Stability of long-chain and short-chain 3-hydroxyacyl-CoA dehydrogenase activity in postmortem liver

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Inherited enzyme defects in mitochondrial fatty acid oxidation (FAO) are associated with acute metabolic crisis and sudden death. Necropsy findings may be subtle, yielding no diagnosis and precluding genetic counseling. Preliminary identification of an FAO disorder requires the use of sophisticated tools (e.g., GC/MS) and specific body fluids, and the diagnosis rests on molecular analysis or enzyme assay. At present, confirmation of long-chain or short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency relies on measurement of enzyme activity. Here, we report our examination of the effect of storage temperature (25, 4, 20, and 70°C) and the postmortem interval on enzyme activities in rat and human liver. Enzyme activity decreases 50% in 30 h in samples stored at 25°C, whereas 55 h at 4°C is required to reach this value; freezing minimizes this loss. Regardless of rate of degradation, however, the short-chain to long-chain activity ratio remains constant—which should make it possible to differentiate postmortem degradation from enzyme deficiency.

INDEXING TERMS: sudden infant death syndrome • fatty acid oxidation • inherited metabolic disease • pediatric chemistry • sample handling • rats

Inherited enzyme defects in mitochondrial fatty acid oxidation (FAO) are a recognized cause of mortality in infants and children who die unexpectedly.4 Affected infants are often given the diagnosis of sudden infant death syndrome (SIDS) [1–4]. Clues to the correct diagnosis can be derived from observations of subtle pathological findings such as hepatic steatosis or from measurements of metabolic intermediates that accumulate in body fluids (blood, urine, bile, vitreous humor) or tissues [5–9]. The pattern of abnormal intermediates can suggest the probable site of defect; however, the confirmation of a defect relies on either direct assay for a particular enzyme in the appropriate tissue or on the demonstration of homozygosity for known disease-causing genetic mutations. Frequently, one cannot confirm a diagnosis enzymatically because of lack of information about the stability of specific enzymes in postmortem tissues. Knowledge of enzyme stability is important because most autopsies are performed several hours, or even days, after the death of a child.

Long-chain and short-chain 3-hydroxyacyl-CoA dehydrogenases (LCHAD and SCHAD, respectively) are chain-length-specific forms of the third enzyme in the mitochondrial β-oxidation sequence. LCHAD activity resides in a membrane-bound multienzyme complex that also contains long-chain 3-enoyl-CoA hydratase and long-chain 3-ketoacyl-CoA thiolase activities (trifunctional protein) [10, 11]. SCHAD is a single activity enzyme found on the mitochondrial matrix. Defects involving these enzymes are increasingly recognized as a significant cause of morbidity and mortality in infancy and childhood [12–19]. Correct identification of the defect in an affected child has clear implications for siblings, present or potential [12]. It also has ramifications for the mother during subsequent pregnancies because LCHAD deficiency in the fetus has been associated with a severe obstetric complication, acute fatty liver of pregnancy [15]. The present study was undertaken to establish the postmor-

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Received May 8, 1996; revised and accepted October 17, 1996.

4 Nonstandard abbreviations: FAO, fatty acid oxidation; SIDS, sudden infant death syndrome; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; and SCHAD, short-chain 3-hydroxyacyl-CoA dehydrogenase.
tem lability of LCHAD and SCHAD activity in liver as a prerequisite for confirming enzyme-based diagnoses in infants dying suddenly. We present data from stability studies of the enzymes in both rat and human liver tissues.

**Materials and Methods**

*Rat liver tissue.* Five adult male Sprague–Dawley rats were fasted overnight and decapitated at 0700. The abdomen was incised and the liver surgically removed. Each liver was divided into six pieces of approximately equal size and each piece was placed on a large glass slide. These pieces were then stored as follows: (a) at room temperature, (b) at room temperature in a humidified chamber, (c) at 4 °C, (d) at 4 °C in a humidified chamber, (e) at −20 °C, and (f) at −70 °C. After 12, 24, 36, 48, 72, and 96 h, a small piece (30–50 mg) was removed from each specimen, weighed, labeled, and frozen at −70 °C. Livers from three additional rats were frozen at −70 °C and sampled repeatedly over 5 years. We also took samples from various lobes of the rat liver (anterior right, posterior right, left, middle) and stored them frozen at −70 °C. Livers from three additional rats were frozen at −70 °C and sampled repeatedly over 5 years. We also took samples from various lobes of the rat liver (anterior right, posterior right, left, middle) and stored them frozen at −70 °C. All procedures performed on animals were in accordance with ethical standards and were approved by the Animal Use Committee of the Children’s Hospital of Philadelphia.

*Human liver tissue.* Although studies similar to those with rat liver cannot be performed with fresh, normal human liver, we examined enzyme activity loss in livers from three SIDS victims in whom the time of death could be determined from the history with a high degree of certainty (±2 h). The time between discovery and sample collection and freezing was short (<4 h after discovery; therefore, 4–6 h after demise). Small portions (0.1–0.2 g) of the livers were sliced off and either prepared for immediate assay or stored at 4 °C under humidified conditions for 6–72 h before assay.

We also examined the relation between the postmortem interval and the change in LCHAD and SCHAD in liver tissue from 42 infants—samples that were part of a series of liver samples collected for a large study of the relation between FAO disorders and sudden death (FAO Study, [1]). Of these children, 22 had a final diagnosis of SIDS, 8 died traumatically, and 12 had a specific nonmetabolic pathological diagnosis; none had organic acids in urine suggestive of an FAO disorder. Determination of the postmortem interval (time from death until sample collection) was based on standard forensic markers and on a direct interview with the family to ascertain the approximate time of demise. All samples were stored at −70 °C from the time of sample collection until the day of assay.

In both the SIDS and FAO studies, liver specimens were routinely taken from the anterior portion of the right lobe of the liver. All studies with human tissue were performed with the approval of the Ethical Committee (Children’s Hospital, Sheffield, UK) or the Institutional Review Board (Children’s Hospital of Philadelphia).

**Sample preparation.** A portion (~20 mg) of tissue was added to 1 mL of chilled (4 °C) 100 mmol/L potassium phosphate buffer (pH 6.5, containing 0.1 mmol/L EDTA) and then was sonicated for 3 × 5 s at 40 W with a tissue sonicator. After each 5-s sonication, the sample tube was returned to the ice bucket for at least 1 min. After the third (final) sonication, the solution was homogeneous. We then centrifuged this homogeneous solution at 30 000g for 20 min at 4 °C and transferred the supernatant liquid to a clean, labeled vial, which we placed on ice until the enzyme assays were performed, usually within 2 h and always within 4 h of tissue preparation. Preliminary experiments demonstrated that enzyme activity toward C16 (3-ketopalmityl-CoA; Sigma Chemical Co., St. Louis, MO) and C4 (acetoacetyl-CoA; Laroden Fine Chemicals, Malmo, Sweden) substrate in liver supernate did not decline for at least 4 h under these conditions.

**Determination of enzyme activity.** LCHAD and SCHAD activities were determined spectrophotometrically at 340 nm with the C4 and C16 substrates and with NADH as the electron donor. The final assay mixture contained, in a final volume of 1 mL, 100 mmol/L potassium phosphate, pH 6.5, 0.1 mmol/L EDTA, and 0.1 mmol/L NADH. Buffer was warmed to 30 °C and all assays were carried out in a water-jacketed chamber maintained at 30 °C. Samples (1–20 µL, containing 2–10 µg of soluble protein) were added to this mixture and the basal rate (for 1–3 min) was determined. The baseline was generally stable and showed minimal change over 10 min. The reaction was initiated by the addition of substrate (final concentrations: C4, 50 µmol/L; C16, 20 µmol/L). Preliminary experiments did not reveal any difference in the basal rate according to whether substrate or enzyme source was added last. The subsequent reaction was linear for at least 5 min.

Each sample was assayed with each substrate 3–6 times. Intraassay variability ranged from 0.3% to 5.7% and interassay variability ranged from 0.5% to 8%. An aliquot of each sample was assayed for protein according to the modified Lowry method, and enzyme activity is expressed as nanomoles of NADH reduced per minute per milligram of soluble protein. The results reported for each liver are the mean of all assays run with a specific substrate.

SCHAD and LCHAD exhibit some overlap in substrate chain-length specificity; in particular, SCHAD contributes ~10% of the total activity measured with C16 [20]. Extramitochondrial enzymes with affinity for C4 may contribute to measured SCHAD activity, but this cannot be evaluated in postmortem frozen liver samples; LCHAD, however, has no activity with C4. For purposes of simplicity and clarity, in the remainder of this paper we refer to activity measured with C16 substrate as LCHAD activ-
ity and to activity measured with C₄ substrate as SCHAD activity.

Statistics. Repeated measures ANOVAs were used to test for mean differences between storage conditions and duration of storage for each enzyme [21]. Storage conditions and enzyme type were tested as the independent groups, and time was the repeated measure. Dunnett’s test for contrasts was used to evaluate specific mean differences for significance of interactions between enzymes, type of storage conditions, and duration of storage conditions. Measurements taken at various times were contrasted with the baseline value at time 0. For some analyses, we examined the rate of change of enzyme activity with time. An ANOVA of the rate of change was used to evaluate differences in rate of loss of enzyme activity with time and storage conditions. We evaluated mean differences in assay values across pathology groups from the FAO study by ANCOVAs and used the Tukey multiple comparison test to examine specific mean differences. Time was the covariate in these analyses. All statistical tests were considered significant at \( P < 0.05 \).

Results
Enzyme activities in rat liver stored at −20 and −70 °C were determined repeatedly over 5 years. Although SCHAD activity in tissue stored at −70 °C varied somewhat, there was no difference between the initial rates and the rates after 5 years. In contrast, there was a slow but steady loss of SCHAD activity at −20 °C over the course of the studies, which was significant at and beyond 24 months (Fig. 1, top). There was essentially no change in LCHAD activity over the first 52 months at either temperature; after 60 months at −20 °C, however, LCHAD activity was significantly different from the initial values in the samples. There was no change in the SCHAD/LCHAD ratio in any specimen over the 5 years of the study (Fig. 1, bottom). Moreover, LCHAD and SCHAD activity in the four sampled regions of the rat liver were not significantly different from each other, and the SCHAD/LCHAD ratio was the same in all regions examined (data not shown).

The temperature of the liver, the storage conditions, and the time from death until sample collection and freezing distinctly affect LCHAD and SCHAD activity. In rat liver, 30–40% of LCHAD and SCHAD activity is lost in the first 24 h when the tissue is stored at 25 °C (Fig. 2, top and middle panels). Storage at 4 °C retards the loss of enzyme activity, and freezing the tissue at −20 or −70 °C prevents any reduction in activity for 96 h. Samples stored in a nonhumidified environment at either 25 or 4 °C showed no change in activity from the basal rate in the first 12 h. Subsequently, the rate at which activity was lost in the nonhumidified sample was similar to that of the humidified specimen. After 12 h, the rate of loss of activity was significantly more rapid at 25 than at 4 °C. However, there was no statistical difference in the SCHAD/LCHAD ratio under any condition (Fig. 2, bottom).

In the three SIDS samples stored in a humidified environment at 4 °C, increased storage time resulted in a loss of both LCHAD and SCHAD activity; freezing the samples prevented any loss of activity. As with rat liver, the SCHAD/LCHAD ratio remained constant throughout the duration of the experiment (Fig. 3). In addition, repeated sampling over the 5 years from four of the frozen (−70 °C) human livers from the FAO study (two with high activity and two with low activity at the time of the original assays; data not shown) showed that neither the individual enzyme activities nor the SCHAD/LCHAD activity ratio changed significantly during that time. We
did not examine the regionality of enzyme activity in human liver.

Access to a large cohort of livers from children dying unexpectedly and in whom the time of death was reasonably well-established (FAO study livers) [1] allowed us to examine changes in LCHAD and SCHAD activity from the perspective of the postmortem interval. Both LCHAD and SCHAD activity decreased linearly with increasing postmortem interval (Fig. 4). Although the temperature of the liver between the time of the child’s death and sample collection and storage was unknowable, the rate of activity loss was slightly more rapid than that of rat liver stored at 4 °C and slower than that stored at 25 °C. The specimens collected within 10 h after death had LCHAD and SCHAD activities comparable with those found in the three closely studied SIDS livers. The SCHAD/LCHAD ratio was constant, regardless of the postmortem interval (Fig. 4), and there were no significant differences in the rate of loss of activity or in the SCHAD/LCHAD ratio between those children dying from traumatic injuries, those with a specific pathological diagnosis, and those having no pathologically identifiable cause of death (SIDS).

Discussion

Data from several studies suggest that the prevalence of the FAO disorders in children is between 1:3000 and 1:10 000 [1, 9, 20, 22, 23]. More accurate estimates of the frequency are not possible because no single tool is currently sufficient to identify all children with FAO defects. The association between inherited FAO disorders and sudden unexpected death is well-documented [1–4, 8–10]; however, the contribution of the FAO disorders to the overall incidence of SIDS is still an area of active investigation [20, 22, 23]. Analysis of metabolic intermediates such as organic acids, acylglycines, and acylcarnitines is not sufficient to confirm a diagnosis; instead, enzyme assays or molecular tests are needed for confirmation.

LCHAD deficiency was first described in 1987 [13]. Presentation of this deficiency is varied and ranges from sudden unexpected death through acute hepatic failure and chronic liver disease [11–18, 25–27]. Muscle dysfunction is also present, with both cardiomyopathy and skeletal muscle involvement being recognized features. And ~50% of known cases develop changes in retinal pigment and a peripheral neuropathy.
LCHAD is part of a multienzyme complex comprising four α- and four β-subunits [10]. LCHAD activity is associated with the α-subunit, as is the activity of long-chain enoyl-CoA hydratase. The β-subunit contains long-chain 3-ketoacyl-CoA thiolase activity. A relatively common mutation (G1528→C) has been identified in children with LCHAD [17, 19] and is thought to affect the binding of NAD⁺ to the 3-hydroxyacyl-CoA dehydrogenase region. This base substitution results in a new PstI cleavage site and consequently permits a simple molecular approach to diagnosis. However, the frequency of this mutation is considerably less than the A985→G mutation in medium-chain acyl-CoA dehydrogenase deficiency; thus, screening for the G1528→C mutation alone will result in considerable underdiagnosis of LCHAD deficiency.

SCHAD deficiency was first described in 1991 [14]; more recently, an apparently liver-specific SCHAD deficiency has been identified [28]. Few patients have been identified to date; therefore, little information on the clinical aspects of this defect is available and no information on the molecular abnormalities causing SCHAD deficiency. Accordingly, there is a need for direct enzyme analyses for the diagnostic confirmation of both LCHAD and SCHAD deficiencies. Our data, the first to be presented on the stability of both enzymes in postmortem and stored tissues, demonstrate that activity of both enzymes is lost at about the same rate. Consequently, the

Fig. 4. Effects of the postmortem interval on (A) LCHAD activity, (B) SCHAD activity, and (C) the SCHAD/LCHAD ratio in livers of children dying from various causes.
ratio of SCHAD to LCHAD remains constant over many hours and in a wide range of conditions—which should permit differentiation of autolytic degradation from specific enzyme deficiency. As regards enzyme degradation, the SCHAD/LCHAD ratio remains constant because both enzyme activities are lost at the same rate. In contrast, the ratio should shift upward in liver in LCHAD deficiency, as it does in fibroblasts [13], and downward in SCHAD deficiency. Using the direct enzyme assay described here gives valid results for at least 40 h in postmortem tissue, a time during which most necropsies are performed.

We acknowledge the assistance of the Philadelphia County Medical Examiner, Haresh Mirchandani, and his staff in obtaining small liver specimens from children dying suddenly. We also thank Sadick Variend and Rodney Pollitt for permitting us access to the liver tissues from the Sheffield (UK) SIDS study. These studies were supported in part by a fund for SIDS Research at The Children’s Hospital of Philadelphia and by the Foundation for the Study of Infant Deaths (London, UK).

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