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METABOLIC DISSERTATION

D-2-Hydroxyglutaric aciduria: Unravelling the biochemical pathway and the genetic defect

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Summary D-2-Hydroxyglutaric aciduria (D-2-HGA) is a neurometabolic inherited disorder first described in 1980. In the following years, it became clear that the clinical phenotype of the disease varies widely from severe neonatal to asymptomatic. However, the sparse biochemical knowledge made D-2-HGA a poorly understood disease. Much progress has been made in the last five years in various studies, revealing two human enzymes that play a role in the metabolism of D-2-hydroxyglutarate (D-2-HG): hydroxyacid-oxoacid transhydrogenase (HOT) and D-2-HG dehydrogenase. HOT is expected to be responsible for the formation of D-2-HG, while D-2-HG dehydrogenase converts D-2-HG into 2-ketoglutarate. We demonstrated pathogenic mutations in the *D2HGD* gene in patients with D-2-HGA, helping to unravel the primary defect causing D-2-HGA. However, in approximately 50% of the patients with D-2-HGA examined, no pathogenic mutations have yet been found.

D-2-Hydroxyglutaric acid, the compound and the disease

2-Hydroxyglutaric acid (2-HG) is a five-carbon dicarboxylic acid in which the second carbon atom carries a hydroxyl group (Fig. 1); it is indirectly named from the Latin word *gluten*. The systematic name of 2-HG according to the International Union of Pure and Applied Chemistry (IUPAC) is 2-hydroxypentanedioic acid.

The second carbon atom (C2) in 2-HG is termed an asymmetric carbon atom or chiral centre, since four different functional groups are attached to it. Owing to the presence of this chiral centre, 2-HG occurs in two optically active forms: D-2-HG and L-2-HG, which are effectively mirror-images of one another (Fig. 2).

D-2-HG and L-2-HG are *enantiomers*, which have identical chemical properties such as melting point and solubility. However, a major difference between them is manifested when plane-polarized light is passed through samples containing either D-2-HG or L-2-HG leading to a different optical rotation of the plane-polarized light. In addition to the D and L nomenclature, the symbols *R* and *S* are also used to indicate chirality: D corresponds to *R* and L corresponds to *S*.

The existence of the different three-dimensional molecular forms of D-2-HG and L-2-HG has huge significance for the recognition of the compounds by enzymes. In general, enzymes will only interact with one specific enantiomer, being inactive for the other enantiomer. Thus, D-2-HG and L-2-HG can be regarded as two individual compounds, each having its own specific metabolism and, as will be described below, associated with different individual disease entities: D-2-HG aciduria (D-2-HGA) and L-2-HG aciduria (L-2-HGA).

In the 1970s, it was recognized that 2-HG is a normal constituent of human urine and amniotic fluid (Gregersen et al 1977; Thompson et al 1977). Mildly increased urinary levels of 2-HG were detected in patients affected with multiple acyl-CoA dehydrogenase deficiency (Goodman et al 1980; Przyrembel et al 1976), pyruvate decarboxylase deficiency (E1) (Chalmers et al 1977), and dihydrolipoyl dehydrogenase

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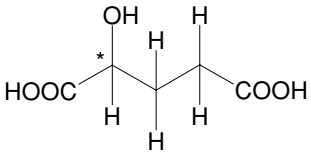

Fig. 1 Molecular structure of 2-HG; the chiral centre is indicated by an asterisk

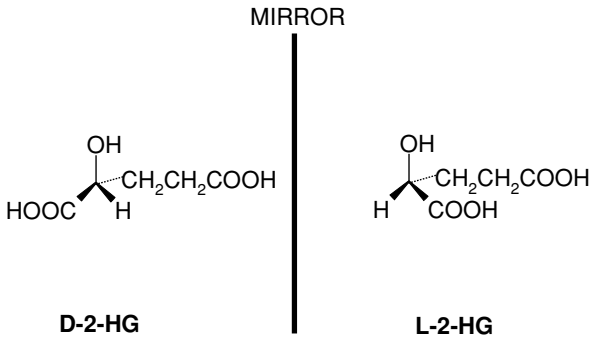

Fig. 2 Stereochemistry of 2-HG. D-2-HG and L-2-HG are not superimposable, making them enantiomers

deficiency (E3) (Kuhara et al 1983), and in a disorder of gluconeogenesis, pyruvate carboxylase deficiency (Van Biervliet et al 1977). In 1980, a patient was reported who excreted 2-HG to a much greater extent than patients with one of the disorders described above (Chalmers et al 1980). The patient was the first child of first-cousin parents, normal at birth but presenting with anaemia and recurrent antibiotic-resistant infections. Investigation with chromium-51 revealed a severe protein-losing enteropathy caused by an allergic response to the dietary intake of eggs. The patient was not retarded and attended a normal school. The increased excretion of 2-HG was detected by urinary organic acid screening after conversion of the organic acids to their trimethylsilyl (TMS) derivatives, followed by gas chromatography (GC) with flame ionization detection (FID). The absolute configuration of 2-HG in the patient's urine cannot be determined by this analytical procedure, since D- and L-2-HG appear as a single peak in the chromatogram (Fig. 3).

Enantiomeric analysis of D- and L-2-HG

Chromatographic separation of a pair of enantiomers can be achieved by two different strategies. First, the compounds can be derivatized with an optically pure chiral reagent, resulting in a set of derivatives that are no longer mirror-images and are called *diastereomers*. These diastereomers have slightly different chemical characteristics, allowing separation of the derivatized enantiomers by GC or liquid chromatography (LC). This strategy was applied to the urine sample of the index patient, in which (*R*)-(−)-2-butanol was used as chiral reagent (Chalmers et al 1980). By the use of enantiomerically pure standards of 2-HG, the increased 2-HG could be identified as D-2-HG. In the same year, a second patient was described who also excreted large amounts of 2-HG. This patient presented with psychomotor retardation and dystrophy. Using the same analytical approach as described above, the 2-HG excreted by this patient was found to be of the L-configuration (Duran et al 1980). Gibson and colleagues improved the method, by introducing stable-isotope-labelled internal standards and using GC with positive chemical ionization mass spectrometry (MS) as for analysis (Gibson et al 1993a). Recently, our group reported a new method based on the above approach, in which (*R*, *R*)-diacetyltartaric anhydride is used as chiral reagent (Struys et al 2004b). The derivatives formed are separated by LC and detected by tandem mass spectrometry (MS/MS), decreasing both costs and sample handling time of the analysis (Fig. 4). Alternatively,

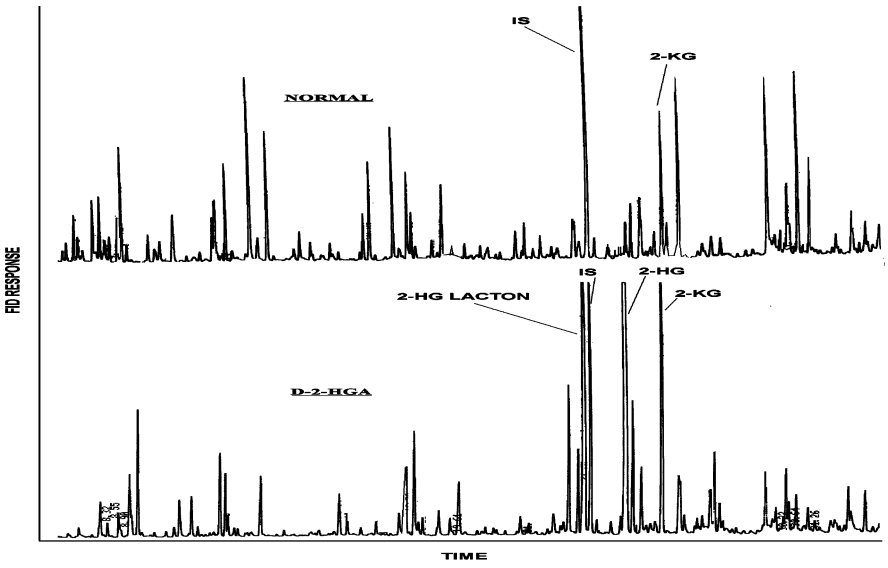

Fig. 3 Normal urinary organic acid profile (upper part) and urinary organic acid profile of a child with D-2-HGA (lower part). The signal designated as 2-HG lactone is formed during sample preparation. IS represents the added internal standard (4-phenylbutyric acid)

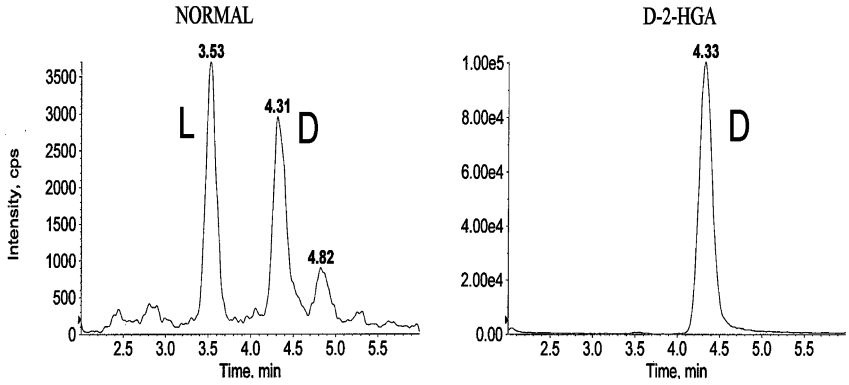

Fig. 4 Enantiomeric separation of D,L-2-HG by LC-MS/MS performed on a normal urine sample (left) and a urine sample from a child with D-2-HGA (right). The intensities of the signals are plotted on the *Y*-axis. Owing to the high level of D-2-HG in the urine sample of the child with D-2-HGA, the signal of L-2-HG is not visible

enantiomers can be separated by the use of a specific analytical column, which is coated (GC) or packed (LC) with a chiral stationary phase. Derivatization with a chiral reagent is not required and the enantiomers are separated on the basis of three-dimensional interactions with the chiral stationary phase of the column. This approach was explored by Rashed and colleagues, and applied to urine samples of patients with L-2-HGA (Rashed et al 2000).

Clinical description of patients with D-2-HGA

The disease has been known for 25 years now and, because of the limited number of patients (approximately 75), it can be concluded that D-2-HGA is a very rare autosomal recessive disorder. The strongly increased level of D-2-HG in urine, plasma and cerebrospinal fluid of patients is the biochemical hallmark of the disease. D-2-HGA has been observed in patients with extremely variable clinical symptoms, creating doubt about the existence of a disease entity related to the biochemical finding. Van der Knaap and colleagues have evaluated clinical and biochemical data obtained from an international survey of 25 patients with D-2-HGA (Van der Knaap et al 1999a,b). Two phenotypes could be distinguished. The severe phenotype is characterized by a neonatal- or early-infantile-onset epileptic encephalopathy with marked hypotonia, cerebral visual failure or delayed cerebral visual development, and serious developmental delay. Cardiomyopathy and facial dysmorphic features are frequently reported in this group of D-2-HGA patients. The facial dysmorphia consist of a flat face with a broad nasal bridge and external ear anomalies (Amiel et al 1999). In addition, spondyloenchondrodysplasia has been reported in three patients with D-2-HGA, all born to nonconsanguineous parents (Bayar et al 2005; Honey et al 2003; Talkhani et al 2000). Magnetic resonance imaging (MRI) of the brain of severely affected patients typically shows signs of delayed cerebral maturation, ventricular white-matter abnormalities, and the presence of subependymal cysts in the first months of life.

The second phenotype of D-2-HGA is the mild variant, which presents much more variably. The most frequent findings are hypotonia and developmental delay. In addition to phenotypically mild patients with D-2-HGA, two asymptomatic siblings with consistent massive D-2-HG elevations in their body fluids have been reported (Korman et al 2004). MRI findings in the group of phenotypically mild D-2-HGA patients were generally in agreement with the findings in the severe group, although the MRI alterations were milder and more variable (Van der Knaap et al 1999a, b). There is no correlation between the levels of D-2-HG in the body fluids of patients and their clinical symptoms, making it impossible to predict the long-term outcome of the disease.

L-2-HGA and combined D,L-2-HGA

Isolated L-2-HGA with normal levels of D-2-HG was first described in 1980 in a 5-year-old boy who presented with psychomotor retardation and dystrophy (Duran et al 1980). To date >100 patients have been diagnosed with L-2-HGA. The clinical phenotype is one of a progressive neurodegenerative disorder manifesting with extrapyramidal and cerebellar signs, seizures, and white-matter alterations visualized by MRI. The enzymatic and molecular bases of the disease have recently been uncovered. The disease is caused by a defect in L-2-HG dehydrogenase, responsible for the conversion of L-2-HG to 2-ketoglutaric acid (2-KG) (Rzem et al 2004; Topcu et al 2004). In addition to the increased levels of L-2-HG in all body fluids, lysine is moderately elevated in cerebrospinal fluid of affected patients (Barth et al 1992). The metabolic origin of L-2-HG has not yet been identified.

Combined D-2-HGA and L-2-HGA was found in four patients who suffered from a severe neonatal-onset encephalopathy (Muntau et al 2000; Read et al 2005). The outcome of this disease is unfavourable: three children died in the first year of life, and a fourth child died at 3.5 years of age. Apart from the elevations of D- and L-2-HG and 2-KG, no other biochemical abnormalities were found in body fluids from these patients.

Metabolism of D-2-HG

To understand the biochemical mechanisms in D-2-HGA, it is necessary to explore the pathways in which D-2-HG is involved. These can be divided into bacterial/yeast and mammalian metabolisms, in which D-2-HG is an intermediate.

Bacterial and yeast metabolism

The main reaction catalysed by the reversible *SerA*-encoded enzyme 3-phosphoglyceric acid dehydrogenase in *Escherichia coli* is the oxidation of 3-phosphoglyceric acid to 3-phosphohydroxypyruvic acid using NAD^+ as cofactor (Fig. 5). Zhoa and colleagues found that, analogously to this reaction, the enzyme exhibited activity for 2-KG resulting in the formation of D- and L-2-HG (Zhao and Winkler 1996). Steady-state kinetics of this enzyme revealed a lower K_m and higher V_{max} for the oxidation of D-2-HG than for the oxidation of L-2-HG. In contrast with the above, rat 3-phosphoglyceric acid dehydrogenase showed no affinity for 2-KG (Achouri et al 1997).

Experiments with [U-^{14}C]glutamic acid in anaerobically grown *Saccharomyces cerevisiae* revealed the production of 2-HG (no enantiomeric specificity has been described) from the labelled substrate (Albers et al 1998). It was suggested that 2-HG is the end product of a metabolic sequence from glutamate via 2-KG to 2-HG, and that 2-HG acts as a local sink for NADH.

In 1980, Buckel described enantiomer-specific dehydration of D-2-HG, resulting in *trans*-glutaconic acid (2,3-pentenedioic acid) during fermentation experiments with whole cells of *Acidaminococcus fermentans* (Buckel 1980) (Fig. 6). D-2-HG is not dehydrated as free acid in this reaction, but is first activated to its CoA ester.

Other bacterial pathways involving 2-HG are the condensation of glyoxylate and propionyl-CoA by *Escherichia coli*, producing D-2-HG (Wegener et al 1968), and the conversion of 2-aminoadipic acid to 2-HG by *Pseudomonas putida* (Kopchick and Hartline 1979). The different bacterial metabolic reactions consuming or producing 2-HG imply that in mammals 2-HG production can be partly the result of intestinal bacterial metabolism (Kumps et al 2002).

Mammalian metabolism

The number of known mammalian metabolic pathways involving 2-HG is limited. In the description of the index case of D-2-HGA it was mentioned that D-2-HG is an intermediate in the succinic acid–glycine cycle with 2-KG semialdehyde and 2-KG being its precursor and product, respectively. It was suggested that the conversion of D-2-HG to 2-KG is defective in the patient described (Chalmers et al 1980) (Fig. 7).

In experiments with rat liver homogenates, it was found that 2-HG is an intermediate in the degradation of 5-hydroxylysine (Lindahl et al 1967), but this has not been confirmed in a later study (Hammerstedt 1968).

In 1988, the isolation and characterization of a hydroxyacid-oxoacid transhydrogenase (HOT) from rat kidney mitochondria was described (Kaufman et al 1988b). This enzyme was uncovered in experiments related to the

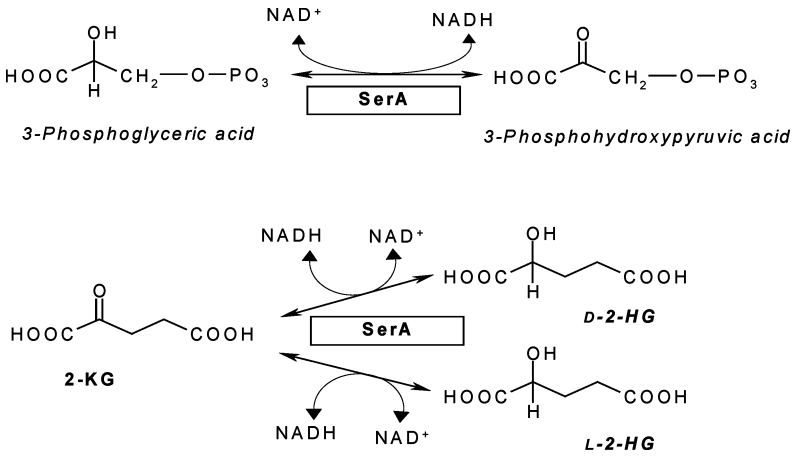

Fig. 5 The oxidation of 3-phosphoglyceric acid to 3-phosphohydroxypyruvic acid, and the analogous reduction of 2-KG to L- and D-2-HG catalysed by SerA-encoded 3-phosphoglyceric acid dehydrogenase of *Escherichia coli*

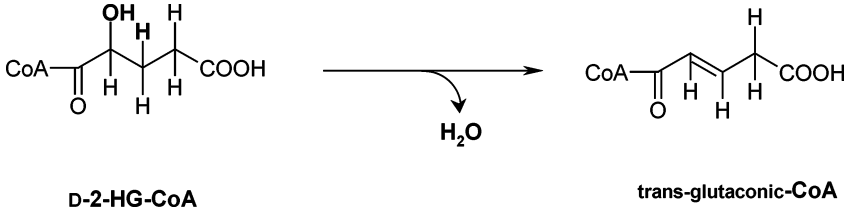

Fig. 6 Dehydration of D-2-HG-CoA during fermentation with whole cells of *Acidaminococcus fermentans* yielding *trans*-glutaconyl-CoA

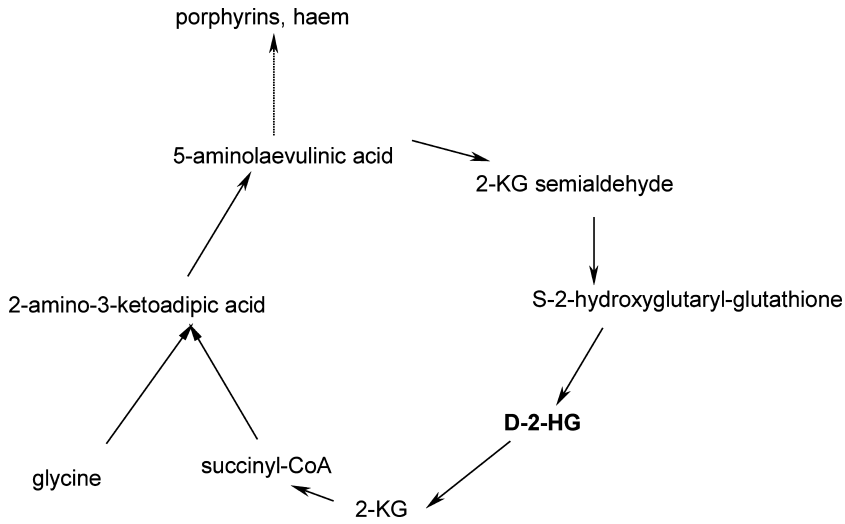

Fig. 7 The succinic acid–glycine cycle in which D-2-HG is involved

oxidation of gamma-hydroxybutyrate (GHB) [36] and HOT activity was found in rat liver, kidney and brain. HOT-catalysed reversible oxidation of a hydroxyacid is obligatorily coupled to the reduction of an oxoacid, and requires no cofactor. Several hydroxyacids and oxoacids have proved to be substrates for HOT, but the main reaction catalysed is the oxidation of GHB in the presence of 2-KG, resulting in the formation of succinic semialdehyde (SSA) and D-2-HG (Fig. 8). Further studies revealed a postnatal increase of HOT activity in rat brain and kidney (Nelson and Kaufman 1994). HOT deficiency has been postulated as a possible cause of D-2-HGA (Craigen et al 1994; Gibson et al 1993b). The existence of human HOT has been demonstrated in homogenates of human liver and fibroblasts (Struys et al 2005a,b). The hypothesis that HOT deficiency is the underlying cause in D-2-HGA was examined by a double stable-isotope-labelled HOT activity assay in fibroblast homogenates. Fibroblasts from patients with D-2-HGA displayed normal HOT activity, implying that there is no link between D-2-HGA and HOT (Struys et al 2005b).

The metabolic origin of D-2-HG has been studied using cultured lymphoblast cells from patients with D-2-HGA, grown on culture medium supplemented with either [U-$^{13}C_6$]glucose or [$^2H_5$]glutamic acid, revealing the metabolic relationship between D-2-HG and the citric acid cycle (CAC) (Struys et al 2004a). In experiments with [U-$^{13}C_6$]glucose, all possible isotopomers of 2-KG, citric acid, and D-2-HG were detected by GC-MS. The isotopomeric distribution is the result of multiple passages of ^{13}C atoms through the CAC. [$^2H_5$]Glutamic acid is converted by the cells to [$^2H_4$]2-KG, which can enter the CAC. During the passage of [$^2H_4$]2-KG through the CAC, ^2H atoms are released during subsequent enzymatic conversions. From quantitative and qualitative interpretations of the ^{13}C and ^2H enrichments measured in D-2-HG, citrate, and 2-KG, it was concluded that 2-KG is the metabolic precursor of D-2-HG.

Recently, a D-2-HG dehydrogenase isolated from rat liver was described (Achouri et al 2004). The enzyme was discovered in experiments using D,L-2-hydroxy[2-^3H]glutarate as substrate, followed by measurement of the released ^3H_2O, resulting from the oxidation of the hydroxy group yielding 2-KG as product. The enzyme was subsequently purified on a DEAE-Sepharose column, a Blue Trisacryl column, and a phenyl-Sepharose column. The remaining proteins were further purified by SDS-PAGE, cut from the gel, digested with trypsin and analysed by electrospray-ionization MS/MS, enabling the determination of the amino acid sequence of the protein. One band showed significant homology with a mouse protein and with a D-lactate dehydrogenase from *Kluyveromyces lactis*. Overexpression experiments of the corresponding putative human D-2-HG dehydrogenase in HEK-293 cells indeed showed increased D-2-HG dehydrogenation activity. The mitochondrial enzyme appeared to be independent of NAD or NADP, was possibly FAD linked, was stimulated by Zn^{2+}, Co^{2+}, and Mn^{2+} and also showed activity towards D-lactic, D-malic, and *meso*-tartaric acids.

The K_m of D-2-HG dehydrogenase for D-2-HG was <10 μmol/L. This very low K_m value makes this D-2-HG dehydrogenase different from previously described D-2-HG dehydrogenases (Tubbs and Greville 1961; Wanders and Mooyer 1995). The low concentration of D-2-HG in human plasma (<1 μmol/L) indicates that the recently found D-2-HG dehydrogenase with its low K_m is likely to be important for D-2-HG metabolism.

Mutation studies in the *D2HGD* gene

Mutation studies were performed in the *D2HGD* gene in two unrelated patients affected with the severe phenotype of D-2-HGA (Struys et al 2005a). The first patient was born to consanguineous (first-cousin) healthy parents. Clinical

assessment of this patient revealed mild facial dysmorphia, psychomotor retardation and nontreatable myoclonic seizures. The second patient was born to nonconsanguineous parents and presented with seizures, hypotonia, a movement disorder, cortical blindness, and developmental delay. In the DNA of the first patient, a homozygous missense mutation (c.1331T > C, *p*.Val444Ala) was found. In the DNA of the second patient, a heterozygous missense mutation (c.440T > G, *p*.Ile147Ser) and a splice-site mutation (IVS1−23A > G) were found. Overexpression studies in HEK-293 cells of proteins encoded by the missense mutations showed marked reduction of D-2-HG dehydrogenase activity.

Pathogenic mutations in the *D2HGD* gene also account for the mild phenotype of D-2-HGA in three patients from two unrelated families (Struys et al 2005c). In one family (first-cousin parents), one child was diagnosed with glutaric aciduria type I (GA I) and two siblings were affected with D-2-HGA, although they were clinically asymptomatic. In DNA of these two patients, a homozygous A-to-G transition was found (IVS4−2A > G; c.687−2A > G), whereas the sibling affected with GA I and the parents were carriers of this mutation. In a second family, with parents from highly consanguineous kindred, a boy was diagnosed with the mild phenotype of D-2-HGA on the basis of his clinical presentation. MRI of brain showed some mild, nonspecific periventricular white-matter changes. In DNA of this patient, a homozygous A-to-G transition was found (c.1315A > G) that results in the replacement of asparagine at position 439 by aspartate (p.Asn439Asp). His brother, father and mother were carriers of this mutation. Overexpression of the mutant protein showed that this amino acid change resulted in 85% decrease of D-2-hydroxyglutarate dehydrogenase activity.

The complexity of genotype–phenotype relationship in D-2-HGA was illustrated by the case of monozygotic female twins affected with D-2-HGA (Misra et al 2005). The twins were compound heterozygous for a duplication in exon 2 (c.326–327dupTC; p.Glu110ArgfsX19) and a missense mutation in exon 7 (c.1123G > T; *p*.Asp375Tyr). The first-born twin (birth weight 1429 g) presented with multiple congenital anomalies, and severe developmental delay. Her sister (birth weight 1701 g) had normal neurocognitive and neuroradiological findings but, like her sister, had mild facial anomalies. The different clinical presentation cannot be attributed to the urinary concentrations of D-2-HG, which were 230 and 430 mmol/mol creatinine for the severely affected girl and the mildly affected girl, respectively. This case implies that the differences in the clinical phenotype arise from postzygotic genetic changes, epigenetic differences, and/or environmental factors that influence the phenotypic response to biochemical dysfunction rather than allelic variation.

The *D2HGD* gene consists of 10 exons, and is depicted in Fig. 9, including the pathogenic mutations found for the seven patients investigated so far.

Pathological implications of D-2-HG

Clinical symptoms in D-2-HGA often involve severe neurological dysfunction and cerebral atrophy, making D-2-HGA a neurometabolic disorder. The primary biochemical abnormality in D-2-HGA is the strongly increased level of D-2-HG in body fluids of affected patients. In addition, increased GABA levels in CSF have been reported (Van der Knaap et al 1999a,b). In urinary organic acid screening, citric acid cycle (CAC) intermediates such as 2-KG, citric, and succinic acids may be moderately elevated in D-2-HGA. In the past five years, several attempts have been made to reveal the pathophysiological mechanisms in D-2-HGA. The main focus in understanding the observed neuropathy in D-2-HGA is to explore (neuro)toxic effects of D-2-HG. In experiments with primary neuronal cultures from chicken and rats, incubation with pathological concentrations of D-2-HG resulted in excitotoxic cell damage by *N*-methyl-D-aspartic acid receptor activation (Kölker et al 2002). In addition, it was concluded that D-2-HG disturbed intracellular calcium homeostasis, elicited the generation of reactive oxygen species and reduced complex V (ATP synthase) activity of the mitochondrial respiratory chain. Studies with cerebral cortex homogenates of young rats revealed that D-2-HG induced oxidative stress (Latini et al 2003). A dose-dependent inhibition of cytochrome-*c* oxidase (COX, complex IV) activity was found in homogenates of rat cerebral cortex and human skeletal muscle (da Silva et al 2002). COX is the

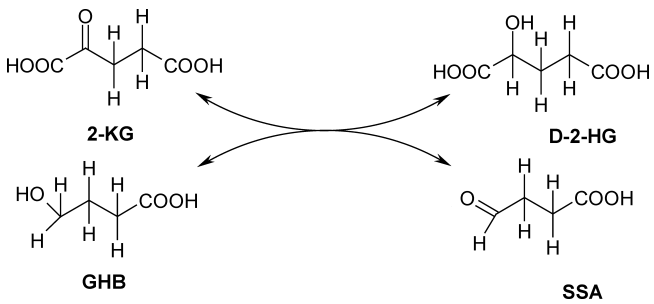

Fig. 8 Main reaction catalysed by HOT: the reversible reduction of 2-KG in the presence of GHB yielding D-2-HG and SSA

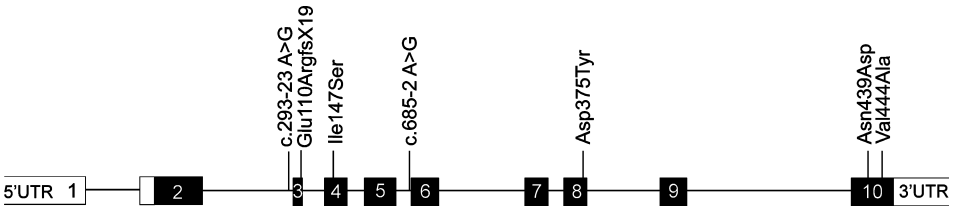

Fig. 9 Mutations in the *D2HGD* gene (see Genbank for genomic DNA [accession number 27465811] and cDNA [accession number 22477763]). Black boxes represent the coding region. The 5′UTR is located in exon 1 and part of exon 2, whereas the 3′UTR is located in exon 10. The figure is drawn to scale. So far, seven pathogenic mutations have been published and these are depicted in the figure: two comprise mutations in the intervening sequences (c.293−23A > G and c.685−2A > G), the others are detected in the coding regions (p.Glu110ArgfsX19; c.326–327dupTC, p.Ile147Ser; c.440T > G, p.Asp375Tyr; c.1123G > T, p.Ans439Asp; c.1315A > G, p.Val444Ala; c.1331T > C)

last component of the respiratory chain and catalyses the transfer of electrons from cytochrome *c* to molecular oxygen. Wajner and colleagues, using cultured fibroblasts from controls and one patient with D-2-HGA, reported similar results (Wajner et al 2002). The authors speculate that inhibition of the respiratory chain *in vivo* by D-2-HG leads to a secondary block of the CAC, explaining the frequently observed accumulation of 2-KG and succinic acid in D-2-HGA. Finally, D-2-HG was found to inhibit creatine kinase in rat cerebellum, cerebral cortex, and cardiac and skeletal muscle (da Silva et al 2003a,b, 2004). The importance of creatine kinase activity for cellular energy homeostasis leads the authors to speculate that their finding might play an important role in D-2-HGA, especially with regard to myopathy and cardiomyopathy.

Therapy in D-2-HGA

Currently, there is no treatment strategy for patients with D-2-HGA, except for the control of such symptoms as seizures. The mutations found in the *D2HGD* gene indicate that the enzymatic defect in the two patients is in the conversion of D-2-HG to 2-KG. The clinical symptoms seen in D-2-HGA cannot be explained by low 2-KG levels, but probably arise from the increased levels of D-2-HG. Future therapies should focus on the reduction of the levels D-2-HG in body fluids from affected patients.

Prenatal diagnosis

Prenatal diagnosis of D-2-HGA can be performed by the quantification of D-2-HG in amniotic fluid from the 16th week of pregnancy (Gibson et al 1993a). Two reports describe the prenatal diagnosis of an affected fetus with D-2-HGA (Clarke et al 2003; Craigen et al 1994). In both cases, the increase of D-2-HG in the amniotic fluid samples was approximately 10-fold when compared to control amniotic fluids samples. Prenatal diagnosis for D-2-HGA can now also include DNA analysis of the *D2HGD* gene in the first or second trimester in those families in which pathogenic mutations have been found. Genetic counselling in D-2-HGA is complicated because of the lack of a clear genotype-phenotype relationship, as is illustrated in the case of the monozygotic twins with D-2-HGA (Misra et al 2005).

Conclusions

After 20 years finally identifying the first patient, the biochemical pathways involved in human D-2-HG metabolism have been partly revealed, resulting in more understanding of the disease. HOT was shown to exist in human using 2-KG and GHB as main substrates, which are converted to D-2-HG and SSA, respectively. Human D-2-HG dehydrogenase showed high affinity for D-2-HG and is responsible for the conversion of D-2-HG to 2-KG using FAD as cofactor. It was hypothesized that an impairment in this enzyme reaction would result in D-2-HGA. Indeed, for seven patients D-2-HGA could be attributed to the finding of pathogenic mutations in the *D2HGD* gene; but mutations were not found in all D-2-HGA patients, requiring additional (DNA) studies. A functional assay for the determination of D-2-HG dehydrogenase activity, preferably in fibroblasts, is desirable.

It has now become clear that there is no genotype–phenotype relationship in D-2-HGA. This was illustrated by monozygotic twins with pathogenic mutations in the *D2HGD* gene, where one girl was severely affected while her sister showed only mild facial dysmorphy.

The pathophysiological mechanisms in D-2-HGA are most likely the result of the accumulating D-2-HG, rather than the decreased formation of 2-KG by D-2-HG dehydrogenase. Treatment strategies should focus on lowering the levels of D-2-HG in the patients. In addition, the different response of patients to the accumulating D-2-HG needs to be studied since there is no correlation between the levels of D-2-HG in the patients and the severity of the disease. The interplay among genetic, epigenetic and environmental influences in D-2-HGA requires further examination.

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