

## HYPERINSULINISM AND HYPERAMMONEMIA IN INFANTS WITH REGULATORY MUTATIONS OF THE GLUTAMATE DEHYDROGENASE GENE

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### ABSTRACT

**Background** A new form of congenital hyperinsulinism characterized by hypoglycemia and hyperammonemia was described recently. We hypothesized that this syndrome of hyperinsulinism and hyperammonemia was caused by excessive activity of glutamate dehydrogenase, which oxidizes glutamate to  $\alpha$ -ketoglutarate and which is a potential regulator of insulin secretion in pancreatic beta cells and of ureagenesis in the liver.

**Methods** We measured glutamate dehydrogenase activity in lymphoblasts from eight unrelated children with the hyperinsulinism–hyperammonemia syndrome: six with sporadic cases and two with familial cases. We identified mutations in the glutamate dehydrogenase gene by sequencing glutamate dehydrogenase complementary DNA prepared from lymphoblast messenger RNA. Site-directed mutagenesis was used to express the mutations in COS-7 cells.

**Results** The sensitivity of glutamate dehydrogenase to inhibition by guanosine 5'-triphosphate was a quarter of the normal level in the patients with sporadic hyperinsulinism–hyperammonemia syndrome and half the normal level in patients with familial cases and their affected relatives, findings consistent with overactivity of the enzyme. These differences in enzyme insensitivity correlated with differences in the severity of hypoglycemia in the two groups. All eight children were heterozygous for the wild-type allele and had a mutation in the proposed allosteric domain of the enzyme. Four different mutations were identified in the six patients with sporadic cases; the two patients with familial cases shared a fifth mutation. In two clones of COS-7 cells transfected with the mutant sequence from one patient, the sensitivity of the enzyme to guanosine 5'-triphosphate was reduced, findings similar to those in the child's lymphoblasts.

**Conclusions** The hyperinsulinism–hyperammonemia syndrome is caused by mutations in the glutamate dehydrogenase gene that impair the control of enzyme activity. (N Engl J Med 1998;338:1352-7.)

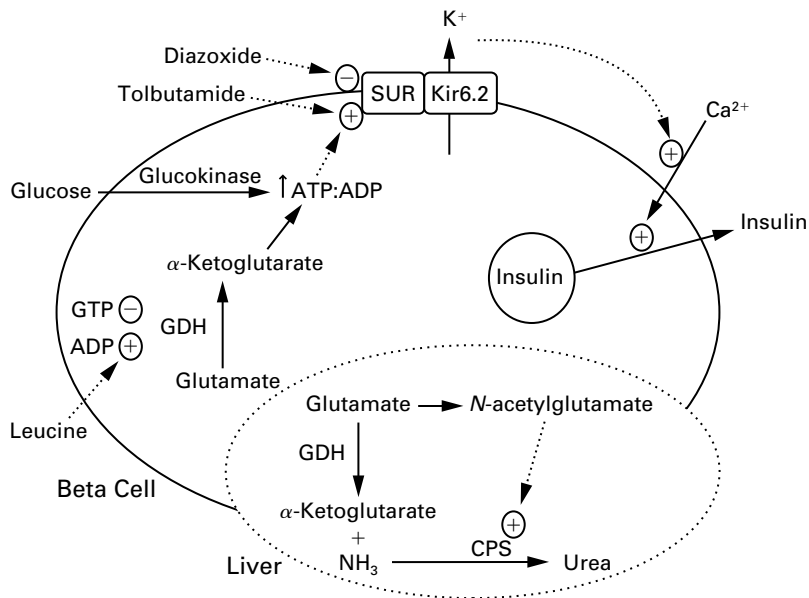
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**C**ONGENITAL hyperinsulinism is the most common cause of recurrent hypoglycemia in early infancy.<sup>1</sup> Affected children present with seizures or coma and are at high risk for permanent brain injury. Treatment consists of diazoxide, octreotide, or subtotal pancreatectomy. Evidence suggests that the majority of cases of congenital

hyperinsulinism are caused by genetic defects in the regulation of insulin secretion by pancreatic beta cells.<sup>2</sup> In some children, recessively inherited mutations have been demonstrated in the gene for the plasma membrane sulfonylurea receptor (*SURI*) or its associated inwardly rectifying potassium channel (*Kir6.2*) of the beta cells.<sup>3-7</sup> Other children have been described with milder, dominantly inherited forms of hyperinsulinism that are not linked to the sulfonylurea receptor locus<sup>8,9</sup>; a mutation in the glucokinase gene has been identified in one of these families.<sup>10</sup> In addition, a third group of children has been described who have an unusual combination of congenital hyperinsulinism and hyperammonemia.<sup>11,12</sup> Plasma ammonium concentrations in these children are persistently three to eight times normal. The hyperammonemia is not affected by changes in blood glucose concentrations and is not associated with a defect in any urea-cycle enzyme.

We hypothesized that the hyperinsulinism–hyperammonemia syndrome was caused by a single inborn error of metabolism shared by the pancreas and the liver. A defect in the mitochondrial enzyme glutamate dehydrogenase appeared to be likely (Fig. 1). Leucine, an amino acid that stimulates the release of insulin, acts by allosterically activating glutamate dehydrogenase to increase the rate of glutamate oxidation in the beta cells.<sup>14,16,17</sup> High concentrations of glutamate are needed for the synthesis of *N*-acetylglutamate, an essential activator of carbamoyl-phosphate synthetase, the first step in the conversion of ammonium to urea.<sup>18,19</sup> Therefore, the hyperinsulinism–hyperammonemia syndrome could be caused by excessive activity of glutamate dehydrogenase, since this would simultaneously increase

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**Figure 1.** Glutamate Dehydrogenase (GDH) and the Regulation of Insulin Secretion and Hepatic Ureagenesis.

Increases in the rate of oxidation of fuels such as glucose or glutamate stimulate the secretion of insulin by increasing the ratio of ATP to adenosine 5'-diphosphate (ADP), which in turn causes closure of potassium channels and ultimately leads to the depolarization of beta cells, calcium influx, and the release of stored insulin granules.<sup>13</sup> Leucine stimulates insulin secretion indirectly by allosterically activating glutamate dehydrogenase (GDH) and increasing the oxidation of glutamate by means of the tricarboxylic acid cycle.<sup>14-17</sup> Diazoxide and tolbutamide have direct effects on the sulfonylurea receptor (SUR) and inwardly rectifying potassium channel (Kir6.2). In the liver, glutamate governs the synthesis of *N*-acetylglutamate, a required allosteric effector of carbamoyl-phosphate synthetase (CPS)<sup>18,19</sup>; the oxidation of glutamate by glutamate dehydrogenase also provides free ammonia derived from the  $\alpha$ -amino nitrogen of amino acids. GTP denotes guanosine 5'-triphosphate.

the release of insulin by pancreatic beta cells and impair the detoxification of ammonia in the liver. We performed enzymatic and molecular studies in eight families in an effort to prove this hypothesis of an abnormality in glutamate enzyme activity as the cause of the syndrome.

## METHODS

### Study Subjects

We studied eight unrelated children, ranging in age from 3 months to 10 years, with the hyperinsulinism–hyperammonemia syndrome. Patients 1 through 6 (five boys and one girl) had sporadic cases, because they had no affected relatives. They were from the United States, Mexico, Italy, and Canada. Two other boys (Patients 7 and 8), one from Italy and one from Canada, were classified as having familial cases, because other family members had hyperammonemia and hypoglycemia. These included the mother of Patient 7 and the mother, a maternal aunt and her daughter, and the maternal grandfather of Patient 8. The mode of inheritance in these families appeared to be autosomal dominant. Clinical descriptions of Patients 1, 2, and 7 have been reported previously.<sup>11,12</sup>

All the children presented with episodes of symptomatic hypoglycemia during the first year of life. The patients with sporadic cases all responded to treatment with diazoxide (Fig. 1); they re-

quired either continuous treatment with diazoxide or subtotal pancreatectomy to prevent hypoglycemia. The patients with familial cases appeared to have milder hypoglycemia; Patient 8 was treated with diazoxide, but Patient 7 was treated with only a low-protein diet. The affected relatives of these two patients had not been treated. None of the affected subjects, whose plasma ammonia concentrations ranged from 112 to 280  $\mu\text{g}$  per deciliter (80 to 200  $\mu\text{mol}$  per liter; normal, <50  $\mu\text{g}$  per deciliter [40  $\mu\text{mol}$  per liter]) had symptoms of hyperammonemia, such as lethargy or coma.

The study protocol was approved by the institutional review board of Children's Hospital of Philadelphia, and informed consent was obtained from all subjects or their parents or guardians.

### Enzymatic and DNA Studies

Peripheral-blood samples were obtained from the patients, 5 affected and 16 unaffected family members, and 10 unrelated normal subjects. Lymphocytes isolated from peripheral blood were transformed with Epstein–Barr virus to establish lymphoblast cultures. The activity of glutamate dehydrogenase in lymphoblast homogenates and the effects of added adenosine 5'-diphosphate (ADP) or guanosine 5'-triphosphate (GTP) were determined spectrophotometrically in triplicate.<sup>20</sup> In some cases, lymphoblast homogenates were subjected to dialysis overnight in the presence of 10 mM potassium phosphate, pH 7.1, to eliminate possible effects of adherent allosteric regulators. Protein was measured according to the method of Lowry et al.<sup>21</sup>

The complementary DNA (cDNA) for glutamate dehydrogenase was prepared from lymphoblast polyA messenger RNA and amplified by the polymerase chain reaction (PCR) for automated fluorescence sequencing (Applied Biosystems). The forward primers spanned nucleotides 137 to 155, 363 to 380, 721 to 741, and 1241 to 1261, and the reverse primers spanned nucleotides 450 to 432, 902 to 883, 1380 to 1360, and 1730 to 1710.<sup>22</sup> Exons 11 and 12 of the gene for glutamate dehydrogenase (*GLUD1*), together with portions of their adjacent introns,<sup>23</sup> were also amplified by PCR from lymphoblast genomic DNA for sequence analysis and restriction-enzyme analysis. For exon 11, the forward primer was TGTAGTGTCTGTCAAGAGAG and the reverse primer was ACACACATGTCACGCACTTAC. For exon 12, the forward primer was ACAGGGACACAAAGCAGGTC and the reverse primer was ACAGTCTGGCGGCTGAGATAG.

Site-directed mutagenesis was used to construct a pcDNA3 plasmid (Invitrogen) capable of expressing in COS-7 cells the mutation identified in Patient 1: a change from histidine to tyrosine at position 454 of the enzyme (His454Tyr). Full-length normal human glutamate dehydrogenase cDNA was obtained through the courtesy of Dr. Roberta Colman (Department of Chemistry, University of Delaware). The corresponding nucleotide substitution of thymidine for cytosine at position 1532 was incorporated with two rounds of overlapping PCR with a pair of internal primers containing the mutant base. After confirmation that the orientation and sequence were correct, the His454Tyr mutant glutamate dehydrogenase pcDNA3 was transfected into COS-7 cells with Lipofectin (BRL) and clones were selected with G418 (Geneticin, BRL). Aliquots of G418-resistant cells were grown in a 96-well plate, and the subclones were tested to determine whether they had increased expression of glutamate dehydrogenase and whether the sensitivity of the enzyme to GTP was altered. Cells transfected with the pcDNA3 vector alone were used as controls. Student's t-test was used to compare the results of these studies in the various groups of subjects.

## RESULTS

### Enzymatic Activity of Glutamate Dehydrogenase

The activity and allosteric responses of glutamate dehydrogenase in lymphoblasts from the patients are shown in Table 1. The activity of the enzyme in the patients with sporadic cases of the hyperinsulinemia-hyperammonemia syndrome was not inhibited by GTP, as shown by the fact that the half-maximal inhibitory concentration (IC<sub>50</sub>) for this effector was nearly four times as high as in the normal subjects. Enzyme activity in the subjects with the clinically milder familial cases was also less sensitive to inhibition by GTP, but the IC<sub>50</sub> was only twice the normal value.

Basal and maximal ADP-stimulated glutamate dehydrogenase activities were similar in the patients with sporadic hyperinsulinism-hyperammonemia and the normal subjects. In the patients with familial cases, basal enzyme activity was 38 percent of normal, although maximally stimulated glutamate dehydrogenase activity was only slightly less than normal (P=0.07). The sensitivity to stimulation with ADP was similar in the three groups. The pattern of differences among the three groups was similar after dialysis of the lymphoblast homogenates, thus ruling out the possibility that the abnormalities were caused by binding of the effector molecules to the enzyme (data not shown). These results are compat-

**TABLE 1.** ACTIVITY AND ALLOSTERIC RESPONSIVENESS OF GLUTAMATE DEHYDROGENASE IN LYMPHOBLASTS FROM CHILDREN WITH SPORADIC OR FAMILIAL HYPERINSULINISM-HYPERAMMONEMIA SYNDROME, THEIR AFFECTED RELATIVES, AND NORMAL SUBJECTS.

VARIABLE	PATIENTS 1-5, WITH SPORADIC CASES	PATIENTS 7 AND 8 AND THEIR AFFECTED MOTHERS, WITH FAMILIAL CASES	NORMAL SUBJECTS
		mean ±SE	
Basal enzyme activity in the absence of effectors (nmol/min/mg of protein)	22±2	9±2*	24±1†
Half-maximal inhibitory concentration of guanosine 5'-triphosphate (nmol/liter)	1560±163*	900±200‡	425±100§
Half-maximal stimulatory concentration of adenosine 5'-diphosphate (μmol/liter)	17±1	22±2	18±1¶
Maximal enzyme activity with 100 μmol of adenosine 5'-diphosphate per liter (nmol/min/mg of protein)	33±1	24±3	33±3¶

\*P=0.001 for the comparison with the normal subjects.

†Ten normal subjects were tested.

‡P<0.036 for the comparison with the normal subjects.

§Nine normal subjects were tested.

¶Four normal subjects were tested.

ible with the presence of intrinsic abnormalities in glutamate dehydrogenase in both groups of patients. Lymphoblast glutamate dehydrogenase from the unaffected parents and siblings of both groups of patients had normal responses to GTP. These results suggested that the defect in glutamate dehydrogenase is dominantly expressed and that the sporadic cases represented spontaneous mutations.

**Mutation Analysis of Glutamate Dehydrogenase**

Each of the eight patients with the hyperinsulinism–hyperammonemia syndrome was found to have a change in a single nucleotide that was predicted to alter 1 of 4 amino acids between residues 446 and 454 of the 505-amino-acid mature glutamate dehydrogenase (Table 2 and Fig. 2). Among the six patients with sporadic cases, four different mutations were found. The two patients with familial cases

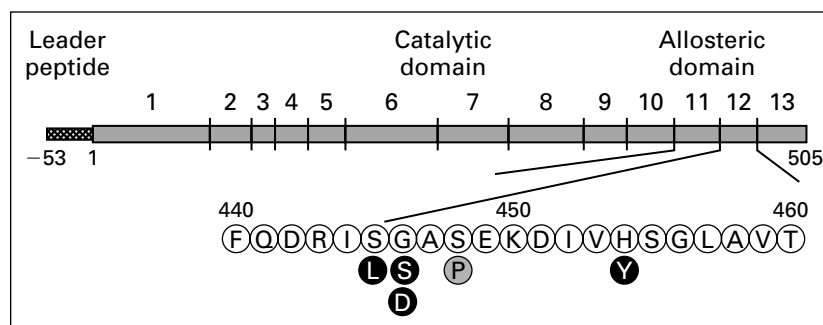
shared a fifth mutation, although there was no evidence that they were related. No other mutations were found in any of the patients when the rest of the cDNA coding region for the enzyme was sequenced.

Each of the five mutations eliminated a restriction-endonuclease digestion site or created a new site, thus making it possible to distinguish readily wild-type and mutant alleles in PCR products from either the cDNA or genomic DNA for glutamate dehydrogenase. All eight patients were heterozygous, with one mutant and one wild-type allele, a pattern consistent with the dominant expression of the mutations. None of the mutations were found on restriction-endonuclease analysis of genomic DNA from 55 normal subjects, suggesting that none of the mutations represented a common silent polymorphism. Similar analyses of genomic DNA from

**TABLE 2.** MUTATIONS IDENTIFIED IN THE GLUTAMATE DEHYDROGENASE GENE IN EIGHT CHILDREN WITH THE HYPERINSULINISM–HYPERAMMONEMIA SYNDROME.

PATIENT No.	CITY OF BIRTH	NUCLEOTIDE CHANGE IN cDNA SEQUENCE	AMINO ACID CHANGE IN MATURE ENZYME	WILD-TYPE ENDONUCLEASE SITE	MUTANT ENDONUCLEASE SITE	PATIENT'S GENOTYPE*	PARENT AFFECTED
<b>Sporadic cases</b>							
1	Mexico City, Mexico	C→T at 1532	His→Tyr at 454	<i>Sma</i> I	<i>Rsa</i> I	Mutant/WT	Neither
2	Cortland, N.Y.	C→T at 1506	Ser→Leu at 445	<i>Eco</i> RV		Mutant/WT	Neither
3	Padua, Italy	C→T at 1506	Ser→Leu at 445	<i>Eco</i> RV		Mutant/WT	Neither
4	Ann Arbor, Mich.	G→A at 1508	Gly→Ser at 446		<i>Taq</i> I	Mutant/WT	Neither
5	Toronto	G→A at 1509	Gly→Asp at 446		<i>Nsi</i> I	Mutant/WT	Neither
6	Chicago	G→A at 1509	Gly→Asp at 446		<i>Nsi</i> I	Mutant/WT	Neither
<b>Familial cases</b>							
7	Florence, Italy	T→C at 1514	Ser→Pro at 448	<i>Sfi</i> NI		Mutant/WT	Mother
8	Winnipeg, Man., Canada	T→C at 1514	Ser→Pro at 448	<i>Sfi</i> NI		Mutant/WT	Mother

\*WT denotes wild type.



**Figure 2.** Location of Mutations in the Glutamate Dehydrogenase Gene in Patients with Sporadic and Familial Cases of the Hyperinsulinism–Hyperammonemia Syndrome.

Shown are the regions encoded by the 13 exons, the leader peptide, and the catalytic and allosteric domains of the enzyme. The mutations in both groups of patients are clustered in an area of 10 amino acid residues in the allosteric domain encoded by exons 11 and 12. Solid symbols indicate clinically more severe mutations in the sporadic cases, and the stippled symbol a clinically milder mutation in the familial cases.

the mothers and fathers of the six patients with sporadic cases showed that none had their child's mutation, confirming that the mutation in these children was spontaneous.

Restriction-enzyme analysis of the PCR products of the cDNA for glutamate dehydrogenase or exon 12 genomic DNA in the parents and other relatives of Patients 7 and 8 showed that all seven affected relatives were heterozygous for the Ser448Pro mutation and the wild-type allele and that none of the six unaffected relatives who were studied had the mutation.

#### Expression of Glutamate Dehydrogenase Mutations in COS-7 Cells

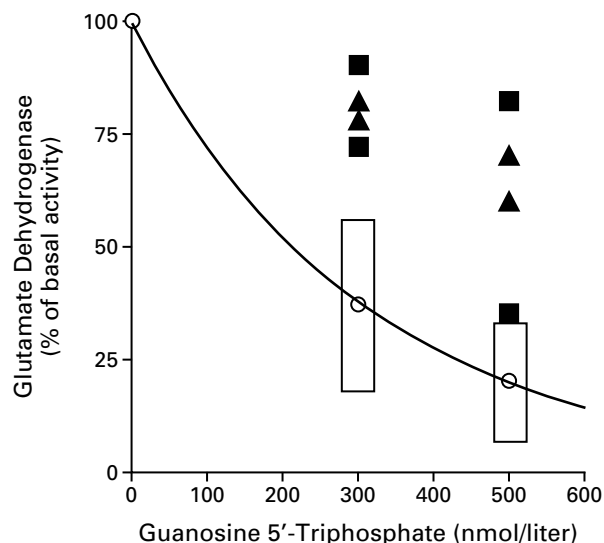
The glutamate dehydrogenase activity of two clones of COS-7 cells transfected with the His454Tyr mutation (from Patient 1) was 57 and 35 nmol per minute per milligram of protein, as compared with a value of 24 nmol per minute per milligram of protein in cells transfected with vector alone. The enzyme in these two clones was less sensitive to GTP-induced inhibition than was the endogenous glutamate dehydrogenase in the control COS-7 cells (estimated  $IC_{50}$ , 200 nmol per liter) (Fig. 3) or in normal human lymphoblasts. These results confirmed that the His454Tyr mutation resulted in decreased sensitivity to GTP-induced inhibition in a manner similar to that found in the patient's lymphoblasts.

#### DISCUSSION

The results of these studies indicate that the hyperinsulinism-hyperammonemia syndrome is associated with dominantly expressed mutations of mitochondrial glutamate dehydrogenase, which is encoded by the *GLUD1* gene on chromosome 10. In all affected patients who were tested, glutamate dehydrogenase had reduced sensitivity to inhibition by GTP. This defect would be expected to result in abnormally high rates of glutamate oxidation, leading to excessive insulin secretion and impaired detoxification of ammonia by the liver (Fig. 1).

The two groups of patients with hyperinsulinism and hyperammonemia had different degrees of impaired responsiveness to GTP inhibition that correlated with differences in the clinical phenotype. The patients with familial cases, in whom enzyme activity was more sensitive to inhibition by GTP than in the patients with sporadic cases, had less severe hypoglycemia. The lower basal enzyme activity in the patients with familial cases may also have contributed to their less severe hypoglycemia. Whether this difference reflects lower intrinsic activity of the enzyme, an altered allosteric effect, or lesser amounts of enzyme protein is not known.

The five mutations in glutamate dehydrogenase identified in these patients were all within exons 11



**Figure 3.** Sensitivity of Mutant Glutamate Dehydrogenase to Inhibition by Guanosine 5'-Triphosphate.

Two clones of COS-7 cells with the His454Tyr mutation (solid symbols) and control cells transfected with vector alone (open symbols) were incubated with various concentrations of guanosine 5'-triphosphate. The mean residual enzyme activity was significantly greater in cells containing the mutant glutamate dehydrogenase than in the control cells for both concentrations of guanosine 5'-triphosphate: at 300 nM guanosine 5'-triphosphate, the mean ( $\pm$ SE) activity of the mutant enzyme was  $80 \pm 3$  percent, and the mean activity of the normal enzyme was  $37 \pm 7$  percent ( $P = 0.002$ ); at 500 nM guanosine 5'-triphosphate, the respective values were  $61 \pm 10$  percent and  $20 \pm 5$  percent ( $P = 0.004$ ). Values are the means of four experiments for the mutant enzyme; for the normal enzyme, means and 95 percent confidence intervals for seven experiments are shown.

and 12 of *GLUD1*.<sup>23</sup> The tertiary structure of mammalian glutamate dehydrogenase has not been determined, but sequence comparisons with the non-allosteric glutamate dehydrogenase of prokaryotes and photoaffinity studies of bovine glutamate dehydrogenase suggest that the GTP allosteric domain of the mammalian enzyme is near the region encoded by these exons.<sup>24-26</sup> This suggestion is consistent with our observation that the mutations in the patients with sporadic cases impaired enzyme sensitivity to GTP-induced inhibition but did not affect basal or ADP-stimulated enzyme activity. All the mutations identified lie within a sequence of 15 amino acids that has been suggested to contain the GTP binding site.<sup>26</sup> Whether the mutations alter responses to other allosteric effectors of glutamate dehydrogenase, such as leucine or palmitoyl-coenzyme A,<sup>27,28</sup> is not known.

Since mature glutamate dehydrogenase is a hexamer of six identical subunits, the enzyme in patients with the hyperinsulinism-hyperammonemia syndrome is likely to be composed of a mixture of heterohexamers containing, on average, equal num-

bers of mutant and wild-type subunits. Protein-protein interactions between these subunits may be an important factor in the dominant effects of the mutations. The glutamate dehydrogenase with the His454Tyr mutation had impaired sensitivity to GTP when transfected into COS-7 cells, a finding similar to those in lymphoblasts from the heterozygous patients.

The existence of the hyperinsulinism-hyperammonemia syndrome highlights the importance of glutamate dehydrogenase in the regulation of insulin secretion<sup>15,16,29</sup> and indicates that the enzyme has an important role in regulating hepatic ureagenesis.<sup>18</sup> Partial deficiency of glutamate dehydrogenase has been reported in some patients with cerebellar degeneration,<sup>30</sup> suggesting that the enzyme is important in brain function. Further studies of glutamate dehydrogenase in beta cells, liver, and brain may provide explanations for three features in patients with the hyperinsulinism-hyperammonemia syndrome that remain a puzzle: sensitivity to leucine or protein-induced hypoglycemia, the ability to ingest protein loads without worsening hyperammonemia, and the apparent absence of central nervous system symptoms due to hyperammonemia.<sup>11,12</sup>

In conclusion, the hyperinsulinism-hyperammonemia syndrome is a distinctive genetic disorder of insulin secretion caused by mutations in the gene for glutamate dehydrogenase. The disorder should be considered as part of the differential diagnosis in children with hyperinsulinism who respond to diazoxide; it can be recognized by the finding of a high plasma ammonium concentration in association with insulin-induced hypoglycemia.

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