Disease-related Metabolites in Culture Medium of Fibroblasts from Patients with D-2-Hydroxyglutaric Aciduria, L-2-Hydroxyglutaric Aciduria, and Combined D/L-2-Hydroxyglutaric Aciduria

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Background: D-2-Hydroxyglutaric aciduria (D-2-HGA), L-2-hydroxyglutaric aciduria (L-2-HGA), and the combined D/L-2-hydroxyglutaric aciduria (D/L-2-HGA) are poorly understood organic acidurias. To investigate the usefulness of cultured human skin fibroblasts for both diagnostic and research purposes, we measured disease-related metabolites in the cell culture medium.

Methods: We measured D-2-hydroxyglutarate (D-2-HG), L-2-hydroxyglutarate (L-2-HG), succinate, 2-ketoglutarate, and citrate in fibroblast cell medium by stable-isotope-dilution gas chromatography–mass spectrometry and glutamine, glutamic acid, and lysine with an amino acid analyzer. We used six cell lines from patients with D-2-HGA, two from patients with L-2-HGA, three from patients with D/L-2-HGA, and seven control cell lines. Culture medium was analyzed after a 96-h incubation period.

Results: Culture media from cell lines from D-2-HGA patients contained D-2-HG at concentrations 5- to 30-fold higher than media from controls, whereas the concentration of L-2-HG in media was not increased. Media from L-2-HGA cell lines showed a fivefold increase in L-2-HG compared with controls. Media containing fibroblasts from D/L-2-HGA patients contained moderately increased amounts of both D-2-HG and L-2-HG. For all cell lines, succinate concentrations in the blank medium were higher than after 96 h of incubation with the exception of two of three D/L-2-HGA cell lines. Media of D-2-HGA cell lines had 2-ketoglutarate concentrations that were 40% of that for controls. Glutamic acid concentrations in media of these cell lines were 60% lower than in controls.

Conclusions: Cell culture media from fibroblasts from patients with D-2-HGA, L-2-HGA, or D/L-2-HGA contain increased amounts the corresponding 2-HGs, demonstrating the suitability of fibroblasts for both diagnosis of and research concerning 2-HGAs.

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Among the organic acidurias, the basic defects in D-2-hydroxyglutaric aciduria (D-2-HGA), L-2-hydroxyglutaric aciduria (L-2-HGA), and the combined D/L-2-hydroxyglutaric aciduria (D/L-2-HGA) remain mysteries. Despite the fact that the first patients with D-2-HGA and L-2-HGA were described in 1980, no major progress has been made since then in revealing the primary cause of these disorders. Clinical and biochemical findings of 25 patients with D-2-HGA have been described, supporting evidence for the existence of severe and mild clinical phenotypes of this disorder (3, 4). Biochemically, these two variants cannot be distinguished. Frequent clinical findings in D-2-HGA include developmental delay, epilepsy, and hypotonia. Movement disorders and cardiomyopathy are also common among patients with D-2-HGA. In 1995, Wanders and Mooyer (5) published the finding of a FAD-dependent D-2-hydroxyglutarate (D-2-HG) dehydrogenase localized in human mitochondria. The involvement of this enzyme in D-2-HGA has not been demonstrated.

In 1993, the clinical and biochemical findings in 12 patients with L-2-HGA were described (6). The clinical

1 Nonstandard abbreviations: D-2-HGA, D-2-hydroxyglutaric aciduria; L-2-HGA, L-2-hydroxyglutaric aciduria; D/L-2-HGA, D/L-2-hydroxyglutaric aciduria; CSF, cerebrospinal fluid; SPE, solid-phase extraction; IS, internal standard; and GC-MS, gas chromatography–mass spectrometry.
phenotype includes white matter disease and mental retardation and appears specific for L-2-HGA. In all seven individuals in whom the cerebrospinal fluid (CSF) and plasma concentrations of L-2-hydroxyglutarate (L-2-HG) had been measured, the CSF/plasma ratio was >1, indicating that L-2-HGA is a neurometabolic disorder. In the same report, in vitro experiments performed with 14C-labeled 2-ketoglutarate revealed the existence of a NAD\(^+\)-dependent L-2-HG dehydrogenase in human liver, but the involvement of this enzyme in L-2-HGA has not been demonstrated. To date, we know of >75 individual cases of L-2-HGA.

Recently, Muntau et al. (7) described a third 2-HGA variant; patients with this variant excrete moderately increased amounts of both D-2-HG and L-2-HG (7).

The enantiomeric analysis of D- and L-2-HG in urine, plasma, and CSF, in many cases preceded by urinary organic acid analysis, is the only reliable biochemical tool for the diagnosis of these organic acidurias (8). Additional biochemical abnormalities include increased t-lysine in the CSF of L-2-HGA patients (9, 10), regularly increased urinary Krebs cycle intermediates (especially succinate, 2-ketoglutarate, and citrate) in D-2-HGA (3, 4), and increased excretion of 2-ketoglutarate in D/L-2-HGA (7).

In many organic acidurias, confirmatory enzymatic studies are performed on cultured skin fibroblasts, followed by DNA/RNA mutational analysis. Often, labeled substrates are used in fibroblast studies or enzyme assays for selective measurement of (impaired) enzyme activity (11, 12). In case of a biochemical diagnosis of D-2-HGA, L-2-HGA, or D/L-2-HGA, these approaches are not feasible because both the precursors and products of D-2-HG and L-2-HG are unknown and the biochemical and genetic defects have not been established.

We measured the concentrations of D-2-HG and L-2-HG in media of cultured human skin fibroblasts taken from patients with D-2-HGA, L-2-HGA, or D/L-2-HGA and controls. In the same culture media, we also measured the concentrations of the Krebs cycle intermediates succinate, 2-ketoglutarate, and citrate and the amino acids lysine, glutamine, and glutamic acid. The two latter amino acids were investigated because they are closely related to 2-ketoglutarate.

**Materials and Methods**

**Patients**

Fibroblasts were from 18 individuals. Six cell lines were from patients who have been biochemically diagnosed as having D-2-HGA, two cell lines were from patients who have been biochemically diagnosed as having L-2-HGA, and three cell lines were from patients with increased concentrations of both D- and L-2-HG in different body fluids. Seven cell lines from patients who were not affected with D-2-HGA, L-2-HGA, or D/L-2-HGA were used as controls.

**Materials**

The fibroblast culture medium (Ham’s F-10) was obtained from Life Technologies. D-2-HG, L-2-HG, 2-ketoglutarate, succinate, and citrate were from Sigma. Stable-isotope-labeled 3,3,4,4-2H\(_4\)-2-ketoglutarate and 2,2,4,4-2H\(_4\)-citrate were from Euriso-Top. 13C\(_6\)-Succinate was from Cambridge Isotope Laboratories. 3,3,4,4-2H\(_4\)-d\(_1\)-2-Hydroxyglutarate was prepared by chemical reduction of 3,3,4,4-2H\(_4\)-2-ketoglutarate as described previously (8). R-(-)-2-Butanol was purchased from Aldrich. N,O-Bis(trimethylsilyl)trifluoroacetamide containing 10 mL/L trimethylchlorosilane was from Pierce. Solid-phase extraction (SPE) cartridges containing 60 mg of Oasis HLB were from Waters. All other chemicals and solvents used were of analytical grade.

**Cell Culture**

Fibroblasts were grown in Ham’s F-10 culture medium containing 100 mL/L fetal bovine serum and 10 mL/L penicillin/streptomycin at 37 °C in a 5% CO\(_2\)-95% air incubator. After the cells reached at least 90% confluency, the medium was removed and replaced by fresh medium. The cells were then cultured for 96 h, after which the medium was collected and stored at −20 °C until further analysis. Fibroblasts were harvested by trypsinization and washed twice with Hank’s balanced salt solution. Dry cell pellets were stored at −20 °C before the protein content measurements.

**Methods**

D- and L-2-HG were quantified by a modified version of the method described by Gibson et al. (8). Instead of the liquid–liquid extraction used in the original procedure, D-2- and L-2-HG were extracted from 1000 μL of the cell medium, supplemented with 10 nmol of 3,3,4,4-2H\(_4\)-d\(_1\)-2-hydroxyglutarate as internal standard (IS), by SPE using cartridges containing a polymeric stationary phase. Briefly, the SPE procedure consisted of conditioning of the cartridge with 750 μL of methanol, followed by the addition of 750 μL of 0.1 mol/L HCl, application of the acidified sample (pH <1), washing of the cartridge with 750 μL of 0.1 mol/L HCl, and elution of the analytes with 850 μL of methanol. The methanolic eluate was evaporated to dryness under nitrogen at 50 °C, after which the analytes were converted to the R-(-)-2-butyl ester forms. From this step, the previously described sample preparation protocol was followed. The gas chromatography–mass spectrometry (GC-MS) measurements were performed with selected-ion monitoring measuring m/z 173 and m/z 177 for endogenous D-2-HG, L-2-HG, and 2H\(_4\)-D,L-2-HG, respectively, in electron-impact ionization mode. The GC-MS system consisted of a Hewlett Packard 5890 series II gas chromatograph equipped with a non-chiral GC column and a 5989 B type Engine mass spectrometer.

Succinate, 2-ketoglutarate, and citrate were analyzed in one combined procedure. A mixture of the corresponding
stable-isotope-labeled IS containing 12.5 nmol of \(^{13}\)C\(_4\) succinate, 1.5 nmol of 3,3,4,4-\(^2\)H\(_4\) 2-ketoglutarate, and 15 nmol of 2,2,4,4-\(^2\)H\(_4\) citrate was added to 500 \(\mu\)L of cell medium, after which 50 \(\mu\)L of hydroxylamine solution (15 g/L) was added, and the samples were acidified by the addition 25 \(\mu\)L of 6 mol/L HCl. The samples were allowed to react for 30 min at 60 °C to obtain oxime derivatives of 2-ketoglutarate. The samples were then cooled to room temperature and subjected to SPE using the same procedure as described for D-2- and L-2-HG. After the SPE eluate was dried under nitrogen, the corresponding trimethylsilyl derivatives were formed by the addition of 80 \(\mu\)L of \(N,O\)-bis(trimethylsilyl)trifluoroacetamide containing 10 mL/L trimethylchlorosilane and 20 \(\mu\)L of pyridine. The derivatization was carried out for 30 min at 60 °C, and 1 \(\mu\)L was injected on the same GC-MS system as described for D-2- and L-2-HG. The GC-MS measurements were performed in electron-impact mode using selected-ion monitoring. A Chrompack Cp-Sil 19-CB capillary GC column was used for separation. The measured ions were \(m/z\) 247.2 and \(m/z\) 251.2 for succinate and the corresponding IS, \(m/z\) 377.4 and \(m/z\) 381.4 for 2-ketoglutarate and the corresponding IS, and \(m/z\) 465.5 and \(m/z\) 469.5 for citrate and the corresponding IS. Calibration curves were constructed with constant amounts of IS and increasing amounts of calibrators for proper quantification.

Glutamine, glutamic acid, and lysine were measured on a Pharmacia Biotech Biochrom-20 amino acid analyzer equipped with postcolumn ninhydrin derivatization followed by detection by ultraviolet absorbance.

The protein content of the fibroblast cell pellets was determined by the biocinchoninic protein assay from Sigma. Samples were prepared by adding 250 \(\mu\)L of demineralized water to the thawed cell pellet, followed by vigorous mixing with a Pasteur pipette to achieve complete lysis of the cell membranes; 50 \(\mu\)L of this solution was used for the protein measurement.

**Results**

Analysis of nonincubated blank culture medium containing 100 mL/L fetal bovine serum and 10 mL/L penicillin/streptomycin (stored at \(-20^\circ\)C) revealed that the medium contained detectable concentrations of all organic acids studied. Concentrations of D-2- and L-2-HG, succinate, 2-ketoglutarate, and citrate in blank culture medium were measured and subtracted from the concentrations measured after 96 h of incubation.

The amounts of D-2- and L-2-HG measured in cell culture media are shown in Table 1. In four of seven controls, the concentration of D-2-HG was equal to that of the blank culture medium. The concentration of L-2-HG after 96 h was in all cases higher than the concentration of L-2-HG in the blank culture medium. The mean amount L-2-HG in the cell media of controls after 96 h of incubation was 30 nmol/mg of protein. Media from fibroblasts from D-2-HGA patients contained distinctly higher amounts of D-2-HG than controls with a mean accumulation of 75 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\), whereas the amount of L-2-HG was within the values measured for the controls. In the media of the two L-2-HGA cell lines, the accumulation of L-2-HG was almost identical: 229 and 232 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\). This is more than fourfold higher than the amounts measured in the controls. D-2-HG concentrations in culture media from D/L-2-HGA cell lines were increased compared with controls, whereas L-2-HG concentrations were at the upper end of the range for controls. Overlaid mass fragmentograms for D- and L-2-HG are shown in Fig. 1.

The amounts of succinate, 2-ketoglutarate, and citrate measured in the different culture media are shown in Table 2. In most cell line media, the concentration of succinate in the blank nonincubated cell medium was higher than after 96 h of incubation, indicating that succinate is actively metabolized by fibroblasts. This phenomenon is displayed as negative “formation” of this compound, with the exception of two of three D/L-2-HGA patients cell lines. In cell media of controls, 2-ketoglutarate concentrations were in a well-defined range: 116–283 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\), with a mean of 196 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\). On average, cell lines from D-2-HGA patients contained lower amounts of 2-ketoglutarate (range, 32–139 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\); mean, 77 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\)). In contrast, culture media from L-2-HGA cell lines patients contained slightly increased amounts of 2-ketoglutarate: 376 and 328 nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\). Cell media from two D/L-2-HGA cell lines contained amounts of 2-ketoglutarate that were similar to the amounts in control cultures, whereas one had a slightly increased 2-ketoglutarate concentration. None of the cell media from the three patients groups had altered amounts of citrate relative to the controls.

Glutamine, glutamic acid, and lysine are ingredients of Ham’s F-10 cell culture medium and were therefore present in considerable amounts. The concentrations of these amino acids are listed in Table 3. Glutamine and

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**Table 1. Concentrations of D-2-HG and L-2-HG measured in cell media from cultured human skin fibroblasts.**

|          | D-2-HG Mean | D-2-HG Range | L-2-HG Mean | L-2-HG Range | D/L ratio Mean | D/L ratio Range |
|----------|-------------|--------------|-------------|--------------|----------------|----------------
| D-2-HGA  | 75          | 25–163       | 30          | 13–69        | 2.5            | 1.4–4.7        |
| (n = 6)  |             |              |             |              |                |                |
| L-2-HGA  | 5           | 6            | 229, 232    | 0.02, 0.03   |                |                |
| (n = 2)  |             |              |             |              |                |                |
| D/L-2-HGA| 26          | 25–27        | 58          | 48–75        | 0.5            | 0.4–0.5        |
| (n = 3)  |             |              |             |              |                |                |
| Controls | 2.1         | 0–4.9        | 33          | 17–59        | 0.1            | 0–0.2          |
| (n = 7)  |             |              |             |              |                |                |

\(^a\) Concentrations of D-2-HG and L-2-HG are expressed as nmol \(\cdot\) 96 h\(^{-1}\) \cdot mg protein\(^{-1}\). D-2-HG and L-2-HG concentrations in the culture medium from each individual cell line were measured in triplicate.

\(^b\) Individual mean values for the two cell lines are shown.
lysine concentrations in the cell media of the L-2-HGA, D-2-HGA, and D/L-2-HGA cell lines were of the same magnitude as for controls. For glutamic acid, the mean concentration in controls was 217 μmol/L (range, 144–315 μmol/L), whereas in media of D-2-HGA cell lines, the measured concentrations were 60% lower (mean, 87 μmol/L; range, 1–201 μmol/L), suggesting net consumption of glutamic acid. The glutamic acid concentrations in L-2-HGA and D/L-2-HGA cell media were comparable to those of controls.

Discussion

The use of cultured human skin fibroblasts is widespread and valuable for both diagnostics as well as biochemical research related to inborn errors of metabolism (13). In the present study we investigated the amounts of several metabolites in cell media from cultured human skin fibroblasts from patients and controls. A similar study has been performed for long-chain l-3-hydroxy-acyl-coenzyme A dehydrogenase deficiency (14). In that study, palmitate was added to the culture medium as substrate, the amounts of the 3-hydroxy fatty acids formed were measured. Because the knowledge of D-2-HGA, L-2-HGA, and D/L-2-HGA biochemistry is limited, we measured the concentrations of D/L-2-HG and related metabolites in culture medium that was not supplemented with substrates.

We cultured fibroblast cell lines from six different D-2-HGA patients. These cell lines clearly accumulated D-2-HG compared with controls. We found no correlation between the previously measured urinary concentrations of D-2-HG and the D-2-HG concentrations in cell media from fibroblasts isolated from these patients (data not shown). This might indicate that urinary excretion of D-2-HG in affected patients depends on other variables. The mean amount of 2-ketoglutarate in media from fibro-

<table>
<thead>
<tr>
<th>Cells</th>
<th>Succinate</th>
<th>2-Ketoglutarate</th>
<th>Citrate</th>
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<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>D-2-HGA (n = 6)</td>
<td>212</td>
<td>-493 to -3</td>
<td>77</td>
</tr>
<tr>
<td>L-2-HGA (n = 2)</td>
<td>-156, -46</td>
<td></td>
<td>328, 376</td>
</tr>
<tr>
<td>D/L-2-HGA (n = 3)</td>
<td>41</td>
<td>-26 to 145</td>
<td>249</td>
</tr>
<tr>
<td>Controls (n = 7)</td>
<td>-211</td>
<td>-465 to 22</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>323</td>
</tr>
</tbody>
</table>

*a Concentrations of succinate, 2-ketoglutarate, and citrate are expressed as nmol · 96 h⁻¹ · mg protein⁻¹. Succinate, 2-ketoglutarate, and citrate concentrations in the culture medium from each individual cell line were measured in duplicate.

*b Succinate concentrations after 96 h of incubation were in most cases lower than succinate concentrations in the blank culture medium. Most concentrations are thus expressed as a negative formation.

*c Individual mean values for the two cell lines are shown.
Table 3. Concentrations of glutamine, glutamic acid, and lysine measured in cell media from cultured human skin fibroblasts.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Glutamine, a µmol/L</th>
<th>Glutamic acid, a µmol/L</th>
<th>Lysine, b µmol/L</th>
</tr>
</thead>
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<tr>
<td>L-2-HGA a (n = 2)</td>
<td>194, 256</td>
<td>262, 365</td>
<td>135, 147</td>
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<tr>
<td>D/L-2-HGA (n = 3)</td>
<td>278, 205–390</td>
<td>255, 229–291</td>
<td>143, 132–154</td>
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<tr>
<td>Control (n = 7)</td>
<td>227, 134–334</td>
<td>217, 144–315</td>
<td>126, 107–146</td>
</tr>
</tbody>
</table>

a Concentrations of glutamine, glutamic acid, and lysine are expressed as µmol/L of cell culture medium. Glutamine, glutamic acid, and lysine concentrations in the culture medium from each individual cell line were measured in duplicate.

b Individual mean values for the two cell lines are shown.
References


