Inherited Propionyl-CoA Carboxylase Deficiency in "Ketotic Hyperglycinemia"

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ABSTRACT Cultured fibroblasts from a young girl with ketotic hyperglycinemia were unable to oxidize propionate-^{14}C to $^{14}CO_2$, but oxidized methylmalonate-^{14}C and succinate-^{14}C normally. This block in propionate catabolism was shown to result from a lack of propionyl-CoA carboxylase activity. The carboxylase deficiency was not due to the presence of an intracellular inhibitor and it was not corrected by biotin, a known cofactor for the enzyme. Both of her parents' fibroblasts had approximately 50% of normal propionyl-CoA carboxylase activity. These results demonstrate that ketotic hyperglycinemia and propionicacidemia are the same disease, caused by a mutation of the propionyl-CoA carboxylase apoenzyme, which is inherited as an autosomal recessive trait. This enzymatic localization provides an explanation for the remarkable clinical and chemical similarity between ketotic hyperglycinemia and methylmalonicaciduria and offers a potential means of antenatal detection of this disorder.

INTRODUCTION

In 1961, Childs, Nyhan, Borden, Bard, and Cooke described a new metabolic disorder in an infant boy, E. G., who presented with vomiting and ketoacidosis in the neonatal period (1). Because glycine concentrations in plasma and urine were distinctly increased, the disorder was called "ketotic hyperglycinemia." Despite extensive investigation (2) the basic defect in this condition remained obscure until we suggested in 1968 (3) that the remarkable clinical and biochemical similarities between this condition and methylmalonicaciduria indicated that ketotic hyperglycinemia was caused by a defect in the propionate-methylmalonate-succinate pathway, perhaps in the carboxylation of propionate to methylmalonate. In 1969 we reported a block in propionate oxidation in the leukocytes of A. G., the affected sister of the index patient E. G. (4). Our present studies, utilizing cultured skin fibroblasts from A. G. and her parents, demonstrate that the basic defect in this condition is a deficiency of propionyl-coenzyme A (CoA) carboxylase and that this defect is inherited as an autosomal recessive trait.

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METHODS

Family report. The parents are healthy, Caucasian, and unrelated. The mother had three spontaneous abortions before the birth of E. G., who had episodic ketoacidosis, hyperglycinemia, leukopenia, throbmocytopenia, seizures, and recurrent infections (1). He died, profoundly retarded, at the age of 7 yr, of pneumonia. A. G., his only sister, also developed vomiting and ketoacidosis in the newborn period, and was found to have hyperglycinemia (4.1 mg/100 ml). She was promptly placed on a special low protein diet under the supervision of Dr. I. K. Brandt and Dr. D. Clement. At the age of 6 yr despite intermittent hyperglycinemia, hyperglycinuria, and a liability to ketonuria with any intercurrent infection or dietary indiscretion, her intellectual development and growth (weight 21 kg, height 120 cm) are normal.

Tissue culture. Punch biopsies of the skin were obtained from A. G., her parents, and normal controls. The skin explants were grown and subcultured in Diploid Growth Medium containing 10% fetal calf serum. Intact fibroblasts were harvested for incubation with propionate-3-^{14}C (1.65 mmoles/liter, 7.8 μCi), methyl-^{14}C-malonate (1.65 mmoles/liter, 1.1 μCi), and succinate-1,4-^{14}C (1.65 mmoles/liter, 1.1 μCi) as described previously (5).

Enzymatic assays. Propionyl-CoA carboxylase (EC 6.4.1.3) catalyzes the following reaction: propionyl-CoA + $HCO_3^- \rightleftharpoons$ D-methylmalonyl-CoA. Biotin, adenosine triphosphate (ATP), and magnesium are required cofactors for this enzyme. Propionyl-CoA carboxylase activity was assayed by a modification of the method of Erfle, Clark, and Johnson (6). Fibroblasts, harvested with 0.25% trypsin, were rinsed with isotonic saline and 0.2 M Tris (pH 7.4), then frozen and thawed three times. 0.4 ml of this whole cell extract (0.3–2.0 mg protein) was incubated in 0.2 M Tris buffer (pH 7.4) containing magnesium chloride (0.01 mole/liter), ATP (0.01 mole/liter), reduced glutathione (0.01 mole/liter), propionyl-CoA (0.0008 mole/liter), and sodium bicarbonate-^{14}C (0.01 mole/liter, 4.1 μCi) in a final volume of 1.0 ml at 30°C, in a metabolic shaker, for intervals

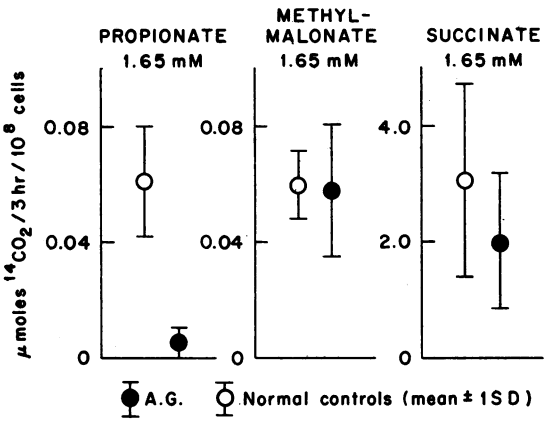

FIGURE 1 Oxidation of ^{14}C-labeled propionate, methylmalonate, and succinate to $^{14}CO_2$ by intact fibroblasts from controls and from A. G. Note the selective block in propionate catabolism. The control data were obtained from more than five different cell lines all studied in duplicate.

ranging from 5 to 180 min. The reaction was terminated by addition of 0.5 ml of 1.0 N potassium hydroxide and the mixture incubated for 15 min at room temperature to hydrolyze all thio-esters. Then 0.5 ml of 5 N hydrochloric acid was added to precipitate proteins and the mixture was centrifuged. The supernatant was heated in a boiling water bath for 4 min (7), a 1.0 ml aliquot was extracted three times with 10 ml of ethyl ether, and the combined ether extracts were evaporated to dryness. The dried extract was redissolved and rinsed successively in 1.0, 0.5, 0.2, and 0.1 ml aliquots of ethyl ether, which were spotted on Whatman 1MM chromatography paper with unlabeled carrier methylmalonate and succinate. Descending paper chromatography (18 hr) was used to separate methylmalonate and succinate, using isoamyl alcohol saturated with 4 N formic acid as the solvent. Methylmalonate and succinate were identified by staining with bromphenol blue (0.04% in ethyl alcohol at pH 6.7). Their spots were cut out, placed in vials containing 10 ml of Liquiflour, and counted in a liquid scintillation spectrometer. Authentic methyl-^{14}C-malonic acid, when subjected to the same procedures, yielded a recovery of 69% and a counting efficiency of 59%. Propionyl-CoA carboxylase activity was expressed in picomoles of methylmalonate-^{14}C formed per minute per milligram cell protein. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (8).

Materials. Propionyl-CoA was prepared from propionyl anhydride and CoA (9). Its purity was confirmed by ascending chromatography (16 hr) on Whatman 1 paper using isobutyric acid : ammonium hydroxide : water (66 : 1 : 33) as the solvent. A spot (R_F 0.54) visible in ultraviolet light and positive to reaction with hydroxylamine hydrochloride was identified as propionyl-CoA. Its concentration was measured by titration of the thio-ester with the hydroxylamine reaction (10).

Coenzyme A (CoA) was purchased from P-L Biochemical Company. D-Biotin was obtained from Nutritional Biochemicals Corp. All ^{14}C-labeled compounds were purchased from New England Nuclear. Other chemicals were obtained from Fisher Scientific Co.

RESULTS

Oxidation of propionate, methylmalonate, and succinate by intact fibroblasts. The ability of A. G.'s cultured fibroblasts to oxidize propionate-3-^{14}C to $^{14}CO_2$ was markedly impaired (Fig. 1). Control fibroblasts produced 0.06 ±0.02 (mean ±1 SD) μmoles $^{14}CO_2$/3 hr per 10^8 cells but her cells produced only 0.005 μmoles $^{14}CO_2$/3 hr per 10^8 cells, although they oxidized methylmalonate-^{14}C and succinate-1,4-^{14}C normally. Thus, her fibroblasts expressed the same block in propionate oxidation as did her uncultured leukocytes (4). Propionate oxidation by her father's leukocytes and fibroblasts was within two standard deviations of mean normal values.

Propionyl-CoA carboxylase activity in control cell extracts. As shown in Table I, no radioactive methylmalonate was formed from bicarbonate-^{14}C in the absence of propionyl-CoA, ATP, magnesium, or cell extract, or if CoA was substituted for propionyl-CoA. The addition of malonate caused no increase in accumulation of methylmalonate or succinate, and addition of the cofactor, biotin, failed to stimulate activity of control cell preparations. Incubation for intervals ranging from 5 min to 180 min revealed that the reaction rate was linear for 30 min and then decreased. In 20 experiments with control cells incubated for periods of 30 min or less, normal activity was 46.5 ±11.9 pmoles methylmalonate formed per minute per milligram cell protein.

Carboxylase activity in A. G.'s fibroblasts. A. G.'s cell extracts had less than 2% of normal activity. Increasing the amount of cell protein in the reaction mixture, varying the duration of the incubation, adding biotin, or growing her cells for several generations in biotin-enriched tissue culture medium all failed to reveal any propionyl-CoA carboxylase activity.

In a mixing experiment (Table II), varying proportions of cell extracts from a control line and from A. G.

TABLE I

Propionyl-CoA Carboxylase Activity in Extracts of Normal Human Cultured Fibroblasts

System	Amount of methylmalonate-^{14}C formed/mg protein
	pmoles
Complete*	1530 ±402*
Complete + biotin (0.0009 M)	1260
Complete + malonate (0.02 M)	1410
No propionyl-CoA	0
No propionyl-CoA, + CoA (0.001 M)	0
No ATP and magnesium	0
No cell extract	0

* See text for details. Incubation duration was 60 min.

were incubated together. The total enzyme activity of each of the mixtures could be attributed to the control line, and there was neither evidence of an inhibitor in A. G.'s cells nor of a stimulating factor in the control cells.

A. G.'s cells were also assayed for activity of methylmalonyl-CoA mutase and cystathionine synthase. The activity of both of these enzymes was normal: mutase, 0.07 nmoles succinate formed/30 min per g wet wt. (normal range 0.05–0.13) (5); synthase, 4.33 nmoles cystathionine formed/135 min per mg protein (normal range 2.6–18.0).

Reduced carboxylase activity in parents' fibroblast extracts. Cell extracts from A. G.'s parents consistently had propionyl-CoA carboxylase activity that was approximately half of normal (Fig. 2). Although her father's leukocytes and intact fibroblasts oxidized propionate at the lower limit of the normal range, extracts of his fibroblasts had propionyl-CoA carboxylase activity of 16.5 ±5.4 pmole/min per mg protein. Similarly A. G.'s mother's fibroblast extracts had carboxylase activity of 19.8 ±5.1 pmole/min per mg protein.

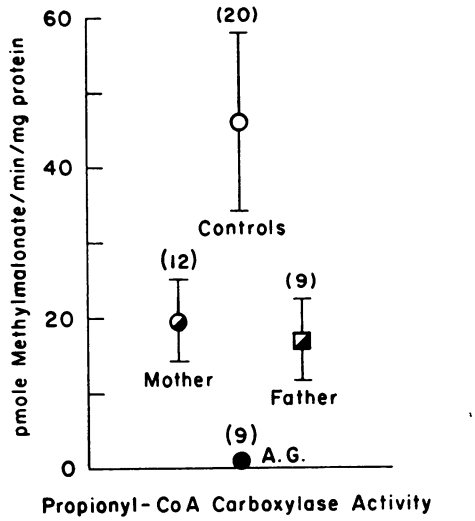

FIGURE 2 Propionyl-CoA carboxylase activity in control fibroblast extracts contrasted with that in A. G. and her parents. Enzyme activity is expressed as picomoles of methylmalonate-^{14}C formed per minute per milligram protein. Incubation duration was 30 min or less. See text for details of experimental procedure.

DISCUSSION

Propionate metabolism. Propionic acid, as its CoA ester, is the end product of beta oxidation of odd numbered carbon chain fatty acids and is a common intermediate in the degradation of the amino acids, isoleucine, valine, methionine, and threonine. Propionyl-CoA, in turn, is converted to succinyl-CoA via D- and L-methylmalonyl-CoA. In retrospect, the vital role that propionate formation and catabolism play in human metabolism was suggested by the intensive investigation of A. G.'s brother, E. G. (1, 2). Oral tolerance tests had revealed that only the amino acid precursors of propionate reproducibly caused vomiting and ketoacidosis in E. G. Subsequently, Menkes (11) reported that E. G.'s urine contained butanone and other longer chain ketones during periods of ketoacidosis. Since butanone is derived, at least in part, from isoleucine catabolism (12), this observation is also consistent with a block in propionate catabolism, leading to an accumulation of propionate and its precursors. Circuitously, it was the study of a child with a defect in methylmalonate catabolism (12) which led us to reinvestigate E. G.'s affected sister, A. G., and to find a block in propionate oxidation by her leukocytes and cultured fibroblasts.

TABLE II

Effect of Mixing Cell Extracts from A. G. and Control Line on Propionyl-CoA Carboxylase Activity

Flask	Cell extract added		Enzyme activity (pmole methylmalonate-^{14}C formed)	% of normal	
	A. G.	Control		Found	Predicted
	ml				
1	—	0.4	2680	100%	100%
2	0.1	0.3	1860	70%	75%
3	0.2	0.2	1310	49%	50%
4	0.3	0.1	640	24%	25%
5	0.4	—	0	0%	0

The total ^{14}C-labeled methylmalonate formed in each flask is compared to the amount formed in flask 1. The percentage predicted in the last column is based on the assumption that only the control cell extract contained propionyl-CoA carboxylase activity and that A.G.'s cell extract was entirely inactive. The results found agree closely with this assumption. Incubation duration was 60 min.

The enzymatic defect. The lack of methylmalonate-^{14}C formation from propionyl-CoA and bicarbonate-^{14}C by A. G.'s cell extracts indicates absence of propionyl-CoA carboxylase activity. If the defect involved methylmalonyl-CoA racemase, the enzyme needed for the conversion of D-methylmalonate to its levo-isomer, L-methylmalonate, one would anticipate only a partial reduction in methylmalonate formation from propionate and would also expect to observe reduced conversion of D,L-methylmalonyl-CoA to succinyl-CoA. Neither of these findings was noted in A. G.'s cells.

Since propionyl-CoA carboxylase activity in A. G.'s cell extracts was not stimulated by addition of biotin, and since carboxylase activity in control cell extracts was not

impaired when mixed with A. G.'s extracts, there is no evidence for either an unusual cofactor requirement or for an intracellular inhibitor causing defective propionyl-CoA carboxylation in A. G.'s cells. Therefore, we conclude that her cells either fail to synthesize propionyl-CoA carboxylase, or, alternatively, make an apoenzyme totaly devoid of biological activity.

Identity with propionicacidemia. Hommes, Kuipers, Elema, Jansen, and Jonxis (13) proposed that propionyl-CoA carboxylase deficiency was the cause of propionicacidemia in an infant who died on the 5th day of life. This child accumulated odd-chain fatty acids in his liver, but never demonstrated hyperglycinemia. Our suggestion (4) that propionicacidemia is the same condition as ketotic hyperglycinemia has now been confirmed by Gompertz, Bau, Storrs, Peters, and Hughes (14). They studied an infant who had not only propionicacidemia and accumulation of odd-chain fatty acids, but hyperglycinemia and long-chain ketonuria as well. He also died, at the age of 8 days, and was found to have only 10% of normal propionyl-CoA carboxylase activity in his liver.

Thus, the diagnosis of propionyl-CoA carboxylase deficiency can now be made by the detection of increased serum concentrations of propionic acid or by the demonstration of a selective block in propionate oxidation in peripheral leukocytes or cultured fibroblasts, and can be confirmed by finding absent or near absent propionyl-CoA carboxylase activity in cultured fibroblasts or liver.

Proof of autosomal recessive inheritance. The occurrence of this condition in both sexes, and its recurrence within a sibship, have suggested autosomal recessive inheritance, as has the report of parental consanguinity (14). The findings of decreased propionyl-CoA carboxylase activity in extracts of fibroblasts from both A. G.'s mother and father provides the first biochemical identification of the heterozygous carrier for this condition and proves that the mutation is inherited as a autosomal recessive trait.

Genetic counselling and prevention. Since this condition can now be diagnosed precisely, and the heterozygous carriers of this mutant gene can be identified, the risk of recurrence can be stated clearly to parents of an affected child. In addition, since normal cells cultured from amniotic fluid early in pregnancy have been found to have propionyl-CoA carboxylase activity which approximates that found in controls,[1] the potential exists for diagnosing this condition *in utero*.

[1] Unpublished observations.

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