Pyrophosphohydrolase Activity and Inorganic Pyrophosphate Content of Cultured Human Skin Fibroblasts

Elevated Levels in Some Patients with Calcium Pyrophosphate Dihydrate Deposition Disease

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Abstract

In calcium pyrophosphate dihydrate (CPPD) crystal deposition disease, metabolic abnormalities favoring extracellular inorganic pyrophosphate (PPi) accumulation have been suspected. Elevations of intracellular PPi in cultured skin fibroblasts from a single French kindred with familial CPPD deposition (19) and elevated nucleoside triphosphate pyrophosphohydrolase activity (NTPPPH), which generates PPi in extracts of CPPD crystal-containing cartilages (14) favor this suspicion. To determine whether NTPPPH activity or PPi content of cells might be a disease marker expressed in extraarticular cells, human skin-derived fibroblasts were obtained from control donors and patients affected with the sporadic and familial varieties of CPPD (CPPD-S and CPPD-F) deposition.

Intracellular PPi was elevated in both CPPD-S ($P < 0.05$) and CPPD-F ($P < 0.01$) fibroblasts compared with control fibroblasts. Ecto-NTPPPH activity was elevated in CPPD-S ($P < 0.01$) but not CPPD-F. Intracellular PPi correlated with ecto-NTPPPH ($P < 0.01$).

Elevated PPi levels in skin fibroblasts may serve as a biochemical marker for patients with familial or sporadic CPPD crystal deposition disease; ecto-NTPPPH activity further separates the sporadic and familial disease types. Expression of these biochemical abnormalities in nonarticular cells implies a generalized metabolic abnormality.

Introduction

In calcium pyrophosphate dihydrate (CPPD)[1] crystal deposition disease, a disorder of inorganic pyrophosphate (PPi) metabolism is suspected because: (*a*) CPPD crystals contain PPi; (*b*) synovial fluid (SF) PPi concentrations are elevated with respect to plasma (1–4); and (*c*) CPPD crystal deposition occurs with increased frequency in adults with hypophosphatasia (5–7), a condition associated with elevated plasma and urinary PPi levels (8, 9). But systemic abnormalities in PPi metabolism have not been identified in most cases of CPPD crystal deposition disease (10). Chondrocytes have been postulated to secrete PPi, since both normal and degenerated articular cartilage in organ culture, but not synovium, subchondral bone, or elastic (ear) cartilage, have elaborated extracellular PPi (11, 12). SF PPi pool and turnover studies in human knee joints suggested PPi production rates of 0.5–1 μmol/h (13), compatible with the quantities liberated from human cartilage slices in organ culture (11, 12).

Tenenbaum et al. found elevated nucleoside triphosphate pyrophosphohydrolase (NTPPPH) activity in Triton X-100 extracts of degenerative CPPD crystal-containing cartilages compared with extracts of normal cartilages or extracts of degenerative crystal-free cartilages (14). This chondrocyte ectoenzyme generated PPi from a number of substrates (15, 16). NTPPPH activity was also increased in SF from patients with CPPD deposition (17).

Lust et al. detected elevated intracellular PPi contents in cultured skin fibroblasts and in Epstein-Barr virus–transformed lymphocytes from 10 affected members of a French kindred with familial CPPD deposition, compared with cells derived from nonaffected family members (18, 19), and cells in a single case of sporadic CPPD crystal deposition (20). These investigators postulated a generalized metabolic aberration of PPi metabolism in patients with CPPD crystal deposition, manifested phenotypically only in chondrocytes. However, interpretation of these data is hampered by the paucity of control values defining normal intracellular PPi and by the considerable overlap between the elevated values in fibroblasts from the French familial cases and the normal values reported in a prior study by the same laboratory (20).

In view of the obvious importance of a biochemical correlate with CPPD crystal deposition, we assayed intracellular PPi in cultured skin fibroblasts from donors in three kindreds affected with CPPD crystal deposition, from patients with sporadic CPPD crystal deposition, and, for comparison, from normal individuals and patients with primary osteoarthritis (OA). Additionally, we studied selected ectoenzyme (5′ nucleotidase, inorganic pyrophosphatase, and NTPPPH) activities in these cells to determine whether the elevation of NTPPPH activity observed in SF and cartilage extracts of patients with CPPD crystal deposition also occurs in nonarticular tissues.

Methods

Patients. Patients with sporadic CPPD crystal deposition ($n = 13$) had definite disease, as defined elsewhere (10). Familial CPPD donors (n

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1. *Abbreviations used in this paper:* CPPD, calcium pyrophosphate dihydrate; CPPD-F, familial CPPD crystal deposition disease; CPPD-S, sporadic CPPD crystal deposition disease; DME, Dulbecco's modified Eagle's medium; NTPPPH, nucleoside triphosphate pyrophosphohydrolase; OA, osteoarthritis; PCA, perchloric acid; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; SF, synovial fluid; UDPG, uridine diphosphate glucose; 10-1-D, 10% fetal calf serum and 1% penicillin-streptomycin-Fungizone.

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= 11) were from three well-studied kindreds expressing CPPD deposition as an autosomal dominant trait. Details of two of these kindreds have been published (21, 22). Simultaneous control biopsies were obtained from spouses of affected familial members. Patients with sporadic CPPD crystal deposition were studied for associated metabolic disease by measurements of serum iron, iron binding capacity, magnesium, free thyroxin, alkaline phosphatase, calcium, and phosphorus. Patients with primary OA (n = 13) had negative joint fluid exams for CPPD crystals and no radiographic evidence of chondrocalcinosis. As nearly half of nonagenarians develop sporadic CPPD crystal deposits, an overlap of values from normal subjects with those from patients with sporadic disease was anticipated. Assay of PPi content and enzyme activities in samples from patients with OA were thought to be a better control since these donors did not develop CPPD crystal deposition, even in stressed cartilage. Laboratory and office personnel without joint symptoms also served as normal donors, in addition to asymptomatic spouses of familial donors (n = 7).

Materials. Tetrasodium pyrophosphate decahydrate, ATP, uridine diphosphate glucose (UDPG), NADP, 5'AMP, trypsin, EDTA, glucose-1,6-diphosphate, phosphoglucomutase, $MgCl_2$, ammonium molybdate, inorganic pyrophosphatase, and Tris were purchased from Sigma Chemical Co., St. Louis, MO; [^{14}C]UDPG and [^{32}P]PPi were purchased from New England Nuclear Corp., Boston, MA. Dulbecco's modified Eagle's medium (DME), Spinner's modified Eagle's medium, and Hank's balanced salt solution (HBSS) were obtained from Gibco, Grand Island, NY. Fetal bovine serum was purchased from MA Bioproducts, Walkersville, MD.

Biopsies. Biopsies were obtained from forearm skin in a manner identical to that described by Lust et al. (18). Explants were transported in DME supplemented with 10% fetal calf serum and 1% penicillin-streptomycin Fungizone (10-1-D).

Explants. The biopsy was washed with 10-1-D. Any hairs were removed. The specimen was cut into 3–4 pieces using a sterile scalpel blade. A 25-cm^2 tissue culture flask which contained 4 ml of 10-1-D was placed upright for 10–15 min before the pieces of tissue were transferred with a Pasteur pipette. The flask was incubated upright for 30 min at 37°C in 10% CO_2 before it was gently placed flat. The flask was left undisturbed for 2–3 d. If the pieces of tissue did not attach, they were transferred to a tissue culture dish with a Pasteur pipette. A sterile microscope coverslip was placed over them and 6 ml of 10-1-D added to the dish. The explants were fed twice weekly with 10-1-D, with care not to dislodge the attached tissues. At confluence, cells were transferred to 2 tissue culture dishes by washing with HBSS and incubating for 5 min at 37° with 0.05% trypsin, 0.2% EDTA in HBSS. 7.5 ml 10-1-D were added to each dish. The second transfer was made to four 100-mm and eight 35-mm diameter tissue culture plates.

Biological variables. Our original attempts at measuring intracellular PPi yielded results which suggested uncontrolled variables. The effect of independently varying cell density, transfer number, and age of culture on intracellular PPi levels was studied. After these studies, all determinations outlined below were performed on cultures of second transfer cells, at 100% visual confluence, ~6 wk after the initial biopsy and 48 h after the most recent media change.

Assay for PPi. PPi formed during the NTPPPH reaction was determined by a modified method of Cheung and Suhadolnik (23). Briefly, [^{14}C]UDPG was quantitatively converted enzymatically to [^{14}C]glucose-1-PO_4 and uridine triphosphate in the presence of PPi. Differential charcoal adsorption of [^{14}C]UDPG before and after the reaction in the assay was directly related to PPi concentration. Standard PPi concentrations from 0.6 to 10.0 μM were measured with each assay.

Intracellular levels of PPi were determined similarly, using standard PPi concentrations from 0.06 to 1.0 μM. Because these samples also contained [^{32}P]PPi, the observed ^{14}C was corrected for the overlap of ^{32}P counts in the ^{14}C channel.

Standard stock PPi solutions (16 mM) were assayed for inorganic phosphate (Pi) content by the method of Fiske and SubbaRow (24) before and after hydrolysis by yeast inorganic pyrophosphatase as described previously (25). This is necessary since stock $Na_4P_2O_7 \cdot 10H_2O$ powder readily gains or loses waters of hydration (25), which makes calculations of molarity by weight inaccurate.

Intracellular PPi. Cells from each of two 100-mm culture plates were harvested and PPi content of each population was measured. The media was decanted and plates were washed twice with 5 ml PPi-free HBSS to remove dead cells and serum. The HBSS had been rendered PPi-free by treating with yeast inorganic pyrophosphatase, then filtering through a Centriflo CF-25 filter (Amicon Corp., Lexington, MA) to remove pyrophosphatase. The sides of the plates were wiped dry. The plates were placed on ice and 0.5 ml cold pyrophosphatase-treated (PPi-free) HBSS containing trace [^{32}P]PPi and 1.5 M perchloric acid (PCA) was then added. The plates were rotated to evenly distribute the PCA and then 0.025 ml cold 3M Tris was added. The cells were scraped off with a rubber policeman and the suspension transferred to a cold glass test tube. The tube was centrifuged for 5 min at 650 g (Clinical Centrifuge; International Equipment Co., Damon Corp., Needham Heights, MA). The sediment was saved for the determination of DNA. The supernatant was neutralized with ~80–95 μl of 5 N KOH. It was again centrifuged and the supernatant was frozen at −20°C for PPi determination. Known amounts (0.1–5.0 μM) of PPi added to neutralized PCA supernatants and frozen at −20°C served as internal standards. PPi recovery was estimated by the recovery of unhydrolyzed [^{32}P] PPi.

NTPPPH activity. NTPPPH activity was determined by measuring the amount of PPi formed during incubation of cells in monolayer culture with 1 mM ATP. A 100-mm culture plate was washed 3 times with DME to remove all of the fetal calf serum. Then 2 ml DME containing 1 mM ATP, 50 mM Tris Cl, pH 7.2, was added. The plate was incubated at 37°. A 200-μl sample of media was removed at 30 min and stored at −20° for PPi determination. At 60 min, the rest of the media was removed and stored at −20° for PPi determination. The cells were released by trypsin treatment for DNA analysis. In preliminary studies we have determined that >95% of the PPi generated results from NTPPPH activity, as we have previously shown in cartilage organ culture studies.

PPi hydrolysis. The amount of [^{32}P]pyrophosphate hydrolyzed during the pyrophosphatase assay was determined by the differential precipitation of phosphorus as phosphomolybdate by triethylamine (26). 200 μl of the sample was placed in a 1.5-ml microfuge tube. 10 μl 10 mM $NaPHO_4$, 40 μl 1.0 M PCA, 40 μl 5% ammonium molybdate, and 10 μl of 0.2 M triethylamine HCl were added sequentially with vortexing. The sample was centrifuged for 10 s at 8,700 g in a microfuge (Brinkmann Instruments Co., Westbury, NY). The supernatant was transferred to a 20-ml scintillation vial which contained 10 ml of 0.5 M HCl. The precipitate was washed, centrifuged twice more, and these supernatants were also added to the scintillation vial. The precipitate was dissolved in 400 μl acetone and transferred to another scintillation vial containing 10 ml 0.5 M HCl. Radioactivity present in each vial was determined by Cerenkov counting as described previously (12). The percentage of hydrolysis in the blank was subtracted from the sample value. PPi hydrolysis in the sample was then recorded.

Pyrophosphatase activity. Two confluent 35-mm culture plates of fibroblasts in their second transfer were used for duplicate determinations. The plates were washed twice with DME and then once with HBSS. 1.5 ml of reaction mixture consisting of 640 μl 20 mM PPi, 40 μl 50 mM Tris Cl buffered Spinner's modified Eagle's medium, pH 7.4, 0.2 ml 40 mM EGTA, 8 ml 2mM $MgCl_2$, and sufficient [^{32}P]PPi to provide 10,000 cpm/100 μl was added to each plate. The plates were incubated at 37°C for 30 min. A blank plate with no cells was also run. The media was removed and stored at −20° for hydrolysis determination. The cells were released by treatment with 0.05% trypsin for DNA determination.

5' Nucleotidase activity. The amount of Pi released when the monolayer fibroblasts were incubated with AMP was measured as described by Edwards (27). The method of Chen (28) was used to measure the Pi. Confluent 35-mm cultures were washed three times with 10 mM Tris HCl, pH 7.4, in 150 mM NaCl. 2 ml containing 50 mM Tris, 4.0 mM $MgCl_2$, 150 mM NaCl, and 0.4 mM AMP were added to each plate and incubated at 37°C. 100-μl aliquots were removed at 5, 10, and 15 min. 100 μl of 0.150 M NaCl was added to each sample in plastic test tubes. 800 μl of a solution consisting of 2% ascorbic acid, 0.5% ammonium molybdate, 1.2 N sulfuric acid was then added and incubated for 1–2 h. The absorbance was measured at 820 nm on a DU 6 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) and activity was expressed

as picomoles Pi per microgram DNA. The cells were then released with 0.05% trypsin for the determination of DNA.

DNA assay. DNA was determined by a modification of the method of Burton (29). The released cells were resuspended in 1 ml HBSS and 100 μl 70% PCA was added. The macromolecular precipitate was removed by centrifuging at 1,700 *g* for 30 min and stored at −20°C. The thawed precipitate was resuspended in 200 μl TrisCl, pH 7.4, and 200 μl 0.4 N PCA, vortexed, and allowed to stand for 5 min. After centrifugation at 1700 *g* for 30 min at 4°C, 600 ml of 1.5 M PCA was added. DNA standards from 5 to 35 μg were prepared in 600 μl of 1.5 M PCA. The glass tubes containing standard or sample were incubated at 70°C for 30 min and allowed to cool at room temperature; 350 μl diphenylamine reagent (4.0 g diphenylamine and 10 μl paraldehyde in 100 ml glacial acetic acid) was added to each tube. The tubes were incubated in the dark for 18 h at room temperature, centrifuged for 30 min at 1700 *g*, and the absorbance at 600 nm was read in a spectrophotometer (model 36; Beckman Instruments, Inc.).

Results

Biological variables. Our first attempts at measuring intracellular PPi content yielded widely discordant values on cells derived from the same explant, suggesting uncontrolled variables. Table I shows the results of PPi measurements in cultures of cells from six donors at different stages of visual confluence during second and third passage. In all samples, intracellular PPi was lower postconfluence than at 50% or 75% confluence. Values at comparable stages of visual confluence were more often lower at third passage than at second. The effect of age in culture was assessed by measuring intracellular PPi in monolayer cells derived from a single explant on second passage at visual confluence and 48 h after media replenishment, but plated on second passage at different cell densities so that confluence was reached at different times. Table II shows the mean of duplicate determinations, which suggest that intracellular PPi decreases with age in culture. After these experiments, cellular PPi and ectoenzymes were routinely measured on cells ~6–7 wk after biopsy, at visual confluence, on second passage, and precisely 48 h after media replenishment.

Table I. Intracellular PPi at Various Stages of Visual Confluence and Passage Number

Patient	Passage no.	50%	75%	95%	Post-confluence
1	T2	144*	125	37	24
	T3	58	65	68	1
2	T2	272	116	46	87
	T3	86	77	139	56
3‡	T2	93	98	68	32
	T3	—	23	34	—
4‡	T2	87	146	46	—
	T3	149	136	72	8
5	T2	—	77	35	9
	T3	25	33	25	—
6	T2	17	18	17	4
	T3	37	51	8	1
Mean±SD	T2	123±95	97±45	46±17	31±33
	T3	71±47	65±40	55±47	17±26

Intracellular PPi was measured in duplicate on fibroblasts derived from skin explants of six donors. On second (T2) or third (T3) passage, cells on duplicate plates at various stages of visual confluence were washed, lysed with 1.5 M PCA, PPi measured on the potassium hydroxide–neutralized supernatant, and DNA measured in the macromolecular precipitate. PPi measurements were by the radiometric UDPG pyrophosphorylase method (23).

* pmol PPi/μg DNA.

‡ Patients with CPPD deposition disease.

*Table II. PPi Content of Fibroblasts Grown from a Single Explant Measured at Different Ages but at Identical Stages of Confluence and Passage**

Age‡	PPi
wk	*pmol/μg DNA*
4	56
6	17
14	7
16	4

Fibroblasts derived from a single explant were passaged and plated on second passage (T2) so as to achieve visual confluence at various intervals. Duplicate plates reached confluence at 4, 6, 14, and 16 wk at which time PPi was measured on the potassium hydroxide–neutralized PCA cell lysate.

* Measured at visual confluence on second passage.

‡ Interval between biopsy and PPi measurement.

Intracellular PPi. Intracellular PPi content was significantly higher in fibroblasts from both sporadic and familial CPPD donors compared with control fibroblasts from normal donors or patients with primary OA ($P < 0.0002$ for all CPPD vs. all others, Wilcoxon's rank sum test). Mean values and statistical findings are listed in Table III. As shown in Fig. 1, there is considerable intergroup overlap despite highly significant differences. The arrows indicate values from two patients enrolled in the primary OA group. However, subsequent joint fluids from the knees of these patients contained CPPD crystals. Differences in PPi content were not related to donor age, unlike our previous findings in human platelets (30).

Ectoenzymes. Ectopyrophosphatase, ecto-5′NT and ecto-NTPPPH activities were present in all cultures. No significant differences in mean values were observed between different diagnostic groups for 5′ nucleotidase or pyrophosphatase ($P = 0.11$

Table III. Intracellular PPi in Skin-derived Fibroblasts

Group	PPi	*P* (Wilcoxon's)
	pmol/μg DNA	
CPPD-S	44.3±77.8*	
CPPD-F	44.6±33.4	
		<0.0002
OA	12.4±9.8	
Normal	19.7±11.6	

<0.05

<0.01

Significance of intergroup differences in intracellular PPi content were determined by a nonparametric method using the Wilcoxon's rank sum test.

* Mean±SD

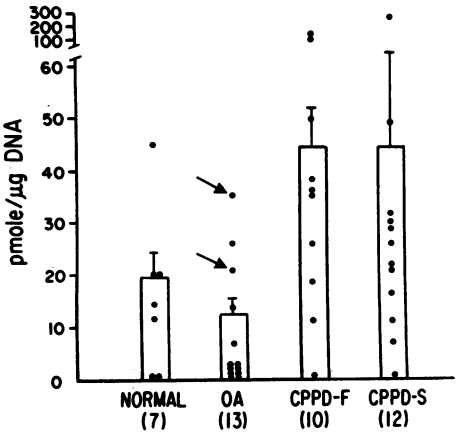

Figure 1. Fibroblast PPi content in cells from normal donors and patients with OA, CPPD-F, and CPPD-S. The arrows identify values from two patients originally classified as having OA in whom CPPD crystals were subsequently found in joint fluid. Values are the mean of duplicate cell samples.

and 0.5, respectively for all CPPD vs. all other). Mean values of NTPPPH±SD for the four groups were: sporadic CPPD (CPPD-S), 9.9±4.8, familial CPPD (CPPD-F), 6.7±3.9; OA, 7.1±10.1; normal, 4.6±3.2. Ecto-NTPPH was significantly elevated in CPPD ($P < 0.002$ for all CPPD vs. all others, Wilcoxon's test). Most of the significant differences resulted from the CPPD-S values which were significantly higher than CPPD-F values ($P < 0.01$, Wilcoxon's test). When CPPD-F values were compared separately with OA or normal values, they were not significantly elevated. The cells derived from two patients entered in the primary OA group and subsequently found to have CPPD deposition contained elevated NTPPPH activity (Fig. 2). Intracellular PPi correlated with ecto-NTPPPH activity ($r_s = 0.49$, $P < 0.05$, Spearman's rank correlate).

Discussion

These studies suggest that two biochemical markers of disordered PPi metabolism are expressed in cultured cells derived from an extraarticular tissue in patients with CPPD crystal deposition disease. Intracellular PPi is a by-product of multiple biosynthetic reactions (31) and its hydrolysis by intracellular pyrophosphatase has long been considered as an important homeostatic mechanism favoring biosynthesis (32). Elevated intracellular PPi levels could result from increased biosynthesis or decreased pyrophosphatase activity. Whether intracellular PPi participates in extracellular CPPD crystal formation is doubtful, since PPi does not readily diffuse across membranes (33). However, it is possible that PPi may be extruded from cells along with materials that are being exported, e.g., proteoglycans or collagen.

The elevated ecto-NTPPPH activity of CPPD-S fibroblasts suggests a systemic counterpart to the previously reported increased activity in CPPD synovial fluid and cartilage (16, 17). The biological role of this enzyme is uncertain but has variously been hypothesized to represent a salvage mechanism for reclamation of purines derived from nucleotides that escape from cells (34), a membrane-bound Ca^{++} pump (35), or a regulator of plasma membrane protein kinase (36). A hypothetical role of ecto-NTPPPH in CPPD pathogenesis is attractive, as NTPPPH can generate extracellular PPi at the extracellular site where crystals most likely form (10). However, an extracellular substrate for this ectoenzyme has not yet been found.

We have no explanation for the observed correlation between ecto-NTPPPH and intracellular PPi content. It is not likely a result of cell size (larger cells would contain more intracellular PPi due to greater volume and more ectoenzyme due to greater surface area) because other surface enzymes (ectopyrophosphatase and ecto-5′ nucleotidase) did not correlate with intracellular PPi and because the cells were all visually confluent at the time of measurement, diminishing potential size variability. Perhaps intracellular PPi may be generated by NTPPPH before its translocation. If this occurs in a cellular compartment where pyrophosphatase activity is low, intracellular PPi may accumulate.

Recently, Yoshida et al. have reported excess NTPPPH in whole-cell detergent extracts of skin fibroblasts from patients with Lowe's syndrome (37). The cellular site of the elevated activity was hypothesized to be the endoplasmic reticulum, not the cell surface. In the endoplasmic reticulum, excess NTPPPH (which degrades 3′-phospho-adenosine 5′-phosphosulfate as well as ATP) would deplete 3′-phospho-adenosine 5′-phosphosulfate. Such depletion could account for the undersulfation of glycosaminoglycans synthesized by culture skin fibroblasts of patients with Lowe's syndrome (38).

In summary, biochemical abnormalities related to PPi and PPi metabolism are identified in cultured extraarticular tissues of patients with CPPD crystal deposition disease. These data support the contention of Lust, Seegmiller, et al. that a generalized metabolic defect expressed phenotypically only in cartilage underlies CPPD crystal deposition disease.

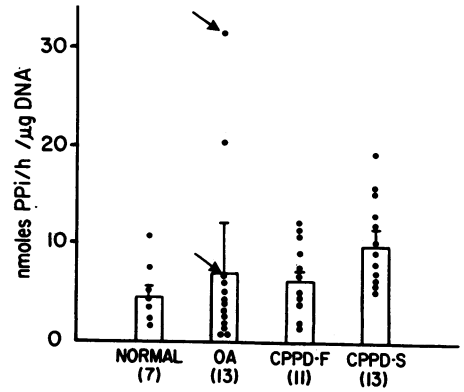

Figure 2. Fibroblast ecto-NTPPPH in cells from normal donors and patients with OA, CPPD-F, and CPPD-S. The arrows identify values from two patients originally classified as having OA in whom CPPD crystals were subsequently found in joint fluid. Values are the mean of enzyme activity found in two plates of cultured cells.

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