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INCREASED PHOSPHORIBOSYLPYROPHOSPHATE SYNTHETASE ACTIVITY IN FIBROBLASTS OF HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE DEFICIENT PATIENTS

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SUMMARY: An increased activity of phosphoribosylpyrophosphate synthetase at physiological levels of inorganic phosphate is demonstrated in extracts of skin fibroblast cultures derived from a patient with Lesch-Nyhan syndrome. This excessive response of the phosphoribosylpyrophosphate synthetase at physiological levels of inorganic phosphate results in increased levels of phosphoribosylpyrophosphate and thus contributes to purine overproduction characteristic of this disorder. The level of enzyme response in skin fibroblast extracts from the carrier mother was between activity of the patient and normals, further suggesting the x-linkage of human phosphoribosylpyrophosphate synthetase.

INTRODUCTION: The Lesch-Nyhan syndrome is an x-linked recessive disorder associated with a severe deficiency of the purine salvage pathway enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) (1). Clinically, it is characterized by spastic cerebral palsy, mental retardation, choreoathetosis, compulsive self-mutilation, hyperuricemia, and excessive excretion of uric acid (2). A partial deficiency of this enzyme has also been described in some patients with severe gout without apparent neurological abnormalities (3,4). According to the Lyon Hypothesis (5), in most eutherian mammals one x-chromosome in each female somatic cell is randomly inactivated at an early stage of embryonic development; females are thus mosaics with respect to the expression of x-linked heterozygous loci (6). As a result cultures of skin fibroblasts from obligate heterozygotes for the severe HGPRT deficiency have shown two populations of cells, one having normal HGPRT activity and the other having deficient HGPRT activity (7). In these heterozygotes red blood cell population normal activity has generally been found (8) and probably as a result of cell selection. On the other hand, in heterozygotes for the partial deficiency state, as in the case of 1-2% of patients with primary metabolic gout (9), HGPRT activities in red cell hemolyzates between 22% and 75% of normal have been found (10).

In the excessive production of uric acid as a consequence of overproduction of purine nucleotides, the role of increased intracellular concentration of 5-phosphoribosyl-1 pyrophosphate (PP-ribose-P), the precursor of the ribose-phosphate

Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyl transferase; PP-ribose-P, 5-phosphoribosyl-1 pyrophosphate; APRT, adenine phosphoribosyl transferase.

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moiety of purine, pyrimidine and pyridine nucleotides has been emphasized in a number of studies (11,12).

PP-ribose-P is an activated sugar phosphate compound which is synthesized from ATP and ribose-5-phosphate in a reaction which is magnesium and inorganic phosphate dependent and catalyzed by the enzyme PP-ribose-P synthetase (E.C. 2.7.6.1). The PP-ribose-P is a substrate in the first reaction of de novo purine synthesis catalyzed by the enzyme phosphoribosylpyrophosphate amidotransferase as well as in the so-called salvage pathway for purine nucleotides in which purine bases are re-utilized to form nucleotides in reactions catalyzed by the x-linked enzyme HGPRT (E.C. 2.4.2.7) and the autosomal-linked adenine phosphoribosyl transferase (APRT) (E.C. 2.4.2.6).

An HGPRT deficient state is considered to promote purine overproduction by two mechanisms: (a) accumulation and elevation of the PP-ribose-P level; this compound serves as the substrate for the first rate-limiting step of de noto purine synthesis, hence its elevation stimulates de novo synthesis; and (b) decrease of feedback inhibition of the same step due to decrease in nucleotide (IMP, GMP) concentrations (13,14). On the other hand, a depletion of the level of intracellular PP-ribose-P by purines, purine analogs or pyrimidines is associated with decreased activity of both de novo and salvage pathway of purine synthesis (15,16). The important regulatory role of PP-ribose-P in these pathways is emphasized by the alterations in the rate of purine synthesis associated with altered intracellular concentration of PP-ribose-P (15,16).

Our studies reveal a new and additional feature of the disorder cells, namely an altered PP-ribose-P synthetase system in fibroblasts derived from both a Lesch-Nyhan patient and his carrier mother, in which increased PP-ribose-P synthetase activity is found at low levels of inorganic phosphate concentration; this is not seen in fibroblast cultures derived from normals.

MATERIALS AND METHODS: Materials: Ribose 5-phosphate and ATP were purchased from Boehringer-Mannheim, yeast orotate phosphoribosyl transferase and phosphoribosyl pyrophosphate were from Sigma Chemical Co. The radioactive compounds were obtained from New England Nuclear. The NCS (trademark) tissue solubilizer and Spectrafluor liquid scintillator were from Amersham-Searle.

Skin fibroblasts from a 12-year-old boy with the Lesch-Nyhan syndrome (GM 2227) and his 45-year-old mother (GM 2226) were obtained from the Institute for Medical Research, Camden, New Jersey. Control skin fibroblasts (GM 730) were also obtained from the same source as well as from a skin biopsy performed in our laboratory (C.A.). The primary explant was cultured in a modified Eagle's Minimum Essential Medium (17) supplemented with 2mM glutamine, 20mM $NaHCO_3$, 15% fetal calf serum and penicillin and Streptomycin. All the cells were subcultured at confluency in the same medium with 10% fetal calf serum and without antibiotics. To determine the number of population doublings the cells were counted using an hemocytometer after each trypsinization and were always seeded at the same cell density. The cells were examined for mycoplasma contamination by the procedures of Levine (18), Chen (19) and by culturing in different media.

The functional activities of the enzymes HGPRT and APRT were determined radiochemically in intact fibroblasts by measuring the rate of conversion of [^{3}H]-hypoxanthine (5 Ci/mmol) and [^{3}H]-adenine (20.2 Ci/mmol) to their ribophosphorylated products. In addition the incorporation of the labelled purine bases into nucleic acids was measured.

PP-ribose-P synthetase in dialyzed cell-free extracts was determined radiochemically at saturating concentrations of the respective substrates. The procedure used is a modification of the method described by Reem (20) which is based on the assay by Kornberg et al. (21). The PP-ribose-P generated from ATP and ribose-5-phosphate is measured by determining [^{14}C]CO_2 release from [^{14}C-carboxyl]-orotic acid (41.25 mCi/mmol). Orotidylate and uridylate synthesis were catalyzed by the addition of a partially purified extract of yeast orotate phosphoribosyl transferase (orotidine-5'-phosphate: pyrophosphate phosphoribosyl transferase, E.C. 2.4.2.10, and oritidine-5'-phosphate decarboxylase, E.C. 4.11.23). This method is suitable for measuring PP-ribose-P synthetase activity in a one-step assay.

The assays were carried out in Warburg flasks containing in the center well a roll of Whatman #3 filter paper (1.6 x 3.3 cm) wetted with 130 µl of NCS tissue solubilizer. One hour prior to harvesting the cells the medium was replaced with fresh medium containing 10% dialyzed calf serum. The medium was separated from the cells, which were washed three times in cold phosphate-buffered saline, pH 7.3 and were harvested by scraping in 1 ml of 1 mM phosphate buffer, pH 7.3, containing 1 mM EDTA and 1 mM β-mercaptoethanol. After the cells were harvested they were sonicated for 20 seconds using a Biosonik III at 20% output, and an aliquot was removed for protein determination. For PP-ribose-P determination the suspension was immediately heated to 95°C for 60 seconds, chilled at 4°C and centrifuged to remove proteins. To measure PP-ribose-P synthetase the suspension after centrifugation was dialyzed for 3 hours against three changes of 1 mM phosphate buffer, pH 7.3 containing 1 mM EDTA and 1 mM β-mercaptoethanol.

The reaction mixture for the determination of PP-ribose-P synthetase was incubated at 37°C at pH 7.3 in a total volume of 500 µl and contained various concentrations of inorganic phosphate ranging from 1 to 50 mM, 2.5 mM ATP, 2.5 mM ribose-5-phosphate, 2.5 mM $MgCl_2$, 1.0 mg of yeast orotate phosphoribosyl transferase and 0.25 µCi in a volume of 10 µl of [^{14}C-carboxyl]-orotic acid. The reaction was started by adding 50λ of the cell extract placed in the side arm to the reaction mix at the bottom of the Warburg flask which had been warmed at 37°C. At the end of the incubation period the reaction was stopped by placing the Warburg flasks in ice. The paper rolls were immediately removed and placed in a scintillation vial containing 0.2 ml of H_2O. After the addition of 15 ml of a toluene-based scintillation fluid the vials were counted in a Beckman Scintillator counter with 94% efficiency. The reaction was dependent on ribose-5-P, ATP and Mg and was stimulated by inorganic phosphate. The reaction was linear up to 30 minutes and proportional to protein added. The amount of protein added in 50 µl volume was maintained practically constant in all the determinations. Suitable blanks containing the same amount of heat inactivated enzyme and for each inorganic phosphate concentration were run in parallel. For the determination of PP-ribose-P content the ATP and ribose-5-phosphate were omitted from the reaction mixture and 50λ of the heated cell extract was used. The amount of PP-ribose-P was calculated from standard solutions of PP-ribose-P which were run in parallel; the results were corrected for the blank values obtained without added PP-ribose-P. Assays were linear with PP-ribose-P concentrations ranging from 0.05 mM to 10 mM and in 15 min incubation recovery of PP-ribose-P was complete. All the studies were carried out on freshly harvested cells. For each cell extract the results represent the average of at least five determinations. The method of Lowry et al. (22) was used for protein determination.

RESULTS: The incorporation studies of [^{3}H]-hypoxanthine in Lesch-Nyhan GM 2227 fibroblasts in the logarithmic phase confirmed the extreme deficiency of HGPRT. Practically no [^{3}H]-hypoxanthine was incorporated in soluble nucleotides or in nucleic acids after 2 hrs. incubation. Fibroblasts from the heterozygous mother (GM 2226) of the Lesch-Nyhan patient showed lower incorporation of [^{3}H]-hypoxanthine in nucleotides and nucleic acids than fibroblasts of normal individuals of approximately the same age and approximately the same population doublings. Nevertheless the incorporation was much higher than in her son's fibroblasts. The incorporation of [^{3}H]-adenine in all cases was within the range observed in normal skin fibroblasts at similar population doublings. The use of intact fibroblasts provides an accurate appraisal of the functioning of the salvage pathway at the cellular level, similar to what has been found by other investigators (23,24). These studies were repeated at different population doublings with similar results.

The PP-ribose-P content and PP-ribose-P synthetase activity levels for the Lesch-Nyhan and heterozygous fibroblasts as well as for normal fibroblasts at different population doublings are shown in Figure 1. It is evident that the PP-ribose-P levels and the activity of the PP-ribose-P synthetase at 5 mM and 50 mM inorganic phosphate concentrations are higher in the fibroblasts of the Lesch-Nyhan patient and in the heterozygous carrier than in the normal fibroblasts. The difference is much more marked at 5 mM than at 50 mM inorganic phosphate concentrations. It is noteworthy that the activities of PP-ribose-P synthetase at both Pi concentrations increased with increasing population doublings in the Lesch-Nyhan and heterozygous carrier fibroblasts. The activity of the PP-ribose-P synthetase in skin fibroblasts was higher during the early logarithmic phase of growth and decreased in early and late stationary phase. Similar observations were reported by Martin and Maler (25). The maximum activity was generally observed 48 hours after seeding, but in some cultures the maximum was observed 24 hours after seeding. These results suggested an abnormality in PP-ribose-P synthetase or its regulation; this enzyme which catalyzed PP-ribose-P formation might contribute to the defect in the Lesch-Nyhan patient. As shown in Figure 2, fibroblast extracts from the Lesch-Nyhan patient, in the presence of saturating amounts of ATP and ribose-5-phosphate and at inorganic phosphate concentrations from 1 to 50 mM, were found to have higher PP-ribose-P synthetase activity than normal fibroblasts. The fibroblast extracts from the heterozygous carrier showed intermediate level between normal fibroblasts and her Lesch-Nyhan son. The difference in activity of the PP-ribose-P synthetase in fibroblasts from the Lesch-Nyhan patient compared with the controls is clearly seen at all Pi concentrations tested and indeed even more significantly in the physiological range.

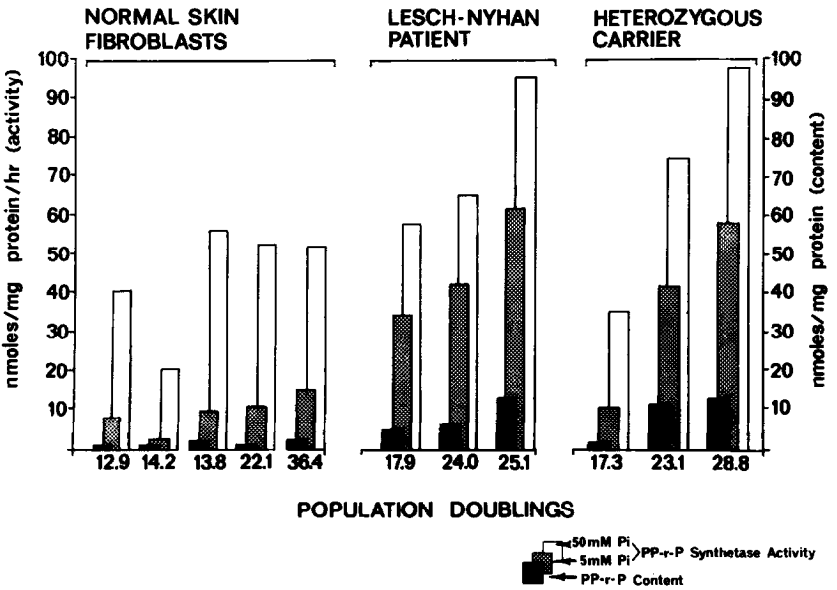

Figure 1: Catalytic activities of PP-ribose-P synthetase in the presence of 5 mM and 50 mM Pi and PP-ribose-P content in normal skin fibroblasts, Lesch-Nyhan and heterozygous carrier skin fibroblasts.

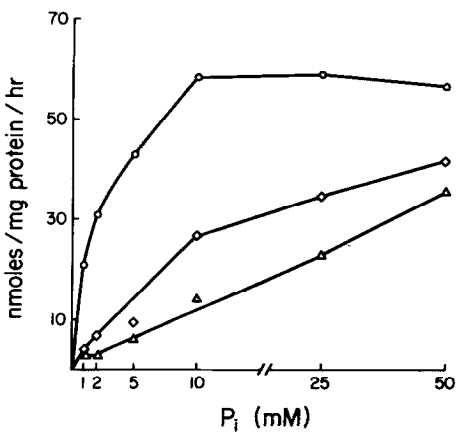

Figure 2: Activity of PP-ribose-P synthetase in normal skin fibroblast extracts (Δ——Δ), Lesch-Nyhan patient (o——o) and his mother (◇——◇) skin fibroblast extracts as a function of added inorganic phosphate (Pi).

DISCUSSION: The stimulatory effect of PP-ribose-P on purine synthesis de novo has been demonstrated in extensive pharmacological, clinical and kinetic studies (26,27) and could be a major part of the biochemical defect underlying purine overproduction. The increased cellular concentration of PP-ribose-P has been ascribed to both excessive PP-ribose-P synthetase activity (20,25,

28,29) and to decreased utilization by HGPRT, the purine salvage enzyme that has been considered to be a major intracellular consumer of PP-ribose-P (30). The Lesch-Nyhan patient and heterozygous carrier fibroblasts studied here have both deficient HGPRT and increased PP-ribose-P synthetase activity. Both enzyme defects may be responsible for the increased levels of PP-ribose-P found in the patient's fibroblasts.

It was reported that there is no direct correlation between the degree of HGPRT deficiency and the overproduction of purines and uric acid levels. Patients with partial deficiencies of HGPRT overproduce purine and uric acid to extents equal to or even greater than many patients with the complete enzyme deficiency (31,32). Graf et al. (29) observed an enhanced rate of de novo purine synthesis in chemically induced HGPRT deficient rat hepatoma cells selected in culture. They proposed that the enhanced rate of the de novo purine synthesis in these mutant cells was due to increased PP-ribose-P synthetase activity rather than to the loss of activity of HGPRT and an associated reduced PP-ribose-P utilization. In our study in the presence of saturating amounts of ATP and ribose-5-P the activity of the patient's PP-ribose-P synthetase in the fibroblast extracts was found greater than normal at all the phosphate concentrations tested; the effect is more marked at low phosphate concentrations. This observation may relate to the increased PP-ribose-P levels in the patient's fibroblasts.

A similar type of mutation has also been found by Becker et al. (33) in erythrocytes of two hyperuricemic brothers and in fibroblasts from one of the patients. In these patients' cells, as is the case in the present study, the increased PP-ribose-P synthetase activity was apparent at all inorganic phosphate concentrations. In the gouty patient reported by deVries and Sperling (34) the increased activity of the PP-ribose-P synthetase was only observed in the physiological range of erythrocyte inorganic phosphate concentration. Both studies (33,34) were on hyperuricemic patients with normal HGPRT and APRT activities. This is in contrast with our patients, one having a severe deficiency of HGPRT and the stigmata of the Lesch-Nyhan syndrome and the other, his mother, an obligate heterozygous with a marked partial deficiency of the same enzyme. Based on the hypothesis that the x-linked HGPRT gene is bifunctional and codes for both catalytic and regulatory functions (29), a mutation that causes a deficiency of HGPRT catalytic activity might also alter the regulatory function that this gene has on PP-ribose-P synthetase making the enzyme less responsive to metabolic control via the concentration of inorganic phosphate. If, as has been proposed (35,36), the PP-ribose-P synthetase gene, like that for HGPRT, is x-linked, the possibility also exists of polar genetic effects of HGPRT mutations. The inability of the PP-ribose-P synthetase en-

zyme, derived from cells with HGPRT deficiency, to be inhibited by physiological levels of inorganic phosphate, the behavior seen with the PP-ribose-P synthetase from normal cells, results in increased levels of PP-ribose-P in Lesch-Nyhan cells which can contribute significantly to augmented de novo purine synthesis.

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