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Detection of Heterozygotes in Maple-Syrup-Urine Disease: Measurements of Branched-Chain α-Ketoacid Dehydrogenase and Its Components in Cell Cultures

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SUMMARY

To detect heterozygotes for maple-syrup-urine disease (MSUD), activities of branched-chain α-ketoacid (BCKA) dehydrogenase and its components in skin fibroblasts of two obligatory heterozygotes and amnion cells of a fetus at risk were measured. Intact heterozygous cells were found to decarboxylate [1-^{14}C]α-ketoisovalerate at rates equal to or only slightly lower than normal subjects. The inability to differentiate heterozygotes from normals with the intact cell assay confirms earlier studies with intact leukocytes using [1-^{14}C]leucine as substrate. By contrast, measurements of BCKA dehydrogenase activity with disrupted cell suspensions showed MSUD heterozygotes with 30%–60% of normal activity. Moreover, biphasic kinetics in heterozygous cells were observed with increasing substrate concentrations. The altered biphasic kinetics probably reflect expression of the normal allele in the early hyperbolic portion of the curve and of the mutant allele in the later secondary rise at high substrate concentrations. Assays of component activities showed concordant E_1 decarboxylase deficiency in both heterozygous- and homozygous-affected cells, whereas the E_3, dihydrolipoyl dehydrogenase-component, activity was normal. The above results taken together appear to provide an approach to detection of the heterozygote in MSUD.

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INTRODUCTION

Maple-syrup-urine disease (MSUD) is an autosomal recessive disease caused by impaired oxidative decarboxylation of branched-chain α-ketoacids (BCKA): α-ketoisovalerate, α-ketoisocaproate, and α-keto-β-methylvalerate. MSUD has been classified into classical (grade 1), intermediate (grade 2), and intermittent (grade 3) forms according to time of onset, severity of symptom, tolerance for dietary protein, and level of residual BCKA dehydrogenase activity [1].

Dancis et al. demonstrated that both the classical and variant forms of MSUD are deficient in the BCKA dehydrogenase activity that is expressed both in peripheral leukocyte preparations [2, 3] and in skin fibroblast cultures [4, 5]. Over the years, homozygous-affected patients have been readily diagnosed using intact cell cultures and low concentrations of [1-^{14}C]leucine or [1-^{14}C]BCKA as substrate [6, 7]. However, these methods have been unable to detect heterozygotes for MSUD. Although the heterozygotes as a group show somewhat lower activity than do normals, overlaps between normals and obligatory heterozygotes were observed with respect to decarboxylation of branched-chain amino acids (BCAA) or BCKA in intact cell cultures [8–12].

Here we compare results of the intact and disrupted cell assays with cultured skin fibroblasts from two obligatory heterozygotes and amnion cells from a fetus at risk for MSUD. Measurements of BCKA dehydrogenase activity with the disrupted cell system clearly differentiate the heterozygotes from normals. With increasing substrate concentration, biphasic kinetics in heterozygous cells were observed, which probably reflects expression of both the normal and mutant alleles at saturating α-ketoacid [13]. Moreover, assays of component activities of the multienzyme complex show that the enzyme defect is confined to the BCKA decarboxylase (E_1) step in both heterozygous- and homozygous-affected cell cultures, while the dihydrolipoyl dehydrogenase (E_3) activity is unaffected.

MATERIALS AND METHODS

Subjects

(1) Fibroblasts: Cultured skin fibroblasts of obligatory heterozygotes GM650 and GM651 and their homozygous infant GM649 were obtained from the Genetic Human Cell Repository, Camden, N.J. GM649 is a classical MSUD patient originally reported by Dancis et al. [2]. Fibroblasts of homozygous MSUD patients DaMa and CoBa were provided by Dr. D. L. Valle, Johns Hopkins University School of Medicine, Baltimore, Md. All the other patients (MeCa, ElMi, Ht, GM612, and RiCh) have been previously reported [8] and defined as classical MSUD by clinical history and intact cell assay using BCAA as substrate. Fibroblasts from normal subjects were selected to provide an age range comparable to the patients. (2) Amnion cells: Cultured amnion cells of normals and a fetus at risk for MSUD (patient Pt) were received from the Cytogenetic Laboratory, Akron Children's Hospital, Akron, Ohio. The brother of patient Pt had been previously diagnosed as a classical MSUD patient. Patient Pt was carried to term and is phenotypically normal.

Cell Culture

Fibroblasts and amnion cells were grown in monolayer culture in Waymouth's medium containing 5% fetal and 5% newborn calf serum and antibiotics (penicillin, 50U; strepto-

mycin, 50 μg; and kanamycin, 30 μg per ml). Both normal and mutant cells were grown to confluency and harvested 1 week after the last medium change. Skin fibroblasts and fibroblastic amnion cells were assayed within 10 to 16 passages at a 1:2 split.

Radioactive Substrate

[1-^{14}C]α-ketoisovalerate ([1-^{14}C]KIV) was prepared from [1-^{14}C]L-valine (specific radioactivity 52.9 mCi/mmol, New England Nuclear, Boston, Mass.) by oxidation with L-amino acid oxidase [14]. The radioactive purity of the [1-^{14}C]α-ketoacid was found to be $>99\%$ by ascending thin-layer chromatography [15]. The labeled α-ketoacid was maintained at pH 4 and stored at −20°C.

Protein Determination

Cell suspensions were precipitated with 7.5% trichloroacetic acid and dissolved in 0.6 N NaOH containing 2% sodium deoxycholate, and protein was determined by the Lowry method [16], using bovine serum albumin as standard.

Intact Cell Assay

Fibroblasts were harvested with Puck's saline A (without glucose) [17] containing 0.02% EDTA and 0.04% trypsin. The harvested cells were washed by centrifugation (650 *g*) once with a Krebs-Ringer buffer [18] containing 20% fetal calf serum and once with the buffer only. The washed cells (1×10^6 in 0.05 ml above buffer) were suspended in 0.35 ml medium containing 0.25 ml of the Krebs-Ringer phosphate buffer, pH 7.4, 0.005 ml thiamine/HCl (100 mg/ml), and [1-^{14}C]KIV (specific radioactivity 172 cpm/nmol). Incubation medium containing equivalent numbers of boiled normal cells were used as a blank. The blank value was within 10%–20% of the total normal sample counts depending on substrate concentrations. The assay was carried out in a Kontes pear-shaped flask (10-ml vol) capped with a serum stopper. Incubations were for up to 80 min at 35°C with $^{14}CO_2$ collected in a center well containing 0.1 ml of 2 N NaOH. After stopping the reaction with 0.05 ml of 15% trichloroacetic acid, the incubation was allowed to continue for 1 hr to recover residual $^{14}CO_2$ from the incubation medium. At the end of the incubation, the NaOH solution was transferred to a scintillation cocktail to count radioactivity. Activity was expressed as nmol $^{14}CO_2$ evolved per min (m unit) per mg cell protein.

Assay for BCKA Dehydrogenase

The overall reaction catalyzed by the multienzyme complex was assayed using disrupted fibroblast suspensions. The assay was essentially that described by Danner and Elsas [19] except for the method of disrupting cells and the inclusion of serum in the assay mixture that protects the enzymes from proteolysis. Using these modifications, the disrupted cell assay has approximately 50% of the intact cell activity. The fibroblasts were harvested and washed as described above and suspended in the Krebs-Ringer buffer (1×10^6/0.05 ml). The cell suspension was rapidly frozen in a Revco freezer (−75°C) and kept for 15 hrs or longer and then thawed for assays. Fibroblasts thus treated were fully disrupted as observed under a phase-contrast microscope. The activity of the frozen cells were stable for at least 2 months at −75°C. The assay mixture contained: 50 mM Tris/HCl, pH 7.5; 0.2 mM EDTA; 0.35 mM $MgCl_2$; 0.2 mM CoA; 0.2 mM NAD^+; 0.2 mM TPP; and 1.4% fetal calf serum; the disrupted cell suspension (1×10^6 cells in 0.05 ml of Krebs-Ringer buffer); and [1-^{14}C]KIV (specific radioactivity 172 cpm/nmol) in a final volume of 0.37 ml. The blank value with Krebs-Ringer buffer was within 10%–20% of the total normal sample counts, depending on substrate concentrations. The incubation and remaining procedure were the same as in the intact cell assay.

Assay for the BCKA Decarboxylase (E_1) Component

The radiochemical assay was modified from an earlier spectrophotometric procedure using $K_3Fe(CN)_6$ as an electron acceptor [20, 21]. The reaction mixture contained: 20 mM phosphate buffer, pH 7.4; 0.54 mM EDTA; 1 mM $MgCl_2$; 3.4 mM $K_3Fe(CN)_6$; 0.2 mM TPP; 1.4% fetal calf serum; [1-^{14}C]KIV (specific radioactivity 800 cpm/nmol); and frozen and thawed cell suspension (0.05 ml) in a final volume of 0.37 ml. The remaining procedure was the same as described in the intact cell assay.

Assay for the Dihydrolipoyl Dehydrogenase (E_3) Component

The dihydrolipoyl dehydrogenase (E_3) component was assayed in the direction of lipoamide reduction [22]. The assay mixture contained: 50 mM potassium phosphate, pH 6.5; 1.5 mM EDTA; 1 mM DL-lipoamide; 0.2 mM NADH; 0.15% Triton X-100; and cell suspension (1 to 5×10^5 cells). The reaction was followed spectrophotometrically at 37° C and 340 nm. Blanks without lipoamide were used. Activity was expressed as nmol NADH oxidized per min (m unit) per mg protein.

RESULTS

Table 1 compares the activity of BCKA dehydrogenase in cultured normal amnion cells with human skin fibroblast cultures. The levels of activities for normal cells from amnion and skin were similar in both the intact and disrupted cell assays. Amnion Bl was an epithelial cell culture while Ad initially showed a mixture of epithelial and fibroblastic cells that later became predominantly fibroblastic. Despite differences in cellular morphology, both intact amnion cell types and intact skin fibroblasts exhibited similar decarboxylation rates (range 0.191–0.233 nmol CO_2/min per mg protein) with [1-^{14}C]KIV as substrate. In the disrupted cell assay, activity was approximately one-half of the intact cell, and the activities were again similar (range 0.096–0.129 nmol CO_2/min per mg protein) for normal cells of skin and amnion of different morphology.

Figure 1 compares the rate of decarboxylation at increasing concentration of [1-^{14}C]KIV by intact cells from normals, eight patients with the classical form of

TABLE 1

BCKA DEHYDROGENASE ACTIVITY IN NORMAL SKIN FIBROBLAST AND AMNION CELL CULTURES

CELL TYPE	SUBJECT	ACTIVITY (NMOL CO_2/MIN/MG PROTEIN)	
		Intact cells	Disrupted cells
Amnion cells	Bl	0.201 ± 0.019*	0.096 ± 0.003†
Amnion cells	Ad	0.210 ± 0.007*	...
		0.205 ± 0.005‡	0.103 ± 0.003‡
Skin fibroblasts	Fs	0.233 ± 0.006	0.129 ± 0.006
Skin fibroblasts	Pa	0.191 ± 0.005	0.107 ± 0.007

NOTE: Concentrations of [1-^{14}C]KIV (specific radioactivity 172 cpm/nmol) were 1 and 2 mM for the disrupted and intact cell assays, respectively. Remaining assay conditions were as described in MATERIALS AND METHODS. Values are expressed in mean ± SD obtained from three to five determinations.

* Mixture of † and ‡.

† Epithelioid amnion culture.

‡ Fibroblastic amnion culture.

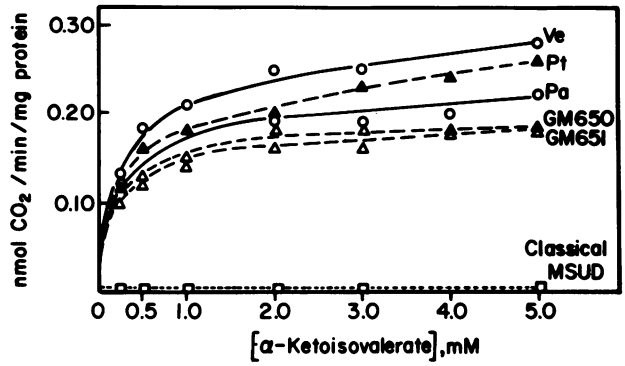

FIG. 1.—Rates of decarboxylation of α-ketoisovalerate by intact cells from normal (———), heterozygous (— — —), and classical MSUD (- - - - -) subjects. Intact cell assays were carried out in the presence of 4 mM thiamine as described in MATERIALS AND METHODS with [1-^{14}C]KIV (specific radioactivity 172 cpm/nmol) as the variable substrate. Protein added was approximately 0.3 mg or 1×10^6 cells. Incubation was at 35°C for 80 min. Viability of intact cells was 95% or greater by the trypan-blue stain. *Ve* and *Pa* are normal fibroblasts, *Pt* are amnion cells of a fetus at risk, *GM650* and *GM651* are fibroblasts of obligatory heterozygous parents of GM649, and *classical MSUD* are fibroblasts of homozygous affected patients: GM649, GM612, DaMa, CoBa, MeCa, ElMi, Ht, and RiCh.

MSUD including an affected infant GM649, the obligatory heterozygous parents of patient GM649 (subjects GM650 and GM651), and an amnion cell culture from a fetus at risk for classical MSUD (patient Pt). As shown in figure 1, intact cells from classical MSUD patients showed no decarboxylating activity up to 5 mM concentration of KIV. In contrast, intact cells from normals, obligatory heterozygotes, and the fetus at risk were similar in decarboxylation rates. The intact cell assay clearly distinguishes homozygous MSUD subjects from normals and heterozygotes but it is unable to separate the heterozygotes from normals.

Figure 2 shows that in the reconstituted disrupted cell assay normals, heterozygotes, and homozygotes for MSUD classical mutation are distinguished. Normal amnion cells (Ad) and skin fibroblasts (Pa and Wa) show hyperbolic kinetics over substrate concentrations from 0.1 to 5 mM with maximal activity at 0.10 ± 0.02 m U/mg of cell protein. Under similar conditions of assay, the heterozygotes (subject GM651 and amnion cells from patient Pt) exhibit biphasic kinetics with enzyme activity 30%–40% of normal at substrate concentrations of 0.1 to 2 mM KIV and approaching 60% of normal at substrate concentrations between 2 to 5 mM of the α-ketoacid (broken middle lines of fig. 2). Disrupted cells from classical patients DaMa and GM649 have BCKA dehydrogenase activity strictly dependent on substrate concentration. At substrate concentrations below 2 mM, there is little or no decarboxylation of substrate, while at 5 mM substrate concentration, the mutant cells exhibit up to 50% of normal activity (fig. 2). The biphasic kinetics in heterozygous cells observed with increasing substrate concentrations probably reflect expression of the normal allele in the hyperbolic early portion of the curve and the mutant allele in the later secondary rise at high substrate concentrations.

Previous studies from this laboratory have shown that in patients with the classical form of MSUD the enzyme defect is confined to the E_1 decarboxylase step

of the reaction, and that the E_2 transacylase and E_3 dehydrogenase steps of the multienzyme complex exhibit normal activity [13]. The activity of the E_1 component in normals, heterozygotes, and homozygous MSUD were measured (table 2). In heterozygous- (Pt, GM650, and GM651) and the homozygous-affected cells (GM649 and Ht), the E_1 decarboxylase activity was reduced. At low substrate concentrations (0.3 mM KIV), the enzyme deficiency was marked, but at high substrate concentrations (1 mM), significant activity was observed (table 2). In contrast, the level of dihydrolipoyl dehydrogenase (E_3) activity in homozygous-affected and heterozygous cells was in the normal range providing a related enzyme baseline for evaluating E_1 activities.

DISCUSSION

Recent studies with highly purified enzyme from bovine kidney [23] and bovine liver [24] have shown unequivocally the existence of a single BCKA dehydrogenase complex catalyzing oxidative decarboxylation of all three α-ketoacids, that is, KIV, α-ketoisocaproate (KIC), and α-keto-β-methylvalerate (KMV). These findings are consistent with the concordant decrease of activity for all three substrates in fibroblasts and leukocytes from MSUD patients [1, 2, 19]. Our study of the heterozygote in MSUD is based on the thesis of a single multienzyme complex in

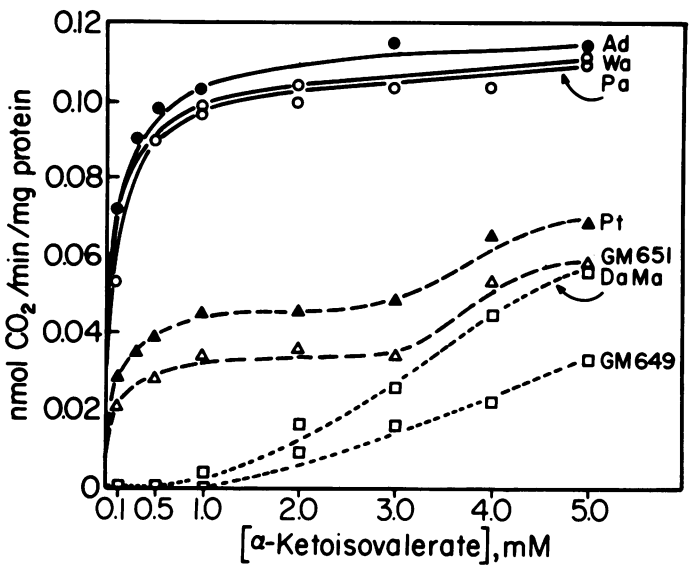

FIG. 2.—Activity of BCKA dehydrogenase in disrupted amnion cells and fibroblasts from normal (———), heterozygous (— — —), and homozygous affected (- - - - -) subjects. Rates of overall reaction catalyzed by multienzyme complex were measured in presence of 0.2 mM TPP as described in MATERIALS AND METHODS. Frozen and thawed amnion cells (*closed symbols*) or fibroblasts (*open symbols*) were used as enzyme source, and [1-^{14}C]KIV (specific radioactivity 172 cpm/nmol) was the variable substrate. Incubation and protein added were the same as in figure 1. *Ad* are normal amnion cells, and *Wa* and *Pa* are normal fibroblasts, *Pt* are cultured amnion cells from a fetus at risk for MSUD, *GM651* are skin fibroblasts from an obligatory heterozygous parent of GM649, and *DaMa* and *GM649* are fibroblast cultures from classical MSUD patients.

TABLE 2

ACTIVITIES OF BCKA DECARBOXYLASE (E_1) AND DIHYDROLIPOYL DEHYDROGENASE (E_3) COMPONENTS IN NORMAL AND MUTANT CELL CULTURES

SUBJECT	GENOTYPE	CELL TYPE	E_1 (μ U/MG PROTEIN)		E_3 (M U/MG PROTEIN)
			0.3 mM KIV	1.0 mM KIV	
Wa	++	SF	6.2 ± 0.3	12.6 ± 1.3	56.8 ± 5.0
Pa	++	SF	5.6 ± 0.7	11.1 ± 0.7	51.3 ± 1.7
Ad	++	AC	...	...	41.7 ± 0.5
GM649	−−	SF	0.7 ± 0.3	3.2 ± 0.7	56.6 ± 4.6
Ht	−−	SF	1.3 ± 0.7	3.9 ± 1.4	47.8 ± 3.0
Pt	+−	AC	2.9 ± 0.4	7.6 ± 1.9	46.6 ± 1.2
GM650	+−	SF	2.6 ± 0.4	7.1 ± 0.8	47.1 ± 3.6
GM651	+−	SF	...	8.6 ± 0.9	48.5 ± 3.3

NOTE: Abbreviations for cell type are: SF = skin fibroblasts, AC = amnion cells. Symbols + and − refer to normal or mutant allele, respectively. Values are mean ± SD and were obtained from five to nine determinations.

human diploid cell cultures. In both intact and disrupted cell assays, KIV is used as a substrate because it gives the highest activity of the three α-ketoacids.

Normal amnion cells of epithelial or fibroblastic morphology and skin fibroblasts have similar decarboxylating activity with KIV as substrate. These findings differ from those of Fensom et al. [25] in which the apparent decarboxylation rate of amnion cells was higher than skin fibroblasts when the amino acid [1-^{14}C]leucine was used as substrate. Differences in the transaminase activity of the two cell types may explain this discrepancy. Similar levels of BCKA dehydrogenase activity in amnion cells of epithelial and fibroblastic morphology is reassuring for prenatal diagnosis in cultures containing various proportions of the two cell types.

Our findings that the intact cell assays with KIV as substrate (fig. 1) are unable to distinguish heterozygotes from normals are in agreement with earlier studies [8–12, 26] in which either [1-^{14}C]leucine or [1-^{14}C]KIC was used as substrate. However, the heterozygote can be reliably differentiated from normal using a reconstituted disrupted cell system (fig. 2). The biphasic kinetics exhibited by the heterozygote suggest that both the normal and mutant alleles are expressed in these cells. Moreover, MSUD variants exhibit altered kinetics similar to that of classical patients except that the enzyme activity is higher (D. T. Chuang, W.-L. Niu and R. P. Cox, unpublished observations). At low α-ketoacid concentration, variants show lower activity than do heterozygotes, but at high substrate concentrations, overlap in activities is observed. Therefore, in the disrupted cell assay, low α-ketoacid concentrations are required to clearly distinguish the phenotypes.

The apparent "discrepancy" in decarboxylation of KIV by mutant cell cultures of MSUD between the intact and disrupted cell assays is probably expected. The close correlations between the patient's phenotype and tolerance for dietary protein with the degree of enzyme deficiency observed in the intact cell assay [5] suggests that this system approximates the metabolic flux through BCKA dehydrogenase in vivo. The correlations would also appear to hold true in the heterozygote whose normal phenotype coincides with the near normal decarboxylating

activity in the intact cell assay. On the other hand, the reconstituted disrupted cell system provides a more direct measurement of enzyme activity. This is shown by the 30%–60% of normal activity in the heterozygote as determined by the disrupted cell assay. Thus the intact and disrupted cell assays complement each other in detecting the heterozygote for MSUD.

The availability of assays for component enzymes of BCKA dehydrogenase may be useful in defining more precisely genetic heterogeneity in MSUD [27]. The concordant reduction of the BCKA decarboxylase (E_1) component activity at low substrate concentration (0.3 mM KIV) in both heterozygotes and the homozygous-affected subjects strengthens our previous conclusions concerning an E_1 deficiency in the classical MSUD patients studied [13]. The significant residual E_1 activity exhibited by the homozygote at high (1 mM) KIV concentration is consistent with a K_m mutation with reduced E_1 affinity for the substrate. Moreover, the presence of normal dihydrolipoyl dehydrogenase (E_3) component activity in heterozygous and homozygous genotypes provides an internal enzyme control for the enzyme deficiency in MSUD. This approach may prove significant since the E_3 component is believed to be a common moiety shared by pyruvate, α-ketoglutarate, and BCKA dehydrogenase complexes [23, 28]. A defect in this component has been reported to result in a combined deficiency in all three multienzymes [29, 30].

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