DECREASED LYMPHOCYTE COPROPORPHYRINOGEN III OXIDASE ACTIVITY IN HEREDITARY COPROPORPHYRIA

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SUMMARY

Using [^{14}C]Coproporphyrinogen obtained from human red blood cells incubated with [4 ^{14}C] δ aminolevulinic acid, we measured lymphocytes Coproporphyrinogen III Oxidase activity in 17 subjects with hereditary coproporphyria. The mean activity was about 50 % of that in lymphocytes from normal subjects. This finding suggests that decreased coproporphyrinogen III oxidase activity reflects the primary genetic defect in Hereditary Coproporphyria. The technique described allows easy detection of asymptomatic carriers.

Coproporphyrinogen III oxidase is a mitochondrial enzyme(E.C.1.3.3.3.) which catalyzes the conversion of Coproporphyrinogen III to Protoporphyrinogen IX by decarboxylation and oxidation of two propionyl groups, yielding two vinyl groups (1). Hereditary Coproporphyria is a genetic disorder of heme and porphyrin biosynthesis (2). It is inherited as an autosomal dominant and clinically resembles two other forms of genetically transmitted Hepatic Porphyria, Intermittent Acute Porphyria and Porphyria Variegata. Excessive excretion of COPRO* III in feces is the most striking biochemical abnormality. Increased excretion of COPRO could result from a hereditary deficiency of the Coproporphyrinogen III oxidase, but measurement of this enzyme activity in Coproporphyric patients has not been made.

In fact, no specific metabolic defect in porphyrin biosynthesis has been described for this Porphyria. Increased hepatic activity of ALA synthetase is known, but it is common to the three hepatic porphyria (3) and it is now admitted (4) that this increased activity is secondary to a partial enzymatic defect in heme biosynthesis which may lead to derepression and/or disinhibition of ALA synthetase.

In the present study we have measured Coproporphyrinogen III oxidase activity in blood lymphocytes of 15 controls and 17 patients (or known carriers) from 4 families with H.C.. In addition Uroporphyrinogen I Synthetase activity has been measured in erythrocytes, to provide informations regarding this enzyme in H.C. (Uroporphyrinogen I Synthetase is the deficient enzyme in I.A.P.).

* Abbreviations used : ALA, δ aminolevulinic acid ; COPRO, coproporphyrin ; COPROgen, Coproporphyrinogen ; I.A.P., Intermittent Acute Porphyria ; H.C., Hereditary Coproporphyria ; P.B.G., porphobilinogen ; PROTO, protoporphyrin ; URO, uroporphyrin.

MATERIALS AND METHODS

Chemicals were obtained from the following sources : [4-^{14}C] ALA-- Amersham ; P.B.G., COPRO and PROTO-- Sigma ; Ficoll-- Pharmacia ; Na Metrizoate-- Nyegaard ; Aquasol-- New England Nuclear ; all other chemicals used were of reagent grade and were obtained from the usual commercial sources.

Lymphocytes were isolated from heparinized blood by centrifugation using the Ficoll-metrizoate mixture (5). For elimination of possible erythrocytes contamination lymphocytes were treated with 0.15 M NH_4Cl during 15 minutes at 37°C. After washing with 0.15 M NaCl, the cells were stored as a pellet at - 20°C until required for enzyme assay (usually 24 hours). They were then thawed (in 0.15 M NaCl) and freezed twice. After centrifugation, supernatant (0.5 to 1 mg protein/ml) was retained for assay.

Erythrocytes were prepared following the method of Blincoe (6). Protein concentration was determined by the method of Lowry et al (7) using human serum albumin as standard. Urinary and fecal porphyrins, ALA and P.B.G. were determined spectrophotometrically after extraction by the usual methods (8, 9).

MEASUREMENT OF COPROPORPHYRINOGEN OXIDASE ACTIVITY. Coproporphyrinogen oxidase activity was determined by measuring the rate of formation of [$^{14}C_8$] PROTO from [$^{14}C_8$] Coproporphyrinogen III.

The standard reaction mixture (2.0 ml) contained 100 μmoles of Tris- HCl buffer (pH 7.4). 5 mg of human albumin, 0.2 ml of enzyme solution, and 10 nmoles of [$^{14}C_8$] COPROgen . In all experiments a reagent blank (no tissue added) was included. After incubation under aerobic conditions for 1 hour at 37°C in the dark, the reaction was terminated by the addition of 4 ml of ethylacetate-glacial acetic acid (3:1, v/v). The reaction mixture was then irradiated for 30 minutes at room temperature from a day- light fluorescent tube to allow the oxidation of the porphyrinogens to porphyrins : 50 nmoles of cold PROTO were then added ; ethyl acetate-acetic acid mixture was evaporated under reduced pressure at 45°C in the dark and the porphyrins treated overnight with 5 ml of methanol-sulfuric acid (95:5, v/v). Porphyrin esters were then extracted into 5 ml of chloroform ; the chloroform phase was washed with 2 x 8 ml of water and evaporated under reduced pressure at room temperature. Porphyrin esters were redissolved in 0.15 ml of chloroform, streaked on silica gel thin layer plastic plates and chromatographed in the solvent system methyl ethyl cetone-benzene (3:40, v/v) to separate COPRO and PROTO (10). After separation, the plate was allowed to dry ; COPRO area was discarded and PROTO again chromatographed in the same solvent system : then the PROTO area was scrapped off into scintillation vials. Ten ml of Aquasol were added and radioactivity measured at 75 percent efficiency with a liquid scintillation spectrometer (Intertechnique, France). Quenching was monitored by the channels ratio method. Radioactivity of blank never exceeded 35 c.p.m.. PROTO concentration was determined in a Hitachi - Perkin Elmer specrophotometer at 409 nm (estimated ε mM in Aquasol : 164)- Calculation of [$^{14}C_8$] PROTO formed was corrected for counting efficiency and recovery of PROTO. Enzyme activity was expressed as the number of [$^{14}C_8$] PROTO picomoles formed per 1 hour per mg of protein at 37° C.

PREPARATION OF [^{14}C] COPROgene III.

[$^{14}C_8$] COPRO isomer III (1,3,5,8 [Methyl ^{14}C] 2,4,6,7 [Propionyl α ^{14}C] porphin) was obtained as follows : 20 ml of human red blood cells hemolysate (not diluted) were added to 40 ml of incubation mixture which contained 2 mmoles of Tris HCl (pH 7.4), 50 μmoles of glutathione and 45.5 μCi of [4 ^{14}C] ALA (6.63 mCi/mmole). Nitrogen was flushed vigorously through the mixture before the flask was evacuated. After incubation for 4 hours at 37°C in the dark, the reaction was stopped by the addition of 400 ml of ethyl acetate - glacial acetic acid (3:1). The precipitated

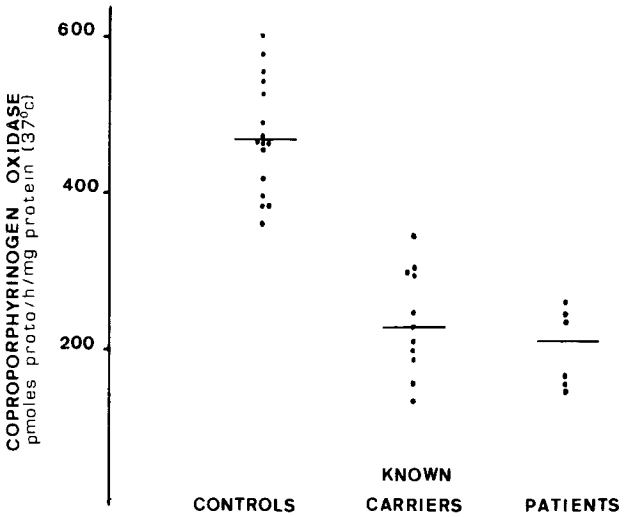


Figure 1 : Lymphocytes Coproporphyrinogen oxidase activity in normal subjects and patients or know carriers with H.C.. The mean enzymatic activity in normal subjects was 473(± 76) whereas in porphyric subjects it was 226(± 59) ($p<0.001$).

protein was removed at a sintered-glass Büchner funnel. The filtrate was washed twice with 3 % (w/v) sodium acetate, and porphyrin extracted with 0.5 N HCl. The pH was adjusted to 4 with cristallized sodium acetate and porphyrin was reextracted with ethyl acetate-glacial acetic acid. After washing with 3 % sodium acetate, the mixture was evaporated and the porphyrin esterified, concentrated in chloroform and streaked on silicagel thin layer plastic plates, as already described. After a first migration in the solvent system methyl ethyl cetone - benzene (3:40) the plate was allowed to dry and an other migration in the Doss system (benzene-ethyl acetate-methanol (85/13/2) was used (11). COPRO area was cutted and scrapped. Porphyrin was extracted with 10 ml of chloroform and filtrated on glass wool. Filtrate was evaporated under reduced pressure and the same chromatography - (in the first solvent system) as well as extraction handling were repeated. COPRO ester was hydrolyzed with 8.N.HCl for 24 hours at room temperature in the dark. [^{14}C] COPRO was stored in 0.5 N HCl. Purity was verified by high pressure liquid chromatography (12) and by thin layer chromatography to separate COPRO isomers (13). COPRO was found to be pure, containing less than 5 % of isomer I. Usually, with red blood cells extracted from 50 ml of whole blood we obtained 250-300 nmoles of [^{14}C] COPRO. Cold COPRO was added to get a specific activity around 30 mCi/mmoles.

[^{14}C] COPROgen was prepared as follows : after neutralisation of COPRO solution by 2.5.N. NH_4OH, COPROgen was obtained by reduction of COPRO with freshly ground 3 % (w/v) sodium amalgam, under nitrogen in the dark, following the technique of Falk (14). Final volume of 5 ml was obtained. COPROgen was immediately used.

Erythrocytes uroporphyrinogen I synthetase activity was measured following a technique already described (15).

Student's test was used to compare data from controls and patients (or carriers) with H.C..

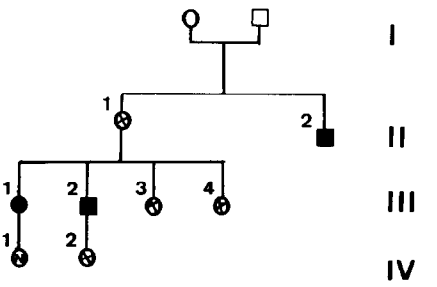
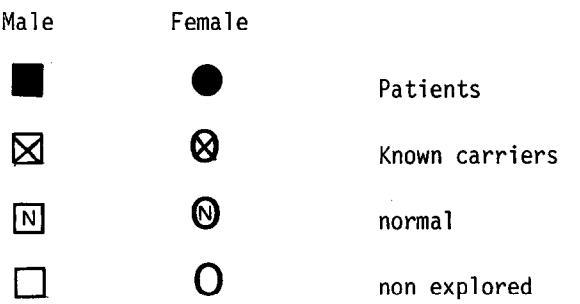


Figure 2 : Pedigree of a family with H.C.



RESULTS

Fig. 1 illustrates the mean lymphocytes coproporphyrinogen oxidase activity for patients, known carriers (members of the families without clinical symptoms, but biochemical abnormalities in feces) and normal subjects studied. No significant difference was found between patients (211 $\pm$ 45) and carriers (235 $\pm$ 66). Patients and carriers had approximately 50 % of the activity of controls (226 $\pm$ 59 versus 473 $\pm$ 76), which was significant at the 0.001 level. Fig. 2 shows the pedigree of a family in which three members had typical crisis of H.C. and Table 1 records the biochemical data of 8 members of this family : all the affected members have a low lymphocytes coproporphyrinogen oxidase activity. In contrast, erythrocytes uroporphyrinogen synthetase activity was found to be normal.

DISCUSSION

The study demonstrates a striking decrease of activity of Coproporphyrinogen oxidase in lymphocytes from patients (or carriers) with H.C.. Peripheral lymphocytes possess the ability to synthesize porphyrins when incubated with ALA (16). We chose these cells because they constitute a more homogeneous cell population than leukocytes, and also because separation of lymphocytes by the method of Boyum (5) yields

Table 1. DATA ON PORPHYRIN FROM A FAMILY STUDY

Subject [x]	Age	URINE [*]			FECES		Coproporphyrinogen Oxidase [**]	Uroporphyrinogen Synthetase [***]
		ALA (mg)	P.B.G. (mg)	COPRO (μg)	COPRO μg/g dry weight	PROTO μg/g dry weight		
II 1	68	4.8	2.8	131	388	28	305	139
II 2	66	4.3	1.2	500	1,466	10	241	122
III 1	44	6.2.	2.3	542	893	56	249	118
III 2	42	2.2	1.3	154	238	81	265	122
III 3	35	4.9	1.8	306	690	8	191	120
III 4	32	not done			330	66	250	153
IV 1	20	0.66	1.5	87	36	20	580	134
IV 2	14	not done			66	12	354	140
Non porphyric controls	13-70	0-2,5	0-1,5	20-120	5-50	5-100	473 ± 76	150 ± 20

x data refer to family illustrated in fig. 2
* results given per g/creatinin
** expressed as pmoles PROTO/mg protein/h. at 37°C
*** expressed as pmoles URO/mg protein/30 minutes at 45°C

less than 5 % of granulocytes and negligible platelets. Furthemore hematological data of all human subjects studied were normal.

Several studies were done to validate the method of determining coproporphyrinogen oxidase activity : product porphyrin was formed linearly with respect to time up to 2 hours, and with respect to protein concentration (0.1 mg - 2 mg/ml). Determinations were run in duplicate on several samples on at least two different days. Results were reproducible within 7 %. For each assay rat liver homogenate (1 % in Tris HCl buffer) was used as control : Results were reproducible within 5 %. Substrate concentration was at least twofold the concentration required to produce maximum velocity of the reaction in samples with normal activity.

The degree of decrease in activity observed (50 % of control) is similar to that observed by Strand et al (17) in liver and by Meyer et al (18) in erythrocytes from I.A.P. patients ; an autosomal structural gene mutation is expected to lead to enzyme activity about 50 % of normal. Mixture of enzyme solutions from control subjects and H.C. patients showed the expected activity, ruling out the presence of an inhibitor in patients cells.

The technique described allows to detect asymptomatic adult carriers. In one of the families studied neither parent of the propositus had abnormal porphyrin excretion in feces or urines: however the father had a very low lymphocytes coproporphyrinogen activity (177 units) ; therefore he carried the gene for H.C.. It will be also probably much easier to know if a young child is carrier (usually they do not show any abnormality in feces or urines before being 10 years old). However our data also show that there is enough variation in the enzyme activity within both normal and porphyric populations to expect a slight overlap between these two groups.

Although a deficiency of coproporphyrinogen oxidase was demonstrated in H.C. patients and carriers, the activity of erythrocytes uroporphyrinogen synthetase was normal within 2 S.D.. This finding excludes a generalized defect in the enzymes of the heme biosynthetic pathway.

ADDENDUM

After this manuscript was written, Elder et al published (Lancet 1976 ii 1217-1219) a study on coproporphyrinogen oxidase in skin fibroblasts from three subjects with H.C., with similar results.

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