>OA=Normal<RA (p<0·001) (p<0·001)
NTPase	PA>OA, Normal, RA (p<0·001) Normal<RA (p<0·002)
Within group comparisons	
PPi	PA Inactive>PA Active (p<0·001)
	RA Active >RA Inactive (p<0·001)
NTPase	RA Inactive>RA Active (p<0·002)

PPi=inorganic pyrophosphate; NTPase=nucleoside triphosphate pyrophosphatase; PA=pyrophosphate arthropathy; OA=osteoarthritis; RA=rheumatoid arthritis.

This study therefore accords with previous reports in showing (*a*) higher synovial fluid PPi concentrations and NTPase activities in pyrophosphate arthropathy than in osteoarthritis and (*b*) lower values in rheumatoid arthritis than in pyrophosphate arthropathy or osteoarthritis. For example, Rachow *et al*,[35] using the radiometric method of Cheung and Suhadolnik,[19] found significant differences in PPi concentrations in synovial fluid from various joints (knees, shoulders, hips) of 40 patients with osteoarthritis and 27 with pyrophosphate arthropathy (mean (SD) 14 (5) and 18 (8) μmol/l respectively); correspondingly higher NTPase activity was found in pyrophosphate arthropathy. Silcox and McCarty, using a differential colorimetric method, measured synovial fluid PPi in 35 patients with osteoarthritis, 29 with pyrophosphate arthropathy, and 12 with rheumatoid arthritis and found mean (range) concentrations of 9·2 (3–35), 9·8 (4–24), and 4·2 (2–8) μmol/l respectively[8]; the differences between rheumatoid arthritis and both osteoarthritis and pyrophosphate arthropathy were significant (Student's *t* test). Although these studies and ours agree about the differences between diagnostic categories, reported numerical values differ. This in part might reflect patient selection, differences in

PPi assay technique, and for NTPase estimations differences in correction to total (present study) rather than specific[26] proteins. Nevertheless, these differences are small and group values are within the same order of magnitude. Overall, such findings support the contention that local production of PPi relates to CPPD deposition, and that increased NTPase activity is a likely mechanism[2]; low values of PPi and NTPase in rheumatoid arthritis may also help to explain the negative association between rheumatoid arthritis and CPPD deposition.[36]

Certain observations, however, are not readily explained by NTPase regulated metabolism of PPi. In rheumatoid arthritis, for example, low concentrations of PPi occur in the presence of normal NTPase activity, and the lack of a direct correlation between PPi and NTPase in any group suggests that other factors may have a major effect on PPi concentrations. The importance of PPi metabolism in predisposition to sporadic CPPD crystal deposition must also be questioned.[37 38] Various factors may inhibit or promote crystal nucleation and growth,[37 39] and rates of crystal clearance and dissolution must be considered: the solubility product (calcium×PPi) may therefore not be the single most important determinant. The significance of factors apart from PPi is further emphasised by the discrepancy between the strong association between aging and CPPD deposition, and the apparent lack of age related change of PPi or NTPase in this study and in a larger cohort of normal subjects.[39] Temporal fluctuation in PPi concentrations is a further factor which has been poorly considered: differing increases of PPi influence not only CPPD but also apatite crystal formation,[40] and estimation at one timepoint may not be relevant to the crystals currently present. For this reason, cross-sectional studies relating single synovial fluid findings to 'radiographic damage'[8] are difficult to interpret, prospective studies being required to determine such relationships more precisely.

We recognise that there are inherent problems with all studies estimating concentrations in synovial fluid due to undetermined joint volumes and synovial mass, the unknown dynamics of production, breakdown and clearance, and the question of correction to other synovial fluid constituent concentrations. Furthermore, throughout such experiments it is generally assumed that synovial fluid concentrations reflect those in cartilage and we accept that this may not be valid.

In the study by Silcox and McCarty paradoxically lower concentrations of PPi in acute pseudogout than in chronic pyrophosphate arthropathy (n=18; mean 5·4 *v* 10·4 μmol/l) suggested an association between inflammation and PPi concentrations.[8] This was attributed to increased synovial flow and faster clearance of PPi. To date, however, few synovial fluid studies have characterised joints according to inflammatory state. There is no generally agreed method of clinically assessing knee inflammation, but the system we chose uses a summated score of six features and has been shown to correlate with complement activation in several arthritides.[28] Interestingly, we found the presence of such inflammation to have different associations in each condition: in rheumatoid arthritis it associated with increased PPi but lowered NTPase; in pyrophosphate arthropathy it associated with reduced PPi; and in osteoarthritis it had no discernible effect. Although such findings are difficult to explain, there are a number of ways in which inflammation might be expected to influence PPi metabolism (table 3) and the relative effects of these may vary in different conditions. The apparent lack of association with inflammation in osteoarthritis is of special interest: unlike rheumatoid arthritis, synovial fluid parameters, such as complement activation products[28] or PPi, were insensitive and did not reflect even florid clinical inflammation in osteoarthritis, suggesting that the mechanisms of inflammation in rheumatoid arthritis and osteoarthritis are different. Such discrepancies indicate that comparative synovial fluid studies should consider degree of inflammation as well as diagnostic categories.

Table 3 Factors influencing synovial fluid component concentration

1 Rate of PPi production
NTPase, cytolysis, other nucleoside pyrophosphatases
2 Rate of PPi destruction
Hydrolysis, enzymic (pyrophosphatase and lysosomal)
Non-enyzmic (temperature and pH dependent)
3 Removal of PPi from synovial fluid
Vascular, blood flow, lymphatic flow/stasis
Leucocyte traffic
4 Effects on NTPase
Proteolytic enzymes, pH, temperature

PPi=inorganic pyrophosphate; NTPase=nucleoside triphosphate pyrophosphatase.

Data on non-cadaveric, normal, human synovial fluids are understandably sparse. For PPi the results for only five normal joints have been published,[5] giving a mean value of 3·6 μmol/l; we are unaware of any data for NTPase. In this study, using a different assay, we found the mean PPi concentration from 50 normal knees to be 9·3 μmol/l; values for normal synovial fluid NTPase activity are also presented. Increases of synovial fluid PPi and NTPase in pyrophosphate arthropathy, compared with normal or osteoarthritis, may reflect increased biosynthetic activity within the joint rather than implying any specific alteration in PPi metabolism. Such an interpretation would accord with the 'hypertrophic response' (florid osteophytosis, cysts, remodelling) and possible good outcome[3] which have been associated with pyrophosphate arthropathy. Conversely, low synovial fluid concentrations of PPi in rheumatoid arthritis may reflect enzymic hydrolysis of PPi to orthophosphate, or an 'atrophic' articular response to insult.

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