Leucine-sensitive hyperinsulinaemic hypoglycaemia in patients with loss of function mutations in 3-Hydroxyacyl-CoA Dehydrogenase

Amanda J Heslegrave1,2, Ritika R Kapoor1,2, Simon Eaton1,2, Bernadette Chafiaux3, Teoman Akcay4, Enver Simsek5, Sarah E Flanagan6, Sian Ellard6 and Khalid Hussain1,2,7*

Abstract

Background: Loss of function mutations in 3-Hydroxyacyl-CoA Dehydrogenase (HADH) cause protein sensitive hyperinsulinaemic hypoglycaemia (HH). HADH encodes short chain 3-hydroxacyl-CoA dehydrogenase, an enzyme that catalyses the penultimate reaction in mitochondrial β-oxidation of fatty acids. Mutations in GLUD1 encoding glutamate dehydrogenase, also cause protein sensitive HH (due to leucine sensitivity). Reports suggest a protein-protein interaction between HADH and GDH. This study was undertaken in order to understand the mechanism of protein sensitivity in patients with HADH mutations.

Methods: An oral leucine tolerance test was conducted in controls and nine patients with HADH mutations. Basal GDH activity and the effect of GTP were determined in lymphoblast homogenates from 4 patients and 3 controls. Immunoprecipitation was conducted in patient and control lymphoblasts to investigate protein interactions.

Results: Patients demonstrated severe HH (glucose range 1.7–3.2 mmol/l; insulin range 4.8-63.8 mU/l) in response to the oral leucine load, this HH was not observed in control patients subjected to the same leucine load. Basal GDH activity and half maximal inhibitory concentration of GTP was similar in patients and controls. HADH protein could be co-immunoprecipitated with GDH protein in control samples but not in patient samples.

Conclusions: We conclude that GDH and HADH have a direct protein-protein interaction, which is lost in patients with HADH mutations causing leucine induced HH. This is not associated with loss of inhibitory effect of GTP on GDH (as in patients with GLUD1 mutations).

Keywords: Hyperinsulinism, Hypoglycaemia, Leucine tolerance

Background

HADH encodes 3-hydroxyacyl-CoA dehydrogenase (HADH), an intra-mitochondrial enzyme that catalyses the penultimate reaction in fatty acid β-oxidation. HADH is highly expressed in pancreatic β-cells [1] and plays an important, but as yet unknown, role in the regulation of insulin secretion [2,3]. The pivotal role of HADH in pancreatic β-cells is supported by the existence of dysregulated insulin secretion (hyperinsulinaemic hypoglycaemia; HH) in patients with HADH mutations. We recently reported that patients with HH due to recessively inherited HADH mutations are protein sensitive [4]; this observation demonstrates that HADH somehow regulates protein-induced insulin secretion.

Protein-sensitive HH, due to leucine sensitivity, is also known to be associated with the hyperinsulinaemia-hyperammonaemia syndrome (HI/HA syndrome) [5], a condition characterised by HH and asymptomatic hyperammonaemia. HI/HA syndrome is caused by missense mutations in the GLUD1 gene that encodes the mitochondrial enzyme glutamate dehydrogenase (GDH), which catalyses the oxidative deamination of glutamate to α-ketoglutarate and ammonia. In the pancreatic β-cell, α-ketoglutarate enters the tricarboxylic acid cycle and leads to insulin secretion. GDH is allosterically activated...
by leucine and ADP and is inhibited by GTP [5]. Activating mutations in GLUD1 reduce the sensitivity of the enzyme to allosteric inhibition by GTP and ATP leading to increased leucine-induced glutamate deamination [5]. Hence patients with HI/HA syndrome develop hypoglycaemia following ingestion of leucine.

A recent study [6] suggests the existence of an interaction between HADH and glutamate dehydrogenase (GDH) in the pancreatic β-cell. Even more recently, studies on hadh−/− mice have confirmed this in pancreas and other organs. [7] It could hence be hypothesized that HADH deficiency causes protein-sensitive HH due to leucine sensitivity via the GDH axis. Hence patients with HI/HA syndrome develop hypoglycaemia following ingestion of leucine.

Methods
Nine patients previously reported by us to have HH due to recessively inherited mutations in HADH were studied [4,8-10]. The clinical characteristics of the patients are summarised in Table 1.

Leucine tolerance test
Nine patients and seven controls (aged-matched children being investigated for other causes of hypoglycaemia) underwent an oral leucine load test. Diazoxide administration to patients was stopped 24 hours before the test and patients were managed with regular feeds or IV dextrose as necessary. Following a four hour fast, 0.1-0.15gm/kg of oral L-Leucine was administered. Blood glucose and plasma insulin concentrations were measured at 15 minute intervals. The test was stopped at 120mins, or earlier if hypoglycaemia (defined as blood glucose concentration of </=3.0 mmol/l) developed.

GDH activity
Peripheral blood samples were obtained from four patients with HADH mutations and lymphocytes from these patients were transformed with Epstein–Barr virus to establish lymphoblast cultures. Glutamate dehydrogenase activity in lymphoblast homogenates was determined spectrophotometrically, as NADH oxidation at 340 nm with α-ketoglutarate as the substrate [11]. The IC50 for GTP was determined by addition of GTP (50nM – 1000nM) to the assay. Protein content was determined by the Bradford assay [12].

Co-immunoprecipitation
GDH was immunoprecipitated from 300 μg lymphoblast mitochondrial lysate (3 controls and 4 patients with mutations in HADH (Refs 10, 4, 8, 9.)) (prepared using a Mitosciences mitochondria isolation kit for cultured cells according to the manufacturer’s instructions) using a rabbit polyclonal antibody and protein A sepharose. Immunoprecipitated proteins were denatured in SDS-sample buffer and separated by SDS-polyacrylamide electrophoresis. 50 μg of whole lymphoblast lysate was also run on one of the gels for control and patient samples. The gel was transferred to a nitrocellulose membrane and subjected to immunostaining with a monoclonal antibody to HADH overnight and peroxidase conjugated secondary antibody for 1 hour. Proteins were visualised using an Amersham Healthcare ECL + kit.

Results
Clinical characteristics
The median birth weight of the patients with a mutation in HADH was +0.07 SDS and patients presented at a median age of 16.5 weeks. The nadir blood glucose concentration was 4.4 mmol/l and the nadir insulin concentration was 9.5 mU/l.

Table 1 Results of the leucine tolerance test performed on the eight patients with HH due to a mutation in HADH

| Subject | Age at presentation | Birth weight SDS | HADH Mutation | Age at testing (years) | Baseline BG (mmol/l) | Nadir BG (mmol/l) | Baseline insulin (mU/l) | Peak insulin (mU/l) | Time to nadir (mins) | Reference |
|---------|-------------------|-----------------|---------------|------------------------|---------------------|-------------------|----------------------|-------------------|---------------------|-----------|
| Pt 1    | 16 weeks          | +0.25           | P258L         | 105                    | 4.4                 | 2.7               | 9.5                  | 45                | 45                  | 8         |
| Pt 2    | 16 weeks          | -1.1            | IV56-2a>g     | 10                    | 4.4                 | 1.7               | 6.5                  | 63.8              | 30                  | 9         |
| Pt 3    | 16 weeks          | -0.11           | M188V         | 4.2                    | 5.1                 | 2.7               | 8.6                  | 31.7              | 30                  | 4         |
| Pt 4    | 5 days            | +1.69           | R236X         | 2.1                    | 4.8                 | 2.8               | 12.9                 | 25.12             | 30                  | 10        |
| Pt 5    | 1 week            | +1.23           | R236X         | 6.6                    | 5.5                 | 2.2               | <2                   | 13.6              | 45                  | 10        |
| Pt 6    | 16 weeks          | -0.71           | K136E         | 3.2                    | 6.6                 | 3.0               | <2                   | 4.8               | 90                  | 10        |
| Pt 7    | 16 weeks          | -1.85           | Q163X         | 7.8                    | 5.0                 | 2.7               | <2                   | 5.6               | 90                  | 10        |
| Pt 8    | 2 weeks           | +1.16           | R236X         | 1.3                    | 4.8                 | 3.0               | 2.2                  | 9.2               | 60                  | 10        |
| Pt 9    | 2 days            | +0.93           | Ex1del/Ex1del | 2                     | 4.2                 | 2.8               | <2                   | 23.9              | 60                  | 10        |
median of 16 weeks. Clinical characteristics are summarised in Table 1. All patients with HADH mutations were diazoxide-responsive and demonstrated normal fasting tolerance on diazoxide.

**Leucine tolerance test**

All nine subjects with HADH deficiency developed hypoglycaemia after oral leucine load, at a median time of 45 minutes (Figure 1a). None of the control subjects became hypoglycaemic after the leucine load (Figure 1b). Table 1 summarises the blood glucose and insulin responses to the oral leucine load in patients.

**GDH activity**

Basal GDH activity was not significantly different in lymphoblast homogenates from 4 patients and 3 controls (Figure 2a), and IC50 for GTP is not higher in patients with mutations in the HADH gene. In patients with HI/HA syndrome caused by mutations in the GLUD1 gene, higher than usual basal enzyme activities can be seen and the inhibitory effect of GTP on GDH is lost [13,14].

**Co-immunoprecipitation**

When GDH is immunoprecipitated from control human lymphoblasts using a polyclonal antibody, HADH is also pulled down as picked up by monoclonal HADH antibody (Figure 2b.1, Lanes 4,5 and 6, Figure 2b.2., Lane 1). This protein interaction between HADH and GDH in normal lymphoblasts does not occur in the 4 patient samples (Figure 2b.1, Lane 3, Figure 2b.2., Lanes 2,3,4). Figure 2b.1 also demonstrates that HADH protein is well expressed in control lymphoblasts (Lane 2).

**Discussion**

Leucine-induced HH is a feature of the HI/HA syndrome and is due to gain of function mutations in GLUD1. In the pancreatic β-cells, α-ketoglutarate enters the tricarboxylic acid cycle and leads to an increase in the concentration of cellular ATP. This rise in the cellular ATP causes closure of the ATP sensitive potassium channel (KATP channel); resulting in cell membrane depolarization, Ca2+ influx via voltage gated calcium channels and insulin exocytosis.

GDH is allosterically activated by leucine and inhibited by GTP [15]. Activating mutations in the GLUD1 gene reduce the sensitivity of the enzyme to allosteric inhibition by GTP and ATP [14] or less frequently cause an increase in the basal GDH activity [13]. The loss of inhibition by GTP leads to increased leucine induced glutamate oxidation to α-ketoglutarate. Hence leucine sensitivity is manifested by hypoglycaemia following protein-rich meals which is a classical feature of this condition [5,16]. The mechanism of persistent hyperammonaemia is not completely understood, although a recent paper by Treberg et al [17] suggests that this is due to renal ammoniagenesis.

Protein sensitivity (but no leucine sensitivity) is also a feature observed in patients with mutations in the KATP channel genes [18]. We recently described severe protein sensitivity in patients with loss of function mutations in the HADH gene [4]. We now show for the first time, in a larger number of patients, that loss of function mutations in the HADH gene causes severe leucine hypersensitivity. These clinical observations suggest that HADH acts in some way to limit leucine induced insulin secretion. Interestingly, in contrast to patients with mutations in the GLUD1 gene the serum ammonia level is not elevated in patients with HADH mutations (data not shown). Our results show that despite the leucine hypersensitivity, mutations in HADH do not cause an increase either in the basal activity of GDH, or a change in the IC50 for GTP. This suggests that HADH mutations cause leucine sensitivity and dysregulated insulin secretion via a novel pathway not involving GTP regulation of GDH.

![Figure 1 Blood glucose concentrations in response to the oral leucine load in the nine patients with a mutation in HADH.](image.png) All the patients demonstrated marked hypoglycaemia (blood glucose concentration of <3.0 mmol/l) in response to the leucine load (1a). In contrast, none of the control subjects developed hypoglycaemia in response to an oral protein load (1b).
Our results show that a protein-protein interaction exists between GDH and HADH thus suggesting that HADH in some way regulates the activity of GDH. This regulatory mechanism would not seem to involve GTP but must occur by another as yet unidentified mechanism. It is possible that an interaction between HADH and GDH has allosteric effects that affect how leucine stimulates GDH activity. Reduced expression of HADH protein, which can be seen in patient lymphoblasts, could allow leucine to over stimulate GDH and hence lead to excessive insulin secretion. In support of this hypothesis Li et al [7] have shown an interaction between HADH and GDH both in liver mitochondria and islets from hadh +/- but not hadh -/- mice. In hadh -/- liver His-tagged HADH was used to immunoprecipitate GDH as identified by mass spectrometry but this was not the only protein pulled down, it may be speculated that this could be true of islet cells also. Interestingly, basal activity of GDH in islets were similar in +/- and -/- islets and also the GTP IC50 which agrees with our data on human lymphoblasts. Li et al [7] also showed a reduced $K_m$ for $\alpha$-ketoglutarate in hadh -/- islets that was not seen in -/- liver or kidney which resulted in a 50 % increase in enzyme efficiency. This increase in enzyme efficiency shown in pancreas may be due to the fact that HADH is so highly expressed [1] in this organ compared to others, that a deficiency of it is detrimental with regard to regulation of GDH. In other organs the expression of HADH is much lower and so does not have the same effect on GDH. This highlights the difference between patients with mutations in GDH where activity of this enzyme is affected in all tissues in the body.

HH on administration of leucine was demonstrated in the hadh -/- mouse by Li et al [7]. This HH was exacerbated by the addition of glutamine and alanine and it is
possible that the HH we see in our patients would also be responsive to these amino acids.

Martens et al [3] showed that expression of HADH in pancreatic β-cells is higher than that of other enzymes of β-oxidation pathway suggesting an important role for this protein in β-cell physiology. Li et al [7] show that the ratio of HADH to GDH mRNA was 5 fold higher in mouse islets than in liver. Martens et al [3] showed however that suppression of the HADH protein resulted in increased basal and glucose stimulated insulin secretion which was not due to increased rates of glucose metabolism or an inhibition of fatty acid oxidation. The data obtained by Li et al [7] suggested that deletion of HADH protein caused an increase in basal islet metabolism but that this did not have a major effect on glucose stimulated insulin secretion. These data appear to contradict each other but may be due to an incomplete KO of HADH in the study by Martens et al [3].

Conclusions

In conclusion, our clinical data indicates that the protein sensitivity seen in patients with HADH mutations is caused by leucine. The biochemical data reveals that this is not due to an increase in GDH activity, and shows for the first time that in control human lymphoblasts a protein-protein interaction exists between HADH and GDH that is not seen in lymphoblasts with HADH mutations. This, in conjunction with the h-adh+/− mouse data [7] suggest that the protein interaction modifies GDH action in some way that allows dysregulated insulin secretion. Further research in this area is required to fully understand the way in which the interaction disrupts insulin secretion and how amino acid metabolism and fatty acid oxidation are linked by these two enzymes.

Abbreviations

HADH: 3-hydroxyacyl-CoA dehydrogenase; GDH: Glutamate dehydrogenase; HH: Hyperinsulaemic hypoglycaemia; HI/HA syndrome: Hyperinsulinism/hyperammonaemia syndrome.

Competing interests

The authors declare no competing financial interests.

Acknowledgements

The authors thank all patients and their family members for their participation in this study. This study was funded by the Wellcome Trust (081188/A/06/Z).

Author details

1The Institute of Child Health, University College London, London, WC1N 1EH, UK. 2London Centre for Paediatric Endocrinology and Metabolism, Hospital for Children NHS Trust, Great Ormond Street, London, WC1N 3JH, UK. 3Metabolic Biochemistry, Hôpital Necker-Enfants Malades, Université Paris Descartes, Paris, France. 4Dept of Endocrinology, Sela Efthal Education and Research Hospital, Istanbul, Turkey. 5Department of Paediatrics and Division of Pediatric Endocrinology Eskişehir, Osmangazi University School of Medicine, Eskişehir, Turkey. 6Institute of Biomedical and Clinical Science, Peninsula Medical School, University of Exeter Ex2 SDW, Exeter, UK. 7Developmental Endocrinology Research Group Molecular Genetics Unit Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH, UK.

Authors’ contributions

AJH wrote the paper and performed the experiments. RRK wrote the paper and performed clinical characterisation. SE reviewed and edited the manuscript, BC reviewed and edited the manuscript, TA contributed to clinical characterisation and reviewed the manuscript, ES contributed to clinical characterisation and reviewed the manuscript, SEF reviewed and edited the manuscript, SE reviewed and edited the manuscript, KH wrote the paper, contributed to the clinical characterisation and reviewed and edited the manuscript. AJH and RRK contributed equally to the manuscript. All authors read and approved the final manuscript.

Received: 23 January 2012 Accepted: 14 May 2012
Published: 14 May 2012

References

1. Agren A, Berg K, Brolin SE, Carlman J, Lundqvist G: Hydroxacyl CoA dehydrogenase, an enzyme important in fat metabolism in different cell types in the islets of Langerhans. Diabeta Metab 1977, 3:169–172.
2. Hardy GT, Hohmene HE, Becker TC, Manduchi E, Dolbta NM, Gupta RK, White P, Stoeckert CJ Jr, Matschinsky FM, Newgard CE, Kaestner KH: Functional genomics of the beta-cell: short-chain 3-hydroxyacyl-coenzyme A dehydrogenase regulates insulin secretion independent of K + currents. Mol Endocrinol 2007, 21:765–773.
3. Martens GA, Vervoort A, Van de CM, Stange G, Helleman K, Van Thi HV, Schult F, Pipeleers D: Specificity in beta cell expression of L-3-hydroxyacyl-CoA dehydrogenase, short chain, and potential role in down-regulating insulin release. J Biol Chem 2007, 282:21134–21144.
4. Kapoor RR, James C, Flanagan SE, Ellard S, Eaton S, Hutton K: 3-Hydroxyacyl-CoA dehydrogenase deficiency and hyperinsulinism-hyperammoniaemia: characterization of a novel mutation and severe dietary protein sensitivity. J Clin Endocrinol Metab 2009, 94:2221–2225.
5. Kelly A, Ng D, Ferry RJ Jr, Grimberg A, Koo-McCoy S, Thornton PS, Stanley CA: Acute insulin responses to leucine in children with the hyperinsulinism/hyperammonaemia syndrome. J Clin Endocrinol Metab 2001, 86(8):3724–3728.
6. Filling C, Keller B, Heschberg D, Marschall HI, Jornvall H, Bennett MJ, Oppermann U: Role of short-chain hydroxyacyl CoA dehydrogenases in SCHAD deficiency. Biochem Biophys Res Commun 2008, 368(1–2).
7. Li C, Chen P, Palladino A, Narayan S, Russell LK, Sayed S, Xiong G, Chen J, Stokes D, Burt YM, Jones PM, Collins HW, Cohen NA, Cohen AS, Nissim S, Smith TJ, Strauss AW, Matschinsky FM, Bennett MJ, Stanley CA: Mechanism of hyperinsulinism in short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency involves activation of glutamate dehydrogenase. J Biol Chem 2010, 285:31806–31818.
8. Clayton PT, Eaton S, Aysen-Green A, Edington M, Hussain K, Krywawaych S, Datta V, Malingre HE, Berger R, van den Berg IE: Hyperinsulinism in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency reveals the importance of beta-oxidation in insulin secretion. J Clin Invest 2001, 108:457–465.
9. Hussain K, Clayton PT, Krywawaych S, Chatziandreou I, Mills P, Gibney DW, Geboers AJ, Berger R, van den Berg IE, Eaton S: Hyperinsulinism of infancy associated with a novel splice site mutation in the SCHAD gene. J Pediatr 2005, 146:706–708.
10. Flanagan SE, Patch AM, Locke JM, Akcay T, Simsek E, Alaei M, Yekta Z, Desai M, Kapoor RR, Hussain K, Ellard S: Genome-Wide homozygosity analysis reveals HADH mutations as a common cause of diazoxide-responsive hyperinsulinemic-hypoglycemia in consanguineous pedigrees. J Clin Endocrinol Metab 2011, 96:E498–E502.
11. Wrzeszczynski KO, Colman RF: Activation of bovine liver glutamate dehydrogenase by covalent reaction of adenosine 5'-O-[S-(4-bromo-2,5-dideoxy-D-ribo-hexofuranosyl)] with arginine-459 at an ADP regulatory site. Biochemistry 1994, 33:11544–11553.
12. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72:248–254.
13. Yorifuji T, Murci J, Uematsu A, Hiramatsu H, Momoi T: Hyperinsulinism-hyperammonaemia syndrome caused by mutant protein.
dehydrogenase accompanied by novel enzyme kinetics. *Hum Genet* 1999, 104:476–479.

14. Stanley CA, Fang J, Kutyna K, Hsu BY, Ming JE, Glaser B, Poncz M: Molecular basis and characterization of the hyperinsulinism/hyperammonemia syndrome: predominance of mutations in exons 11 and 12 of the glutamate dehydrogenase gene. *Diabetes* 2000, 49:667–673.

15. Fahien LA, MacDonald MJ, Kmiotek EH, Metz RL, Fahien OM: Regulation of insulin release by factors that also modify glutamate dehydrogenase. *J Biol Chem* 1988, 263:13610–13614.

16. Hsu BY, Kelly A, Thornton PS, Greenberg CR, Dilling LA, Stanley CA: Protein-sensitive and fasting hypoglycemia in children with the hyperinsulinism/hyperammonemia syndrome. *J Pediatr* 2001, 138:383–389.

17. Treberg JR, Clow KA, Greene KA, Bronnn ME, Bronnn JT: Systemic activation of glutamate dehydrogenase increases renal ammoniagenesis: implications for the hyperinsulinism/hyperammonemia syndrome. *Am J Physiol Endocrinol Metab* 2010, 298:E1219–E1225.

18. Fourtner SH, Stanley CA, Kelly A: Protein-sensitive hypoglycemia without leucine sensitivity in hyperinsulinism caused by K(ATP) channel mutations. *J Pediatr* 2006, 149:47–52.

doi:10.1186/1750-1172-7-25
Cite this article as: Heslegrave et al.: Leucine-sensitive hyperinsulinaemic hypoglycaemia in patients with loss of function mutations in 3-Hydroxyacyl-CoA Dehydrogenase. *Orphanet Journal of Rare Diseases* 2012 7:25.