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MANAGEMENT OF A PATIENT WITH HOLOCARBOXYLASE SYNTHETASE DEFICIENCY

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Abstract

We investigated in a patient with holocarboxylase synthetase deficiency, the relation between the biochemical and genetic factors of the mutant protein with the pharmacokinetic factors of successful biotin treatment. A girl exhibited abnormal skin at birth, and developed in the first days of life neonatal respiratory distress syndrome and metabolic abnormalities diagnostic of multiple carboxylase deficiency. Enzyme assays showed low carboxylase activities. Fibroblast analysis showed poor incorporation of biotin into the carboxylases, and low transfer of biotin by the holocarboxylase synthetase enzyme. Kinetic studies identified an increased K_m but a preserved V_{max} . Mutation analysis showed the child to be a compound heterozygote for a new nonsense mutation Q379X and for a novel missense mutation Y663H. This mutation affects a conserved amino acid, which is located the most 3' of all recorded missense mutations thus far described, and extends the region of functional biotin interaction. Treatment with biotin 100 mg/day gradually improved the biochemical abnormalities in blood and in cerebrospinal fluid, corrected the carboxylase enzyme activities, and provided clinical stability and a normal neurodevelopmental outcome. Plasma concentrations of biotin were increased to more than 500 nM, thus exceeding the increased K_m of the mutant enzyme. At these pharmacological concentrations, the CSF biotin concentration was half the concentration in blood. Measuring these pharmacokinetic variables can aid in optimizing treatment, as individual tailoring of dosing to the needs of the mutation may be required.

Introduction

Holocarboxylase synthetase deficiency (OMIM 253270) is a rare autosomal recessive disorder of biotin metabolism leading to multiple carboxylase deficiency [1]. Holocarboxylase synthetase (HLCS; OMIM 609018, E.C. 6.3.4.10) covalently links biotin to the five biotin-

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dependent carboxylases: the three mitochondrial enzymes propionyl-CoA carboxylase (E.C. 6.4.1.3), 3-methylcrotonyl-CoA carboxylase (E.C. 6.4.1.4), and pyruvate carboxylase (E.C. 6.4.1.1), and both the mitochondrial and the cytosolic acetyl-CoA carboxylases (E.C. 6.4.1.2). Patients with holocarboxylase synthetase deficiency present in the neonatal period or early infancy with metabolic acidosis, hyperammonemia, tachypnea, skin rash, feeding problems, hypotonia, seizures, developmental delay, alopecia, and coma. Diagnosis is suggested by urine organic acids analysis and confirmed by holocarboxylase synthetase enzyme assay or DNA mutation analysis. Treatment with biotin doses ranging between 3 and 200 mg per day has been reported [2,3]. In this case report we discuss the pharmacobiologic factors that possibly contributed to the excellent results of treatment.

Case Report

After an uncomplicated pregnancy, this girl was born at term by cesarean section for breech position with normal birth weight and Apgar scores. She had a taut, shiny, and thickened skin with a cellophane-like appearance and perioral creases. She developed respiratory distress requiring intubation and ventilation. Chest x-ray showed a ground-glass picture with air bronchogram consistent with respiratory distress syndrome despite 40 weeks gestation and lacking physical signs of prematurity. A brain MRI on day 8 showed immature myelination with slight dilatation of the ventricles. Laboratory evaluation showed metabolic acidosis with pH 7.20, anion gap 28 mEq/L, plasma lactate 15.4 mM (normal 0.5–2.0), pyruvate 0.32 mM (normal 0.034–0.102) with an elevated lactate:pyruvate ratio 48 (normal <25). Blood ammonia was 192 μ M (normal 64–107). Serum amino acids were normal. Urine organic acids showed increased lactate, 3-hydroxybutyrate, 3-hydroxyisovalerate, 3-hydroxypropionate, propionylglycine, methylcitrate, and 3-methylcrotonylglycine. Serum acylcarnitines showed increased propionylcarnitine and 3-hydroxyisovalerylcarnitine. These data suggested multiple carboxylase deficiency, which was later confirmed (see results).

Empiric treatment with biotin 10 mg/day in two doses begun on day 3 did not result in improvement. After diagnosis on day 6, treatment was initiated with enteral biotin 100 mg/day, glycine 100 mg/kg/day, and L-aspartate 4 mmol/kg/day, and intravenous L-carnitine 300 mg/kg/day. Due to ileus, biotin could only be administered in 3 mL/day, limiting its soluble amount to 0.7 mg/day given biotin's solubility of 22 mg/100 mL. Feedings gradually increased and became substantial on day 15. As more enteral intake of biotin-containing formula was possible, the metabolic abnormalities corrected. The skin blistered, desquamated, and normalized. The respiratory distress syndrome responded to surfactant allowing extubation. On day 27, cerebrospinal fluid (CSF) had a lactate of 2.1 mM, increased alanine of 44 μ M (control < 35), and no abnormal organic acids, showing metabolic control behind the blood-brain barrier. The initial leucine-restricted diet was gradually lifted over 6 months, but protein intake remained restricted at 2 g/kg/day. Aspartate was discontinued within a month. She remains treated with 100 mg/kg/day of glycine and 50 mg/kg/day of L-carnitine.

The initial serum propionylcarnitine was 10.9 μ M, and during follow-up, these levels remained mildly elevated between 1.9 and 3.7 μ M (average 2.5 μ M, normal < 1.8 μ M). The 3-hydroxyisovalerylcarnitine was 1.2 μ M at presentation, and during treatment averaged 0.30 μ M, range of 0.10 to 0.87 μ M (normal < 0.12 μ M). At 1 year 9 months, her protein intake had increased to 5.15 g/kg/day and 3-hydroxyisovalerylcarnitine increased up to 0.87 μ M, but propionylcarnitine did not elevate. After reinstituting dietary protein restriction to 2.5 g/kg/day, 3-hydroxyisovalerylcarnitine levels have remained below 0.23 μ M. During treatment, urine organic acids showed continued traces of 2-methylcitrate and 3-hydroxyisovalerate. Her plasma lactate has remained normal. At 14 months of age, she had normal brain MRI with normal brain magnetic resonance spectroscopy without a lactate signal. Cerebrospinal fluid showed normal concentrations of lactate 1.1 mM and pyruvate 0.102 mM.

On long-term follow-up, she continues to show metabolic stability including over intercurrent illnesses. Her neuropsychomotor development has been age-appropriate. At age 3 years, she is talking in sentences, can copy a circle, can hop on one leg, dresses by her self, and knows some colors and body parts.

Methods

Informed consent was obtained from the parents on an Institutional Review Board-approved study. Biotin concentrations in plasma and CSF were quantified as total avidin-binding substances (TABS) using an avidin-binding assay as previously described [4]. Activities of carboxylases (propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, and pyruvate carboxylase) were assayed in lymphocytes by measuring the incorporation of ^{14}C from $\text{NaH}^{14}\text{CO}_3$ into the appropriate substrate to give labeled non-volatile products, as described previously [5].

Fibroblasts were grown from a punch skin biopsy. The biotinylation status of carboxylases was studied in fibroblasts grown for up to 13 days in biotin-replete (biotin approximately 0.40 nM) or in biotin-deficient medium as previously described [6]. Carboxylase biotinylations was determined by Western blot analysis of 100 μg of total protein in polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were incubated with 1:2000 solution of streptavidin-AP conjugate (Roche Applied Science), and the bands visualized using a BM chemiluminescence Western blotting kit (Roche Applied Science), and quantified using an FX image analyzer (Bio-Rad).

The biotinylation activity was determined using p-67, a peptide containing the last 67 amino acids of the alpha subunit of propionyl-CoA carboxylase as modified from a previously published method [7]. A vector encoding p67 with an 8 amino acid amino-terminal FLAG sequence was used to transform biotin-auxotroph *E. coli* strain C-124. Unbiotinylated p-67 was purified with anti-flag M2 affinity gel (Sigma). Cell extracts (100 μg total protein) from normal and mutant fibroblasts were incubated for 1 hour with affinity gel containing 1 μg of p67 in PBS buffer containing 10 mM ATP, 8 mM MgCl_2 and 1 μCi ^3H -biotin (Amersham). The biotinylation of p67 was determined using a scintillation counter (LS-6500 Beckman) [8].

The kinetic constants of holocarboxylase synthetase activity were determined in fibroblasts cultured in low biotin medium [9]. The rate of incorporation of ^3H -biotin into acid-precipitable material was followed upon addition of the fibroblast lysate to a mixture composed of 22.7 nM ^3H -biotin, 3 mM ATP, 8 mM MgCl_2 , and 2.5 mM reduced glutathione, 0.5 mM EDTA and rat liver apocarboxylases in 100 mM Tris, pH 8, prepared from livers of rats that were fed a biotin-free diet [10]. Varying the concentration of ^3H -biotin, the K_m and V_{max} were determined from the Eadie-Hofstee plot.

For sequencing, all exons were amplified by PCR using intronic primers with standard M13 sequence tags (primers sequences available upon request), and the obtained PCR fragments sequenced on both the sense and anti-sense strands with standard M-13 sequence primers on an ABI3730 automatic capillary sequencer. Sequence analysis was performed using the CodonCode™ Aligner software (CodonCode Corp.), and results compared to the sequence for human holocarboxylase synthetase (Ensembl gene OTTHUMG00000086636 and transcript OTTHUMT00000194686) [11]. Variations were reported according to the consensus statement with +1 as the A of the ATG start codon [12]. Multi-species alignment of the peptide sequence for analysis of the missense mutation was performed based on proteins of human, macaque, snake, ox, mouse, frog, and cat (www.ensembl.org/), and further to the alignment

for birA and avidin as published [13]. The new mutation was verified in a set of controls and compared to the databank of human variation at Ensembl [11].

Results

At diagnosis prior to treatment, lymphocyte carboxylase activities were deficient: pyruvate carboxylase 6 pmol/min/mg protein (normal range 160–447), 3-methylcrotonyl-CoA carboxylase 4 pmol/min/mg protein (normal range 62–228), and pyruvate carboxylase 0.0 pmol/min/mg protein (normal range 6.5–40.7). The first biotin samples were obtained at age 14 months while she received 100 mg/day of biotin, or 10.4 mg/kg/day. The concentration of total avidin-binding substances in plasma at trough level after a period of fasting was 1800 nM; at peak level, the plasma total avidin-binding substances level was 4900 nM. Each of these values is more than 1000 fold increased over the normal range of 0.4 to 1.5 nM, observed in a study of 49 children consuming either a general or vegetarian diet [14]. These data provide evidence that the high doses of biotin were absorbed by this child and produced plasma concentrations substantially greater than the K_m of the mutant HLCS. The biotin concentration in CSF obtained simultaneously with the trough sample was 1000 nM. This value is increased approximately 600 fold over the normal mean of 1.6 nM (range from undetectable (< 0.03 nM) to 5.9 nM), obtained from 55 diagnostic CSF samples [15]. These data provide evidence that at these high supraphysiologic plasma concentrations, biotin was able to cross the blood-brain-barrier in this child, and exceeded the K_m for biotin in CSF as well. Of total avidin-binding substances, biotin is estimated to be at least 60% in serum at normal concentrations and 50% in CSF. During supplementation with large amounts of biotin, the molar percent of biotin increases modestly in serum [16]. Thus total avidin-binding substances should accurately reflect biotin concentrations under these circumstances. A new sampling of biotin was taken at age 4 year while she received 100 mg/day or 7.14 mg/kg/day. The total avidin-binding substances concentration was 835 nM. With this, the lymphocyte carboxylase activities were (on two separate samples): propionyl-CoA carboxylase 68.1 and 154 pmol/min/mg protein (normal range > 70), 3-methylcrotonyl-CoA carboxylase 44.0 and 56 pmol/min/mg protein (normal range > 31), and pyruvate carboxylase 4.6 and 6.1 pmol/min/mg protein (normal range > 6).

We studied the biotinylation status of the following carboxylases: pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase. Patient fibroblasts and control fibroblasts were grown in biotin-replete medium and the biotin content in carboxylases visualized. In normal cells, propionyl-CoA carboxylase- α subunit (72 kDa) and 3-methylcrotonyl-CoA carboxylase- α subunit (76 kDa) appear as a doublet, distinguishable from the fainter pyruvate carboxylase band (128 kDa), whereas acetyl-CoA carboxylase is not detected [6] (Figure 1, lane 1). The holocarboxylase synthetase deficient cells showed a strong reduction in carboxylase biotinylation in biotin-replete medium to about one tenth of the levels observed in the normal fibroblasts (Figure 1, lane 3). Incubation of cells in a biotin-deficient medium for 13 days reduced carboxylase biotinylation in normal cells to about one third of the control levels (Figure 1, lane 2), while in mutant cells biotinylation of carboxylases was undetectable (Figure 1, lane 4). To verify that the reduced carboxylase biotinylation in cultured cells was a direct result of mutant HLCS, we determined the biotinylation activity of the patient cells and controls cells using unbiotinylated p-67 as a substrate. The amount of ^3H -biotin incorporated in p-67 was taken as a measure of holocarboxylase synthetase activity. Extracts from mutant cells catalyzed the incorporation of ten times less biotin into p-67 than normal fibroblasts (Figure 2). Kinetic analysis of holocarboxylase synthetase activity using biotin-deficient apocarboxylases showed an elevated K_m for biotin of 163.7 nM and a normal maximal velocity of 1511 fmol/mg protein/h (parallel control fibroblasts had K_m 11.6 nM and V_{max} 618 fmol/mg protein/h, control range of fibroblasts K_m was 1.0–12 nM).

Mutation analysis of the HLCS gene showed a novel paternal missense mutation Y663H (c.1987T>C) in exon 8 at the end of the biotin-binding domain, and a novel maternal nonsense mutation Q379X (c.1135C>T) in exon 6. The parents were shown to be carriers for the respective mutations. This Q379X mutation introduces a stop codon most probably resulting in production of a truncated protein, since it is too close (44 bp) to the exon-intron junction to trigger nonsense-mediated mRNA decay [17]. The tyrosine 663 involved in the missense mutation is conserved in all examined vertebrates in which the sequence extends this far (Figure 3), and in the related biotin binding BirA gene of E.coli it is replaced by tryptophan, a conservative change [13]. The mutation was not recorded as a recognized human variation in Ensembl, and was not present in 104 alleles of control samples.

Discussion

This patient presented with a typical clinical and metabolite profile consistent with multiple carboxylase deficiency, including the skin findings and their resolution with biotin treatment [1,18]. A new symptom was the development of respiratory distress syndrome of the neonate despite a normal gestational age, which responded to surfactant treatment. Many patients have shown either tachypnea or breathing difficulties [19]. It is unclear whether the reported respiratory distress was due to the metabolic acidosis or if neonatal respiratory distress syndrome was present.

In the literature, patients with holocarboxylase synthetase deficiency have responded to a variable degree to treatment with pharmacologic doses of biotin. Three possible mechanisms can explain the effect of biotin treatment [3]. Many mutations in the HLCS gene increase the K_m of the holocarboxylase synthetase enzyme for biotin, and higher concentrations of biotin are required for proper enzymatic function. Biotin also increases the transcription of the HLCS gene, resulting in more holocarboxylase enzyme being produced [3,6,20]. This mechanism can explain improvement even in patients in which the K_m is normal but the V_{max} is decreased. It was also postulated that the holocarboxylase enzyme is not saturated at physiologic concentrations of biotin, leaving room for increased activity at higher biotin concentrations. If the causative mutation affected the V_{max} of the enzyme, then limited benefit was observed from biotin treatment, but if the mutation affected the K_m of the holocarboxylase synthetase for biotin, then substantial improvement to normalization of the carboxylases was observed [21].

In the patient described here, the residual activity almost certainly arose from the Y663H missense mutation, since the second allele contained a nonsense mutation. Most missense mutations that affect the K_m for biotin are located in the biotin-binding domain. The mutation in our patient, Y663H, is located 3' beyond the avidin-binding domain of D562 to P609, but is still within the birA homologues region, which ranges from amino acid 448 to 701 [13]. It is beyond the thus far most 3' reported missense mutation, which involved D634 [22,23]. The amino acid Y663 is preserved across a wide range of vertebrates indicating its probable functional significance. The effect of the mutation on the K_m of the enzyme indicates that even this extreme 3' end of the birA homologous domain influences biotin binding or biotin activity. In fibroblasts the V_{max} of the enzyme was preserved but the K_m was raised 40 fold to 164 nM. At physiologic biotin concentrations of 0.4 to 1.5 nM, the activity is insufficient to provide full biotinylation of the apocarboxylases, and the activities of all three enzymes tested in leucocytes were deficient.

However, at a dose of 100 mg/day, delivering a dose of 38 to 7 mg/kg/day decreasing as the child was growing up, the carboxylases corrected with all leucocyte carboxylase activities to within, or just under, the normal range. The metabolites corrected as well, although slight elevations of 3-hydroxyisovaleric and of 3-hydroxyisovalerylcarnitine persisted, as seen in

other well-treated patients [3]. A high protein intake resulted in a striking increase in metabolites, which disappeared again with a mild protein restriction, suggesting that the diet could not be completely unrestricted. A similar need for protein restriction has been reported before [5]. At a dose of 10 mg/kg/day of biotin the plasma total avidin binding substances concentrations varied between 1800 and 4900 nM reflecting a biotin concentration of about 1080 and 2940 nM. At a biotin dose of 7 mg/kg/day a plasma concentration of 835 nM TABS, reflecting a biotin concentration of at least 500 nM was documented. These concentrations far exceed the measured K_m of the enzyme in fibroblasts of 164 nM. Despite this apparent excess, not all carboxylases were always fully reconstituted in leucocytes, and slight metabolite elevations always persisted, and there remained a limitation to the protein intake that could be metabolized. These plasma biotin concentrations are similar to that seen in an infant receiving 10 mg/day and another receiving 20 mg/day, which had plasma biotin levels of 1300 and 4830 nM respectively [18]. The plasma concentrations are higher than the plasma biotin levels of 87 to 445 nM reported in a patient taking from 5 to 40 mg/day (0.8–4 mg/kg/day) [24], and at the upper end of that recorded in 4 patients receiving between 1.8 and 10.1 mg/kg/day of biotin in whom plasma biotin levels between 112 and 684 nM respectively were reported [25]. This shows a substantial range of plasma biotin levels with currently employed doses of biotin ranging from 87 to 4830 nM. The lower levels would not be sufficient to saturate the K_m of our mutant enzyme. Thus, our observations suggest that it may be prudent to measure the biotin plasma levels to ensure that the dosing used is indeed sufficient to saturate the K_m of the mutant holocarboxylase synthetase enzyme.

The metabolic abnormalities required over two weeks to correct in our patient. Two mechanisms could be involved. First, the solubility of biotin limited the dissolved amount that could be given to less than 2 mg/day. If biotin is provided in a sufficiently large volume, consistency of the bioavailability for oral uptake will be ensured. Also, an intravenous formulation of biotin would have been helpful to overcome this obstacle during severe illness. Second, biotin induces the sodium-dependent multivitamin transporter SMVT, which is required for uptake and distribution of biotin to all non-neurological tissues. This increase is dependent on biosynthesis of biotinyl-5'-AMP, which is formed in the first half reaction of the holocarboxylase synthetase enzyme [7,20], and then further via cGMP formation. This mechanism forms a positive feedback loop whereby as more biotin is taken up, through a higher expression of the SMVT more biotin will become bioavailable to the tissues [8], resulting also in a higher expression of HLCS and thus again a higher production of biotinyl-5'-AMP. This mechanism is impeded in holocarboxylase synthetase deficiency where a high K_m for biotin will limit the formation of biotinyl-5'-AMP. A slow gradual increase in the production of both SMVT and of biotinyl-5'-AMP may have prolonged the time of recovery.

Biotin is taken up in the brain via a facilitated transport by a different sodium-independent biotin transporter [26–28]. Uptake is linear at concentrations above 60 nM. At pharmacological concentrations, biotin apparently passed the blood-brain-barrier and resulted in CSF concentrations about half of the blood concentration, and a thousand fold higher than physiologic concentrations. The normal CSF lactate, organic acids, and the absence of a lactate peak on MRS all provide evidence for correction of the enzyme activity behind the blood brain barrier. These pharmacologic and biochemical findings explain the excellent neurodevelopmental outcome.

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Figure 1. Biotinylation of the carboxylases

Legend: In the first lane, control normal fibroblasts grown in normal medium (NF-B) show a band of pyruvate carboxylase PC at 128 kDa, and a doublet of 3-methylcrotonyl-CoA carboxylase- α subunit (MCC) (76 kDa) and propionyl-CoA carboxylase- α subunit (PCC) (72 kDa). These bands are reduced in intensity in patient's fibroblasts (B-PF). In biotin depleted medium (D), the signal intensities are further reduced in control fibroblasts (D-NF), and virtually unnoticeable in patient's fibroblasts (D-PF).

Figure 2. Biotinylation activity

Legend: The amount of ³H-biotin incorporated into using p-67, a peptide containing the last 67 amino acids of the alpha subunit of propionyl-CoA carboxylase. Extracts from mutant cells catalyzed the incorporation of ten times less biotin into p-67 than normal fibroblasts.

QDKGPNSVLPL	Y	RYWVHSGQQ	human	ENSG00000159267
QDKGPNSVLPL	Y	RYWVHSGQQ	Macaca mulatta (Macaque)	ENSMUG00000015566
QDQGPSGLPL	Y	KYWVHSGQQ	Microcebus murinus (Snake)	ENSMICG00000013188
QDKGPNSVLPL	Y	RYWVHSGQQ	Bos Taurus (Ox)	ENSBTAG00000033679
QDQGPDLPL	Y	KYWVHGGQQ	Mus musculus (Mouse)	ENSMUSG00000040820
QSKGPDGLPA	Y	KYWLHSGAK	Xenopus tropicalis (Frog)	ENSXETG00000013028
QDKGPNGILPL	Y	KYWVHSGQR	Felis catus (Cat)	ENSFCAG00000002943
EQEGLAPYLSRWEKLDNFNRP		BirA (E. coli)		from reference [13]

Figure 3. Alignment of Holocarboxylase synthetase

Legend: Multi-species alignment of the peptide sequence according to the Ensemble website at www.ensembl.org/, and from reference 13. The highlighted amino acid is where the Y663H mutation occurred.