Investigations by mass isotopomer analysis of the formation of D-2-hydroxyglutarate by cultured lymphoblasts from two patients with D-2-hydroxyglutaric aciduria

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Abstract **D-2-Hydroxyglutaric aciduria is an inborn error of metabolism first described in 1980. To date, more than 40 patients have been diagnosed with this disease. To identify the metabolic precursor of D-2-hydroxyglutarate (D-2-HG), cultured human lymphoblasts from two patients with D-2-HG aciduria were grown in culture medium supplemented with [U-$^{13}C_6$]-glucose or [$^{2}H_5$]glutamate. Mass isotopomer distribution measurements of D-2-HG, 2-ketoglutarate (2-KG) and citrate were performed by gas chromatography-mass spectrometry. The mass isotopomer distributions in D-2-HG, 2-KG and citrate, following [U-$^{13}C_6$]glucose and [$^{2}H_5$]glutamate incubations, revealed that 2-KG interconverts rapidly to D-2-HG and that D-2-HG is formed within the mitochondria.**

Key words: D-2-Hydroxyglutaric aciduria; D-2-Hydroxyglutarate; Origin; Citric acid cycle; Mass isotopomer analysis; 2-Ketoglutarate; Citrate; GC-MS

1. Introduction

2-Hydroxyglutarate (2-hydroxypentanedioic acid, 2-HG) is a five carbon dicarboxylic acid in which the α carbon carries a hydroxy group. Therefore 2-HG occurs in two enantiomeric configurations: D (*R*), and L (*S*). In the 1970s, it was recognized that 2-HG is a normal constituent of human urine [1,2]. Since 1980, the interest in 2-HG has increased markedly as patients were described who excreted large amounts of 2-HG [3,4]. By using chiral derivatization followed by gas chromatographic analyses, the two enantiomeric forms of 2-HG could be individually detected, resulting in the finding of two new organic acidurias: D-2-HG aciduria (D-2-HGA) [3] and L-2-HG aciduria (L-2-HGA) [4]. The present report focuses on D-2-HGA.

At this moment more than 40 patients have been diagnosed with D-2-HGA. Clinical symptoms of these patients are: developmental delay, epilepsy and hypotonia and, less frequently, movement disorders and cardiomyopathy. Some patients show dysmorphic features. Magnetic resonance imaging (MRI) of the brain reveals disease-specific alterations in all patients. The clinical and biochemical features of 25 D-2-HGA cases were reviewed by Van der Knaap et al. [5,6].

Urinary 2-HG is elevated not only in D-2-HGA or L-2-HGA but also in combined D- and L-2-HG aciduria [7], glutaric aciduria type II (D-2-HG) [8], deafness-onychodystrophy-osteodystrophy-retardation syndrome [9], pyruvate decarboxylase deficiency [10], dihydrolipoyl dehydrogenase deficiency [11], malonic aciduria [12], and α-amino/α-ketoadipic aciduria [13]. With the exception of glutaric aciduria type II, enantiomeric differentiation of the elevated 2-HG has not been reported.

The origin of D-2-HG in D-2-HGA is still not clear, in spite of the fact that 2-HG is known to be involved in different metabolic pathways. Three mammalian pathways involving 2-HG are known. First, a mitochondrial enzyme that dehydrogenates D-2-hydroxyacids to their 2-keto-analogs was identified in rabbit kidney by Tubbs et al. [14]. Second, a hydroxyacid–oxoacid transhydrogenase, present in rat kidney, liver, and brain converts 2-ketoglutarate (2-KG) to D-2-HG in a reaction in which γ-hydroxybutyrate is converted to succinic semialdehyde [15]. Third, D-2-HG is an intermediate in the metabolism of 5-hydroxy-L-lysine and 2-keto-5-hydroxyadipate is its direct precursor [16]. In addition to these three mammalian pathways, D-2-HG-involving pathways have been described in bacteria. First, a 3-phosphoglycerate dehydrogenase in *Escherichia coli* also has a 2-KG reductase activity yielding 2-HG [17]. Second, the condensation of glyoxylate and propionyl-CoA by *E. coli* produces D-2-HG [18]. Third, 2-aminoadipate is converted to D-2-HG by *Pseudomonas putida* [19].

Analysis of urinary organic acids from D-2-HGA patients shows that in addition to the massive excretion of D-2-HG, the concentrations of citric acid cycle (CAC) intermediates are frequently elevated, implying a link between D-2-HG and the CAC [5,6].

Here, we present a study in which lymphoblasts of a control and two D-2-HGA patients were grown on media supplemented with either [U-$^{13}C_6$]glucose or [$^{2}H_5$]glutamate. The ^{13}C and ^{2}H mass isotopomer distributions [20] of citrate, 2-KG and 2-HG were used to identify the precursor of D-2-HG, and the subcellular location of its formation.

2. Materials and methods

2.1. Chemicals

[U-$^{13}C_6$]glucose (>97% isotopic purity), D-2-HG, L-2-HG, 2-KG,

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and ethoxylamine HCl were purchased from Sigma (St. Louis, MO, USA). [2,3,3,4,4-$^2H_5$]Glutamate (98% isotopic purity) was from CDN Isotopes (Quebec, QC, Canada). *R*-(−)-2-Butanol was purchased from Aldrich (St. Louis, MO, USA). Solid-phase extraction cartridges containing 60 mg OASIS HLB were from Waters (Milford, MA, USA). Lymphoblast culture medium RPMI 1640 was purchased from Gibco (Grand Island, NY, USA). Diazomethane was prepared according to the procedure of De Boer [21]. All other chemicals and solvents used were of analytical grade.

2.2. Cell lines

Human lymphoblast cell lines were established by Epstein–Barr virus transformation of leukocytes derived from whole blood. The lymphoblast cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. [U-$^{13}C_6$]Glucose incubations were performed by the addition of the sterilized labelled substrate to the complete medium in a 1:1 ratio towards the native unlabelled D-glucose content: 11 mM. [$^2H_5$]Glutamate incubations were performed by the addition of the sterilized labelled substrate (adjusted to pH 7.4) to the complete medium in a 10:1 ratio towards the native unlabelled glutamate content: concentration [$^2H_5$]glutamate 1.4 mM. The amounts of labelled substrates added to the culture medium were chosen after pilot experiments identified the minimal substrate enrichments needed to allow accurate measurements of (small) enrichments in the products.

One D-2-HGA lymphoblast cell line was from a male patient with neonatal onset of the disease and affected with the severe phenotype (patient 3 in Van der Knaap et al.'s review [5]). Clinically, the boy suffered from hypotonia, seizures, epilepsy and developmental delay. MRI revealed major alterations including enlarged lateral ventricles and delayed myelination. Laboratory analysis of urine, plasma and cerebrospinal fluid showed strongly elevated levels of D-2-HG (>200 times normal) and normal levels of L-2-HG.

The second D-2-HGA lymphoblast cell line was from a female patient with onset of the disease at 11 months and affected with the mild phenotype (patient 6 in Van der Knaap et al.'s review [6]). The girl suffered from psychomotor and mental retardation and cardiomyopathy. MRI, obtained at the age of 9 years, revealed multifocal small white matter lesions, spread over the cerebral white matter in both periventricular and subcortical areas. Laboratory analysis of urine and plasma showed elevated levels of D-2-HG (>40 times normal) and normal levels of L-2-HG.

The control lymphoblast cell line was originally obtained for carrier screening for an autosomal recessive disorder not related to D-2-HGA, and found to be normal.

Each cell line was cultured in (1) regular RPMI 1640 medium, (2) [U-$^{13}C_6$]glucose-supplemented medium and (3) [$^2H_5$]glutamate-supplemented medium. All incubations were performed in triplicate. At the start of the incubation, each 25-cm^2 flask contained approximately 100 000 cells/5 ml of the appropriate medium. After 96 h of incubation in a 5% CO_2–95% air incubator at 37°C, the medium was collected by centrifugation for 5 min at 1500×*g*. The cell-free medium was stored at −20°C until further analysis. The cell pellet was washed three times with Hanks' balanced salt solution, and the dry cell pellet was stored at 20°C until protein determination [22].

2.3. Determination of the mass isotopomer distributions of D- and L-2-HG

The analytical procedure for the determination of D- and L-2-HG was according to Gibson et al. [23] with some modifications. After the addition of 50 μl 6 M HCl to 1 ml cell medium (pH < 1), D- and L-2-HG were extracted using OASIS HLB solid-phase extraction cartridges. Briefly, the cartridge was pre-conditioned with 750 μl of methanol followed by 750 μl of 0.1 M HCl. After loading the sample by gravity, the cartridge was washed with 750 μl of 0.1 M HCl. The analytes were eluted from the cartridge with 800 μl of methanol. After drying the methanolic eluate under nitrogen at 50°C, a two-step derivatization procedure yielded the di-*R*-(−)-butyl-*O*-acetyl derivatives of D- and L-2-HG. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Hewlett Packard 5890 series II gas chromatograph, equipped with a CP-Sil 88 CB (Chrompack, The Netherlands) capillary column, and linked to a Hewlett Packard Engine 5989B mass spectrometer. The mass spectrometer was used in the positive chemical ionization mode using 5% NH_3 in methane as reagent gas. The distribution of the M to M5 mass isotopomers was monitored at *m/z* 303–308.

2.4. Determination of the mass isotopomer distributions of 2-KG

To 500 μl of cell medium 200 μl (100 g/l) ethoxylamine in H_2O was added after which the mixture was heated at 60°C for 30 min at pH < 1, to obtain the stable ethoxime derivative of the unstable α-keto group. The derivative was extracted using the same solid-phase extraction procedure as described for 2-HG. The methanolic eluate was blown to dryness under nitrogen at 50°C. Dimethyl derivatives were prepared by treatment of the residue with 20 μl of methanol and 180 μl diazomethane in ether. After a period of 5 min at room temperature, the solution was blown to dryness and the residue dissolved in 50 μl of ethyl acetate. GC-MS analysis was performed using the same set-up as described for the measurement of 2-HG. The distribution of the M to M5 mass isotopomers was monitored at *m/z* 218–223.

2.5. Determination of the mass isotopomer distributions of citrate

Citrate was extracted by the above-mentioned solid-phase extraction procedure, from 500 μl of culture medium, which was acidified by the addition of 25 μl of 6 M HCl. After the methanolic eluate was blown to dryness under nitrogen at 50°C, treating the dry residue with 20 μl of methanol and 180 μl diazomethane in ether formed the trimethyl derivative of citrate. After 5 min at room temperature, the solution was blown to dryness and the residue dissolved in 50 μl of ethyl acetate. GC-MS analysis was performed using the same set-up as described for the measurement of 2-HG. The $[M+NH_4]^+$ ion was found to be the most abundant ion for the GC-MS measurement of trimethylated citrate. The distribution of the M to M6 mass isotopomers was monitored at *m/z* 252–258. Unlabelled standards of D- and L-2-HG, 2-KG, and citrate were included in each sample set to verify the GC-MS mass calibration, and to assess the absence of contaminants at all *m/z* monitored.

2.6. Quantitative measurement of unlabelled 2-HG, 2-KG, and citrate in regular medium

The analytical work-up for 2-HG, 2-KG, and citrate in regular medium without the addition of labelled substrates, was performed as described in Sections 2.3, 2.4, and 2.5. Prior to the analysis, the following stable isotope-labelled internal standards were added to the corresponding samples: α-keto[3,3,4,4-$^2H_4$]glutarate and [2,2,4,4-$^2H_4$]citrate (Euriso-Top, Gif-sur-Yvette, France) and D/L-2-hydroxy-[3,3,4,4-$^2H_4$]glutarate, prepared by chemical reduction of α-keto-[3,3,4,4-$^2H_4$]glutarate as described previously [21]. Calibration curves were prepared with increasing amounts of analyte and a constant amount of the stable isotope-labelled internal standard. In the GC-MS analysis, the following single-ion monitoring settings were performed (*m/z* analyte; *m/z* internal standard): 2-HG (*m/z* 303; *m/z* 307), 2-KG (*m/z* 218; *m/z* 222), and citrate (*m/z* 252; *m/z* 256).

2.7. Calculations of enrichments in 2-HG, 2-KG, and citrate

The enrichments were calculated based on the results of the GC-MS measurements of the analytes of interest. Enrichments are expressed as molar percentage excess (MPE) and for the incubations performed with [U-$^{13}C_6$]glucose as the sum of the enrichments defined as total enrichment (TE).

In formula:

$$\mathrm{MPE}i = \frac{\text{peak area of isotopomer corrected for natural enrichment}}{\sum(\text{all corrected peak areas of the analyte})} \times 100$$

$$\mathrm{TE} = \frac{\sum(1 \text{ to } i) \text{ of } (\mathrm{MPE}i \times i)}{\text{number of C atoms in the underivatized molecule}}$$

where *i* represents the number of carbon atoms which can be labelled in the underivatized molecule and MPE*i* represents the MPE of the specific isotopomer. Note that TE represents the average fraction of carbon atoms that are labelled in the underivatized analyte. For an extensive discussion of these calculations, see [20].

In the nomenclature of mass isotopomers, the unlabelled species is designated M. Isotopomers containing 1 to *n* heavy atoms (^{13}C or ^2H) are designated M1 to M*n*.

Table 1
Formation of 2-HG, 2-KG, and citrate measured in cell culture media in nmol/96 h/mg protein

	Control cell line	D-2-HGA cell line, severe phenotype	D-2-HGA cell line, mild phenotype
L-2-HG	nd[a]	nd	nd
D-2-HG	nd	52 ± 9	34 ± 4
2-KG	11 ± 1.5	69 ± 14	10 ± 2
Citrate	108 ± 24	144 ± 29	81 ± 10

All the results were from three incubations per cell line, with single GC-MS measurement per flask, and are expressed as mean ± S.D.
[a]nd, not detectable.

3. Results

3.1. Levels of 2-HG, 2-KG, and citrate in media of cells grown on unsupplemented medium

The amounts of 2-HG, 2-KG, and citrate formed by the cultured lymphoblasts grown in regular RPMI 1640 medium are displayed in Table 1. The values were corrected for the levels of 2-HG, 2-KG, and citrate present in blank unincubated culture medium. The D-2-HGA cell lines accumulated D-2-HG with an average amount of 52 nmol and 34 nmol per mg protein after a 96-h incubation for the severe and mild cell line respectively. No D-2-HG could be detected in the culture medium of the control cell line. This shows that D-2-HGA lymphoblasts are suitable for biochemical investigations related to D-2-HGA. The levels of citrate in the cell medium were comparable for all cell lines, while the level of 2-KG in the medium of the severe D-2-HGA cell line was more than six times that in the media of the control cell line and the mild D-2-HGA cell line.

3.2. ^{13}C mass isotopomer distributions of metabolites labelled from [U-$^{13}C_6$]glucose

The ^{13}C mass isotopomer distributions of 2-HG, 2-KG, and citrate labelled from [U-$^{13}C_6$]glucose in the control cell line and the D-2-HGA cell lines are presented in Table 2. The mass isotopomer distributions of 2-KG and citrate were identical in the three cell lines. All possible mass isotopomers (M0 to M5 for 2-KG, and M0 to M6 for citrate) were detected. The most abundant isotopomer of 2-KG and citrate was the doubly labelled M2 mass isotopomer.

No D- or L-2-HG was detected in the control cell line, thus the enrichments of these compounds could not be measured. The mass isotopomer distribution of D-2-HG in the media of the two D-2-HGA cell lines strongly resembles that of 2-KG and citrate in which also the M2 isotopomer was the most abundant. The other isotopomers of D-2-HG found were M1, M3, M4 and M5. L-2-HG was not detectable.

3.3. ^{2}H mass isotopomer distributions of metabolites labelled from [$^{2}H_5$]glutamate

L-Glutamate is closely related to 2-KG via transamination and via glutamate dehydrogenase. In the three cell lines, high proportions of the M4 isotopomer of 2-KG were found (Table 3). The proportions of the M3 isotopomer were also measured in all cell lines with MPEs of approximately 7%. No other isotopomers of 2-KG were detected. The only detected mass isotopomer of citrate was M1, with MPEs of 2.1%, 2.3% and 0.9% for the control, severe and mild D-2-HGA cell line respectively.

For D-2-HG formed by the D-2-HGA cell lines, the M4 isotopomer (MPEs 9.1% and 10.1% for the severe and mild cell line respectively) was the most abundant. Next to M4, the M3 isotopomer was also present.

4. Discussion

The ongoing research into the underlying defect in D-2-HGA has not been very successful since the first description of the disease in 1980. The lack of knowledge about the metabolic origin of D-2-HG hampers biochemical studies focussed on the cause of its accumulation in affected patients. Recently, we reported that cultured human fibroblasts from patients with D-2-HGA released high amounts of D-2-HG in the culture medium [24]. Further experiments showed that this was

Table 2
^{13}C enrichments expressed as MPE and TE in D-2-HG, 2-KG, and citrate measured in media of a control and two D-2-HGA cell lines grown on [U-$^{13}C_6$]glucose-supplemented medium

	Isotopomer	Control cell line (MPE, %)	D-2-HGA cell line, severe phenotype (MPE, %)	D-2-HGA cell line, mild phenotype (MPE, %)
D-2-HG	M1	nd[a]	7.8 ± 0.6	8.9 ± 0.4
	M2	nd	20.2 ± 0.2	19.2 ± 0.8
	M3	nd	7.2 ± 0.4	6.9 ± 0.5
	M4	nd	4.4 ± 0.6	3.6 ± 0.4
	M5	nd	1.0 ± 0.4	1.1 ± 0.2
	TE	nd	0.18 ± 0.004	0.18 ± 0.01
2-KG	M1	6.5 ± 0.5	7.2 ± 0.1	8.9 ± 1.0
	M2	17.1 ± 0.5	18.7 ± 0.1	16.0 ± 0.7
	M3	5.5 ± 0.9	6.1 ± 0.2	6.0 ± 0.7
	M4	4.0 ± 0.2	3.9 ± 0.2	3.1 ± 0.2
	M5	0.7 ± 0.2	1.2 ± 0.1	0.8 ± 0.1
	TE	0.15 ± 0.007	0.17 ± 0.001	0.15 ± 0.005
Citrate	M1	5.6 ± 0.1	6.0 ± 0.03	3.6 ± 0.4
	M2	16.7 ± 0.2	18.0 ± 0.1	13.6 ± 0.4
	M3	6.3 ± 0.1	6.6 ± 0.03	5.2 ± 0.3
	M4	4.7 ± 0.1	5.2 ± 0.1	3.9 ± 0.1
	M5	2.1 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
	M6	0.43 ± 0.05	0.35 ± 0.05	0.30 ± 0.02
	TE	0.15 ± 0.001	0.16 ± 0.001	0.14 ± 0.002

All the results were from three incubations per cell line, with single GC-MS measurement per flask, and are expressed as mean ± S.D.
[a]nd, not detectable.

Table 3
^{2}H enrichments expressed as MPE in D-2-HG, 2-KG, and citrate measured in media of a control and two D-2-HGA cell lines grown on [$^{2}H_5$]glutamate-supplemented medium

	Isotopomer	Control cell line (MPE, %)	D-2-HGA cell line, severe phenotype (MPE, %)	D-2-HGA cell line, mild phenotype (MPE, %)
D-2-HG	M3	nd[a]	2.2 ± 0.2	3.2 ± 0.3
	M4	nd	9.1 ± 0.5	10.1 ± 0.1
2-KG	M3	7.7 ± 0.1	6.7 ± 0.03	6.2 ± 0.5
	M4	43 ± 0.2	38 ± 0.6	22 ± 1
Citrate	M1	2.1 ± 0.1	2.3 ± 0.3	0.9 ± 0.1

All the results were from three incubations per cell line, with single GC-MS measurement per flask, and are expressed as mean ± S.D.
[a]nd, not detectable.

also true for human lymphoblasts, with the difference that these cells accumulated even more D-2-HG.

We took advantage of the availability of these cell lines and of recent developments in mass isotopomer analysis of metabolic pathways [20] to conduct a study on the origin of D-2-HG. The physiological metabolite with a structure closest to that of D-2-HG is 2-KG. 2-KG can be formed in mitochondria via the CAC, glutamate dehydrogenase, or transamination of glutamate. It can also be formed in the cytosol via transamination of glutamate. We designed a two-pronged study to label 2-KG either via the mitochondrial reactions of the CAC (using [U-$^{13}C_6$]glucose), or via a combination of mitochondrial and cytosolic reactions (using [$^{2}H_5$]glutamate). The mass isotopomer distributions of D-2-HG, 2-KG and citrate labelled from the two tracers reveals the mitochondrial origin of D-2-HG.

4.1. Experiments with [U-$^{13}C_6$]glucose

The mass isotopomer distributions of D-2-HG, 2-KG and citrate labelled from [U-$^{13}C_6$]glucose (Table 2) result from multiple passages of labelled molecules through the CAC (Fig. 1). Briefly, [U-$^{13}C_6$]glucose is first metabolized by glycolysis to [$^{13}C_3$]pyruvate, which is converted to [$^{13}C_2$]acetyl-CoA and [$^{13}C_3$]oxaloacetate by pyruvate dehydrogenase and pyruvate carboxylase respectively. At the level of succinate, the ^{13}C carbons originating from [$^{13}C_2$]acetyl-CoA are located either on carbon positions 1 and 2 or on positions 3 and 4 as a result of the symmetry in succinate. In three subsequent bio-

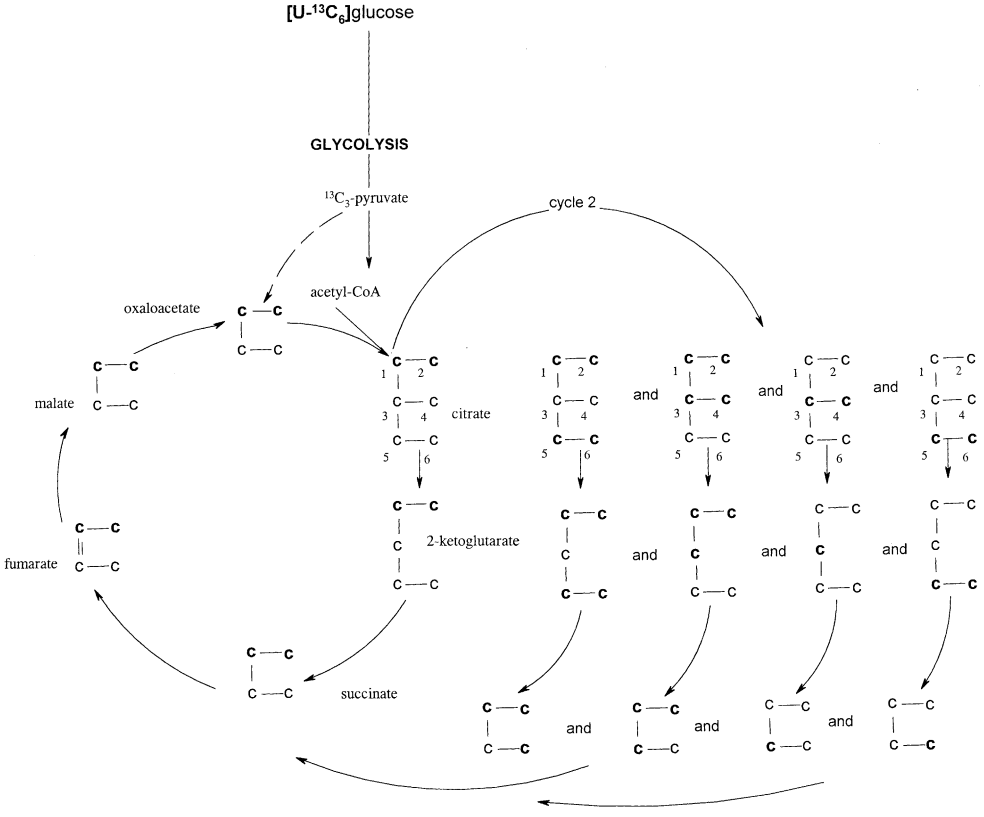

Fig. 1. The metabolic fate of ^{13}C deriving from [$^{13}C_6$]glucose is represented in this figure in which only the carbon backbones of the intermediates are shown. ^{13}C atoms that are incorporated in the CAC intermediates are displayed in bold. The dashed arrow indicates the conversion of [$^{13}C_3$]pyruvate to [$^{13}C_3$]oxaloacetate. For reasons of simplicity, ^{13}C incorporation in CAC intermediates originating from [$^{13}C_3$]oxaloacetate is not shown. See Sections 3 and 4 for explanation.

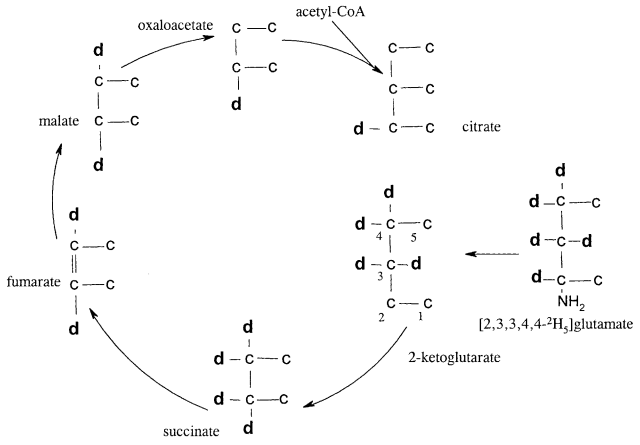

Fig. 2. The metabolic fate of ^2H (represented as **d**) deriving from [$^2H_5$]glutamate is represented in this figure starting with the conversion of [$^2H_5$]glutamate to [$^2H_4$]2-KG. Subsequent losses of ^2H atoms during the passage through the CAC lead to [^2H]oxaloacetate in which the remaining ^2H atom is fixed on the third C atom of the molecule.

chemical reactions, doubly labelled oxaloacetate is obtained, which can start its second cycle by condensation with the pool of acetyl-CoA, consisting of both unlabelled and doubly labelled molecules. In parallel, [$^{13}C_3$]oxaloacetate, formed by carboxylation of [$^{13}C_3$]pyruvate by pyruvate carboxylase, condenses with the pool of labelled+unlabelled acetyl-CoA resulting in the M3 and M5 isotopomers of citrate. Lastly, after multiple turns of the cycle, all possible mass isotopomers of citrate are formed, provided that the enrichment of the [U-$^{13}C_6$]glucose precursor is sufficiently high (in our case, it was 50%).

In both D-2-HGA cell lines cultured with [U-$^{13}C_6$]glucose, we found a strong identity between the distributions of the M1 to M5 isotopomers of D-2-HG and 2-KG (Table 2). This suggests that one of the two metabolites is the precursor of the other, or that the two compounds are interconverted. Also, the distribution of the M1 to M5 isotopomers of citrate and 2-KG is very similar. This results from the fact that, in the conversion of citrate to 2-KG, very little label is lost from carbon 6 of citrate. Because of the reversibility of the reactions catalyzed by aconitase and isocitrate dehydrogenase [25], much of the label on the C6 of citrate is lost by isotopic exchange with unlabelled CO_2. Therefore, the almost identical distributions of M1 to M5 isotopomers of D-2-HG, 2-KG and citrate show that the three compounds are part of a metabolic sequence. This is confirmed by the almost identical total enrichment fractions of D-2-HG, 2-KG and citrate. Since citrate is formed only in the mitochondria, the identity of the distributions of the M1 to M5 isotopomers of citrate and 2-KG shows that the labelling of the latter is not diluted by unlabelled carbon derived from glutamate in the culture medium (at least under our conditions). Then, the identity of the distributions of the M1 to M5 isotopomers of 2-KG and D-2-HG shows that D-2-HG is formed in mitochondria from 2-KG.

4.2. Experiments with [$^2H_5$]glutamate

In orientating experiments with physiological concentrations of [$^2H_5$]glutamate, we could not detect the labelling in citrate. This is why we conducted all subsequent experiments with 90% enriched [$^2H_5$]glutamate at a 10 times physiological concentration. The unphysiological concentration of [$^2H_5$] glutamate did not affect the conclusions of the study (see below). The interpretation of the ^2H mass isotopomer distribution of citrate, 2-KG and D-2-HG labelled from [$^2H_5$]glutamate (Table 3) is based on the sequence of reactions represented in Fig. 2. First in the conversion of [$^2H_5$] glutamate to 2-KG catalyzed by glutamate dehydrogenase and transaminases, ^2H on the C2 of glutamate is lost to water. This is why M4 2-KG is the most abundant labelled isotopomer of 2-KG. At the time of harvesting the MPEs of M4 and M5 glutamate, measured by GC-MS as their dimethyl-*N*-methylcarbamate derivatives, were 35 and 11% respectively for the control and severe D-2-HGA cell line and 25 and 1% for the mild D-2-HGA cell line, showing the interconversion between glutamate and 2-KG.

In addition to M4 2-KG, there was a substantial abundance of M3 2-KG in the media of the control and the D-2-HGA cells (Table 3). The abundance of the M3 2-KG, about one fifth that of M4 2-KG, cannot be explained by the presence of substantial amounts of M4 glutamate in the tracer. Indeed, the abundance ratio M5/M4 in the glutamate tracer, 13, was greater than the corresponding ratio M4/M3 in 2-KG. The production of M3 2-KG results most likely from the reaction of keto-enol tautomerism between C2 and C3 of 2-KG, resulting in partial loss of ^2H on C3 (Fig. 3).

In the D-2-HGA cell lines, the abundances of the M4 and M3 isotopomers of D-2-HG were two to five times lower than those of the M4 and M3 isotopomers of 2-KG (Table 3). In addition, the abundance ratios M4/M3 for the severe and mild D-2-HGA cell lines, i.e. 4.1 and 3.8, were not very different from the M4/M3 abundance ratios in 2-KG, i.e. 5.6 and 3.5. This supports the previous conclusion that M4 and M3 2-KG are related to M4 and M3 D-2-HG. However, when 2-KG is labelled from [$^2H_5$]glutamate, there is a dilution of unlabelled carbon between 2-KG and D-2-HG. In cells incubated with a high concentration of 90% labelled [$^2H_5$]glutamate, there must be a high concentration of highly labelled cytosolic 2-KG derived from transamination of [$^2H_5$]glutamate. As cytosolic 2-KG enters the mitochondria via reversible transamination, it gets diluted by unlabelled 2-KG. The origin of this unlabelled 2-KG is evident from the very low enrichment of citrate. In the steps between 2-KG and oxaloacetate (the precursor of citrate), most of the label of 2-KG is lost at the rapidly reversible reactions catalyzed by succinate dehydrogenase, fumarase and malate dehydrogenase. Since the labelling of citrate is very low, it generates unlabelled 2-KG in the next turn of the CAC, thus diluting the label of 2-KG derived from [$^2H_5$]glutamate.

In conclusion, the mass isotopomer distribution of citrate, 2-KG and D-2-HG released by cell cultured with [U-$^{13}C_6$]-glucose or [$^2H_5$]glutamate demonstrates that mitochondrial 2-KG interconverts with D-2-HG. In addition, the similarity of the isotopomer patterns of citrate, 2-KG and D-2-HG labelled from [U-$^{13}C_6$]glucose demonstrates the close relation-

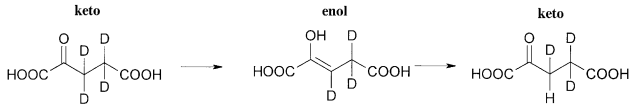

Fig. 3. The keto-enol tautomerism of 2-KG, resulting in the loss of one ^2H atom, non-enzymatically generates [$^2H_3$]2-KG from [$^2H_4$]2-KG.

ship of the CAC reactions with the reaction(s) forming D-2-HG. The knowledge obtained in this study provides a step forwards in the elucidation of the basic defect causing D-2-HGA.

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