Familial Hyperinsulinemic Hypoglycemia Caused by a Defect in the SCHAD Enzyme of Mitochondrial Fatty Acid Oxidation

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Inappropriately elevated insulin secretion is the hallmark of persistent hyperinsulinemic hypoglycemia of infancy (PHHI), also denoted congenital hyperinsulinism. Causal mutations have been uncovered in genes coding for the β-cell's ATP-sensitive potassium channel and the metabolic enzymes glucokinase and glutamate dehydrogenase. In addition, one hyperinsulinemic infant was recently found to have a mutation in the gene encoding short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD), an enzyme participating in mitochondrial fatty acid oxidation. We have studied a consanguineous family with severe neonatal hypoglycemia due to increased insulin levels and where well-established genetic causes of hyperinsulinism had been eliminated. A genome-wide, microsatellite-based screen for homozygous chromosomal segments was performed. Those regions that were inherited in accordance with the presupposed model were searched for mutations in genes encoding metabolic enzymes. A novel, homozygous deletion mutation was found in the gene coding for the SCHAD enzyme. The mutation affected RNA splicing and was predicted to lead to a protein lacking 30 amino acids. The observations at the molecular level were confirmed by demonstrating greatly reduced SCHAD activity in the patients' fibroblasts and enhanced levels of 3-hydroxybutyryl-carnitine in their blood plasma. Urine metabolite analysis showed that SCHAD deficiency resulted in specific excretion of 3-hydroxyglutaric acid. By the genetic explanation of our family's cases of severe hypoglycemia, it is now clear that recessively inherited SCHAD deficiency can result in PHHI. This finding suggests that mitochondrial fatty acid oxidation influences insulin secretion by a hitherto unknown mechanism. *Diabetes* 53:221–227, 2004

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MIM, Mendelian Inheritance in Man; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; SCHAD, short-chain 3-hydroxyacyl-CoA dehydrogenase.

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In most populations, persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Mendelian Inheritance in Man [MIM] no. 601820), also referred to as neonatal or congenital hyperinsulinism, is a rare disorder. Its incidence has been estimated to be ~1:40,000 live births (1), but can reach 1:3,000 in areas of high consanguinity (2). Studies of the disease may, however, clarify principles of insulin secretion in the pancreatic β-cell and thereby contribute significantly to our knowledge about common disorders, in particular type 2 diabetes.

During the past decade, there has been substantial progress in the understanding of the genetic background as well as pathophysiology of PHHI (1,3). Autosomal recessive hyperinsulinism is caused by loss-of-function mutations in one of two adjacent genes on chromosome 11, encoding the high-affinity sulfonylurea-receptor and the inwardly rectifying potassium channel of the β-cell (4–6). These mutations lead to reduced or abolished activity of the β-cell's ATP-sensitive potassium channel (7). Hyperinsulinism with autosomal dominant inheritance can be caused by loss-of-function mutations in the sulfonylurea receptor gene (8) or by gain-of-function mutations in two genes encoding metabolic enzymes (9–11): glucokinase, the hexokinase controlling the rate-limiting step of β-cell glucose metabolism, and glutamate dehydrogenase, an enzyme central in oxidative deamination of amino acids. There also exists a non-Mendelian form of inherited hyperinsulinism due to focal adenomatous hyperplasia of the pancreatic islet cells (12).

Thus, in the different types of PHHI recognized today, a causal relationship has been established to mutations in altogether four genes. Still, in about half of pediatric hyperinsulinism cases, the genetic basis remains to be identified (3). We have studied a consanguineous family with autosomal recessive hyperinsulinemic hypoglycemia and where the segregation of known hyperinsulinism genes did not fit with the presumed inheritance model (13). Here we present molecular genetic, enzymatic, and metabolic evidence that implicate a fifth gene in PHHI, the gene encoding the mitochondrial enzyme short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD). The findings in our family, and in a single, recently described patient (14), suggest an important but still uncharacterized link between fatty acid oxidation and insulin secretion.

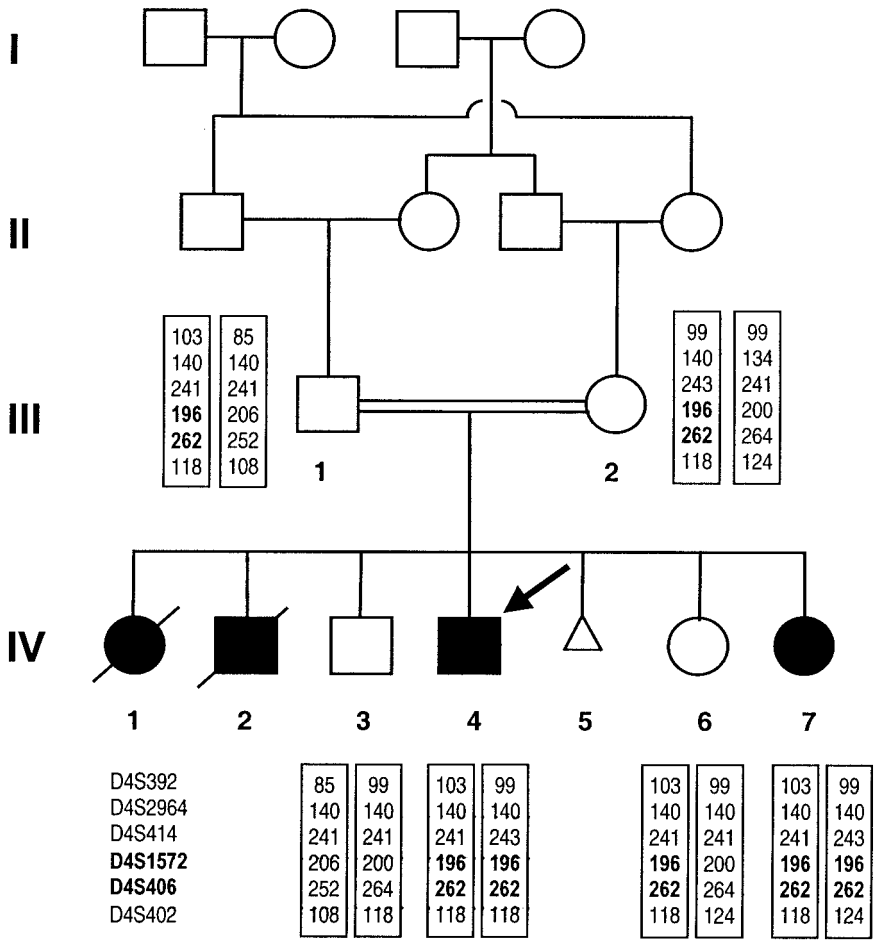

FIG. 1. Pedigree and chromosome 4 haplotypes of a consanguineous family with severe hypoglycemia due to hyperinsulinism. The arrow indicates the proband originally reported in 1977 by Vidnes and Øyasæter (15). Filled symbols denote subjects with hypoglycemia, of whom subjects IV-1 and IV-2 are deceased. There was also one miscarriage (IV-5). Haplotypes from chromosome 4q13–4q26, determined as allele sizes of microsatellite markers, are listed for the parents and the four living siblings. The distribution of the chromosomal segment (bold face) defined by the 196–262 allele of the markers D4S1572 and D4S406, respectively, is consistent with the presumed inheritance pattern.

RESEARCH DESIGN AND METHODS

Clinical findings. A detailed clinical study of the male proband (Subject IV-4 in Fig. 1) and his healthy parents was originally published in 1977 as a case of severe neonatal hypoglycemia, erroneously attributed to glucagon deficiency (ref. 15 and MIM 231530; see also the discussion in ref. 13). The family was of Pakistani origin, and the parents were double first cousins (Fig. 1). Two children had died at about 4 months of age with a diagnosis of hypoglycemia. In brief, the proband was admitted to hospital on his third day of life due to hypotonia, convulsions, and cyanosis. His blood glucose was 0.8 mmol/l. Serum free fatty acids were normal, and ketone bodies were in the lower normal range. During the first month of life, normoglycemia could be maintained by frequent feedings with extra glucose. In this period, seven measurements of insulin and blood glucose gave mean values of 9.6 ± 1.5 μU insulin/ml at 1.9 ± 0.2 mmol/l glucose, with normal insulin being <4 μU/ml at this blood glucose concentration. The proband's condition gradually worsened after 1.5 months of age, with an increasing number of convulsive attacks. When glucagon treatment (0.4 mg s.c. twice daily) was started at 3.5 months of age, a striking improvement of his condition was observed. During follow-up, glucagon was replaced by diazoxide. The patient is currently 28 years old and mentally retarded, probably due to the long-lasting, severe hypoglycemia he suffered as an infant. His blood glucose is controlled by diazoxide at a daily dose of 150 mg.

The proband's younger sister (Subject IV-7 in Fig. 1) had an elective blood glucose of 1.6 mmol/l 1.5 h after birth. Preliminary genetic and clinical data for this patient have been presented (13). Free fatty acids and ketone bodies were in the lower normal range. Extra feedings with 10% glucose were introduced. At a dose of 13.3 mg glucose · kg^{-1} · min^{-1}, hypoglycemic episodes did still occur (blood glucose values between 1.6 and 2.0 mmol/l were measured on several occasions). On day 4, the patient's plasma insulin was 21 μU/ml at a blood glucose of 2.5 mmol/l. Diazoxide treatment was started on day 11, with a satisfying clinical response. Since then, diazoxide and occasionally extra oral glucose have sufficed to control her hypoglycemia. Today, this patient is 20 years old and healthy, with no complications from her hyperinsulinism or diazoxide medication (daily dose 100 mg).

The study was performed according to the Helsinki Declaration, and informed written consent was obtained from the family.

Homozygosity mapping. Blood samples were obtained from the six living family members, and genomic DNA was purified by standard methods. DNA from the two affected subjects was screened with a panel of 382 microsatellite markers (ABI Prism Linkage Mapping Set MD10; Applied Biosystems, Foster City, CA) covering chromosomes 1–22. Subsequently, chromosomal regions found to be homozygous and identical in the affected subjects were screened in their parents and healthy siblings. Allele sizes were determined by capillary electrophoresis using an ABI Prism 310 Genetic Analyzer and the GeneScan Analysis software (Applied Biosystems).

Molecular genetic studies. The SCHAD enzyme is encoded by the gene *HADHSC* on chromosome 4q24–4q25 (ID 3033 in the Locus Link Database on http://www.ncbi.nlm.nih.gov/). PCR products from the eight *HADHSC* exons (16) were amplified from all available family members. DNA sequencing was performed using the BigDye Terminator Cycle Sequencing kit version 2 (Applied Biosystems) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Details are available upon request.

After identifying the family's mutation, mRNA was isolated by the QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden) from fibroblast cultures obtained from skin biopsies of the affected subjects and their parents. Complementary DNA was synthesized by the TaqMan Gold RT-PCR kit (Applied Biosystems), and a region covering exons 4–6 of the *HADHSC* gene was PCR-amplified by the primers 5′-CCTCCATT TCTTCAACCCAGTG (forward) and 5′-CGTTCATACAGCCTGATTGCTTC (reverse), followed by analysis by agarose gel electrophoresis and sequencing of the product.

Enzymatic studies and metabolite screen. SCHAD activity was measured spectrophotometrically at 340 nm using acetoacetyl-CoA as substrate exactly as described (17). As reference enzyme, the activity of long-chain 3-hydroxyacyl-CoA dehydrogenase was measured using 3-ketopalmitoyl-CoA as substrate (17).

Plasma acylcarnitines were analyzed by electrospray tandem mass spectrometry of their butyl esters following simultaneous extraction and deproteinization using a 10-fold excess of acetonitrile (18). Calculations were made using deuterium-labeled internal standards. Reference ranges were obtained from >500 hospital-based control subjects who were free of any inherited disease.

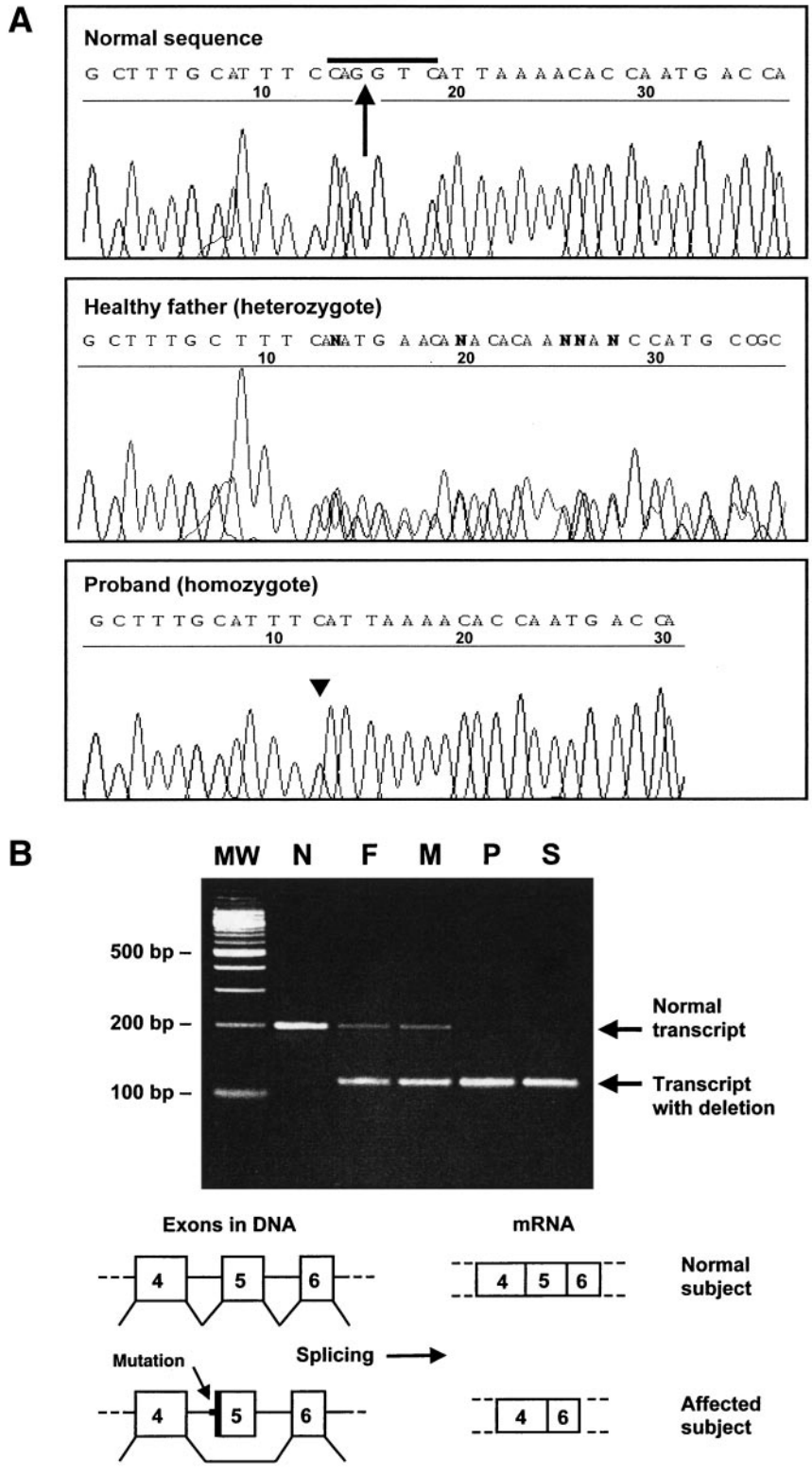

FIG. 2. Molecular genetic studies demonstrating a splice site mutation in the SCHAD-encoding gene. *A*: The results from sequencing the exon 5 region of the *HADHSC* gene, coding for the enzyme SCHAD. A black bar over the upper normal sequence indicates the six bases, including an acceptor splice site, that are missing in the mutated allele. The arrow shows the position where exon 5 starts. The lower sequence is the same region from an affected subject with the site of deletion marked by a triangle. The sequence from a heterozygous subject (middle) is a mixture of the normal and mutated patterns. *B*: The effect of the mutation on *HADHSC* transcription. A PCR product covering exons 4–6 was amplified from reverse-transcribed fibroblast mRNA and analyzed by agarose gel electrophoresis. Lane MW contains a molecular weight standard with sizes in base pairs given to the left. Lanes N, F, M, P, and S contain samples from a normal subject, the father (subject III-1), the mother (III-2), the proband (IV-4), and his affected sister (IV-7), respectively. The mutation leads to shortened transcripts with exon 5 missing.

For metabolite screening, urine samples were acidified and extracted with diethyl ether (19). The extract was methylated with diazomethane before injection into an HP 5972 (Hewlett-Packard, Palo Alto, CA) combined gas chromatograph and mass spectrometer. The peaks of the gas chromatogram were identified by matching their mass spectra against a library of known spectra.

RESULTS

Genome-wide homozygosity mapping. The proband and his sister were originally diagnosed with inherited glucagon deficiency (15), but a clinical examination of the sister at age 14 years and a re-evaluation of the medical records of the proband resulted in a diagnosis of autosomal recessive hyperinsulinism (13). Because of closely consanguineous parents, we assumed that the affected subjects would be homozygous for the chromosomal segment containing the mutation.

Previously, the four known genetic causes of PHHI, as

TABLE 1
Fibroblast enzyme activities and plasma acylcarnitines in healthy and affected family members

	Father (subject III-1)	Mother (subject III-2)	Proband (subject IV-4)	Sister (subject IV-7)	
3-hydroxyacyl-CoA dehydrogenase activity					Healthy control subjects
C4-substrate* (acetoacetyl-CoA) ($nmol \cdot min^{-1} \cdot mg^{-1}$)	61	57	8	9	104 ± 29 (105)
C16-substrate (3-ketohexadecanoyl-CoA) ($nmol \cdot min^{-1} \cdot mg^{-1}$)	62	52	50	30	79 ± 20 (102)
Activity ratio (C16/C4)	1.02	0.91	6.25	3.33	0.79 ± 0.20 (102)
Plasma acylcarnitines (μmol/l)					Reference values
Free carnitine	56.1	57.6	44.1	33.7	22.3–54.8
C2-carnitine	5.46	6.35	5.54	4.73	3.40–13.0
C3-carnitine	0.54	0.53	0.46	0.42	0.14–0.94
C4-carnitine	0.18	0.24	0.28	0.22	0.07–0.58
C8-carnitine	0.08	0.04	0.11	0.07	0.04–0.22
3-hydroxy-C4-carnitine	0.03	0.03	0.93	1.09	0.00–0.03

Data of healthy control subjects are means ± SD (*n*). *Cn refers to the number of carbon atoms in the acyl group.

well as >20 other candidate genes coding for pancreatic enzymes, hormones, receptors and transcription factors, had been excluded as a cause of the family's disease (13). We therefore chose a genome-wide approach and screened chromosomes 1–22 of the two affected subjects with a panel of microsatellite markers to identify shared homozygous regions. Next, the microsatellite genotypes of such regions were determined for the parents (who should be heterozygous for the segment containing the mutation) and for the two healthy siblings (who should differ from the affected subjects). The homozygosity mapping resulted in the identification of two chromosomal regions that were inherited in the presupposed pattern: one segment on chromosome 7q defined by the markers D7S630, D7S657, and D7S515 and one segment on chromosome 4q defined by the markers D4S1572 and D4S406 (Fig. 1).

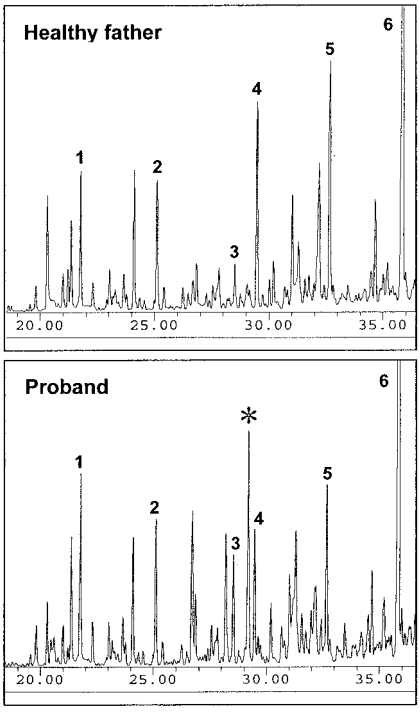

FIG. 3. Metabolite screen of urine samples from the family with SCHAD deficiency. The section between 20 and 35 min of the gas chromatogram is shown. The peaks were identified by mass spectrometry. The numbers 1–6 denote typical peaks in this part of the chromatogram: 3-methyladipic acid; cyclopropane dicarboxylic acid; 2-ketoglutaric acid; azelaic acid; 2,5-fructose dicarboxylic acid; and citric acid, respectively. Only in the proband and his affected sister (not shown) was a strong peak at 29.2 min (*), corresponding to 3-hydroxyglutaric acid, detected.

Molecular genetic studies pinpointing the mutation. For the identification of the mutated gene, we used a candidate gene approach. The majority of autosomal recessive diseases with no congenital malformations and with onset within the first year of life are caused by mutations in enzyme genes (20). Therefore, when evaluating the two positive chromosome regions, we focused on genes coding for enzymes with a role in energy metabolism. First, the pyruvate dehydrogenase kinase IV gene between markers D7S657 and D7S515 was sequenced in the two affected subjects, but no mutations were observed. Our second candidate was the gene *HADHSC* between markers D4S1572 and D4S406, encoding the enzyme SCHAD involved in fatty acid metabolism. A deletion of the six base pairs CAGGTC was discovered at the start of *HADHSC* exon 5 (Fig. 2*A*). As expected from the microsatellite marker analysis (Fig. 1), the proband and his affected sister were homozygous and the parents and subject IV-6 heterozygous for the mutation, while subject IV-3 had no mutation. The complete gene was sequenced in all six available family members and compared with sequences obtained from two unrelated, healthy individuals. No other changes in the *HADHSC* gene were observed.

The six–base pair deletion removes the acceptor splice site adjacent to exon 5 of *HADHSC*. To examine the molecular consequence, we analyzed mRNA from fibroblasts obtained from the two patients and their parents. Our data showed that exon 5 is skipped during the mRNA splicing process, so that exon 4 is coupled directly onto exon 6 (Fig. 2*B*). This leads to an in-frame deletion of 90 nucleotides in the mature mRNA, resulting in a protein product predicted to lack 30 amino acids.

Effects on enzyme activity and metabolite distribution. The impact of the mutation on protein function was examined by determining SCHAD activity in fibroblast cultures from subjects IV-4 and IV-7 and their parents. In the two affected subjects and the parents, enzyme activity was <10% and <60% of control values, respectively (Table

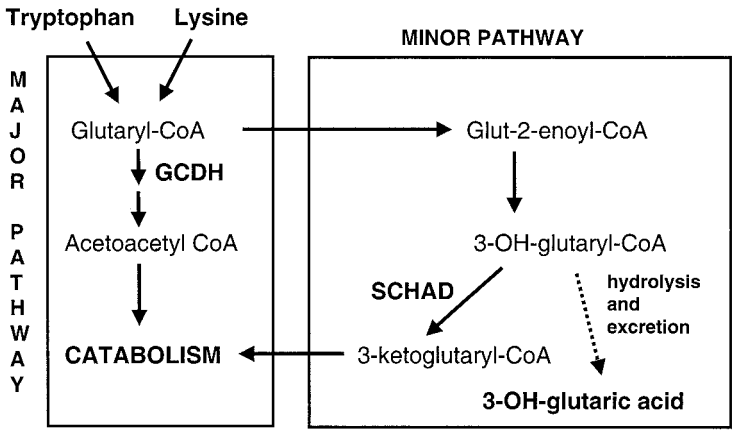

FIG. 4. Possible explanation for the excretion of 3-hydroxyglutaric acid by SCHAD-deficient patients. The metabolite glutaryl-CoA is central in the degradation of tryptophan and lysine and is metabolized via a major pathway that includes glutaryl-CoA dehydrogenase (GCDH). Glutaryl-CoA may also to some extent be metabolized through a pathway where 3-hydroxyglutaryl-CoA is converted to 3-ketoglutaryl-CoA by SCHAD. If the latter step is blocked, excretion of 3-hydroxyglutaric acid may occur.

1). For comparison, long-chain 3-hydroxyacyl-CoA dehydrogenase activity was assayed, and abnormal activity ratios were found for both siblings, but not their parents. A plasma analysis of the distribution of fatty acyl groups conjugated to the transport molecule carnitine showed a normal pattern, except for 3-hydroxybutyryl(C4)-carnitine (Table 1). This substance is barely detectable in normal control subjects, but reached a plasma concentration around 1 μmol/l in both patients. Finally, we performed metabolic screening using gas chromatography–mass spectrometry on urine samples from the family. One strong peak, corresponding to 3-hydroxyglutaric acid, was observed in both patients, but not in their parents or healthy siblings (Fig. 3).

DISCUSSION

We describe a family where a defect in mitochondrial fatty acid oxidation causes autosomal recessive and diazoxide-responsive hyperinsulinemic hypoglycemia. Following genome-wide homozygosity mapping, a mutation in the gene encoding the SCHAD protein was revealed. This mitochondrial enzyme catalyzes the conversion of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA, the third of four repetitive steps in the fatty acid oxidation spiral. Findings at the genetic level were supported by low activities of SCHAD in cultured fibroblasts from the two living patients. Furthermore, their plasma acylcarnitine profiles showed strongly increased 3-hydroxybutyryl-carnitine and normal C2-carnitine levels, in contrast to the situation in ketotic subjects, in whom both of these metabolites are increased (21).

The clinical findings in the family did not suggest a problem in fatty acid oxidation. In particular, there were no signs of hepatic dysfunction, cardiomyopathy, or skeletal muscle affection. Admittedly, the patients presented with hypoketotic hypoglycemia, commonly found in fatty acid oxidation defects (22), but their circulating fatty acids were in the lower normal range. In fact, this pattern of metabolites is entirely consistent with hyperinsulinism, since insulin suppresses both ketogenesis and lipolysis.

TABLE 2
Clinical and genetic findings in patients with PHHI and SCHAD deficiency

Patient	FS (ref. 14)	IV-4 (this report and ref. 15)	IV-7 (this report and ref. 13)
Sex	Female	Male	Female
Gestation age	38 weeks	42 weeks	40 weeks
Birth weight	3.2 kg	3.8 kg	3.9 kg
Age when hypoglycemia was first diagnosed	4 months	3 days	2 h*
Blood glucose at diagnosis	1.4 mmol/l	0.8 mmol/l	1.6 mmol/l*
Plasma insulin (blood glucose)	18 μU/ml (1.3 mmol/l)	9.6 μU/ml† (1.9 mmol/l)†	21 μU/ml (2.5 mmol/l)
Ketosis	No	No	No
Serum free fatty acids	0.17 mmol/l	0.31 mmol/l‡	“Lower normal range”
Diazoxide-responsive hypoglycemia	Yes	Yes	Yes
Concentration of 3-hydroxybutyryl-carnitine in blood	1.78 μmol/l¶	0.93 μmol/l§	1.09 μmol/l§
Presence of 3-hydroxyglutaric acid in urine	Yes	Yes§	Yes§
Mutation in the *HADHSC* gene	Homozygous C773T substitution in exon 7	Homozygous deletion of CAGGTC at the exon 5 acceptor splice site	Homozygous deletion of CAGGTC at the exon 5 acceptor splice site
Predicted effect on the SCHAD protein	Pro258Leu substitution	Deletion of amino acids 183–212	Deletion of amino acids 183–212

*Elective blood glucose was measured shortly after birth, treatment was started before symptoms of hypoglycemia appeared; †mean of 7 determinations; ‡mean of 3 determinations; ¶mean of 16 determinations; §determined as adults

By identifying the family's mutation, enzyme defects in the catabolic breakdown of all three major energy-rich substrates of the body—carbohydrates, proteins, and fatty acids—have been linked to PHHI. Activating glucokinase and glutamate dehydrogenase mutations are thought to stimulate insulin secretion by increasing the β-cell's ATP production, but a similar explanation for the elevated insulin level seems unlikely when fatty acid oxidation is inhibited. Intriguingly, the 3-hydroxybutyryl-carnitine accumulating in our patients will, as a derivative of disturbed fatty acid metabolism, belong stereochemically to the L-form, in contrast to the D-form that occurs during ketosis. We speculate that the unusual L-form has distinct effects, possibly interfering with potassium channel function or with the proposed ATP-independent and lipid-sensitive mechanism of insulin secretion (23). The mechanism should now be studied in model systems of insulin secretion, for example, by monitoring the effect from inactivating the SCHAD enzyme or from adding specific metabolites elevated in SCHAD-deficient patients.

The presence of 3-hydroxyglutaric acid in the patients' urine is another enigma, since glutaric acid is a five-carbon acid and the organic acids involved in fatty acid metabolism are even-numbered. The only other known metabolic disease characterized by excretion of 3-hydroxyglutaric acid is glutaricacidemia type I (MIM 231670), which is caused by mutations in the gene encoding glutaryl-CoA dehydrogenase (24). We have considered the possibility that the two affected siblings may suffer also from glutaricacidemia, although their phenotype is different from that of such patients and their haplotypes are inconsistent with a common recessive disease on chromosome 19p13.2 (not shown), the region where the glutaryl-CoA dehydrogenase gene is located. An assay of fibroblast glutaryl-CoA dehydrogenase activity confirmed the expected normal values for both the parents and their two SCHAD-deficient children (not shown).

Glutaryl-CoA dehydrogenase is a mitochondrial enzyme required for the catabolic degradation of tryptophan and lysine, amino acids that presumably can be degraded also by a minor pathway that includes conversion of 3-hydroxyglutaryl-CoA to 3-ketoglutaryl-CoA by SCHAD. We suggest that when this step is blocked, hydrolysis of 3-hydroxyglutaryl-CoA followed by excretion of 3-hydroxyglutaric acid will occur (Fig. 4).

While this work was in progress, Clayton et al. (14) described a patient with a similar, but milder, clinical presentation than reported here, including an older age of onset of hypoglycemic episodes (4 months). Their patient was homozygous for a single-base substitution mutation in the *HADHSC* gene, causing a change from proline to leucine at amino acid 258 in the SCHAD protein. The mutation in our family, leading to a loss of amino acids 183–212, may explain the more severe phenotype with two neonatal deaths and one mentally retarded subject.

Clayton et al. (14) mentioned the possibility that their patient could have two genetic defects, one causing SCHAD deficiency and the other causing hyperinsulinism. By our genome-wide screen of the affected family, and in light of the phenotypic similarities observed (summarized in Table 2), there should be no doubt that mutations in the *HADHSC* gene can cause hyperinsulinism. Moreover, the clinical presentation of such cases appears to be clearly different from that of some patients previously reported as SCHAD deficient, but for whom mutational evidence was not presented (25–27). We assume that other enzymes with SCHAD activity were affected in the latter patients.

Mutations in the *HADHSC* gene should be sought in hyperinsulinemic subjects in whom autosomal recessive inheritance can be presumed and defects in the sulfonylurea receptor and the inwardly rectifying potassium channel of the β-cell have been ruled out. The presence of 3-hydroxyglutaric acid in urine, raised plasma levels of 3-hydroxybutyryl-carnitine, and responsiveness to diazoxide may aid the diagnostic evaluation. The elucidation of SCHAD deficiency as a cause of hyperinsulinism, along with other novel aspects of fatty acids in a β-cell context (28–30), illustrates the many ways fatty acids may contribute to the regulation of insulin secretion.

ACKNOWLEDGMENTS

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