Three patients presented with evidence of a fatty acid oxidation disorder. Analysis of urinary organic acids by gas chromatography/mass spectrometry demonstrated the presence of medium-chain (C₆-C₁₂) dicarboxylic, 3-hydroxydicarboxylic, and 3-ketodicarboxylic acids in all three urines. 3-Ketodicarboxylic aciduria is reported for the first time here, as are the mass spectra for 3-keto- suberic, 3-ketosebacic, and 3-ketodecanedioic acids and the oxidized spectrum for 3-ketoacipic acid. The presence of 3-ketodicarboxylic acids suggests a defect at the level of a long-chain 3-ketoacyl-CoA thiolase, an enzyme for which a deficiency state has not previously been described. Our patients may represent the first cases of a long-chain thiolase defect.

Indexing Terms: heritable disorders • metabolism • urine • organic acids • gas chromatography/mass spectrometry

Inborn errors of mitochondrial fatty acid oxidation are a recently described group of metabolic diseases (1). The process of fatty acid oxidation involves the sequential removal of two carbon fragments in the form of acetyl-CoA from the carboxyl-terminal of the fatty acyl-CoA. This involves the concerted action of four enzymatic processes. The first reaction involves reduction across the 2,3-position of the acyl-CoA by a FAD-dependent acyl-CoA dehydrogenase. Mammals have four acyl-CoA dehydrogenases, each with different chain-length specificities (very long-, long-, medium- and short-chain acyl-CoA dehydrogenases). The second reaction involves hydration across the double bond in the 2,3-position to form an L-3-hydroxyacyl-CoA by a 2,3-enoyl-CoA hydratase, of which two enzymes have been identified in mammalian systems with long- and short-chain-length specificity. Further reduction by an NAD+-requiring L-3-hydroxyacyl-CoA dehydrogenase produces a 3-ketoacyl-CoA. Two L-3-hydroxyacyl-CoA dehydrogenases have been described to date, with long- and short-chain-length specificities. The final enzymatic step involves thiolytic cleavage of the 3-ketoacyl-CoA to produce acetyl-CoA and an n-2 chain-length-shortened acyl-CoA that reenters the oxidation cycle at the first reaction. At least three mitochondrial 3-ketoacyl-CoA thiolases exist in mammalian systems, with long-, short-, and branched-chain specificities. Much of the characterization of the β-oxidation enzymes is recent and it remains possible that our knowledge of both the nature of these enzymes and their tissue distribution is incomplete (1).

Most disorders involving these enzymes present with acute infection- or fasting-related metabolic decompensation with primarily hepatic symptoms ranging from symptomatic hypoglycemia through a Reye-like illness to sudden and unexpected death (2–4). Recently, a phenotype has been described in which the primary presenting features include both hepatic symptoms and chronic cardiac and skeletal muscle myopathy. Included in this group are disorders of long- and short-chain L-3-hydroxyacyl-CoA dehydrogenases (LCHAD, EC 1.1.1.211; and SCHAD, EC 1.1.1.35) (1, 5) and disorders of carnitine transport (6). The major biochemical markers for both LCHAD and SCHAD deficiency are urinary L-3-hydroxydicarboxylic acids. We present here three patients presenting with evidence of a fatty acid oxidation defect, whose urine contained both 3-hydroxydicarboxylic and 3-ketodicarboxylic acids of chain lengths C₆ to C₁₂. The presence of 3-ketodicarboxylic acids has not been previously reported in urine. Their occurrence in our patients suggests a metabolic defect at the level of a long-chain 3-ketoacyl-CoA thiolase.

Materials and Methods

Subjects

The patients were three infants who came to our attention during investigation for suspected metabolic disease. Patient 1 presented to us at age 23 months with a history of chronic motor delay culminating in a rapidly lethal episode of Reye-like hepatic coma. Autopsy revealed marked fatty accumulation in liver, suggesting a fatty acid oxidation defect. Patient 2 presented to us at age 1 year with nonspecific failure to thrive, weight loss, and hypotonia with muscle weakness. Patient 3 presented at age 9 months with muscle weakness, intractable vomiting, and weight loss and a diagnosis of gastroesophageal-reflux. Evidence for a defect in fatty acid oxidation in patients 2 and 3 came from the results of a 21-h fasting study, which demonstrated hyperfatty acidemia and an increased ratio of esterified to free

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1 Nonstandard abbreviations: LCHAD, SCHAD, long- and short-chain L-3-hydroxyacyl-CoA dehydrogenases; TMS, trimethylsilyl; GC/MS, gas chromatography/mass spectrometry; and MU, methylene unit.
serum carnitine, and from the analysis of urine organic acids as described below. The clinical histories in patients 2 and 3 did not include periods of metabolic decompensation or Reye-like illness.

Urine Organic Acid Analysis

Urine was obtained at autopsy in patient 1 and at the end of 21-h fasting studies in patients 2 and 3. After acidification to pH 2.0 of a volume of urine containing 0.1 mg of creatinine, organic acids were extracted by discontinuous extraction (three times) into ethyl acetate (5 volumes each time).

The organic acids were analyzed as their trimethylsilyl (TMS) derivatives by gas chromatography/mass spectrometry (GC/MS) with a Hewlett-Packard HP-5 capillary column, 25 m × 0.2 mm (0.33-μm film thickness), in a HP 5970 GC/MS (Hewlett-Packard, Palo Alto, CA) (7). Further analysis of samples from patient 2 was undertaken with use of a similar column in a VG Quatro GC/MS/MS in the single quadrupole mode (V.G. Instruments, Danvers, MA). The retention times of novel compounds were determined as methylene unit (MU) equivalents (8). The oxime derivatives of keto acids in urine collected from patient 2 were made by the addition of hydroxyamine hydrochloride (Mallinkrodt, Paris, KY), 10 g/L, for 1 h at 37 °C before the solvent extraction described above and were reanalyzed as TMS derivatives with the HP-5 column in the HP 5970 GC/MS system.

Organic acids were quantified by comparison with authentic standards, when available, or with dicarboxylic acids of equal chain-length when authentic standards were unavailable (e.g., 3-hydroxyadipic and 3-ketoacidic acids were quantified from a calibration curve of adipic acid by use of relative peak areas). Recovery of adipic, suberic, and sebamic acids was near 100% in this extraction system. For quantification purposes, the recoveries of other dicarboxylic acids were presumed to be similar.

Urinary acylglycines were quantified by stable isotope dilution analysis as previously described for n-hexanoyl- and 3-phenylpropionyl glycine (7). Suberyl [13C, 15N]glycine was added at a concentration of 50 mg/g (32.6 mmol/mol) of creatinine.

Results

Concentrations of urinary acylglycines were normal in all patients. Organic acid analysis did not demonstrate the presence of excess glutaric or ethylmalonic acids. All four urines contained substantial quantities of dicarboxylic and 3-hydroxydicarboxylic acids (Table 1). In addition we detected significant excretion of 3-ketoacidic acid in all samples (0.062–0.320 mmol/L). Figure 1 (top) demonstrates the mass spectrum obtained for 3-ketoacidic acid in our three patients’ samples; this spectrum was identical to the National Institute of Standards and Technology library spectrum and to that for an authentic standard (Sigma, St. Louis, MO). The

<table>
<thead>
<tr>
<th>Acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>3-Hydroxybutyrate</th>
<th>2.54</th>
<th>3.51</th>
<th>0.18–6.46</th>
<th>&lt;0.01</th>
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<tr>
<td>Glutaric</td>
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<td>ND</td>
<td>ND</td>
<td>1.38</td>
<td>2.54</td>
<td>3.51</td>
<td>0.18–6.46</td>
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<td>Adipic</td>
<td>20.13</td>
<td>7.78</td>
<td>4.05</td>
<td>2.11</td>
<td>0.86</td>
<td>0.16</td>
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<tr>
<td>Pimelic</td>
<td>10.62</td>
<td>3.40</td>
<td>1.55</td>
<td>1.25</td>
<td>3.12</td>
<td>0.66</td>
<td>0.019–0.23</td>
<td>0.019–0.23</td>
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<tr>
<td>Suberic</td>
<td>0.49</td>
<td>0.082</td>
<td>&lt;0.01</td>
<td>1.45</td>
<td>1.61</td>
<td>0.067</td>
<td>0.09–0.031</td>
<td>&lt;0.01–0.13</td>
</tr>
<tr>
<td>Sebamic</td>
<td>0.06</td>
<td>0.57</td>
<td>0.32</td>
<td>6.06</td>
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<td>0.96</td>
<td>0.35</td>
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<td>0.033</td>
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<tr>
<td>3-Ketododecanedioic</td>
<td>0.100</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

* Range in five age-matched control subjects suspected of having, but subsequently not found to have, a fatty acid oxidation defect. Patients with long-chain acyl-CoA dehydrogenase deficiency do not excrete large amounts of 3-hydroxydicarboxylic acids (7). ND, not detected.
identification of 3-ketosuberic, 3-ketosebacic, and 3-ketododecanedioic acids, which have not previously been described, are assigned on the presence of the ion m/z 231, representing the TMS fragment COO–CH=CH–O(TMS) characteristic for 3-keto compounds, and the expected M–15 ions of m/z 389, 417, and 445, respectively. Monounsaturated 3-hydroxydicarboxylic acids would be expected to have identical M–15 ions but would also be expected to have the characteristic ion of m/z 233, representing a 3-hydroxy compound (9). The retention times of 3-ketosuberic, 3-ketosebacic, and 3-ketododecanedioic acids correspond to MU values of 19.2, 21.0, and 22.9, respectively, on the HP-5 column. Oximation of urine before extraction of urine from patient 2 resulted in the loss of these peaks, confirming the presence of a reactive keto group.

We also detected the appearance of a compound with the expected molecular mass of oximated 3-ketoacidic acid (Figure 1, bottom). However, we could not detect compounds with the predicted molecular masses for the oximated TMS derivatives of 3-ketosuberic, 3-ketosebacic, and 3-ketododecanedioic acids—possibly because of the lack of sensitivity in the HP 5970 GC/MS system. This is a potential problem for laboratories that routinely analyze organic acids as the oximated derivatives. The finding of oximated 3-ketoacidic acid should therefore alert such laboratories to rerun samples in the nonoximated form to identify longer-chain 3-ketodicarboxylic acids. Alternatively, it may become possible to synthesize stable-isotope-labeled 3-ketodicarboxylic ac-

ids and reanalyze all samples for 3-hydroxydicarboxylic aciduria by stable isotope dilution/selected ion monitoring mass spectrometry. Figure 2 shows the tentative spectra obtained for the nonoximated TMS derivatives of 3-ketosuberic, 3-ketosebacic, and 3-ketododecanedioic acids. Figure 3 shows the total ion chromatogram of organic acids from postmortem urine collected from patient 1.

Analysis of urine from patients with previously characterized fatty acid oxidation defects (disease controls) did not detect 3-ketodicarboxylic acids in the following conditions: multiple acyl-CoA dehydrogenation defects (n = 2), medium-chain acyl-CoA dehydrogenase deficiency (n = 6), long-chain acyl-CoA dehydrogenase deficiency (n = 1), short-chain acyl-CoA dehydrogenase deficiency (n = 2), and LCHAD deficiency (n = 1)—although all of these patients excrete variable amounts of medium-chain dicarboxylic and (or) 3-hydroxydicarboxylic acids. 3-Ketodicarboxylic acids have not been reported in carnitine palmityl transferase deficiency or in the plasma membrane carnitine uptake defect. The

Fig. 1. Electron impact mass spectrum of (top) nonoximated and (bottom) oximated tri-TMS derivatives of 3-ketoacidic acid identified in urine from three patients with evidence for a new defect in fatty acid oxidation

Fig. 2. Electron impact mass spectra of tri-TMS derivative of (top) 3-ketosuberic acid, (middle) 3-ketosebacic acid, and (bottom) 3-ketododecanedioic acid, identified in urine from the three patients
Detection limits in our system are ~10 mmol/mol creatinine.

Discussion

We have identified metabolic markers in three patients with evidence for a previously undescribed defect in fatty acid oxidation. These patients had excretion of dicarboxylic, 3-hydroxydicarboxylic, and 3-ketodicarboxylic acids. Known disorders of fatty acid oxidation were ruled out. The absence of abnormal acylglycines and glutaric and ethylmalonic acids excludes the possibility of multiple acyl-CoA dehydrogenation defects and of medium-chain and short-chain acyl-CoA dehydrogenase deficiency. No abnormal acylcarnitines were detected by radioisotope exchange HPLC acylcarnitine analysis in patient 2, performed by Eberhard Schmidt-Sommerfeld, University of Chicago (the utility of acylcarnitine in disorders of long-chain fatty acid oxidation has yet to be established). The detection of 3-ketodicarboxylic acids suggests a metabolic block beyond the level of acyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-hydroxy acyl-CoA dehydrogenase; the indication from our findings is that the primary defect is likely to be in a 3-ketoacyl-CoA thiolase enzyme. The detection of 3-ketodicarboxylic acids of chain lengths up to 12 carbon atoms suggests a long-chain fatty acid oxidation defect. The 3-ketoacyl-CoA thiolase enzymes have not been fully characterized. It was originally suggested that there was a long-chain mitochondrial thiolase and two short-chain thiolases, one cytosolic and the other mitochondrial (10, 11). The substrate used to define the long-chain enzyme in pig heart, 3-ketodecanoyl-CoA (11), was probably inappropriate to define long-chain thiolase status because the natural 3-ketoacyl-CoA substrates begin at chain lengths of C_{18} or greater. Recently, a new mitochondrial trifunctional enzyme containing long-chain 2,3-enoyl-CoA hydratase, long-chain L-3-hydroxyacyl-CoA dehydrogenase, and long-chain L-3-ketoacyl-CoA thiolase activities has been described in rat liver with substrate activity to C_{16} compounds (12). Human hepatic LCHAD reportedly also contains the same trifunctional activities (13). There is also a specific mitochondrial thiolase for 3-methylacetoacetyl-CoA, the so-called β-ketothiolase, and a peroxisomal 3-ketoacyl-CoA thiolase enzyme (14).

Genetic defects have been described for the peroxisomal thiolase, which presents as pseudo-Zellweger syndrome (15), and for 3-methylacetoacetyl-CoA thiolase deficiency (16) and the mitochondrial short-chain acetoacetyl-CoA thiolase (17) deficiency, which both present as metabolic acidemia. Cytosolic acetoacetyl-CoA thiolase deficiency presents as a neurological disorder, this enzyme being essential forsterol synthesis (18). No defect of a long-chain 3-ketoacyl-CoA thiolase has been described to date. Given our present knowledge of thiolase enzymes and the biochemical findings in our patients, the likely site for the defect is either in a specific long-chain thiolase or in the thiolase subunit of the trifunctional protein.

Urine samples collected from patients 2 and 3 when they were clinically well did not demonstrate any abnormalities of dicarboxylic, 3-hydroxydicarboxylic, or 3-ketodicarboxylic acid, even when we used more-sensitive selected ion monitoring of urine organic acids. Therefore, this disorder will be missed in any screening program when an unstressed urine sample is analyzed. Investigation of the disorder should therefore be indicated primarily from clinical history. However, in our patients the clinical history was variable and in two patients included relatively nonspecific clinical symptoms such as myopathy, motor delay, vomiting, and failure to thrive—suggesting a greater involvement of muscle fatty acid oxidation. Biochemical evidence for a fatty acid oxidation defect should be sought by the analysis of urine samples collected when the patient is either acutely ill or after a carefully controlled fasting study. Further understanding of the nature of this disorder may help us to better characterize the enzymes responsible for 3-ketoacyl-CoA thiolysis and further the understanding of disorders of fatty acid oxidation.

Fig. 3. Regenerated total ion chromatogram of postmortem urine organic acids in patient 1

Peak identities: 1, lactate; 2, 3-hydroxybutyrate; 3, adipate; 4, unsaturated sebacate; 5, 3-hydroxyadipate; 6, suberate; 7, 3-keto adipate; 8, 9, unsaturated sebacate; 10, 3-hydroxysebacate; 11, sebacate; 12, 3-keto sebacate; 13, unsaturated 3-hydroxy sebacate; 14, 3-hydroxy sebacate; 15, dodenicadecanolate; 16, 3-ketosebacate; 17, 18, 3-hydroxydodecanolate; 19, 3-hydroxydodecanolate; 20, 3-ketododecanolate; 21, 22, 3-hydroxytetradecanolate; 23, 3-hydroxytetradecanolate.
We thank Cindy Mamer (VG Instruments) for additional GC MS/MS analysis.

References