Endocrine and Metabolic studies in children with hypoglycaemia

by

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Abstract

Hypoglycaemia is one of the most common clinical problems seen in paediatric practice. Normoglycaemia is maintained by the interaction of various hormones such as insulin, glucagon, adrenaline, growth hormone (GH) and cortisol. GH and cortisol play important roles in the counter-regulatory hormonal response to hypoglycaemia. Although the GH and cortisol responses to hypoglycaemia induced by an Insulin Tolerance Test (ITT) are well defined there is very little literature on the GH and cortisol responses to spontaneous hypoglycaemia in childhood. Children and neonates referred with hypoglycaemia underwent a diagnostic fast. Serum GH and cortisol were measured before hypoglycaemia, at the time of hypoglycaemia and at ten-minute intervals for fifty minutes after the hypoglycaemia was corrected. The neonatal group included only babies with Hyperinsulinism of Infancy (HI). The serum GH and cortisol responses generated to spontaneous hypoglycaemia were compared with those in sixteen children undergoing the ITT. The results of this study showed that children with spontaneous hypoglycaemia generate inappropriately low serum GH responses at the time of hypoglycaemia which persists for fifty-minutes after hypoglycaemia. Serum GH levels were not influenced by physiological changes in serum Non-Esterified-Fatty-Acid or serum Ketone body concentrations. In contrast all children in the ITT group had appropriate serum GH responses at the time of hypoglycaemia. Serum cortisol responses were appropriate in children with spontaneous and ITT induced hypoglycaemia. Neonates with HI generated inappropriately poor serum cortisol responses. This was related to low serum Adreno-Corticotrophic Hormone (ACTH) levels. These neonates mounted a vigorous serum cortisol response when administered ACTH at the time of hypoglycaemia.
Acknowledgements

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I would like to thank Jane Pringle and Branca from the Cobbold Laboratories in the Middlesex Hospital for teaching me the techniques of radioimmunoassay and for helping me in the measurement of serum cortisol and growth hormone.

I am also grateful to the Camelia Botnar laboratory staff at Great Ormond Street Hospital for teaching me the techniques of measuring plasma glucose as well as the intermediary metabolites.

Most of all I want to thank all the children and their parents who consented to taking part in this research project, without whom it would not be possible to undertake this type of clinical project. Last but not least I want to express my sincere gratitude to my beloved parents who gave me the precious gift of education.
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<td>AcAc</td>
<td>Acetoacetate</td>
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<tr>
<td>ACTH</td>
<td>Adreno-Corticotropic Hormone</td>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CPT</td>
<td>Carnitine Palmitoyl Transferase</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotrophin Releasing Factor</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dihydroepiandrosterone</td>
</tr>
<tr>
<td>FAOD</td>
<td>Fatty Acid Oxidation Disorder</td>
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<tr>
<td>Fruc-1-6-Phos</td>
<td>Fructose-1-6-bisphosphatase</td>
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<td>GH</td>
<td>Growth Hormone</td>
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<td>GHRH</td>
<td>Growth Hormone Releasing Hormone</td>
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<td>Glu-6-Pase (G-6-P)</td>
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<td>GSD</td>
<td>Glycogen Storage Disease</td>
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<td>HI</td>
<td>Hyperinsulinism of Infancy</td>
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<tr>
<td>HMG-CoA</td>
<td>Hydroxymethyl-GLutaryl Coenzyme A</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Axis</td>
</tr>
<tr>
<td>IGF BP</td>
<td>Insulin Growth Factor Binding Proteins</td>
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<tr>
<td>IGF</td>
<td>Insulin Growth Factors</td>
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<tr>
<td>ITT</td>
<td>Insulin Tolerance Test</td>
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<td>KIR 6.2</td>
<td>Potassium Inward Rectifier 6.2</td>
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<tr>
<td>LHA</td>
<td>Lateral Hypothalamic Area</td>
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<td>NEFA</td>
<td>Non-Esterified Fatty Acids</td>
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PC............................................................... Pyruate Carboxylase
PEPCK...................................................... Phosphoenoylpyruvatecarboxykinase
PK...............................................................Pyruvate Kinase
SUR 1......................................................... Sulphonylurea Receptor 1
TCA........................................................... Tricitric acid cycle (Citric acid cycle)
VMA......................................................... Ventro-Medial Hypothalamic Area
3β-HOBH.................................................. 3Beta-Hydroxybutyrate
Preface

Hypoglycaemia is one of the most common metabolic and endocrine abnormalities observed in infancy and childhood. Despite the high prevalence of hypoglycaemia there is still controversy as to the definition of hypoglycaemia, as to the association between brain function and blood glucose concentrations and its management (Cornblath M et al 2000).

Hypoglycaemia results in a reduced supply of glucose to the vital organs such as the brain. The brain is critically dependent on a continuous supply of glucose (Siesjo BK. 1988). Recurrent and persistent episodes of hypoglycaemia can cause significant morbidity and mortality with the risk of sudden death or long term neurological damage (Siesko BK 1988). Specific treatment will depend on understanding the exact underlying cause of the hypoglycaemia.

The diagnosis of hypoglycaemia is best achieved by measuring the hormones and intermediary metabolites at the time of the presenting episode (Aynsley-Green A et al 2000). If this is not possible the child will then need to be subjected to a diagnostic fast in order to induce the stressful stimulus.

Great Ormond Street Hospital for Children is a tertiary referral Centre for the diagnosis and management of children with complex medical problems including hypoglycaemia. Children with hypoglycaemia are referred to either the Endocrine or Metabolic units.
The child then undergoes a complex set of investigations in order to establish the underlying etiology of the hypoglycaemia. Treatment is then initiated depending on the cause.

As a clinical research fellow, funded by the Nationwide Children's Fund, I was given the opportunity to investigate the complex physiological mechanisms regulating blood glucose concentrations in infants and children. A normal blood glucose concentration is maintained by a complex interaction between the hormone insulin and the counter-regulatory hormones including growth hormone (GH) and cortisol. These two hormones play an essential role in preventing hypoglycaemia and their levels in the blood are thought to increase at or before the time of hypoglycaemia (Bolli GB et al 1999). In the standard Insulin Tolerance Test (ITT) there is a brisk rise in the serum levels of both GH and cortisol at the time of hypoglycaemia (Hindmarsh P et al 1995). Very little is known about the response of these two hormones to spontaneous hypoglycaemia in childhood. Data from adults have been extrapolated to interpret serum GH and cortisol changes seen in childhood during hypoglycaemia. It has been observed that children with spontaneous hypoglycaemia have inappropriately low serum GH responses at the time of hypoglycaemia (Aynsley-Green A et al 1991), but the underlying mechanism/s of this has not been explored. Whether this is due to the pulsatile nature of GH secretion or some other factor is not clear. This finding of a low serum GH response to spontaneous hypoglycaemia is sometimes thought to be the underlying cause of the hypoglycaemia. Some of these children are then labelled as being GH deficient. These children are then subjected to potentially dangerous provocation tests such as the ITT or the Glucagon.
provocation test to stimulate growth hormone secretion from the anterior pituitary gland (Shah A et al 1992).

It has also been observed that infants with hyperinsulinaemic hypoglycaemia have poor cortisol counter-regulatory responses at the time of hypoglycaemia (Morris AAM et al 1996). Here again the underlying mechanism of the poor cortisol response is unclear. Whether this poor cortisol response is due to a lack of drive from the hypothalamic-pituitary axis or due to a poor adrenal response is also not clear. Based on the poor serum cortisol response to the hypoglycaemia these infants are given hydrocortisone replacement therapy. There are no data to suggest that the hydrocortisone replacement therapy benefits this group of children. There have been no previous studies monitoring the sequential changes in serum GH and cortisol secretion in response to spontaneous hypoglycaemia in infants and children. There have also been no studies in childhood or even in adults comparing the counter-regulatory responses of GH and cortisol to different forms of hypoglycaemia. Hence this study was designed to try and understand how serum GH and cortisol are secreted in response to hypoglycaemia in childhood under normal physiological conditions which prevail at the time of hypoglycaemia.

With reference to hyperinsulinaemic hypoglycaemia work related to this thesis has revealed several newly recognised syndromes and new protocols have been put in place for the management and diagnosis of children with Hyperinsulinism of Infancy.
Chapter 1

The physiology of blood glucose control and the roles of insulin, cortisol and GH.

1.0 Glucose metabolism.

1.0.1 Introduction.

Glucose is the most important metabolic substrate for normal functioning of the central nervous system, and although it is important at all ages, it is of special importance for the neonate and young child since a normal supply of glucose is essential for the normal neurological development. Conditions, which lead to hypoglycaemia, are therefore of clinical significance for the young child since hypoglycaemia is a potent cause of severe and permanent brain damage if the condition is not suspected and if there is delay in diagnosing the cause and in restoring normoglycaemia through effective management (Aynsley-Green A et al 1995).

The maintenance of a normal blood glucose concentration involves a complicated interaction between plasma glucose, insulin and the various counter-regulatory hormones including cortisol and GH. Glucose metabolism accounts for approximately half of the daily energy needs (Kelly D et al 1988). Glucose can be stored in the form of glycogen and fat and it can also be used for the synthesis of proteins and structural components such as cell membranes via the recycling of its carbon atom. A normal blood glucose concentration is maintained by a balance between glucose production and glucose utilization. Any factor which alters this equilibrium will lead to hypoglycaemia. Insulin decreases glucose production and increases glucose utilization, whereas both serum
cortisol and GH increase glucose production and decrease glucose utilization (Bolli GB et al 1999).

Figure 1.1 The apposing actions of insulin, cortisol and GH on glucose utilization and glucose production. 🥁 Inhibitory  🧡 Stimulatory.
1.0.2 Glucose production.

In addition to ingested glucose, glucose can be produced from three sources, from fat, from protein via gluconeogenesis and from the release of stored glycogen in the liver. The breakdown of stored glycogen from the liver results in a readily available source of glucose for the brain and other neural tissues, which are obligate glucose users.

The liver and the kidneys are the only two tissues that are able to release glucose into the circulation. Their ability to do this is dependent upon the fact that they have significant amounts of the enzyme glucose-6-phosphatase. The liver is able to provide glucose by two processes, glycogenolysis (breakdown of stored glycogen) and gluconeogenesis.

It is estimated that the liver glycogenolysis accounts for about 30-40% of overall hepatic output of glucose in adults (Rothman DL et al 1991). No such data are available for children.

Glycogenolysis occurs as a result of the actions of several enzymes. During fasting glycogen phosphorylase initiates glycogen breakdown by cleaving glucose-1-phosphate, which is converted to glucose-6-phosphate by a debranching enzyme. Glucose-6-phosphatase then converts glucose-6-phosphate to free glucose.

The metabolism of glycogen is predominately controlled by the activities of glycogen synthase and phosphorylase. From the hormonal point of view insulin and glucagon are the major factors controlling the activities of these two enzymes.

Hepatic gluconeogenesis involves the synthesis of glucose from non-carbohydrate sources. The major gluconeogenic precursors after an overnight fast are lactate and
alanine. The majority of the lactate and alanine generated after an overnight fast originate from plasma glucose and represent recycling of carbon atoms. The first reaction in gluconeogenesis involves the conversion of pyruvate to oxaloacetate to phosphoenolpyruvate. The second of these reactions converts fructose-1, 6-biphosphate to fructose-6-biphosphate. This is the rate-limiting step for the process of gluconeogenesis. The final step involves the conversion of glucose-6-phosphate to free glucose.

Sunehag (Sunehag AL et al 2001) have shown that the rate of gluconeogenesis, on a body weight basis, is greater in children between the ages of 8 and 9 years than in adolescents between the ages of 14 and 16 years, whereas the fraction of glucose production derived from gluconeogenesis was essentially identical between the two groups of subjects. The same study showed that gluconeogenesis contributed to 50% of glucose production in the childhood period.

1.0.3 Glucose utilization.

The factors which determine glucose utilisation by the tissues include the plasma glucose concentration, the tissue requirement for glucose, the availability of alternative substrates and in certain tissues the sensitivity of the tissues to insulin. Glucose uptake by tissues occurs by facilitated diffusion. The transport of glucose into tissues will depend on the presence of specific glucose transporters, of which five have been identified, GLUT 1 to GLUT5. GLUT 1 is an insulin-independent transporter found in all cells (Thorens B, 1996). GLUT 1 is responsible for glucose transport across the blood brain barrier.
GLUT 2 is mainly present in liver and pancreatic β-cells (Bell GI et al 1990). The function of GLUT 2 is insulin-independent, being a low affinity transporter that is not easily saturated at even high plasma glucose concentrations. Tissues that use GLUT 2 as glucose transporter experience a rise in cellular glucose with increases in plasma glucose, which allows the pancreatic β cells and hepatocytes to act as glucose sensors (Matschinsky FM et al 1996).

GLUT 3 is distributed in the central nervous system and is an insulin independent glucose transporter that has the highest affinity for glucose (Thorens B, 1996). GLUT 4 is an insulin-dependent transporter in muscle and adipose tissue. GLUT 5 is primarily expressed in the jejunal brush border and is mainly a fructose transporter (Thorens B, 1996).

Insulin regulates the steady state concentration of these insulin-dependent transporters by promoting their synthesis but also causes mobilisation of these transporters to the cell membrane when the plasma glucose concentration increases (Thorens B, 1996).

Glucose taken up by cells has three potential fates, storage as glycogen or fat, oxidation to carbon dioxide and conversion to lactate. The percentage of glucose, which contributes to these different fates, will depend upon the degree of fasting, the hormonal milieu and the presence of alternative energy substrates (Kelley D et al 1988). Figure 1.2 gives a simplified overview of glucose metabolism. The key enzymes involved in controlling glucose metabolism and the intermediary metabolites are also shown.
Figure 1.2 Outline of glucose metabolism

- Glucose
- Glucose-6-phosphate
  - Glucokinase
  - Phosphorylase
- Glucose-1-phosphate
  - Glucose-6-phosphatase
  - Glycogen synthase
- Fructose-6-phosphate
  - Phosphofructokinase
  - Fructose 1,6 bisphosphatase
- Fructose 1,6 bisphosphate
- Glyceraldehyde 3-phosphate
- Phosphoenolpyruvate
  - Phosphoenolpyruvate carboxykinase
  - Pyruvate carboxykinase
- Pyruvate
- Oxaloacetate
- Citrate
- α-ketoglutarate
- Succinyl-CoA
- Malate
- Fumarate
- Citric acid cycle

Mitochondria
1.1 The synthesis, secretion and physiological actions of insulin.

1.1.0 Introduction.

Insulin has numerous actions on the regulation of body metabolism. These actions of insulin are generally geared towards anabolism. Thus insulin promotes the storage of fuels in the form of glycogen and triglycerides and stimulates the synthesis of proteins while inhibiting their catabolism.

Insulin is a small globular protein with a molecular weight of 5.3kDa, consisting of two peptide chains, designated as A and B linked together by disulphide bridges. It is synthesised by the β-cells of the islets of the Langerhans as a larger precursor molecule, pre-proinsulin, which undergoes post-translational processing during its transit through the secretory pathway. Pre-proinsulin contains an NH2-terminal 24-amino acid hydrophobic signal peptide. The signal peptide is removed in the endoplasmic reticulum to generate proinsulin, in which the insulin B chain and the A chain are linked by a connecting peptide, C-peptide. Insulin is a member of the superfamily of structurally related peptides, which include the insulin like growth factors I and II (IGF-I and IGF-II) (Steiner DF et al 1985).

1.1.1 Insulin gene and biosynthesis of insulin.

In humans the gene encoding pre-proinsulin, the ultimate precursor of insulin in located on the short arm of chromosome 11, close to the gene encoding the Insulin-like Growth factor II (IGF II). The insulin gene is highly conserved amongst species and consists of three exons and two introns (Bell GI et al 1980). Exon 1 is at the 5' untranslated region of the gene. Exon 2 contains sequences encoding the signal peptide, the insulin B chain,
and part of the C-peptide. Exon 3 encodes the reminder of the C-peptide, the A chain, and the 3' untranslated sequences. Transcription and splicing to remove the sequences encoded by the introns yields a messenger RNA of 600 nucleotides, translation of which gives rise to preproinsulin, an 11.5-Kda polypeptide. This is then rapidly (within a minute) discharged into the cisternal space of the rough endoplasmic reticulum, where proteolytic enzymes immediately cleave it into proinsulin, then removing the signal peptides. Proinsulin is a 9kDa peptide containing A and B chains of insulin joined by the C-peptide. Proinsulin is then transported by microvesicles to the Golgi apparatus where it is packaged in vesicles that are enclosed by the membrane containing an ATP-dependent proton pump.

1.1.2 Insulin secretion.

Insulin and C-peptide are stored together in the granule sac and are ultimately released in equimolar amounts. Under normal conditions 95% of the hormone is secreted as insulin and less than 5% as unconverted proinsulin (Bell GI 1980). Insulin, together with the C-peptide is released from the pancreatic β-cell by exocytosis. The insulin containing granules first move close to the cell membrane and then fuse together releasing the granule contents (Gerber SH et al 2002). Following the incorporation of the granule membrane the newly expanded plasma membrane is partly reabsorbed into the cell by endocytosis and recycled back to the Golgi complex to be used again (Gerber SH et al 2002).
The whole process of exocytosis is extremely complicated. Multiple components are involved in the late stages of the stimulus-secretion coupling and exocytosis. The secretary granules express several different surface proteins including synpasin 1, synaptophysin, VAMP (vesicle associated membrane protein), synaptobrevin and synaptotagmin (Shepherd RM et al 2000). In order for these proteins to release the contents of their granules they have to be able to recognise their counterparts located on the surface of the plasma membrane. The proteins on the plasma membrane include syntaxin and SNAP-25. For docking to occur all these proteins must form a complex (Gerber SH et al 2002).

The secretion of insulin is controlled by numerous signals including those from nutrients (Prentki M 1987), hormones (Porte D et al 1970) and the autonomic nervous system (Ahren B 2000). Each pancreatic β-cell functions as a miniature fuel sensor, integrating all the various signals, which stimulate insulin secretion, and responding appropriately (Prentki M 1996). The pancreatic β-cell possesses a unique signal transduction system, which links the metabolism of the fuel stimulus to initiate secretion (Malaisse WJ 1979), so called stimulus-response coupling. Glucose is the most important fuel involved in the stimulus-response coupling mechanism. This stimulus-response coupling event is controlled by potassium channels located in the pancreatic β-cell membrane (K\textsubscript{ATP}) which are sensitive to intracellular nucleotides, in particular the ratio between ATP and ADP (Dunne MJ 1991). As the intracellular glucose concentration increases, pancreatic β-cell glycolysis increases the ratio of ATP to ADP. This closes the K\textsubscript{ATP} sensitive channel resulting in depolarisation of the pancreatic β-cell membrane. This phenomenon leads to
the influx of calcium through voltage-gated calcium channels, which triggers exocytosis (Dunne MJ 1991). Thus, the K\textsubscript{ATP} channel functions as a "on off" switch for triggering insulin secretion.

Although K\textsubscript{ATP} channels have an essential role in linking the metabolism of glucose to the secretion of insulin, there is now evidence that there may well be other mechanisms of insulin secretion, the so-called K\textsubscript{ATP} channel independent pathways of insulin secretion (Aizawa T et al 1994, Gembal M et al 1993). This pathway leads to the augmented insulin release in the presence of raised cytosolic calcium concentrations. Increases in the intracellular Ca\textsuperscript{2+} concentration in the pancreatic \(\beta\)-cell cause modest increases in insulin secretion, which can be dramatically increased by modulators of protein kinases and phosphatases (Ammala C et al 1994). This suggests that steps distal to the elevation of Ca\textsuperscript{2+} are of greater quantitative importance in controlling insulin secretion (Ammala C et al 1994). It has also been shown that glucose can cause pronounced insulin secretion in Ca\textsuperscript{2+} depleted islets in the presence of activators of protein kinases A and C (Yajima H et al 1999).

Glucose also stimulates the production of new insulin molecules by the translation of preformed mRNA encoding insulin (within minutes) and over longer periods by increasing the transcription of the insulin gene (Melloul D et al 1993). The Homeodomain transcription factor PDX1 plays an essential role in linking the cytosolic events to nuclear signalling (Melloul D et al 1993). Glucose metabolism causes phosphorylation of PDX1 and translocation of this transcription factor into the nucleus.
In addition to binding to the promoter sequences of the insulin gene PDX1 also binds to the coding sequences of genes, which are specifically expressed in the pancreatic β-cell such as GLUT2 and Glucokinase (Shepherd RM et al 2000). Figure 3 is a schematic model showing how the metabolism of glucose is linked to regulation of insulin secretion.

Insulin is released in two phases in response to an intravenous bolus of glucose (Gerich JE et al 1976). The first phase is rapid and plasma levels of insulin peak at 3-5 minutes. The second phase of insulin secretion persists as long as the elevation of plasma glucose.

The insulin response to an oral glucose load is always greater than that due to an intravenous glucose load (Fehmann HC et al 1995). This is related to the concept that gut factors modulate insulin secretion and a variety of gut hormones participate in promoting insulin secretion (Fehmann HC et al 1995, Ahren B 2000).
Figure 1.3. Glucose stimulus secretion coupling. The metabolism of glucose is intricately linked to insulin secretion. The metabolism of glucose causes changes in the intra-cellular ratio of ATP/ADP which bind to the $K_{ATP}$ channels causing depolarization. This allows the entry of calcium which triggers the exocytosis of insulin. The transcription factor PDX1 translocates to the nucleus to increase transcription of the insulin gene. The metabolism of glucose is also thought to be important for the “augmentation” pathway of insulin secretion which is $K_{ATP}$ channel independent.
1.1.3 Insulin actions on metabolism.

Insulin has numerous effects on metabolism, which are geared towards anabolism. The major effects of insulin on metabolism can be broadly divided into:

a) Metabolism of fat - Triglycerides are a major form of stored fuel in the human body, and are a more efficient depot of energy than glycogen (Cahill GF et al 1976). Triglycerides are essentially composed of glycerol and fatty acids. The enzyme hormone sensitive lipase plays an essential role in lipolysis and release of free fatty acids from stored adipose tissue. Insulin is inhibitory to hormone sensitive lipase (Carey GB et al 1999), hence in the presence of insulin lipolysis is inhibited. In the absence of insulin, hormone sensitive lipase hydrolysis triglycerides into glycerol and free fatty acids. During this process for every one molecule of glycerol released three molecules of free fatty acids are released. Glycerol diffuses out of the adipose tissue into the circulation and is transported into the liver to be incorporated into the Embden-Mayerhof pathway as a gluconeogenic source or to be used for the resynthesis of fat. The liberated free fatty acids are transported to the liver bound to albumin, where they can either undergo beta-oxidation in the mitochondria or be re-esterified to triacylglycerols and phospholipids. The process of beta-oxidation yields acetyl-Co which can then be converted to ketone bodies (acetoacetate and 3 beta-hydroxybutyrate) via the hydroxymethylglutaryl-CoA (HMG-CoA) pathway or it can undergo complete oxidation in the tricarboxylic acid cycle. Figure 1.4 shows the metabolic pathways linking carbohydrate, fat and protein metabolism.
Figure 1.4 The integration of glucose metabolism with carbohydrate and fat metabolism.

Key: CT = carnitine translocase, CPT = carnitine palmitoyl transferase, CoQ = Coenzyme Q, OAA = Oxaloacetate, TCA = Tricyclic acid cycle, ETF = Electron Transport Chain, LCAD, MCAD, SCAD = Long, medium and short chain acyl-CoA enzymes.
Among the many metabolic and hormonal factors involved in controlling ketone body production insulin and glucagon are considered to be very important (Johnston DG et al 1982). Glucagon is thought to activate ketogenesis by acting on the liver only whereas insulin inhibits ketogenesis by reducing the supply of free fatty acids but also may have an independent action on the liver (Witters LA et al 1979). Basal glucagon levels are thought not to affect ketone body production to any significant extent, but in states of insulin deficiency glucagon exerts a marked stimulatory effect (Keller U et al 1977).

b) Carbohydrate metabolism- the major pathways for glucose metabolism include storage of glucose in the form of glycogen, metabolism through the glycolytic pathway and through the pentose cycle. Insulin plays a critical role in regulating all these pathways. The first step in the synthesis of glycogen from glucose involves the phosphorylation of glucose to glucose-6-phosphate. This is carried out by the enzyme glucokinase. This is then first converted to glucose-1-phosphate and then to UDP-glucose. The final step in the pathway of glycogen synthesis involves the enzyme glycogen synthase. This last reaction is thought to be the rate-limiting step. In the reverse direction glycogen undergoes phosphorylation to glucose-6-phosphate this step being catalysed by glycogen phosphorylase. The enzyme glucose-6-phosphatase is only present in the liver and kidney; hence these two tissues are the only ones able to release stored glycogen. Insulin activates the enzyme glycogen synthase allowing the synthesis of glycogen (Larner J 1988) and inhibits glycogen breakdown by inactivating the enzyme glycogen phosphorylase (Cohen P 1985).
In the glycolytic pathway glucose is metabolised to pyruvate, with ATP being generated as a by-product. Once glucose enters a cell it is converted into glucose-6-phosphate. This can either be converted into glycogen or rapidly converted into fructose-6-phosphate. The next step involves the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate the reaction being catalysed by phosphofructokinase. The activity of this enzyme is induced by insulin (Pilkis SJ et al 1992). Fructose-1, 6-bisphosphate is then converted through a sequence of steps to eventually yield pyruvate.

The reverse reactions of glycolysis are what happens in gluconeogenesis. The only exception to this is the three reactions catalysed by the enzymes hexokinase, phosphofructokinase and pyruvate kinase. In order to circumvent this and to have a continuous supply of gluconeogenic precursors there are additional enzymes, which allow a net flux along the gluconeogenic pathway (Pilkis SJ et al 1985).

The pentose cycle is a side pathway that leads to the generation of NADPH as a consequence of oxidative decarboxylation of glucose-6-phosphate. Insulin increases flux through this cycle.

c) Protein metabolism-Amino acid transport into the cell is stimulated by insulin as well as assembly of amino acids into proteins (Kimball SR et al 1988). The major amino acids released into the circulation for gluconeogenesis are alanine and glutamine. Amino acids enter the energy pathway through the citric acid cycle or by conversion to pyruvate.

**Figure 1.5** opposite summarises the main actions of insulin on carbohydrate, fat and protein metabolism.
1.1.4 Integration of the physiological changes associated with feeding and fasting.

Insulin plays a major role in regulating glucose production and utilisation during both the fed and fasted state. Following the ingestion of a meal the plasma glucose concentration starts to increase within 15 minutes (Mitrakou A et al 1990). This increase in the plasma glucose level and the stimuli from neurogenic and entero-insular axis stimulates insulin secretion from the pancreatic β-cells. Peak levels of plasma glucose are reached around 30-60 minutes following ingestion after which it starts to decrease until absorption is complete usually about 4-5 hours later, with plasma insulin concentrations following a similar time course (Mitrakou A 1990). Following the ingestion of a meal there is a marked suppression of endogenous glucose production from the liver, the magnitude of which is largely determined by the insulin and glucagon responses (Mitrakou A et al 1992). Endogenous glucose production may be suppressed up to 50-60% with about 25 grams less glucose being delivered into the systemic circulation (Firth RG et al 1986, Kelly D et al 1988).

Postprandially plasma glucose levels are determined by balance between the rates of glucose removal from the systemic circulation and the rate of delivery of glucose into the systemic circulation. Also postprandially the processes of lipolysis, ketogenesis, glycogenolysis and gluconeogenesis are all suppressed. The tissues mainly responsible for the removal of glucose from the systemic circulation include the liver, small intestine, brain, muscle and adipose tissue (Martin P et al 1987). The magnitude of glucose uptake by the tissues, except for the brain, is largely determined by the plasma insulin
concentration. Glucose uptake by the brain is determined by the plasma glucose concentration and is independent of the plasma insulin concentration. The physiological changes in glucose metabolism during the fed and fasted state are shown in figure 1.6.

The 4-6 hours interval following the ingestion of a meal is sometimes referred to as the post absorptive state. During this interval a steady state is reached whereby glucose production is equal to the rate of glucose consumption and plasma glucose concentrations are maintained within a normal range. During this state it is estimated that glucose turnover (glucose production and utilisation) is approximately 10micromol/kg/min (Bolli G et al 1984). In this state non-insulin dependent utilisation of glucose accounts for 80%, mainly by the brain (which accounts for 50% of the total), red blood cells, kidneys and the gastro-intestinal system (Kelly G et al 1988). During this phase glucose concentrations are maintained by interactions between insulin and the various counter-regulatory hormones including glucagon, cortisol, growth hormone, adrenaline and nor-adrenaline. Glucagon allows the controlled release of stored glycogen from the liver and insulin restrains the effects of glucagon by preventing accelerated lipolysis and proteolysis. The counter-regulatory hormones including cortisol and GH play permissive roles in setting the sensitivity of the peripheral tissues to glucagon and insulin.
Figure 1.6 The integration of the physiological changes associated with feeding and fasting. The liver plays a central role in regulating glucose metabolism during the fed and fasted state. Lactate and alanine provide important substrates for gluconeogenesis. Gluconeogenesis principally takes place in the liver.
As the period of the fast is lengthened utilisation of glucose by the tissues decreases while utilisation of free fatty acids and ketone bodies increases (Harvel R 1972). There is a reduction in hepatic glucose output, which is accounted for mainly by a decrease in glycogenolysis, with an increase in the rate of gluconeogenesis (Consoli A et al 1987). The increased gluconeogenesis is thought to be related to the increased secretion of glucagon and other counter-regulatory hormones, as well as the reduction in insulin secretion. The increased glucagon secretion associated with a reduced insulin secretion allows stored fats to be converted to glycerol and fatty acids and proteins to be converted to amino acids for gluconeogenesis. The liberated free fatty acids are transported to the liver bound to albumin, where they can either undergo beta-oxidation in the mitochondria or be re-esterified to triacylglycerols and phospholipids. The process of beta-oxidation yields acetyl-Co which can then be converted to ketone bodies AcAc and 3β-HOBH via the hydroxymethylglutaryl-CoA (HMG-CoA) pathway or it can undergo complete oxidation in the tricarboxylic acid cycle.

Muscle and other tissues become progressively more dependent on free fatty acids and ketone bodies (Harvel R 1972) for their continued energy requirements as the period of the fast is prolonged. Ketone bodies are produced in the liver, mainly from the oxidation of fatty acids, and are exported to peripheral tissues for use as an energy source. They are particularly important for the brain, which has no other substantial non-glucose-derived energy source. Ketone bodies replace glucose as the predominant fuel for nervous tissue, thereby reducing the obligatory requirement of the brain (Patel MS et al 1975).
During the period of a fast there is a complex interaction of metabolic and hormonal mechanisms, which produces significant variations in the concentrations of various hormones and intermediary metabolites. Children differ in their response to fasting in comparison to adults (Raymond MW et al 1982). For example, studies in adults have shown that the levels of free fatty acids, glycerol, and ketones in the blood gradually increase as the period of starvation is extended (Cahill GF 1976). During a brief period of fasting in the childhood period ketosis and ketonuria develop readily, suggesting that children convert more rapidly to a fuel economy based largely on fat (Mitchell GA et al 1995).

1.2 The roles of Growth Hormone and Cortisol in blood glucose regulation.

1.2.1 Introduction.

GH and cortisol play an essential role in the regulation of normal blood glucose concentration. Both of these hormones counter-act the actions of insulin on glucose metabolism (Gerich JE et al 1980). Both hormones reduce the peripheral utilisation of glucose as well as increasing the rates of gluconeogenesis (Gerich JE et al 1980). The secretion of both of these hormones is controlled by different physiological mechanisms.

1.2.2 Developmental biology of the adrenal gland and physiology of cortisol secretion.

The primordium of the fetal adrenal gland can first be recognised just cephalad of the developing mesonephros at 4 weeks of gestation. By about 8 weeks an inner foetal zone of cells becomes surrounded by a sub capsular rim of immature cells referred to as the outer or definitive zone. The foetal gland grows rapidly and progressively in mass and at
term it is estimated that the foetal zone occupies about 80% of the mass of the gland (Johannison E et al 1968). At birth there is rapid involution of the foetal zone with each gland losing approximately 25% of its mass within the first 4 days (Winter JDS 1992). Associated with these morphological changes there are also physiological changes in the pattern of steroid biosynthesis during the foetal and neonatal periods.

During fetal development hormone activity can first be detected at about twelve weeks of gestation (Mesiano S et al 1997). Corticotrophin Releasing Factor (CRF) is produced from the foetal hypothalamus. This controls the production of adrenocorticotropic hormone (ACTH) from corticotrophs in the anterior pituitary gland. CRF regulates the growth of the corticotrophs, adrenocortical differentiation and steroidogenic maturation of the fetal hypothalamic-pituitary axis (Mesiano S et al 1997).

There are differences in the relative activities of the different steroidogenic enzymes in the foetal adrenal gland accounting for the pattern of steroids produced during this period. The steroidogenically active fetal zone expresses abundant amounts of P450<sub>sc</sub> and P450<sub>c17</sub> but no 3β-HSD mRNA or protein (Winter JDS 1992), hence its major secretion products are dehydroepiandrosterone (DHEA) and DHEA-S (sulphate). These are biologically inactive products and can be converted by the placenta to oestrogens.

The major stimulus for the functioning of the fetal adrenal gland appears to be the drive from the foetal pituitary ACTH, although other factors specific to the intra-uterine environment may also play a role (Mesiano S et al 1997).
Serum cortisol concentrations at thirteen weeks of gestation are about 20nmol/L, rising to 150nmol/L at term, with even higher values after spontaneous delivery (Winter JDS 1992). These concentrations represent a net input from both the foetal and maternal sources. At birth there is a rapid involution of the foetal adrenal zone with increased activity of the 3β-HSD with a corresponding increase in the capacity to secrete cortisol. This is reflected in a reduction in the plasma and urine concentrations of DHEA-S.

Cortisol and aldosterone are the two most active hormones secreted by the adrenal gland. Cortisol is synthesised from cholesterol in the zona fasciculata of the adrenal gland. Cholesterol is firstly converted to pregnenolone, which is then converted by a sequence of enzymatic steps to cortisol. These enzymatic steps are all controlled by ACTH. Cortisol secretion from the adrenal gland is regulated by ACTH (figure 1.7 shows the mechanisms involved in regulating serum cortisol secretion). This is cleaved from a larger precursor protein (pro-opiomelanocortin, POMC). The release of ACTH from the anterior pituitary is regulated by CRF from the hypothalamus. The factors that affect CRF release from the hypothalamus include stress, trauma, and drugs. CRF is transmitted by the hypophyseal portal vessels to the adenohypophysis evoking the release of ACTH. ACTH is secreted in regular pulses of variable amplitude over 24 hours with most secretion occurring during the night. This forms the basis for the circadian rhythm for cortisol secretion (Wallace WHB et al 1991). The circadian rhythm of cortisol secretion is not established until about three months of age (Economou G et al 1993). Cortisol feeds back at the level of the pituitary, with high levels inhibiting further ACTH
secretion. There is evidence that ACTH may itself exert a negative feedback at the level of the pituitary on its own secretion (Beckford U et al 1983).

Figure 1.7 The regulation of serum cortisol secretion by factors such as stress acting on the level of the hypothalamus. Serum cortisol regulates its own secretion by a negative feedback.
1.2.3 The physiology of GH secretion.

The fetal pituitary starts synthesizing and secreting GH from eight to ten weeks gestation (Silverman BL et al 1989). GH concentrations measured in cord blood are 1 to 4 mU/L during the first trimester and progressively increase to a mean peak value of 6 mU/L at midgestation. At term plasma GH levels decrease to between 2 to 5mU/L (Kaplan SL et al 1972). This pattern of ontogenesis of plasma GH reflects the progressive maturation of the hypothalamic-pituitary axis.

GH is produced and secreted by specialised cells in the anterior pituitary called the somatotrophs. GH is secreted in a pulsatile fashion with a half-life of approximately ten minutes (Faria AC et al 1989). The secretion of GH from the pituitary somatotrophs is controlled by a complex interaction of the two neuropeptides, GHRH (growth hormone releasing hormone) and SS (somatostatin). GHRH stimulates GH release as well as increasing the rate of GH synthesis. SS inhibits GH release from the somatotrophs. It is thought that pulses of GHRH are secreted into the hypophysial portal blood during troughs of SS release (Plotsky POM et al 1985) but the main determinant of pulsatile GH release is the GHRH pulse occurring at the times of a nadir in the SS release (Frohman LA et al 1990). Apart from these two neuropeptides influencing GH secretion other factors also influence GH secretion. Amongst these glucose is an important regulator of GH secretion. Changes in the level of blood glucose are thought to act via hypothalamic mechanisms with no evidence of any direct pituitary stimulation (Page MD et al 1987, Berelowitz M et al 1982). GH secretion is also influenced by pharmacological changes in other intermediate metabolites such as Non-Esterified Fatty Acids (NEFA) which
when elevated suppresses GH release (Casanueva FF et al 1987, Alvarez C et al 1991). The exact mechanism by which changes in the plasma NEFA concentration influence GH secretion is not clear.

GH released from the anterior pituitary stimulates the production of Insulin Growth Factors (IGFs) from the liver. Of these Insulin Growth Factors, IGF-I is probably the most important. It exhibits a high sequence homology to proinsulin. IGF-I plays an integral role in regulating GH secretion as it feeds back at the level of the pituitary to control GH secretion. IGF-I is thought to be an important signal for many cell types integrating information on both GH secretion and nutritional status. Only about 1% of circulating IGF-I is free whilst the rest is bound to specific binding proteins (IGFPB). IGFPB-I has an inverse relation to food intake and plasma insulin levels. IGFPB-I levels increase during the period of a fast and binds more circulating IGF-I thus reducing the effects of IGF-I at the insulin receptor and preventing hypoglycaemia.

Another newly discovered natural endogenous peptide, which controls GH secretion, is Ghrelin (Takaya K et al 2000). Ghrelin is synthesized in the human stomach and stimulates GH release from the pituitary cells in a dose-dependent manner (Takaya K et al 2000). Ghrelin levels increase following fasting and decrease post-prandially (Ariyasu H et al 2001).

The neonatal period is characterised by a physiological hypersecretion of GH. The exact reason for this is not clear but thought to be related to several factors. The somatotrophs
may have a higher sensitivity to GHRH than somatostatin, there may be an amplifying effect of GH secretion caused by oestrogens of placental origin or by a neonatal surge in thyroid hormones, and the fall in the serum levels of IGF-I within hours of birth will remove the inhibitory effect on GH secretion (Boguszewski CL et al 2000). Human GH is a complex mixture of different molecular isoforms, the most abundant form is the 22 kDa GH with other monomers such as 20kDa, 27kDa, 17kDa and 5kDa also being found in the circulation and pituitary extracts (Lewis UJ et al 1994). The 20 kDa is a variant produced from an alternatively spliced mRNA, which lacks amino acids 32-46 of the 22kDa GH molecule. It comprises 5-10% of the pituitary GH and its physiological role is not known (Lewis UJ et al 1978). Neonates produce the same fraction of the 20kDa GH as adults (Boguszewski CL et al 2000).

1.2.4 The actions of cortisol and growth hormone on glucose metabolism.

Both cortisol and GH have numerous effects on glucose metabolism. These two hormones play crucial roles in the maintenance of a normal blood glucose concentration. Deficiency of GH has been recognised as a cause of hypoglycaemia for many years (Brasel JA et al 1965), and hypoglycaemia due to ACTH deficiency and cortisol deficiency has also been reported (Hochberg Z et al 1985 + Hinde FRJ et al 1984). Hypoglycaemia may also occur due to malformations of the hypothalamic area in which case it is more likely to be due to a combination of multiple pituitary hormone deficiencies, associated with microphallus and hypogonadism.

At the time of spontaneous hypoglycaemia cortisol levels are raised if the cause of the hypoglycaemia is not due to cortisol deficiency, whereas the levels of GH do not
correlate with the secretary reserve measured after pharmacological provocation tests (Aynsley-Green A et al 1991). It is not known if this is related to the time of collection of the blood sample or to the pulsatile nature of growth hormone release.

GH antagonises the actions of insulin, with insulin insensitivity to peripheral tissues developing within 2 to 12 hours after a transient increase (Bratusch-Marrain P et al 1982). The impairment of glucose metabolism is caused by a post-receptor defect in insulin action, whereas insulin binding to its receptor is only slightly diminished after prolonged exposure to GH (Bratusch-Marrain P et al 1982). GH decreases insulin stimulated glucose utilisation, increases hepatic glucose production, stimulates lipolysis and stimulates protein catabolism. Children with GH deficiency are more sensitive to insulin than those with sufficient GH concentrations and this difference is attenuated with age and puberty, possibly secondary to pubertal sex steroids (Husbands S et al 2001). Figure 1.8 shows the apposing actions of GH, cortisol and insulin on glucose metabolism.

Cortisol also antagonises the actions of insulin. The anti-insulin effect of cortisol is also thought to be mediated at the post-receptor level (Rizza RA et al 1982). There is evidence that glucocorticoids induce a rapid and persistent reduction of insulin to its receptor, indicating that receptor alterations may also be occurring (DePirro R et al 1980). Cortisol as with GH decreases insulin induced peripheral utilisation of glucose, increases hepatic glucose production and stimulates protein breakdown. This has the effect of providing amino acids for gluconeogenesis. In adult humans it has been shown
that cortisol administration increases glucose production by stimulating gluconeogenesis (Khani S et al 2001).

The effects of insulin, GH and cortisol on glucose metabolism

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Blood glucose

Figure 1.8 summarising the actions of cortisol and GH on glucose metabolism.

Normoglycaemia is maintained by an interaction between insulin and the counter-regulatory hormones. Cortisol and GH have opposite effects on glucose metabolism in relation to insulin.
Chapter 2

Causes of hypoglycaemia in childhood and the serum GH and cortisol response to stress.

2.0 Hypoglycaemia in childhood.

2.0.1 Definition of hypoglycaemia.

The definition of hypoglycemia especially in the newborns is confusing and controversial (Comblath M et al 2001). This is because there is poor correlation between plasma glucose concentrations, the onset of clinical symptoms, and the long-term neurological sequelae. It is difficult to define a blood glucose level that will require intervention (especially in neonates) since there is uncertainty over the level and duration of hypoglycaemia that can cause neurological damage. Four different approaches have been used to define hypoglycaemia (Comblath M et al 2001). Firstly hypoglycaemia may be defined statistically with the disadvantage that blood glucose levels below a certain derived centile are not always associated with the presence or absence of symptoms. Secondly hypoglycaemia may be defined by a functional definition which relates hypoglycaemia to evidence of concurrent physiological counter-regulatory hormone responses or to evidence of neurological dysfunction. In this case there is very little data on the counter-regulatory hormonal responses to specific blood glucose levels in different subjects. Thirdly hypoglycaemia may be defined by symptoms in relation to a specific blood glucose level. The disadvantage here is that there is very poor correlation between symptoms and blood glucose concentrations. Lastly hypoglycaemia may be defined in relation to adverse neurological outcome, in the context of previous experiences of
hypoglycaemia. However the predictive value of neurodevelopmental tests is not well established. Hence for these reasons none of these definitions are satisfactory and all have been misinterpreted at some stage. The current accepted definition is based on the work of Koh et al (Koh TH et al 1988) who measured sensory evoked potentials in relation to blood glucose concentrations and showed that abnormal evoked potentials were recorded from children whose blood glucose concentration fell below 2.6 mmol/l.

2.0.2 Importance of diagnosis.

Hypoglycaemia is of particular importance in childhood since it is a potent cause of neurological damage when it is persistent or recurrent (Aynsley-Green A et al 1985). Data from Lucas et al suggest that even mild hypoglycaemia, at levels which were previously thought to be innocuous may in fact be associated with serious long-term effects in preterm infants (Lucas A et al 1988). Hence the diagnosis and treatment of hypoglycaemia is extremely important.

Hypoglycaemia can be due to many causes in the neonatal and childhood period. These are summarized in Table 2.1 at the end of this chapter. It is important when confronted with a child with hypoglycaemia to obtain a blood sample for the detailed investigations prior to giving enteral feeds or intravenous glucose. The blood sample taken at the time of hypoglycaemia will allow one to decipher the defect in the many metabolic and endocrine pathways involved in the aetiology of the hypoglycaemia. A urine sample should also be saved at the time of the hypoglycaemia.
When interpreting a blood glucose result, the method of collection of the blood sample will be important. Whole blood glucose values are about 15% less compared to those in the serum and plasma. On the other hand venous blood glucose concentrations are 10% lower than arterial. For measurement of blood glucose blood samples are collected into fluoride containing tubes to inhibit glycolysis.

2.0.3 Glucose and the brain.

The brain is critically dependent on a continuous supply of glucose (Cryer PE et al 1991). The entry of glucose into the brain is not regulated by insulin. Certain parts of the brain seem more susceptible to hypoglycaemia than others. The characteristic ultrastructural appearance is that of damage to the dendrites with relative sparing of the axons. In adult rats subjected to hypoglycaemia there is neural necrosis, which is different in distribution and appearance to that caused by ischaemia (Auer RN et al 1993). The study by Auer also showed that the areas of the brain which are damaged by severe and prolonged hypoglycaemia are those that may be exposed to toxic neurotransmitters such as glutamate and aspartate. Severe hypoglycaemia in animals causes a neuronal loss in a characteristic distribution (superficial cortex, the dentate gyrus, the hippocampus, and the caudate nucleus) with relative sparing of the brainstem and posterior fossa structures (Auer RN et al 1993).

Studies in humans (Frackowiak RSJ et al 1989) using positron emission tomography have shown minute-minute regional changes in cerebral glucose consumption and blood flow during a variety of sensory and motor activities. Anwar et al (Anwar M et al 1988) using
radioactive micropheres have shown that the brain has the ability to alter regional blood flow to ensure that vital parts of the brain continue to receive adequate glucose. Similar increases in cerebral blood flow have been documented in human preterm infants during hypoglycaemia (Pyrd O et al 1988, Skov L et al 1992). The utilisation of alternative substrates may provide another possible mechanism by which the brain protects itself against the effects of hypoglycaemia. Ameil et al (Amiel SA et al 1992) infused healthy adults with beta-hydroxybutyrate during insulin induced hypoglycaemia and showed that the counter-regulatory hormonal response to hypoglycaemia were lowered as was the delay in the cognitive dysfunction.

For obvious reasons it is very difficult to study ketone body utilisation in children. Nonetheless studies in the past have shown that the human fetus and neonate can take up and oxidise ketone bodies (Jones MD et al 1979, Edmond J et al 1985). This uptake of ketone bodies by the human brain is proportional to the circulating concentration and in neonates the uptake is higher than in adults (Edmonds J et al 1985).

2.0.4 Differences in blood glucose regulation between children and adults.

Differences in glucose homeostasis between adults and children have been known since 1921 (Spence JC 1921). Young children differ from adults in that they have limited glycogen stores which are only adequate for a period of starvation of approximately 12 hours after which the maintenance of a normal blood glucose concentration is dependent on gluconeogenesis. Haymond et al (Haymond MW et al 1982) showed that children fasted for 30 hours had the lowest glucose and alanine concentrations as compared to adult men and women. For this reason children are unable to tolerate prolonged periods
of starvation

Children have higher glucose production rates in comparison to adults in order to meet the increased metabolic demands of the brain, that, relative to body size is much larger than in adults. Bier et al measured glucose production rates in infants and children using 6,6-dideuteroglucose and showed that the brain size was the principal determinant of factors that regulate hepatic glucose output throughout life (Bier DM et al 1977). The fasting newborn and young children demonstrate a high glucose utilisation rate per kilogram body weight relative to adult requirements (Haymond MW et al 1983). Hence for these reasons children are more susceptible to hypoglycaemia in comparison to adults.

2.1 GH and Cortisol as counter-regulatory hormonal responses to hypoglycaemia.

2.1.0 Introduction.

Counter-regulatory hormonal responses to hypoglycaemia refers to all the physiological changes in hormonal levels that occur in response to a low blood glucose concentration. This counter-regulatory system serves an extremely important role in ensuring a continuous supply of glucose to vital organs such as the brain. In this process hormones may participate either by immediate actions or by chronic (permissive) effects, which may alter the responsiveness of target tissues (Gerich JE et al 1980).

In response to insulin-induced hypoglycaemia plasma levels of glucagon, catchelomines, growth hormone and cortisol all increase (Garber AJ et al 1976). In contrast to insulin-induced hypoglycaemia the response and the evolution of these hormones to spontaneous hypoglycaemia is not clear. The mechanism of the counter-regulatory response to an experimental model of prolonged hypoglycaemia, closely mimicking the clinical scenario
may well be different from that of hypoglycaemia due to acute cause (Bolli GB et al 1983).

In the hierarchy of counter-regulatory responses the first signal is the switching "off" of insulin secretion (Gerich JE et al 1991). This has the effect of reducing the portal vein insulin concentration, hence allowing the counter-regulatory hormones to manifest their metabolic effects. In adults there is a well-defined hierarchy of counter-regulatory hormonal responses to hypoglycaemia (Gerich JE et al 1979). It is thought that glucagon and adrenaline are the two hormones that are important in the immediate restoration of the blood glucose concentration, whereas cortisol and growth hormone are thought to have permissive roles in restoring normoglycaemia (Bolli GB et al 1999). The glycaemic threshold for cortisol and GH refers to the concentration of blood glucose which stimulates the release of these hormones. For cortisol and GH the glycaemic thresholds (in adults) lie just within the physiological range of plasma glucose and are thought to be 3.6± 0.06mmol/L and 3.6-3.7mmol/L respectively (Schwartz NS et al 1987, Fanelli C et al 1994). Hence the release of both of these hormones is stimulated even before the blood glucose reaches 3mmol/L. Figure 2.1 illustrates the changes in the counter-regulatory hormones (again in adults) at different blood glucose concentrations.
Figure 2.1. The changes in counter-regulatory hormones in response to reductions in blood glucose concentrations in adults. Serum cortisol and GH concentrations start to increase even before the blood glucose concentration reaches 3mmol/L. Insulin release is inhibited when blood glucose concentration drops to around 4mmol/L.
Following a bolus of insulin, plasma glucose reaches a nadir at about 20-30 minutes, with significant changes in the levels of cortisol and GH values reached at 30 minutes (Gerich JE et al. 1980). Maximum levels of GH and cortisol occur at 40 and 60 minutes respectively after a bolus dose of insulin (Gerich JE et al. 1980). The restoration of normoglycaemia after insulin-induced hypoglycaemia is mainly due to an increase in glucose production with the waning effect of insulin on glucose utilisation.

Although the release of both cortisol and GH is stimulated at the glycaemic threshold, their metabolic effects on glucose are not manifest until several hours after the hypoglycaemic stimulus is corrected (DeFeo P et al. 1989, DeFeo P et al. 1989). Increases in GH secretion participate in glucose counter-regulation by enhancing glucose production and limiting glucose utilisation. Using the PAP clamp technique DeFeo et al. (DeFeo P et al. 1989) were able to show that by preventing a rise in GH secretion glucose production was reduced by 15% and glucose utilisation increased by 8%. This greater effect of a lack of GH on glucose production compared to glucose utilisation suggests that during hypoglycaemia GH exerts its counter-regulatory action predominantly by altering glucose production. GH antagonises the actions of insulin and this usually takes more than 2 hours to manifest (MacGorman J et al. 1981). The lack of GH during hypoglycaemia also has an effect on the intermediary metabolites. Plasma free fatty acids, glycerol and β-hydroxybutyrate concentrations are all reduced during GH deficiency suggesting that GH stimulates lipolysis during hypoglycaemia (DeFeo P et al. 1989). These intermediary metabolites may provide more gluconeogenic substrates for ketone body synthesis thereby reducing the requirement for glucose.
A lack of cortisol response during hypoglycaemia is associated with diminished rates of glucose production with increased rates of glucose utilisation. The effects of cortisol on glucose metabolism generally take several hours to become manifest (Baxter J et al 1972). The effects of cortisol on glucose production occur earlier than its effects on glucose utilisation during glucose counter-regulation (DeFeo P et al 1989). Cortisol has numerous effects on glucose metabolism the most important of which include stimulating gluconeogenesis and antagonizing the actions of insulin. Cortisol deficiency during hypoglycaemia also causes changes in the intermediary metabolites. Plasma free fatty acids, plasma alanine and ketone bodies concentrations are reduced during hypoglycemia in the absence of a cortisol response.

2.1.1 The effect of stress on the hypothalamic-pituitary adrenal axis.

Stress is a powerful stimulus for the activation of the hypothalamic-pituitary adrenal (HPA) axis. Pain and hypoglycaemia are considered to be stressful stimuli. Stress induced activation of the HPA axis is associated with release of hypothalamic CRF, which is the principal regulator of ACTH from the anterior pituitary corticotrophs. The adrenal gland has an astonishing capacity to adapt to various form of acute stress (Pigatelli D et al 1998). Following central activation of the HPA axis ACTH triggers physiological, molecular and morphological changes in the adrenal gland. This leads to increased glucocorticoid release with up-regulation of steroidogenic cytochrome P450 messenger ribonucleic acid (Simpson ER et al 1988).
2.1.2 Cortisol response to stress.

At birth, mixed cord blood cortisol concentrations are relatively high, around 880nmol/L, (Stevens JF 1970) this reflecting the maternal transfer of steroids and the stress of delivery. By 24 hours of age cortisol concentrations fall rapidly to around 270nmol/L (Stevens JF 1970). By 3 days of age the normal cortisol values range between 46.93 to 385.4nmol (Weiner DJ et al 1987). From birth both cortisol and ACTH are released in a pulsatile fashion with secretory bursts at intervals of 1 to 2 hours. Initially these secretory bursts have a high frequency, low amplitude pattern followed by low frequency high amplitude pattern. The circadian rhythm of ACTH and cortisol release is not established until about 3 months of age.

There is no doubt that neonates can generate an adequate serum cortisol response to stress. A study by Anand et al (Anand KJS et al 1985) showed that neonates undergoing cardiac surgery have serum cortisol responses in excess of 750nmol/L. Another study by Hughes et al (Hughes D et al 1987) showed preterm neonates undergoing intensive care to have cortisols levels above of 2200nmol/L. Term babies undergoing major surgery with halothane anaesthesia had serum cortisol concentrations increased by 400nmol/L compared to their pre-operative values (Anand KJS et al 1988)

Since hypoglycaemia is considered to be a severe form of a stressful stimulus (Mantagos S et al 1991) it would be expected that neonates with hypoglycaemia especially if accompanied with symptoms are able to generate an adequate serum cortisol response.
In adults a serum cortisol rise of more than 500nmol/L at the time of hypoglycaemia is accepted as an adequate serum cortisol response. A similar definition is used for infants and children (Hughes IA 1986, Savage DCL et al 1978).

2.1.3 The glucose sensor.

The brain is the key organ for sensing the stimulus of hypoglycaemia. Studies in dogs in which insulin-induced hypoglycaemia was allowed to occur peripherally while glucose infusions were maintained in the carotid and vertebral arteries showed a complete lack of response from the counter-regulatory hormones, whereas this response was normalised in dogs with brain neuroglycopaenia (Biggers DW et al 1989).

The parts of the brain which are thought to play a role in this sensing mechanism are the ventromedial hypothalamic area (VMA) and the lateral hypothalamic area (LHA), (Borg W et al 1994, Oomura Y et al 1974). The concentration of blood glucose in the brain is thought to be an important endogenous regulator of the activity of these two centres, with 30-45% of the neurons in the hypothalamic LHA and VMH having exquisite sensitivity to increases and decreases in the glucose concentration (Silver IA et al 1998). These sensing centres have the ability to respond to increments/decrements in blood glucose concentration as low as 0.2-0.3 mmol/L (Silver IA et al 1998). Not all cells in the central nervous system exhibit the same degree of sensitivity, there being a continuous spectrum of responsiveness and the stronger the stimulus (i.e. the greater the decrease in the blood glucose concentration) the larger are the number of cells recruited (Silver IA et al 1998).
2.1.4 The Insulin Tolerance Test (ITT) as a test used to assess the integrity of the hypothalamic-pituitary adrenal axis.

A number of provocation tests are available for assessing the function of the anterior pituitary axis (Hindmarsh PC et al 1995). Each of these tests involves administrating a certain stimulus and measuring the hormonal response produced to that stimulus. For assessment of GH secretion and ACTH secretion and hence serum cortisol the ITT is most often used. The ITT was first described by Fraser et al (Fraser R et al 1941) as a diagnostic test for the evaluation of pituitary disorders. Stress resulting from the hypoglycaemia induced by this test is thought to be the stimulus for the release of GH and ACTH. The precise mechanism of how hypoglycaemia induces the release of GH and ACTH is unclear. It is possible that the ITT induces the release of readily releasable stores of GH within the pituitary as observed with GHRH provocation testing (Korbonitis M et al 1996), but this has never been clearly documented. This is regarded as the gold standard for determining the integrity of the hypothalamic-pituitary-adrenal axis (Hindmarsh PC et al 1995). The ITT is not without hazard with reports of children dying as a consequence of the test (Shah A et al 1992). Nonetheless it continues to be used in Centres who have the necessary experience and expertise.

The ITT involves giving a bolus dose of intravenous insulin (0.1-0.15U/kg) after an overnight fast. During the test adequate hypoglycaemia has to be achieved either by the child becoming symptomatic (sweaty, clammy, drowsy, headache, nausea or vomiting) or the laboratory blood glucose concentration must be <2.6mmol/l or reduced to 50% of baseline value (Gale EAM et al 1983). Blood samples are taken at -30 minutes, 0, +15,
+30, +45, +60 and +90 minutes. If the hypoglycaemic is inadequate then the GH stimulation may be inadequate.

In an ITT the cortisol response is dependent on the ACTH drive from the anterior pituitary (Littley MD et al 1989). A normal cortisol response is defined as a rise of cortisol above 500nmol/L at the time of hypoglycaemia (Hughes IA 1986). A normal growth hormone response is defined as a rise in the growth hormone of >15mU/L (Hughes IA 1986), with <7mU/L suggesting growth hormone deficiency.

When a bolus of insulin is given intravenously peak plasma insulin levels are achieved within the first ten minutes and then fall exponentially (Gale EAM et al 1983). The nadir of plasma glucose following an intravenous bolus dose of insulin is reached at about twenty minutes (Gale EAM et al 1983). The rate of recovery of blood glucose is slower the greater the dose of insulin used and the severity of hypoglycaemia achieved.

Following an intravenous bolus of insulin there is suppression of the NEFA plasma concentration, with recovery being more rapid the severe the hypoglycaemia.

2.2 Overview of the different causes of hypoglycaemia in childhood.

Hypoglycaemia in childhood can be due to many causes. These can be broadly summarised into those due to hyperinsulinism, hormonal abnormalities such as, cortisol or growth hormone deficiency, defects of hepatic glycogen release/storage, defects in gluconeogenesis, defects in fatty acid oxidation, and unknown causes such as ketotic hypoglycaemia. These are summarised in Table 2.1.
2.2.0 Hypoglycaemia due to Hyperinsulinism of Infancy (HI).

2.2.1 Physiology and biochemistry of HI.

Under normal physiological conditions the metabolism of glucose is intricately linked to regulated insulin secretion in pancreatic β-cells. Glucose enters the β-cell and is converted to glucose-6-phosphate by the enzyme glucokinase. The enzyme glucokinase plays a unique role in acting as a glucose sensor (Glaser B et al 2000) providing a link between the extracellular glucose concentration and the metabolism of glucose in the β-cell. When the blood glucose concentration is increased the activity of glucokinase is also increased hence increasing insulin production from the β-cell. Similarly as the blood glucose concentration decreases serum insulin becomes undetectable at plasma glucose concentrations below 3mmol/L.

In HI there is the inappropriate and excessive release of insulin from the pancreatic β-cell in relation to the plasma glucose concentration. It is now clear that the uncontrolled release of insulin is the final manifestation of a number of different processes that either alter intracellular biochemical pathways of the pancreatic β-cell thereby generating abnormal signals for the secretion of insulin (Dunne MJ et al 1991), or alter the transport of cations across the cell membrane. These abnormalities perturb the stimulus-secretion coupling mechanisms that normally ensure that the amount of insulin secreted is directly related to the ambient blood glucose concentration (Dunne MJ et al 1991). HI is characterised, therefore, by the presence of insulin concentrations that are inappropriately high for the level of blood glucose. A 'normal' insulin level for normoglycaemia is inappropriate in the presence of hypoglycaemia (Aynsley-Green A et
al 2000). The biochemical hallmark of HI is hyperinsulinaemic, hypoketotic, hypofattyacidaemic hypoglycaemia. These biochemical abnormalities reflect the metabolic actions of insulin. These children also have increased glucose requirements (normal 4-6mg/kg/min) in order to maintain a normal blood glucose concentration. Classically HI presents in the neonatal period usually within the first afew days of birth (Aynsley-Green A et al 2000). HI can be persistant or transient. The transient form is associated with maternal diabetes mellitus, intra-uterine growth retardation and perinatal asphyxia (Colins JE et al 1984) and tends to resolve spontaneously. The mechanism/s causing HI in these conditions is not clear. In these cases the HI tends to be transient. The treatment of the persistent form is discussed in the next section.

2.2.2 Brief review of the molecular biology and genetics of HI.

Pancreatic β-cells have numerous channels or pores embedded in their cell membrane. Of these the $K_{\text{ATP}}$ channels are unique in playing a pivotal role linking the metabolism of glucose to regulated insulin secretion (Dunne MJ 1991). Each $K_{\text{ATP}}$ channel consists of a heteromultimeric complex of at least two proteins designated SUR1 and KIR 6.2 (Inagaki N et al 1995). The functional integrity of both of these proteins is necessary for potassium channel movement and the genes responsible for them have been localised very closely to each other on the short arm of chromosome 11(11p14-15.1). Under normal physiological conditions the $K_{\text{ATP}}$ channels maintain the electrical potential of the β-cell membrane. The metabolism of glucose in the β-cell increases the ratio of ATP/ADP which has the effect of closing the $K_{\text{ATP}}$ channels (figure 2.2). This in turn causes the opening up of voltage gated calcium channels which regulate the entry of
calcium into the β-cell. The entry of calcium is the thought to be the final stimulus for insulin exocytosis (Dunne MJ et al 1991).

The commonest genetic cause of persistent HI is an abnormality of the K<sub>ATP</sub> channel. A number of mutations in the SUR1 and Kir 6.2 genes have been defined, particularly in children with the familial forms of HI (Nestarowicz A et al 1996, Thomas P et al 1996). So far more than 40 mutations have been described in the SUR 1 component and three in the KIR 6.2 (Glaser B et al 2000). Some of these mutations destroy the function of the K<sub>ATP</sub> channel whereas some alter channel density or channel activity in response to changes to intracellular nucleotide concentration (Glaser B et al 2000). So far no mutations have been described in about 60% of patients with HI.

Two other recent discoveries have emphasised further the complexity of HI. Thus, abnormal activation of glucokinase (Glaser B et al 1998) and of glutamate dehydrogenase (Stanley A et al 1998) both lead to increased intracellular concentrations of ATP which trigger insulin secretion in the absence of any defect in membrane polarisation. It has been proposed that the glutamate dehydrogenase syndrome, which leads to hyperammonaemia with hypoglycaemia, may be the cause of the so called "leucine-sensitive" hypoglycaemia described in previous years. Figure 2.2 show a schematic illustration of the different mechanisms leading to HI.

Histology two types of HI have been described so far (De Lonlay P et al 1997). The "focal" form of HI is found in about 30% of children and appears to be localised to one region of the pancreas. The focal form appears to be associated with a different genetic background, namely, genetic imprinting (Delonlay P et al 1997). This is not
found in diffuse disease. In this circumstance there is loss of heterozygosity with paternal imputing. There is loss of maternal chromosome 11p in the focal lesion. The loss of the chromosome apparently occurs during development of the pancreas and only affects β-cells in one particular region of the pancreas. Verkarre et al (Verkarre V et al 1998) reported the loss of the maternal chromosome 11p in the focal lesion associated with a germ line mutation on the paternal SUR 1 allele. This results in reduction to hemizygosity or homozygosity for the maternal allele in the affected β-cells. This allows the β-cells in this region to proliferate and they will also lack functional K\textsubscript{ATP} channels.

The treatment of persistent HI involves medical therapy and surgery. The mainstay of medical therapy is diazoxide. This drug works by locking onto the intact SUR component of the K\textsubscript{ATP} channel. By keeping the channel open it stops insulin secretion. Hence patients with mutations in the K\textsubscript{ATP} channel will not respond to diazoxide.

Chlorothiazide is used in conjunction with diazoxide for its hyperglycaemic action as well as counter-acting the fluid retaining properties of diazoxide (Barnes PD et al 2000). Nifedipine, glucagon and somatostatin are also used in some children (Lindley KJ et al 1996, Aynsley-Green A et al 2000). For those children unresponsive to medical therapy surgery is the treatment of choice. It is now imperative to identify those children who have the focal form of the disease as management will be radically different these children requiring only a limited pancreatectomy to remove only the focal lesion (De Lonlay P 1997). Those children with diffuse disease will require a subtotal pancreatectomy. HI has also been reported in association with rare metabolic conditions such as the carbohydrate deficient glycoprotein syndrome (De Lonlay P et al 1999) and in defects of fatty acid metabolism (Clayton P et al 2001).
Exocytosis of Insulin

Voltage gated Ca^{2+} channels

Depolarisation

Increased Ca^{2+} causing exocytosis of insulin

Fatty acids

abnormal metabolites

SCHAD

Ketoglutarate + NH3

GDH

Glutamate Glucose -6-phosphate

Glucokinase

Glucose

Sites of known mutations

| SUR 1: Sulfonylurea Receptor 1 |
| KIR 6.2: Potassium Inward Rectifier 6.2 |
| GDH: Glutamate Dehydrogenase |
| Glucokinase |
| Short Chain 3-HydroxyAcyl CoA |

\[ \text{K}_{\text{ATP}} \text{ channel} \]

Figure 2.2 The different mechanisms leading to HI. The most common cause is a defect in the \( \text{K}_{\text{ATP}} \) channel which causes continuous depolarisation of the plasma membrane with unregulated insulin secretion.
2.3 Hypoglycaemia due to hormone deficiency.

Glucagon, adrenaline, GH and cortisol play an important role in the counter-regulatory responses to hypoglycaemia. The deficiency of any one of these hormones can cause hypoglycaemia. Glucagon and adrenaline deficiency is extremely rare (Kollee LA et al 1978).

On the other hand the incidence of hypoglycaemia due to panhypopituitarism can be as high as 20% (Brasel JA et al 1965). The etiology of the hypoglycaemia due to cortisol and GH deficiency is due to a combination of factors including reduced gluconeogenic substrate availability (decreased mobilisation of fats and proteins) and increased glucose utilisation due to increased insulin sensitivity of tissues in the absence of these two hormones (Gerich JE et al 1980).

Hypoglycaemia associated with hypopituitarism is a cause of sudden death in the childhood period (Nanao K et al 1999). Very rarely congenital hypothyroidism in association with congenital hypopituitarism may cause hypoglycaemia (Kauschansky A et al 1979).

2.4 Hypoglycaemia due to defects in hepatic glycogen release/storage.

Glucose is stored as glycogen mainly in the liver but also in the muscle and in the kidneys. Defects in the storage or release of hepatic glycogen can also cause hypoglycaemia. Glucose-6-phosphatase deficiency (Glycogen storage disease type 1) is the commonest of the glycogen storage diseases causing hypoglycaemia. The deficiency of this enzyme results in the inability to release free glucose from glucose-6-phosphate, with resultant hepatomegaly due to stored glycogen. The two other glycogen storage diseases causing hypoglycaemia are due to deficiencies of the enzymes Amylo-1, 6-
Glucosidase (Glycogen storage disease type 3) and Liver Phosphorylase (Glycogen storage disease 6). The enzyme hepatic glycogen synthase plays an important role in the storage of glycogen in the liver. Hepatic glycogen synthase deficiency is a rare cause of hypoglycaemia in childhood (Aynsley-Green A et al 1977). The characteristic features include fasting hypoglycaemia, with hyperketonaemia but with normal lactate. After a meal the plasma lactate will increase as glucose is channelled along the glycolytic pathway.

2.5 Hypoglycaemia due to defects in gluconeogenesis.
Gluconeogenesis is the process by which glucose is produced from the breakdown products of proteins and fats. The main gluconeogenic precursors include amino acids especially alanine and glutamine, lactate and glycerol. Gluconeogenesis can essentially be viewed as a reversal of glycolysis but with few important differences. These differences include three pathways, which are regulated by different enzymes (Pilkis SJ et al 1988).

2.6 Hypoglycaemia due to defects of fatty acid oxidation.
Fatty acids are derived from the breakdown of stored triglycerides. They are transported to the liver where they undergo a process called β-oxidation to yield energy and ketone bodies. Before a fatty acid can be utilised in the mitochondria it has to combine with a transporter molecule called carnitine forming a fatty-acid carnitine ester. This allows the fatty acids to diffuse cross the outer mitochondrial membrane and is converted to the corresponding acyl-CoA esters in the intermembranous space. Fatty acylcarnitines are formed by the action of carnitine palmitoyltransferase 1 (CPT 1) bound to the inner face
of the outer mitochondrial membrane (Murthy MS et al 1990). The resultant acylcarnitines then cross the inner mitochondrial membrane in exchange for free carnitine and are converted back to acyl-CoA esters by CPT II (Eaton S et al 1996). The carnitine-acylcarnitine shuttle is regulated by malonyl-CoA which modulates the activity of CPT I (McGarry JD et al 1978). In the fed state the concentration of malonyl-CoA is high and this inhibits CPT I with flux through the β-oxidation pathway being reduced (Eaton S et al 1996). Hence in the fed state the delivery of fatty acids to the tissues is diminished due to low rates of lipolysis and the entry of fatty acids into the mitochondria is also inhibited. In the fasted state the rate of fatty acid synthesis is low with low concentrations of malonyl-CoA allowing more efficient transport of fatty acids across the inner mitochondrial membrane (McGarry JD et al 1978).

Once inside the mitochondria each fatty acid undergoes the process of β-oxidation whereby there is progressive shortening of the carbon chain of the fatty acid. Each of these reactions is catalysed by intramitochondrial enzymes (Acyl-CoA dehydrogenases). Each of these enzymes acts upon a specific chain length fatty acid, medium chain, long chain and short chain. The deficiency of these enzymes results in defective fatty acid metabolism with increased fatty acids and reduced or absence ketone bodies in the plasma. Hence any defect of carnitine, CPT I CPT II and Acyl CoA dehydrogenases can cause hypoglycaemia (Bougneres PF et al 1981, Bartlett K et al 1991).

2.7 Hypoglycaemia due to defects in ketone body synthesis/utilization.

Ketone bodies are an alternative form of fuel to glucose especially for the brain. Each ketone body is synthesised from the combination of Acetyl-CoA and Acetoacetyl-CoA to from hydroxymethylglutaryl-CoA (HMG-CoA), which is then split by HMG-CoA lyase
to yield acetoacetate. Acetoacetate is then converted to \( \beta \)-hydroxybutyrate.

Hypoglycaemia may occur either due to defects in the synthesis or utilisation of ketone bodies (Mitchell GA et al 1995).

### 2.8 Hypoglycaemia due to miscellaneous causes.

Ketotic hypoglycaemia is a common form of hypoglycaemia (Chaussain JL et al 1973). It usually presents between the ages of 18 months and 5 years and remits spontaneously by the ages of 9 to 10 years. The typical history is of a child who may miss a meal and develops hypoglycaemia usually following an upper respiratory tract infection. The hypoglycaemic episodes seem to be unpredictable, only developing sometimes.

Biochemically the hypoglycaemia is associated with raised ketone bodies and free fatty acids with suppressed insulin levels. Ketotic hypoglycaemia is characterised by low levels of plasma alanine but the precise mechanism responsible for the hypoglycaemia is not understood. The hormones such as glucagon and cortisol seem to be appropriately raised but the role of GH is unclear (Aynsley-Green A et al 1991). Ketotic hypoglycaemia is a poorly defined term and may include groups of conditions in which there is no clear cause of the hypoglycaemia. Conditions such as hepatic glycogen synthase deficiency (Rutledge SL et al 2001) and acetoacetyl CoA thiolase deficiency have been reported as presenting with ketotic hypoglycaemia (Leonard JV et al et al 1987). Ketotic hypoglycaemia is a diagnosis of exclusion.

Hypoglycaemia can also occur due to a number of metabolic conditions including galactosaemia, fructosaemia, tyrosinaemia, organic acidaemias and in maple syrup urine disease (Saudabray JM et al 1990).
Hypoglycaemia can also be induced pharmacologically, either intentionally as a diagnostic tool, accidentally as a complication of the treatment of diabetes mellitus, or as a consequence of poisoning either with insulin itself (Dershewitz R et al 1976), or with drugs such as sulphonylureas, which stimulate insulin release (Teale JD et al 1989).

The transport of glucose into the cerebral cells is an insulin-independent process that requires the presence of glucose transport proteins. The first glucose transporter in this process is GLUT 1 which is localised in microvessels of the blood brain barrier. This protein moves glucose from the capillary lumen to the brain interstitium and is transported into the neurons and glial cells by GLUT 3 and GLUT 1 transporters. Defects in these glucose transporters can cause hypoglycorrhachia with relatively normal plasma glucose levels (DeVivo DC et al 1991).
Hyperinsulinism
Congenital: SUR1 / KIR6.2 / Glucokinase / Glutamate dehydrogenase.
Carbohydrate deficient glycoprotein syndrome (CDG)
Defects in the metabolism of fatty acids (SCHAD)

Hormonal deficiency
Cortisol / Growth hormone / ACTH / Glucagon / Adrenaline / Rarely hyothyroidism.

Defects in hepatic glycogen release/storage
Glycogen storage diseases: Glucose-6-phosphatase, Amylo 1-6 glucosidase deficiency, Liver phosphorylase deficiency.
Hepatic glycogen synthase deficiency.

Defects in gluconeogenesis
Fructose-1, 6-bisphosphatase deficiency, phosphoenolpyruvate carboxykinase (PEPCK) deficiency.

Defects of fatty acid oxidation.
Medium Chain Acyl-CoA Dehydrogenase Deficiency
Long Chain Acyl-CoA Dehydrogenase Deficiency (+ VLCAD)
Short Chain Acyl-CoA Dehydrogenase Deficiency
Carnitine deficiency (primary and secondary)
Carnitine palmitoyl transferase deficiency (CPT 1 and 2)

Defects in ketone body synthesis/utilisation
HMG CoA synthase deficiency / HMG CoA Lyase deficiency

Metabolic conditions (relatively common ones).
Organic acidaemias (Propionic / Methylmalonic)
Maple syrup urine disease, galactosaemia, fructosaemia, tyrosinaemia
Glutaric aciduria type 2

Defects in glucose transport
Defects of transporters Glut 1/2/3

Drug induced
Sulphonylurea / Insulin / Beta-blocker / Salicylates

Miscellaneous causes (mechanism may not be so clear)
Ketotic hypoglycaemia - The commonest cause of hypoglycaemia.
Infections (sepsis, malaria), congenital heart disease

Table 2.1 Summarising different causes of hypoglycaemia in childhood.
2.9 Aims of the study.

The aims of this study were:

1) To investigate the serum GH and cortisol responses to spontaneous hypoglycaemia in infants and children and compare these responses to those observed in the standard ITT.

2) To investigate the effect of different physiological changes in NEFA and ketone bodies concentrations on serum GH response, and to investigate whether the aetiology of the hypoglycaemia alters the serum GH and cortisol response.

3) To investigate the serum cortisol and GH responses in neonates with hyperinsulinaemic hypoglycaemia.
Chapter 3

3.0 Methods.

3.1 Patient recruitment and patient details.

All the patients recruited into the study were referred to Great Ormond Street Hospital for investigations of persistent hypoglycaemia. Great Ormond Street Hospital is a tertiary referral Centre for the diagnosis and management of complicated forms of hypoglycaemia. All these patients were referred to Professors Aynsley-Green, Leonard and Clayton. Older children with non-acute hypoglycaemia were firstly referred to the outpatients by their local Paediatrician or General Practitioner, where an initial assessment of the cause of their hypoglycaemia was made. If further investigations were required then they were admitted to the Programmed Investigation Unit at Great Ormond Street Hospital. Neonates referred with acute hypoglycaemia were admitted directly to Frederick Still Ward at Great Ormond Street Hospital. These referrals included patients from district General Hospitals and from other sub-specialties within the Great Ormond Street Hospital site. Over an eighteen-month period a total of 80 consecutive patients were referred for investigation of hypoglycaemia. Since the aim of the study was to investigate how serum GH and cortisol were released in response to hypoglycaemia, it was important to identify those children or neonates in whom hypoglycaemia may be due to either GH or cortisol deficiency and exclude them from the study. Hence the following groups of children / neonates were excluded from the study:

a) Children with known GH or cortisol deficiency/insufficiency.

b) Children on replacement therapy with either GH or cortisol.
c) Any child with a midline anatomical lesion indicating the possible involvement of the anterior pituitary gland and hence GH or cortisol deficiency.

d) Children with a history suggestive of psychological dwarfism and chronic illnesses, which may affect GH and or cortisol secretion.

e) Neonates with ambiguous genitalia

e) Neonates on hydrocortisone or diazoxide therapy for hyperinsulinism.

After applying the above exclusion criteria 65 patients were eventually recruited into the study. Before the commencement of the study, the investigator discussed the study details with parents, explained what the study entailed and how it was to be conducted. The investigator gave parents the information sheet (Appendix 1) and explained the practical procedures involved. The initial contact with the parents was made 24 hours before commencement of the study. The parents were left with the information sheet. Once the parents had read this information sheet the investigator would let them ask any questions about the study. Once they understood the research project, the investigator would ask for their written consent. Of the sixty-five children recruited into the study only thirty-five demonstrated hypoglycaemia during fasting studies. These thirty-five children were divided into two groups. These included thirteen neonates with hyperinsulinaemic hypoglycaemia and twenty two older children with various categories of hypoglycaemia. Thirty children did not become hypoglycaemic during the fasting studies. The data from these thirty children is not included in the thesis. All these thirty children had demonstrated hypoglycaemia at their referring hospitals at some stage but
when investigated under controlled fasting conditions at Great Ormond Street Hospital no hypoglycaemia was documented.

Children having the ITT test were admitted under Dr. Peter Hindmarsh at the Middlesex Hospital.

The study was approved by the Ethical Committee of the Institute of Child Health and Great Ormond Street Children’s Hospital.

For the twenty-two older group of children the ages, weights, heights, Body Surface Area (BSA) and the underlying cause of the hypoglycaemia are shown in table 3.1. The age range was 0.5-10 years, with twelve males and ten females. For the children who had the ITT performed their age range was 5 to 11 years with ten males and six females. A total of sixteen children had the ITT. Table 3.2 summarises the mean heights, weights and BSA for both groups.

Of the twenty two children with spontaneous hypoglycaemia twelve had “ketotic” hypoglycaemia, two had a disorder of fatty acid metabolism (MCAD, Medium Chain Acyl-CoA Dehydrogenase), two had Glycogen Storage Disease Type 1A, five had hyperinsulinism and in one child the diagnosis of hypoglycaemia was thought to be due to a ketone body utilization defect although the this has not been established yet. The diagnosis of “ketotic” hypoglycaemia was made by exclusion of any other cause of hypoglycaemia and by the typical biochemical findings of raised plasma NEFA concentration with appropriately raised ketone bodies at the time of hypoglycaemia. The
ratio between the plasma NEFA concentration and the 3β-HOBH was normal in these patients. No other biochemical abnormality was noted in these patients.

The diagnosis of MCAD was made by the typical biochemical findings of an abnormal NEFA / Ketone body ratio with an abnormal acylcarnitine profile in the presence of hypoglycaemia.

The diagnosis of glycogen storage disease was clinically suggested with these children having marked hepatomegaly, with the classical biochemical findings of hypoglycaemia associated with a raised plasma lactate, urate and triglycerides concentrations.

The diagnosis of HI was established biochemically by the typical features of hypoglycaemia associated with hypofattyacidaemia and hypoketonaemia. All these five children had a raised glucose requirement (between 8-10mg/kg/min) which is another marker for HI. The serum ammonia concentration was normal for this group of children. In one child the hypoglycaemia was associated with probable diagnosis of a ketone body utilization disorder but this has still not been confirmed.
<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Weight (Kg)</th>
<th>Height (CM)</th>
<th>BSA</th>
<th>Diagnosis</th>
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<tbody>
<tr>
<td>3</td>
<td>16</td>
<td>91</td>
<td>0.63</td>
<td>Ketotic</td>
</tr>
<tr>
<td>4.5</td>
<td>13.8</td>
<td>87</td>
<td>0.57</td>
<td>Ketotic</td>
</tr>
<tr>
<td>2</td>
<td>11.6</td>
<td>86</td>
<td>0.52</td>
<td>Ketotic</td>
</tr>
<tr>
<td>4</td>
<td>19.8</td>
<td>107.5</td>
<td>0.76</td>
<td>Ketotic</td>
</tr>
<tr>
<td>5</td>
<td>16.8</td>
<td>90</td>
<td>0.64</td>
<td>Ketotic</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>103.8</td>
<td>0.65</td>
<td>FAOD</td>
</tr>
<tr>
<td>3</td>
<td>10.8</td>
<td>85</td>
<td>0.5</td>
<td>Ketotic</td>
</tr>
<tr>
<td>3</td>
<td>19.1</td>
<td>110.9</td>
<td>0.76</td>
<td>Ketotic</td>
</tr>
<tr>
<td>4</td>
<td>17.3</td>
<td>110</td>
<td>0.72</td>
<td>Ketotic</td>
</tr>
<tr>
<td>4</td>
<td>11.6</td>
<td>94.4</td>
<td>0.55</td>
<td>?Diagnosis</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>111</td>
<td>0.84</td>
<td>GSD 1A</td>
</tr>
<tr>
<td>3</td>
<td>14.7</td>
<td>90.7</td>
<td>0.6</td>
<td>Ketotic</td>
</tr>
<tr>
<td>9</td>
<td>19.7</td>
<td>117</td>
<td>0.8</td>
<td>Ketotic</td>
</tr>
<tr>
<td>0.5</td>
<td>7.6</td>
<td>70</td>
<td>0.38</td>
<td>Hyperinsulinism</td>
</tr>
<tr>
<td>3.5</td>
<td>12.1</td>
<td>115</td>
<td>0.62</td>
<td>Hyperinsulinism</td>
</tr>
<tr>
<td>1.2</td>
<td>9</td>
<td>80</td>
<td>0.44</td>
<td>Hyperinsulinism</td>
</tr>
<tr>
<td>2</td>
<td>13.6</td>
<td>86.2</td>
<td>0.57</td>
<td>FAOD</td>
</tr>
<tr>
<td>10</td>
<td>30.2</td>
<td>132.1</td>
<td>1.05</td>
<td>Ketotic</td>
</tr>
<tr>
<td>0.5</td>
<td>8.3</td>
<td>68</td>
<td>0.39</td>
<td>Hyperinsulinism</td>
</tr>
<tr>
<td>3</td>
<td>11.4</td>
<td>87</td>
<td>0.52</td>
<td>Hyperinsulinism</td>
</tr>
<tr>
<td>0.7</td>
<td>10.6</td>
<td>82</td>
<td>0.49</td>
<td>GSD 1A</td>
</tr>
<tr>
<td>5</td>
<td>17.8</td>
<td>103</td>
<td>0.71</td>
<td>Ketotic</td>
</tr>
</tbody>
</table>

**Table 3.1** Summarising the weight, height, BSA and aetiology of hypoglycaemia for each child with spontaneous hypoglycaemia (excluding neonates).

Key: BSA: Body Surface Area. GSD: Glycogen storage disease. FAOD: Fatty Acid Oxidation Disorder.
Patients with spontaneous hypoglycaemia  (N=22)  Patients having ITT  (N=16)

<table>
<thead>
<tr>
<th></th>
<th>Patients with spontaneous hypoglycaemia</th>
<th>Patients having ITT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years range)</td>
<td>0.5-10</td>
<td>5-11</td>
</tr>
<tr>
<td>Males</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Females</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Mean Height (cm)</td>
<td>100</td>
<td>113.6</td>
</tr>
<tr>
<td>Mean Weight (Kg)</td>
<td>15.3</td>
<td>19.7</td>
</tr>
<tr>
<td>Mean BSA(m²)</td>
<td>0.416</td>
<td>0.621</td>
</tr>
</tbody>
</table>

BSA: Body surface area

Table 3.2 Summarising the details of the patients with spontaneous hypoglycaemia and patients undergoing ITT (excluding neonates).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>no. of children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketotic hypoglycaemia</td>
<td>12</td>
</tr>
<tr>
<td>Fatty acid oxidation disorder (MCAD)</td>
<td>2</td>
</tr>
<tr>
<td>Glycogen storage disease (Type 1A)</td>
<td>2</td>
</tr>
<tr>
<td>Hyperinsulinism</td>
<td>5</td>
</tr>
<tr>
<td>? No diagnosis</td>
<td>1</td>
</tr>
</tbody>
</table>

MCAD: Medium Chain Acyl-CoA Dehydrogenase Deficiency.

Table 3.3 Summarising aetiology of hypoglycaemia in the twenty two children.
The neonatal group included thirteen neonates with HI. All these neonates had markedly raised glucose requirements (between 12-21 mg/kg/min, normal being 4 to 8 mg/kg/min). Their gestational age ranged between 34 to 42 weeks. Their birth weights ranged from 2 kg up to 5.6 kg. Five of these babies required a subtotal / total pancreatectomy to control their hypoglycaemia whereas the rest responded to medication with diazoxide +/- chlorothiazide. The clinical details of these babies are shown in table 3.4. In one of these babies the serum ammonia concentration remained elevated suggesting the possible diagnosis of hyperammonaemic hyperinsulinaemic hypoglycaemia.
### Table 3.4. The clinical details of 13 babies with Hyperinsulinism. Key: H = hours, CS = caesarean section, NVD = normal vaginal delivery, P = pancreatectomy, D/C = diazoxide and chlorothiazide.
3.2 The diagnostic fast and ITT:

The biochemical and physiological changes in glucose metabolism that occur during a fast have already been outlined in chapter 1. The diagnostic fast was the standard provocation test used to induce hypoglycaemia (Morris AAM et al 1996). The aim of the fast was to induce a length of period without food so that the physiological mechanisms regulating glycogenolysis, gluconeogensis, fatty acid oxidation and ketone body formation are normally switched on. If there was a defect in any of these pathways this should lead to hypoglycaemia during the fast.

This test was carefully explained to all the parents/children who participated in the study. Prior to the start of the diagnostic fast each child had an intraveous cannula inserted or in the case of the neonates a central venous line such as a Hickman line under general anaesthesia in the operating theatre was inserted. The child was then fasted according to unit protocol. The length of this was determined by the age of the child (Table 3.4). The fast involved stopping all enteral and intravenous fluids. In the older children blood glucose (BM stix and laboratory blood glucose) was measured hourly to begin with and at half hourly intervals if hypoglycaemia was anticipated. In the neonates blood glucose was measured at 10-15 minute intervals. The fast was terminated if the laboratory blood glucose concentration was <2.6mmol/L or if the child showed symptoms of hypoglycaemia (drowsy, confusion, nausea, vomiting, headaches). During the fast each of the infants and children were closely monitored by investigator for signs and symptoms of hypoglycaemia. In the older children blood was withdrawn for serum cortisol and GH measurements at the beginning of the fast, at hourly intervals throughout
the fast, at the time of hypoglycaemia and at 10 minute intervals for 50 minutes after the hypoglycaemic stimulus was corrected. The 10 minute sampling interval was chosen in order to detect pulses of GH secretion during a total sampling interval of 50 minutes from the time of the hypoglycaemia. The hypoglycaemia was corrected either with intravenous fluids (1-2 ml of 10\% dextrose bolus followed by a continuous infusion of dextrose to maintain normoglycaemia) or oral feeds if the child was tolerating enteral feeding.

In the neonatal group blood was drawn from the central line for serum cortisol and GH measurements at the beginning of the fast, at 10 minute intervals during the fast and then at the time of hypoglycaemia. The hypoglycaemia was then corrected with 1-2ml/kg of 10\% dextrose followed by a continuous infusion of dextrose to maintain normoglycaemia. Blood for serum cortisol and GH measurements was again withdrawn at ten minute intervals for fifty minutes after correction of the hypoglycaemia.

Six of the neonates with HI had serum ACTH measured at the time of hypoglycaemia. They were then given 62.5micrograms of Synacthen intravenously after correction of the hypoglycaemia. Blood was withdrawn again at ten minute intervals for fifty minutes after correction of the hypoglycaemia for serum cortisol measurement.

None of the children and neonates fasted were hypoglycaemic forty eight hours before the diagnostic fast was performed. Prior to the fast normoglycaemia was maintained in all the neonates using a combination of concentrated dextrose infusion and enteral feeds.
In total approximately 3mls of blood was taken for the complete hypoglycaemic profile at the time of hypoglycaemia. The intermediary metabolites measured at the beginning of the fast and at the time of hypoglycaemia are shown in table 3.5. At the bedside the blood samples for acetoacetate and pyruvate were precipitated by the addition of perchloric acid to the blood specimen. The ACTH sample was collected into cold EDTA tubes and immediately transported to the laboratory. All the other specimens were then transported to the Biochemistry Laboratory at Great Ormond Street Hospital. The GH and cortisol blood samples were centrifuged for three minutes, separated and serum stored at -20 until analysed. The blood samples for plasma glucose, lactate and pyruvate were centrifuged and plasma separated for analysis immediately. The blood samples for plasma NEFA, Ketone bodies, amino acids and ACTH were centrifuged, separated and serum stored at -20 until analysis.
The maximum length of fast according to the age of the child.

<table>
<thead>
<tr>
<th>Age</th>
<th>Length of fast (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>6</td>
</tr>
<tr>
<td>6-8 months</td>
<td>8</td>
</tr>
<tr>
<td>8-12 months</td>
<td>12</td>
</tr>
<tr>
<td>1-2 years</td>
<td>16</td>
</tr>
<tr>
<td>2-8 years</td>
<td>18</td>
</tr>
<tr>
<td>&gt;8 years</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3.5 Length of fast according to age of child. The length of the diagnostic fast was performed according to the age of the child. The neonatal group was fasted for less than six hours as they became hypoglycaemic very quickly upon withdrawal of exogenous glucose.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Volume of blood sample (mls)</th>
<th>Collected into (container)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.2</td>
<td>flouride</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.5</td>
<td>lithium heparin</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.3</td>
<td>clotted</td>
</tr>
<tr>
<td>ACTH</td>
<td>0.5</td>
<td>ice cold EDTA</td>
</tr>
<tr>
<td>GH</td>
<td>0.5</td>
<td>clotted</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.2</td>
<td>flouride</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.2</td>
<td>precipitate by bedside</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.1</td>
<td>lithium heparin</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>0.1</td>
<td>precipitate by bedside</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>0.1</td>
<td>lithium heparin</td>
</tr>
<tr>
<td>Branch chain amino acids</td>
<td>1ml</td>
<td>lithium heparin</td>
</tr>
<tr>
<td>Urine organic acid</td>
<td>5 mls of urine</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.6** The hormones and intermediary metabolites measured at the beginning of the fast and at the time of hypoglycaemia. Urine sample was collected at the end of the fast. Acyl-carnitine was measured on Guthrie card blood samples.
child was then fasted overnight prior to the test. On the morning of the test at time -30 minutes a blood sample was taken for glucose, cortisol and GH. Insulin (0.1 units/kg) was then administrated intravenously. Blood samples for glucose, cortisol and growth hormone were again taken at +15, +30, +45, +60 and +90 minutes.

3.3 Measurements of hormones and intermediary metabolites.

Both serum GH and cortisol were measured using a different radioimmunoassay. The basic principles used in radioimmunoassays are shown in figure 3.1.

3.3.1 Immunoradiometric Assay for measuring serum GH.

Serum GH concentrations were measured using an immunoradiometric assay (Tandem-R HGH, Hybritech Assay, Leige, Belgium). This assay is a solid phase two-site immunoradiometric assay. Samples containing hGH are reacted simultaneously with a plastic Bead (solid phase) coated with a mouse monoclonal IgG (anti hGH) antibody directed toward a unique site on the hGH molecule and with a radiolabelled mouse monoclonal IgG (anti-hGH) antibody labeled with $^{125}$ Iodine, directed against a distinctly different antigenic site on the same hGH molecule. Following the formation of the solid/hGH/labelled antibody sandwich, the Bead is washed to remove unbound labelled
**Figure 3.1.** Illustrating the basic principles used in a radioimmunoassay.
antibody. The radioactivity bound to the solid phase is then measured with a gamma counter. The amount of radioactivity measured is directly proportional to the concentration of hGH present in the test sample. The Hybritech assay is highly specific for GH with cross reactivity of less than 1% with other hormones. The sensitivity of the assay was 0.2ng/ml. This assay is highly specific for the 22kda form of the hGH with very little cross-reactivity with other isoforms of GH.

The serum GH samples were allowed to be thawed to room temperature. Plastic test tubes were labeled with controls, standards and patient samples. One antibody coated bead was placed into each test tube. Then 100μL of the patients samples, standards and controls were placed into the labeled test tubes. To each of these test tubes was added 100μL of the Anti-hGH tracer antibody. This was them mixed on a vortex and incubated in a water bath for four hours at a temperature of 37° C. After four hours the beads are washed with wash solution by pipetting 2mls of wash solution into each tube. The wash solution was then removed by using a decant rack. This procedure was repeated three times. The tubes were then put into a gamma counter (model NE 1600) which was connected to a computer. The standard curve for GH measurement is shown in figure 3.2.

The inter-assay reliability was determined by replicate determinations of three different serum samples in one assay. The % coefficient of variation was 3.5-3.8%.

The intra-assay reliability was determined by duplicate measurements of three different serum samples over a series of individually calibrated assays. The % coefficient of variation ranged between 3.4-5.2%.
Figure 3.2 Standard curve for measurement of GH.
3.3.2 Radioimmunoassay for measuring serum cortisol.

The radioimmuno assay used to measure cortisol was the Coat-A-Count assay (Diagnostic Products Corporation, Los Angeles, CA). The Coat-A-Count Cortisol procedure is a solid phase radioimmunoassay, wherein $^{125}$I-labelled cortisol competes for a fixed time with cortisol in the patients sample for the antibody sites. Because the antibody is immobilised to the wall of a polypropylene tube, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabelled cortisol. Counting the tube in a gamma counter then yields a number, which is converted by means of a calibration curve to give the amount of cortisol present in the patient sample. The incubation period for the procedure was 45 minutes.

The patient's serum cortisol samples were allowed to thaw to room temperature. Specially provided Cortisol Ab-Coated test tubes were labeled for patient samples and controls. Plain tubes were labeled for standards. 25μL of the patient's serum was added to the appropriate test tubes and mixed. Then 1 ml of $^{125}$I-labelled cortisol tracer was added to each test tube. The mixture was mixed on a vortex for five seconds and incubated in a water bath for 45 minutes at 37° C. The mixture was then decanted thoroughly and each tube was counted in a gammer counter (Model NE1600) for sixty seconds. The standard curve for the measurement of cortisol is shown in figure 3.3.

The reliability of the Coat-A-Count Cortisol procedure was assessed by examining its reproducability on samples selected to represent a range of cortisol levels.
The % coefficient of variation for the intra-assay was between 3.0-5.1%, and that in the inter-assay was between 4.0-6.4%. This procedure could detect as little as 0.2µg/dl of cortisol. The antiserum is highly specific for cortisol, with very low cross-reactivity to other compounds that might be present in the patient’s samples. This assay has a cross reactivity of 0.94% with corticosterone, 0.98% with cortisone, and 0.26% with 11-Deoxycorticosterone. Neither protein, lipaemia, bilirubin, nor haemolysis has any clinically significant effect on the assay.

Figure 3.3 Standard curve for the measurement of cortisol.
Hormones and Intermediary metabolites measured in the Biochemistry Department of Great Ormond Street Hospital.

3.3.3 Measurement of serum insulin.

Serum insulin as well as ACTH were measured in the Biochemistry Laboratory of Great Ormond Street Hospital. A very sophisticated method (outlined below) was used to measure these two hormones. I learned how this method worked and was helped by the laboratory staff in performing the practical procedures.

Serum insulin was measured using the Immulite immunometric assay with the Immulite analyser. The IMMULITE Automated Immunoassay Analyser is a continuous random access instrument which performs automated chemiluminescent immunoassays. The IMMULITE system utilises assay-specific antibody or antigen-coated plastic beads as the solid phase, alkaline phosphatase-labelled reagent and a chemiluminescent enzyme substrate. The bead is housed in a proprietry Test Unit. This Unit serves as the reaction vessel for the immune reaction, the incubation and the washing process and the signal development. The IMMULITE System automates the entire assay process. After incubating the patient sample with the alkaline phosphatase reagent, the liquid reaction mixture in the Test Unit is rapidly separated from the bead when the bead is washed and the Test Unit is spun at a high speed in its vertical axis. The entire fluid contents (the sample, excess reagent and wash solution) are transferred to a coaxial waste chamber in the Test Unit. The bead is left with no residual unbound label. The bound label is then quantified with a dioxetane substrate which produces light. Light emission is detected by a photomultiplier tube (PMT) and printed reports for each sample are generated by the systems computer. The system has a calibration range of 400mU/l and an analytical
sensitivity of 2mU/l. The test is specific for insulin with no cross-reactivity with c-peptide, glucagon or proinsulin. The % coefficient of variation in the within-run varied between 3.8-5.4%.

3.3.4 ACTH measurement.

The serum ACTH concentration was also measured using the Immulite ACTH. This is a sequential immunometric assay used in conjunction with the IMMULITE analyser. The IMMULITE system utilises assay-specific antibody or antigen-coated plastic beads as the solid phase, alkaline phosphatase-labelled reagent and a chemiluminescent enzyme substrate. The bead is housed in a proprietry Test Unit. This Unit serves as the reaction vessel for the immune reaction, the incubation and the washing process and the signal development. The IMMULITE System automates the entire assay process. Samples from patients are collected into ice-cold EDTA tubes and the plasma separated. After incubating the sample with the alkaline phosphatase reagent, the liquid reaction mixture in the Test Unit is rapidly separated from the bead when the bead is washed and the Test Unit is spun at a high speed in its vertical axis. The entire fluid contents (the sample, excess reagent and wash solution) are transferred to a coaxial waste chamber in the Test Unit. The bead is left with no residual unbound label. The bound label is then quantified with a dioxetane substrate which produces light. Light emission is detected by a photomultiplier tube (PMT) and printed reports for each sample are generated by the systems computer. The system has a calibration range of up to 125pg/ml and an analytical sensitivity of 9pg/ml.
3.3.5 Measurement of plasma glucose concentration.

Plasma glucose was measured using the Kodak Vitrous 750 instrument. A 10 microlitre drop of the patient’s plasma was deposited on the Vitrous Glu slide (this is a dry multilayered analytical element coated on a polyester support used for the quantitative measurement of plasma glucose). This causes the spreading layer to promote uniform distribution of the sample and permit an even penetration of the solute molecules into the underlying reagent layer. The oxidation of sample glucose is catalysed by glucose oxidase to form hydrogen peroxide and gluconate. This reaction is followed by an oxidative coupling catalysed by peroxidase in the presence of dye precursors to produce a dye. The intensity of the dye is measured by the reflected light.

Reaction sequence:

\[
\text{B-D-glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Glucose oxidase}} \text{D-glucuronic acid} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + 1, 7\text{-dihydroxynaphthalene} \xrightarrow{\text{peroxidase}} \text{red dye}
\]

3.3.6 Measurements of Ketone bodies.

a) β-hydroxybutyrate- The plasma concentration of β-hydroxybutyrate was determined using the Cobas Mira Plus analyser. The method is based on the oxidation of D-3-hydroxybutyrate to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase. Comcomitant with this oxidation the cofactor NAD⁺ is reduced to NADH. The corresponding change in the absorbance can be directly correlated with the D-3-hydroxybutyrate concentration.
Sequence reaction:

\[
B\text{-hydroxybutyrate} + \text{NAD}^+ \xrightarrow{3\text{-hydroxybutyrate dehydrogenase}} \text{Acetoacetate} + H^+ + \text{NADH}^+
\]

b) Acetoacetate- This is measured using the reaction catalysed by \(\beta\)-hydroxybutyrate dehydrogenase (HBDH). NADH when excited by light at 340nm emits light at 450nm by fluorescence. The associated decrease in fluorescence emission at 450nm caused in NADH during the reaction can be directly correlated with the acetoacetate concentration. The instrumentation used for performing the analysis was the Roche Cobas Bio. Immediately after the blood sample was collected from the patient 100 microlitres of the blood was added to 500 microlitres of ice cold 0.46 M perchloric acid in a Luckham LP3 tube. This was thoroughly mixed and separated on the centrifuge.

\[
\text{Acetoacetate} + \text{NADH} \xrightarrow{\text{HBDH}} \beta\text{-hydroxybutyrate} + \text{NAD}
\]

3.3.7. Measurements of Non Esterified Fatty Acids (NEFA).

The plasma concentration of NEFA was determined using the instrument called the Cobas Mira Plus analyser. The principle of the method is based on the fact that NEFA when reacted with acyl CoA synthase in the presence of adenosine triphosphate, magnesium cations and CoA, form the thiol esters of CoA known as acyl CoA as well as the by products adenosine monophosphate and pyrophosphate. In the second part of the reaction the acyl CoA is oxidised by added acyl CoA oxidase to produce hydrogen.
peroxide which in the presence of added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(b-hydroxyeth)-aniline with 4-aminoantipyrine to form a purple coloured adduct with an absorption maximum at 550nm. Hence the amount of NEFA in the sample can be determined from the optical density measured at 550nm.

3.3.8. Measurement of lactate.

The plasma lactate concentration was measured using the Kodak Vitrous 750. This is used for the quantitative measurement of plasma lactate in plasma. A 10 microlitre plasma sample was deposited on Vitros LAC Slide and evenly distributed. Lactate in the sample is oxidised by lactate oxidase to pyruvate and hydrogen peroxide. The hydrogen peroxide generated oxidizes the 4-aminoantipyrine, 1, 7-dihydroxynaphthalene dye system in a horsesh-ad-peroxidase-catalysed reaction and results in a dye complex. The slide is incubated and the intensity of the dye complex is measured spectrophotometrically.

Reaction sequence:

\[ \text{L - (+) - lactic acid + O}_2 \xrightarrow{\text{Lactate oxidase}} \text{pyruvate + H}_2\text{O}_2 \]

\[ 2\text{H}_2\text{O}_2 + 4\text{aminoantipyrine + 1, 7 dihydroxynaphthalene} \xrightarrow{\text{peroxidase}} \text{red dye} \]

3.3.9. Measurement of pyruvate.

Pyruvate measurement was made based on the principle of the reaction catalysed by lactate dehydrogenase (LDH). NADH when excited by light at 340nm emits light at 450nm by flourescence. The corresponding decrease in flourescence emission of light at
450nm caused by the decrease in NADH during the reaction can be directly correlated with the pyruvate concentration. Immediately after the blood sample was taken from the patient 100 microlitres was added to 500 microlitres of ice cold 0.46M perchloric acid in a Luckham LP 3 tube. This was thoroughly mixed and then spun down in the centrifuge at 3000 RPM for 5 minutes. The instrumentation used for measuring the acetoacetate concentration was the Roche Cobas Bio.

Reaction sequence:

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}
\]

3.3.10 Measurement of Ammonia.

The plasma ammonia concentration was measured on the Kodak Vitrous 750 instrument. The blood sample from the patients was collected into a special ammonia free container. The Vitrous AMON Slide quantitatively measures the ammonia concentration in plasma. A 10 microlitre of patient's serum was deposited on the Vitrous slide and spread evenly. Water and nonproteinaceous components travel to the underlying buffered reagent layer and the ammonium ions are converted into gaseous ammonia. The semipermeable membrane allows only ammonia to pass through and prevents buffer or hydroxyl ions from reaching the indicator light. After a fixed incubation period the reflection density of the dye is measured using the white background of the spreading layer as a diffuse reflector.

Reaction sequence:

\[
\text{NH}_3 + \text{bromophenol blue (ammonia indicator)} \xrightarrow{\text{}} \text{blue dye}
\]
3.4 Statistical analysis

The following statistical analyses were performed on the data:

1) Mean. The mean serum cortisol and GH were calculated for both hormones at different time intervals.

2) Standard Error of the Mean (SEM). The SEM was calculated for serum cortisol and GH.

3) Student's T-test was used to compare the mean differences of serum cortisol and GH to assess degree of significance. The Paired-Samples T Test procedure compare the means of two variables for a single group. It computes the differences between values of the two variables for each case and tests whether the average differs from 0. P values of <0.01 were taken as significance.

4) Analysis of Variance (ANOVA). The One-Way ANOVA procedure produces a one-way analysis of variance for a quantitative dependent variable by a single factor (independent) variable. Analysis of variance was used to test the hypothesis that several means are equal. This technique is an extension of the two-sample t test.

5) Linear regression was used to assess the dependence of one variable on another. Linear Regression estimates the coefficients of the linear equation, involving one or more independent variables, that best predict the value of the dependent variable.

The statistics package used for the analysis was SPSS 10 Microsoft for Windows.
Chapter 4

Results: part 1

4.1 Serum GH and cortisol responses in children with spontaneous and ITT induced hypoglycaemia.

This section analyses the serum GH and cortisol responses in the twenty two children with spontaneous hypoglycaemia and compares these responses in children with ITT induced hypoglycaemia. This section also describes the relationship between changes in the physiological concentrations of NEFA and ketone bodies on the serum GH response. The impact of the underlying aetiology of hypoglycaemia on the serum GH and cortisol response is also assessed.

Serum GH was measured at sixty minutes before hypoglycaemia, at the point of hypoglycaemia and at ten minute intervals for fifty minutes after hypoglycaemia. The individual serum GH values at these different time points and the plasma glucose values are shown in table 4.1. Two children (patients 6 and 8) had plasma glucose concentrations of 2.8mmol/L and 2.9mmol/L when blood was taken for serum GH measurement. Both of these children were symptomatic (sweaty, pallor and tachycardia) at these glucose concentrations and their fast was therefore terminated. In both of these children the hypoglycaemia was treated with a small bolus (1ml/kg) of 10% dextrose. Despite having symptoms of hypoglycaemia these two children had inappropriately low serum GH responses of 3.6mU/L and 4.6mU/L respectively at the time of hypoglycaemia.
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Table 4.1. The plasma glucose concentrations and serum GH profile for the twenty two children with spontaneous hypoglycaemia.
Sixteen patients underwent the ITT. Table 4.2 shows the levels of blood glucose reached during the ITT as well as the baseline and thirty minute serum GH and cortisol values. All patients showed increments in serum GH at the time of hypoglycaemia except patient 12. This patient had similar levels of serum GH before and at hypoglycaemia. All patients had appropriate serum cortisol responses to ITT induced hypoglycaemia.

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<th>Plasma glucose (mmol/L)</th>
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<th>Serum GH (+30 min)</th>
<th>Serum cortisol (time 0)</th>
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Table 4.2 Serum GH and cortisol responses to ITT
The individual serum GH responses to spontaneous and ITT induced hypoglycaemic episode are shown Figures 4.1 and 4.2 respectively. The expected serum GH response to hypoglycaemia is indicated by the reference line at 15mU/L. Only three children (patients 1, 10 and 18) showed a serum GH response greater than 15mU/L at the time of spontaneous hypoglycaemia. Patient 18 had an elevated serum GH value of 17.7mU/L at -60 minutes before the onset of the hypoglycaemia. Patient 10 was the only patient with a clear cut rise in the serum GH at the time of hypoglycemia. This rise in the serum GH persisted for only ten minutes.

In contrast in the ITT all sixteen children tested had a serum GH value greater than 15mU/L at the time of hypoglycaemia.

The mean (± SEM) serum GH response at the time of spontaneous hypoglycemia was 6.73 (± 1.32) mU/L, (with a range of 0.4 to 25.4mU/L) whereas in the ITT group it was 29.23 (± 7.30). Only one child in the spontaneous hypoglycaemia group had an adequate response of 25.4mU/L, with two other children having serum GH responses of 17.5mU/L and 17.2mU/L at the time of spontaneous hypoglycemia, which were just acceptable values. All the other children had inappropriately low serum GH responses at the time of spontaneous hypoglycemia. Two children had symptoms of hypoglycemia when their blood glucose concentrations were between 2.8-2.9mmol/L. These two children failed to generate any significant serum GH responses to symptomatic hypoglycemia.

Blood glucose concentrations at the time of hypoglycaemia were comparable in both the ITT and spontaneous hypoglycaemia groups.
Figure 4.1 Serum GH responses at time of spontaneous hypoglycemia.

Only one child showed a significant serum GH response at the time of hypoglycaemia.

Figure 4.2 Serum GH responses at time of ITT induced hypoglycaemia. All the children generated a serum GH response greater than 15mU/L. There was no correlation between serum GH and level of blood glucose at time of hypoglycaemia.
4.1.1 Serum GH profile to spontaneous and ITT induced hypoglycaemia.

To understand the changes in the serum GH levels before, at and after hypoglycaemia in both groups of children the mean (± SEM) serum GH levels were compared at various time points in the twenty two children with spontaneous hypoglycaemia with the children in the ITT group. The ITT group only had serum GH measured at baseline and at the time of hypoglycaemia.

The mean serum GH value at sixty minutes before the onset of spontaneous hypoglycaemia was 6.85 (± 1.11) mU/L. The mean serum GH value at the point of spontaneous hypoglycaemia was 6.73 (± 1.32) mU/L. The mean serum GH values at +10, +20, +30, +40 and +50 minutes after hypoglycaemia were 5.39 (± 1.02) mU/L, 4.70 (± 0.68) mU/L, 4.57 (± 0.95) mU/L, 5.42 (± 1.38) mU/L, 5.46 (± 1.29) mU/L respectively. Figure 4.3 shows the serum GH profile over time to spontaneous hypoglycaemia. Using the Students paired T-test and ANOVA the mean serum GH values were compared at the different time intervals. There was no significant difference between these mean serum GH values (range of P value 0.37- 0.9) over the period of sampling. Interestingly the mean serum GH levels actually decreased over the time of sampling. In contrast the baseline mean serum GH value in the ITT was 5.12 (± 1.28) mU/L. This reflects the serum GH response to an overnight fast. These children maintained normal blood glucose concentrations throughout the overnight fast. Hypoglycaemia was then induced by the administration of insulin and the mean serum GH value rose to 29.23 (± 7.30) mU/L at thirty minutes. There was a significant difference between these means (P<0.01). These changes are shown in Figure 4.4.
Figure 4.3 Serum GH profile before, at and after spontaneous hypoglycaemia
There were no significant changes in the serum GH levels before, at and for fifty minutes after spontaneous hypoglycaemia in the whole group.

Figure 4.4 Serum GH profile to hypoglycaemia induced by the ITT.
The mean serum GH was significantly raised from baseline at the time of hypoglycaemia induced by the ITT.
4.1.2 Analysis of serum GH and cortisol responses according to aetiology of spontaneous hypoglycaemia.

It is not known if the underlying disease process leading to hypoglycaemia influences the serum GH or cortisol responses to hypoglycaemia. For this reason the serum GH and cortisol responses to spontaneous hypoglycaemia were further analysed according to the underlying aetiology of the hypoglycaemia. Twelve children had "ketotic" hypoglycaemia, five children had hyperinsulinaemic hypoglycaemia, two children had a fatty acid oxidation disorder and two children had glycogen storage disease. The one child with no diagnosis for the hypoglycaemia was excluded from this analysis.

The highest mean serum GH response was seen in children with ketotic hypoglycaemia with a mean value of 6.61 mU/L (corresponding cortisol value was 645.72 ± 68.15 nmol/L). There were only two children with disorders of fatty acid oxidation and two children with glycogen storage disease. The two children with defects in fatty acid oxidation showed the poorest serum GH responses with a mean value of 3.2 mU/L (corresponding cortisol value was 938.0 ± 224 nmol/L). The serum GH and cortisol responses to different causes of hypoglycaemia are shown in Figures 4.5 respectively.

For the serum GH there was no significant difference between the GH responses to different causes of hypoglycaemia (P = 0.34 to P = 0.66). Children with hyperinsulinism had the poorest serum GH response (corresponding cortisol response was also the poorest with a mean serum cortisol of 309.2 ± 54.69 nmol/L. The child where the underlying diagnosis of hypoglycaemia was unclear was excluded from this analysis.
Although the number of children in the present study are relatively small these results suggest that the underlying disease processes did not affect the serum GH response to spontaneous hypoglycaemia. Serum GH responses to spontaneous hypoglycaemia seemed to be unaffected by the mechanism by which spontaneous hypoglycaemia was produced.

On the other hand children with hyperinsulinism produced inappropriately low serum cortisol responses at the time of hypoglycaemia. Serum cortisol responses were appropriate in children with ketotic hypoglycaemia, in children with glycogen storage disease and in children with fatty acid oxidation disorders.
Figure 4.5 A/B Comparing the serum cortisol and GH responses according to aetiology of hypoglycaemia. Key: FAOD: Fatty acid oxidation disorder, GSD: Glycogen storage disease, HI: Hyperinsulinism of Infancy. Serum GH response was inappropriately low in all different causes of hypoglycaemia.
4.1.3 Serum GH responses to physiological changes in serum NEFA and ketone body concentrations.

Pharmacological elevations and reductions in the serum NEFA concentration are known to decrease and increase serum GH respectively. The precise mechanism by which these changes in the plasma NEFA concentration affects serum GH is unclear.

On the other hand the effect of physiological changes in the ketone body concentrations upon serum GH secretion is not known. Ketone bodies are a normal physiological response to fasting and a decreasing blood glucose concentration.

To understand if changes in the physiological concentrations of NEFA and ketone bodies generated by the various conditions leading to hypoglycaemia affect serum GH secretion, the serum GH response was assessed to physiological changes in NEFA and ketone body concentrations. The effect of NEFA concentration generated by the spontaneous hypoglycaemia on serum GH levels is shown in Figure 4.6. The lowest serum NEFA concentrations were found in the children with HI whereas the highest NEFA concentrations were present in the children with a fatty acid oxidation disorder. Regression analysis was performed to assess if there was any correlation between the serum GH response and changes in serum NEFA concentration. There appears to be no significant correlation (R = 0.06) between the endogenous NEFA concentration achieved and the serum GH value in response to spontaneous hypoglycaemia. These results suggest that physiological changes in serum NEFA concentrations do not have any significant effect on serum GH secretion.
As for the ketone bodies children with hyperinsulinism had the lowest serum ketone body concentrations whereas children with ketotic hypoglycaemia had the highest serum ketone body concentration. On comparing the serum GH response to different physiological changes in the ketone body concentration there was no significant correlation between between serum GH levels and total ketone body concentration generated (R = 0.13, figure 4.7). Physiological changes in the ketones body concentrations therefore do not seem to have any significant effect on serum GH secretion.
Figure 4.6 Serum GH response to NEFA. Physiological changes in serum NEFA concentration generated to spontaneous hypoglycaemia have no significant effect on serum GH responses.

Figure 4.7 Serum GH response to ketone bodies. Physiological changes in total ketone body concentrations generated to spontaneous hypoglycaemia have no significant effect on serum GH responses.
4.1.4 Serum cortisol responses to spontaneous and ITT induced hypoglycaemia.

Serum cortisol was measured at sixty minutes before hypoglycaemia, at the point of hypoglycaemia and at ten minute intervals for fifty minutes after hypoglycaemia. The individual serum cortisol values at these different points are shown in table 4.3 with the corresponding plasma glucose values at the time of hypoglycaemia.

As with the serum GH two patients were symptomatic at plasma glucose concentrations of 2.8 and 2.9mmol/L. Blood for serum cortisol was taken at these plasma glucose values and the fast terminated. In contrast to the serum GH responses seen in these two children the serum cortisol responses were appropriate at the time of spontaneous hypoglycaemia. All the other patients had plasma glucose concentrations at or below 2.6mmol/L.
Table 4.3. Summarising the serum cortisol values before, at and after spontaneous hypoglycaemia in the twenty two patients.
The individual serum cortisol responses to spontaneous hypoglycaemia and ITT induced hypoglycaemia are shown in Figures 4.8 and 4.9 respectively. In the spontaneous hypoglycaemia group eight children had a serum cortisol responses below 500nmol/L at the time of hypoglycaemia. These eight children included five children with hyperinsulinism. One of these children with hyperinsulinism had the lowest serum cortisol of 116nmol/L in the whole cohort. The other three children included two with “ketotic” hypoglycaemia and one with GSD 1A. In the ITT group only one child had a serum cortisol response below 500nmol/L.

The appropriate serum cortisol response at time of hypoglycaemia is shown by the reference line at 500nmol/L.
Figure 4.8 Serum cortisol responses at time of spontaneous hypoglycaemia. Eight children had serum cortisol values below 500nmol/L at the time of hypoglycaemia.

Figure 4.9 Serum cortisol responses at time of hypoglycaemia induced by ITT. Only one patient had a serum cortisol below 500nmol/L at the time of ITT induced hypoglycaemia.
4.1.5 Serum cortisol profile to spontaneous and ITT induced hypoglycaemia.

The mean serum cortisol value sixty minutes before spontaneous hypoglycaemia was 424.13 (± 51.10) nmol/L. The mean serum cortisol value at the time of hypoglycaemia was 601.09 ± (65.77) nmol/L. The mean serum cortisol values at +10, +20, +30, +40 and +50 minutes after spontaneous hypoglycaemia were 633.13 (± 69.13) nmol/L, 645.13 (± 71.07) nmol/L, 668.04 (± 70.76) nmol/L, 680.22 (± 72.05) nmol/L and 662.22 (± 77.76) nmol/L respectively. Using the paired Students T-Test to compare the mean serum cortisol changes from sixty minutes before hypoglycaemia to the point of hypoglycaemia the P value was 0.04. The changes in the rest of the mean values were not significant. Of the eight patients with a serum cortisol below 500nmol/L at the time of hypoglycaemia only one of these patients failed to generate a serum cortisol above 500nmol/L at thirty minutes. The increase in serum cortisol persisted for the whole period of sampling. These changes are shown in figure 4.10.

These results suggest that the maximum incremental rise in serum cortisol in response to spontaneous hypoglycaemia had already occurred at the time of hypoglycaemia. The maximal serum cortisol values were reached at forty minutes after hypoglycaemia. This increase in serum cortisol continued for at least fifty minutes from the time of correction of the hypoglycaemia.

In the ITT the mean baseline serum cortisol value before the onset of hypoglycaemia was 396.18 (± 63.25) nmol/L. At the time of hypoglycaemia the mean serum cortisol value increased to 777.68 (± 66.26) nmol/L. The difference between these means was highly significant (P<0.01). These results suggest that the hypoglycaemia induced by the ITT was sufficient to produce an appropriate serum cortisol response.
Figure 4.10 Serum cortisol profile to spontaneous hypoglycaemia. The highest incremental rise in serum cortisol during the whole profile was between sixty minutes before the onset of hypoglycaemia and the time hypoglycaemia.

Figure 4.11. Serum cortisol response to ITT induced hypoglycaemia.
There was a significant increase in serum cortisol levels at the time of ITT induced hypoglycaemia.
4.1.6 Comparison of serum GH and serum cortisol responses to spontaneous hypoglycaemia.

Serum GH and cortisol were compared to assess if there was any correlation between the responses of these two hormones at the time of hypoglycaemia. There was no correlation (R=0.07) between the serum cortisol and GH responses to spontaneous hypoglycaemia. Figure 4.12 shows the corresponding serum GH and cortisol values at the time of spontaneous hypoglycaemia.

Those children with low serum GH response to spontaneous hypoglycaemia showed a wide range of cortisol responses. The three children who demonstrated normal GH responses also had appropriate cortisol responses to spontaneous hypoglycaemia. Hence it appears that there is no correlation between GH and cortisol in response to spontaneous hypoglycaemia.
Figure 4.12 Comparison of serum cortisol and GH responses to spontaneous hypoglycaemia. There is no significant correlation between serum GH and cortisol at the time of spontaneous hypoglycaemia. High serum cortisol levels at the time of spontaneous hypoglycaemia do not affect the serum GH response.
Chapter 5

5.0 Results part II

5.1 GH and cortisol responses in neonates with HI.

This chapter describes the serum cortisol and GH responses observed in babies with hyperinsulinaemic hypoglycaemia. Table 5.1 summarises the individual serum cortisol and GH responses at the time of hyperinsulineamic hypoglycaemia in thirteen neonates. The serum insulin and ACTH levels as well as the urine organic acids and the other intermediary metabolites generated at the time of hyperinsulinaemic hypoglycaemia are also shown. One child had an elevated serum ammonia concentration and was thought to have the hyperammonaemic hyperinsulinaemic syndrome.

All of these neonates had a blood glucose concentration of <2.6mmol/l at the time of hypoglycaemia. All had inappropriately detectable serum insulin levels at the time of hypoglycaemia. All of these neonates had inappropriately low serum NEFA and ketone body concentrations as well as branched chain amino acids for the level of blood glucose concentration.

Individual serum GH and cortisol responses to hyperinsulinaemic hypoglycaemia are shown in figures 5.1 and 5.2 respectively.

All thirteen neonates generated adequate serum GH levels at the time of hypoglycaemia. In contrast serum cortisol levels was inappropriately low at the time of hyperinsulinaemic hypoglycaemia in all of the babies studied.
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<td>41  135 39  22.8 93 35  58.3 26.2 57.3 54 93.1 29.5 26.5</td>
</tr>
<tr>
<td>Ammonia (&lt;40μmol/L)</td>
<td>43  40 38  41  35  70(H) 45  28 38 34 40 32 42</td>
</tr>
<tr>
<td>Lactate (1-2mmol/L)</td>
<td>1.2 1.5 1.1 1.6 1.1 1.4 1.0 1.2 1.4 1.1 1.2 1.3 1.1</td>
</tr>
</tbody>
</table>

Table 4.5. Results of the diagnostic fast and intermediary metabolites at the time of hypoglycaemia in 13 neonates with HI. Patient 6 had an elevated plasma ammonia concentration. All patients had normal acyl-carnitine and suppressed branch chain amino acids. 1 (Stanely CA et al 1979)
Serum GH levels were elevated in all thirteen neonates at the time of hypoglycaemia.

All thirteen babies had an inappropriately low serum cortisol level at the time of hypoglycaemia.
5.1.1 Serum GH profile to hyperinsulinaemic hypoglycaemia.

The changes in serum GH over time in response to hyperinsulinaemic hypoglycaemia are shown in figure 5.3. The mean serum GH value fifteen minutes before the hypoglycaemic episode was 48.82 (± 9.01) mU/L. The mean serum GH value at the time of hyperinsulinaemic hypoglycaemia was 56.36 (± 9.24) mU/L. The mean GH values at +10, +20, +30 +40 and +50 minutes after correction of the hypoglycaemia were 70.40 (± 9.67) mU/L, 72.51 (± 10.17) mU/L, 82.35 (± 9.74) mU/L, 77.93 (± 10.82) mU/L and 73.32 (± 10.21) mU/L respectively. There was no statistical difference between any of these mean values using the Students T-test (P=0.17 to P=0.39). The serum GH levels remained elevated throughout the period of sampling. Hence neonates with HI had elevated serum GH levels even before the onset of hyperinsulinaemic hypoglycaemia.
Figure 5.3 Mean serum GH profile to hyperinsulinaemic hypoglycaemia.

Serum GH levels were raised even before the onset of hypoglycaemia. There was no significant incremental rise in serum GH at the time of hyperinsulinaemic hypoglycaemia. The raised serum GH levels persisted throughout the period of sampling.
5.1.2 Serum cortisol profile to hyperinsulinaemic hypoglycaemia in babies with and without Synacthen administration at the time of hypoglycaemia.

The serum cortisol profile to hyperinsulinaemic hypoglycaemia was investigated according to whether ACTH was administered at the time of hypoglycaemia. For this the neonates were categorised according to those who received ACTH and those who did not receive ACTH at the time of hypoglycaemia. Seven babies with hyperinsulinaemic hypoglycaemia had serum cortisol profile without ACTH administration. In these seven babies the mean serum cortisol value fifteen minutes before the onset of the hypoglycaemia was 102.85 (± 16.52) nmol/L. At the time of the hyperinsulinaemic hypoglycaemia the mean serum cortisol value was 149.42 (± 32.52) nmol/L. At +10, +20, +30, +40 and +50 minutes post hyperinsulinaemic hypoglycaemia the mean serum cortisol values were 202.85 (± 47.45) nmol/L, 223.42 (± 49.01) nmol/L, 210.71 (± 50.65) nmol/L, 206.71 (± 46.51) nmol/L, and 220.85 (± 34.59) nmol/L respectively. There was no significant difference between these means (P=0.04 to P= 0.69). These changes in the serum cortisol levels in the group of babies not given ACTH at the time of hypoglycaemia are shown in figure 5.4.

Six babies were administered intravenous Synacthen (62.5μg) at the point of hyperinsulinaemic hypoglycaemia. The mean serum cortisol value in these six babies at 15 minutes before the hypoglycaemic episode was 207.33 (± 39.68) nmol/L. The mean serum cortisol value at the time of hyperinsulinaemic hypoglycaemia was 219 (± 45.50) nmol/L. The mean serum cortisol values at +10, +20, +30, +40 and +50 minutes after the episode of hypoglycaemia were 378.16 (± 56.81) nmol/L, 664.0 (± 61.60) nmol/L, 905 (±
120.99) nmol/L, 1048.66 (± 101.02) nmol/L and 1175.83 (± 99.84) nmol/L respectively. There was a significant increase in the serum cortisol value from time 0 to time 20 minutes (P<0.01) after ACTH administration. These changes in the serum cortisol levels are shown in figure 5.5. The rise in the serum cortisol continued throughout the period of sampling.
Figure 5.4 Serum cortisol profile in babies not given Synacthen at time of hyperinsulinaemic hypoglycaemia. There was no significant incremental rise in serum cortisol over the period of sampling for fifty minutes.

Figure 5.5 Serum cortisol profile in babies with hyperinsulinaemic hypoglycaemia given Synacthen at the time of hypoglycaemia. There was a significant increase in serum cortisol levels when ACTH was administered from the time of onset of hyperinsulinaemic hypoglycaemia to twenty minutes after ACTH administration.
5.1.3 Serum ACTH at the time of hyperinsulinaemic hypoglycaemia.

Six babies had serum ACTH measured at the time of hyperinsulinaemic hypoglycaemia. This group was administered ACTH at the time of hypoglycaemia. All demonstrated inappropriately low serum ACTH levels for the degree of hypoglycaemia. The serum ACTH values for these babies are shown in figure 5.6.

![Figure 5.6 Serum ACTH levels at the time of hyperinsulinaemic hypoglycaemia](image)

All six neonates had extremely low serum ACTH levels at the time of hyperinsulinaemic hypoglycaemia. The appropriate rise in the serum level of ACTH at the time of hypoglycaemia is shown by the reference line at 50pg/ml.
5.1.4 Serum insulin levels at the time of hyperinsulinaemic hypoglycaemia.

Serum insulin levels ranged between 3.7-18.7mU/L at the time of hypoglycaemia. The lower limit of detection for serum insulin was 2mU/L. All infants had detectable insulin at the time of hypoglycaemia. As shown in Figure 5.7 for a given level of blood glucose the insulin levels were variable. For example at a blood glucose concentration of 2.5mmol/L the insulin levels ranged between 7mU/L up to 18.7mU/L. Whether this difference in the serum insulin level at the time of hypoglycaemia reflects the pulsatile nature of insulin secretion is unclear.

Figure 5.7 Serum insulin levels at time of hyperinsulinaemic hypoglycaemia.

The lower limit of detection of serum insulin (<2mU/L) by the Immulite system is indicated by the reference line.
5.1.5 Comparison of serum cortisol and GH in babies with HI.

To analyse if there was a correlation between serum GH and cortisol levels at the time of hyperinsulinaemic hypoglycaemia the levels of these two hormones were compared at the time of hyperinsulinaemic hypoglycaemia. There was a very poor correlation ($R = -0.09$) between these two hormones at the time of hypoglycaemia. Serum GH levels did not seem to have any impact upon the cortisol response. These results suggest that there was no correlation between these hormones in terms of counter-regulation at least in babies with hyperinsulinaemic hypoglycaemia.

![Graph](image)

Figure 5.8 Comparing the serum cortisol and GH responses to hyperinsulinaemic hypoglycaemia. These two hormones do not appear to influence the secretion of each other at the time of hyperinsulinaemic hypoglycaemia.
5.1.6 Serum Insulin in relation to serum cortisol in babies with hyperinsulinaemic hypoglycaemia.

Since it is possible that the poor serum cortisol response to hyperinsulinaemic hypoglycaemia may be related to the inappropriately raised serum insulin levels, serum insulin levels were compared against serum cortisol responses to assess if the poor serum cortisol response to hyperinsulinaemic hypoglycaemia was related to the inappropriately raised serum insulin levels. There was no significant correlation between serum insulin and cortisol (R=0.12). These results suggest that the inappropriately low serum cortisol responses were not related to the insulin levels at the time of hypoglycaemia.

Figure 5.9 Comparing serum cortisol against serum insulin levels. There is no significant interaction between serum levels of insulin and cortisol.
5.1.7 Comparison of serum GH levels against birth weight in babies with HI.

Serum GH levels were raised in all neonates with hyperinsulinaemic hypoglycaemia. Since babies with HI are usually macrosomic serum GH values at the time of hypoglycaemia were compared against birth weight of babies with HI. There was no significant correlation between birth weight and serum growth hormone levels at time of hypoglycaemia ($R=0.4$).

This suggests that the high serum GH levels in babies with HI were not necessarily related to their birth weight.

![Graph showing correlation between serum GH levels and birth weight in babies with HI.](image)

**Figure 5.10 Serum GH levels in relation to birth weight of babies with HI.**

There was no significant correlation between serum GH levels and birth weight in babies with HI.
Chapter 6

Discussion

6.1 Important critical and ethical points on the scope of the present investigation.

Although this is the first study to analyse the serial serum GH and cortisol responses to different causes of hypoglycaemia in childhood there are a few critical aspects of the study.

The criticisms of the study are:

a) The population of children studied was highly selective. This reflects the nature of referrals to Great Ormond Street Hospital.

b) The numbers of children studied were relatively small especially those with fatty acid oxidation disorders, glycogen storage disorders and other rare conditions causing hypoglycaemia. This reflects the rarity of these conditions where even in a tertiary referral Centre there is likely to be only 1 to 2 cases over a two year period.

c) Lack of proper control data. This applies especially to the neonatal group where there were no “control” neonates. It would be difficult to get control data as it would not be ethically possible to measure cortisol and GH on "normal" neonates. Comparisons of the GH and cortisol responses can be made with other studies where neonates have been subjected to different forms of stress. The neonatal group includes only those with hyperinsulinaemic hypoglycaemia and hence comparisons with other conditions causing hypoglycaemia in the neonatal period cannot be strictly made.
d) The ITT was performed in 16 "normal" children and not in those children with spontaneous hypoglycaemia. Again ethically it would not be possible to perform ITTs in all those children who demonstrated hypoglycaemia to measure GH response.

6.2 Serum GH and cortisol responses to spontaneous and ITT induced hypoglycaemia.

6.2.1 GH and Cortisol responses to spontaneous hypoglycaemia.

Serum GH and cortisol play an essential role in glucose counter-regulation following an episode of hypoglycaemia (Defeo P et al 1989 + Defeo P et al 1989). Although GH and cortisol are released immediately in response to hypoglycaemia the counter-regulatory metabolic effects of these hormones do not become manifest for several hours (Defeo P et al 1989).

GH and cortisol have numerous effects on glucose metabolism including increasing the rates of gluconeogenesis and glycolysis and antagonizing the effects of insulin. In adults the glycaemic thresholds for the activation of glucose counter-regulatory hormones such as GH and cortisol lies within or just below the physiological blood glucose concentration and slightly higher than the threshold for symptoms (Schwartz NS et al 1987). This implies that GH and cortisol start to rise in response to blood glucose concentrations within the normoglycaemic range and it is thought that these increases are inversely proportional to the nadir in blood glucose (Santiago JV et al 1980).

The present observations on the GH and cortisol responses to spontaneous hypoglycaemia have shown that there is an appropriate cortisol response to the
hypoglycaemic stimulus with a paradoxically poor GH response. This lack of GH response to spontaneous hypoglycaemia has been noticed before (Aynsley-Green A et al 1991) but the underlying mechanism for this poor serum GH response has not been explored further. This study has shown that the serum GH responses are different to that observed in spontaneous hypoglycaemia and in hypoglycaemia induced by the ITT.

The poor serum GH response observed in spontaneous hypoglycaemia could be due to several different factors. Given that GH is secreted in a pulsatile fashion one possibility is that the pulses of GH secretion may not be detected. This is unlikely as the sampling interval of ten minutes excludes the possibility of missed GH pulses given that the half-life of GH is approximately 10 minutes (Faria AC et al 1989). Further the timing of the samples with respect to the point of hypoglycaemia ensured that fifty minutes post hypoglycaemia was covered. The ten minute sampling interval was specifically chosen in order to detect GH pulsatility.

It is also possible that the GH response to spontaneous hypoglycaemia may be delayed. The fact that sampling for serum GH was continued for fifty minutes after the hypoglycaemic stimulus should be able to detect any delayed response. It is possible but very unlikely that the GH response to hypoglycaemia may be delayed beyond fifty minutes.

Another possible reason for the difference in serum GH response to spontaneous hypoglycaemia as compared to the ITT may be related to the mechanism by which
hypoglycaemia is achieved. In our cohort of children with spontaneous hypoglycaemia there was a gradual spontaneous reduction in the level of blood glucose concentration (minimum of 1 hour in the hyperinsulinaemic children to a maximum of 18 hours in the ketotic hypoglycaemic children), whereas in the ITT the blood glucose is rapidly lowered with significant hypoglycaemia induced within 15-20 minutes. This suggests that the rate of fall of the blood glucose concentration with respect to time may be an important determinant in signalling GH secretion. Ameil et al (Amiel SA et al 1987) using the glucose - patch clamp techniques showed that the rate of fall of blood glucose concentration does not affect the counter-regulatory responses to hypoglycaemia in normal and diabetic adults. This study suggested that the counter-regulatory hormone response to hypoglycaemia was triggered by the glucose level per se and not by the rate of fall of the blood glucose. No such studies have been performed in children with spontaneous hypoglycaemia. Studies in children with insulin dependent diabetes mellitus when hypoglycaemic show a vigorous growth hormone and cortisol counter-regulatory hormone response (Ameil SA et al 1987). The rate of fall of the blood glucose concentration does not affect the hierarchy of the counter-regulatory hormonal responses to hypoglycaemia (Mitrakou A et al 1993).

The ITT is an unusual test in that the child has to be fasted before hypoglycaemia is induced. During the period of this fast under normal physiological conditions there will be an appropriate increase in the free fatty acid concentration and an appropriate rise in the ketone body concentration. This is achieved by the pancreatic β-cells recognising the decrease in the blood glucose concentration and switching off insulin secretion. The
decrease in the plasma insulin concentration allows an increase in all the counter-regulatory hormones. Fasting studies in adults have shown that the serum cortisol increases during the fast whereas the serum GH levels are not significantly elevated (Bergendahl M et al 1996). The counter-regulatory hormones counteract the effects of insulin causing an increased release of free fatty acids from the adipose tissues with a concomitant increase in the ketone body concentration. By giving a bolus of insulin during the standard ITT this prevailing metabolic milieu is rapidly changed with insulin suppressing the synthesis and release of fatty acids from the adipose tissue and hence inhibiting ketone body formation. It is possible that this sudden change in the metabolic milieu caused by the administration of insulin may also be a trigger factor for the glucosensors in the hypothalamus.

The other major difference between hypoglycaemia induced spontaneously and that induced by the ITT is related to the administration of intravenous insulin. This raises the question whether intravenous insulin administration itself has a role in modulating the release of GH from the anterior pituitary somatotrophs. It is now well established that insulin rapidly crosses the blood-brain barrier by a receptor mediated transport mechanism that involves insulin receptors expressed by brain microvessels (Baskin DG et al 1999). Insulin receptor substrate-1 (IRS-1) and insulin receptors are also co-expressed in discrete populations of neurons suggesting probable transduction mechanisms by which insulin may influence metabolism in the brain (Baskin DG et al 1994). It is suggested that specific insulin receptors are located on the arcuate and paraventricular nuclei of the hypothalamus and when stimulated with insulin send
inhibitory impulses to the vagus and excitatory impulses to the sympathetic nuclei (Ferrannini E et al 1999). This is then thought to trigger the release of CRF, which stimulates cortisol release but suppresses GH secretion (Schwartz MW et al 1992). This central action of insulin might explain why the low serum GH levels were observed in our group of older children with hyperinsulinism, since they all had elevated serum insulin levels at the time of hypoglycaemia. However this mechanism does not explain the poor serum GH responses observed in the ketotic group as well as in the case of the other non-hyperinsulinaemic hypoglycaemic groups where serum insulin levels were undetectable at the time of hypoglycaemia.

The precise mechanism by which the ITT induces GH response is not clear, nor is it known what concentrations of insulin are achieved in the plasma or cerebrospinal fluid when an intravenous bolus of insulin is given during the ITT. West et al (West TET et al 1977) reported that it was the hypoglycaemic stimulus per se which was the triggering factor for the GH release rather than insulin. Shibasaki et al (Shibasaki T et al 1985) suggested that the ITT stimulates GH release through a mechanism, which is largely independent of GHRH. Page et al (Page MD et al 1987) showed that hypoglycaemia induced by the ITT releases GH through inhibition of SS secretion and data from animal work have demonstrated that glucose can alter hypothalamic SS release through a direct action (Berelowitz M et al 1982).
6.2.2 Comparison of serum GH and cortisol responses to different diseases causing hypoglycaemia.

Could the underlying disease processes causing the hypoglycaemia influence the serum GH response generated at the time of hypoglycaemia? In our cohort of children twelve had "ketotic" hypoglycaemia, two had a fatty acid oxidation disorder, two had Glycogen storage disease type 1A, five had hyperinsulinism and in one case there was the possibility of a ketone body synthesis disorder, although the diagnosis is not confirmed in this child. Although the numbers of children with glycogen storage disease and fatty acid oxidation disorders were small, serum GH concentrations were low at the time of hypoglycaemia in all diagnostic categories. The mean serum GH concentration at the time of hypoglycaemia in children with ketotic hypoglycaemia was 6.6mu/L. The mean serum GH concentration at the time of hypoglycaemia in children with hyperinsulinism was 5.7mu/L. The mean serum GH concentrations for GSD1A and fatty acid oxidation disorder were 4.25mu/L and 3.2mu/L respectively. Interestingly the mean serum cortisol concentration at the time of hypoglycaemia was highest in those children with the fatty acid oxidation disorder (mean 938nmol/L) and lowest in those children with hyperinsulinism (mean 309nmol/L).

Hence since the serum GH concentrations were low in all categories (except in 1 child where the cause of the hypoglycaemia is unclear) it seems highly unlikely that the underlying aetiology of the hypoglycaemia has any significant effect on the GH response observed at the time of hypoglycaemia.
6.2.3 GH response to severity of hypoglycaemia.

Could the poor serum GH response in our cohort of patients be related to the degree of hypoglycaemia attained during the fast? The degree of hypoglycaemia achieved in our cohort of patients ranged from 1.3-2.9mmol/L. Two patients became symptomatic before the blood glucose was allowed to drop to <2.6mmol/L. These patients did not have high serum GH levels. In the ITT group the levels of hypoglycaemia ranged from 1.2-2.9mmol/L. Hence similar levels of hypoglycaemia were observed in both groups of patients. Therefore the paradoxically poor serum GH response and appropriate cortisol responses to spontaneous hypoglycaemia could not be related to the severity of hypoglycaemia.

6.2.4 GH, IGF-I and Ghrelin.

Since Insulin Growth Factor 1 (IGF-I), and Ghrelin play an integral role in regulating GH secretion could alterations in the levels of these proteins be in any way different during spontaneous hypoglycaemia as compared to be hypoglycaemia induced by the ITT? Changes in Insulin Growth Factor Binding Protein-1 (IGFBP-1) are inversely correlated to fasting and serum insulin levels and directly correlated with serum cortisol levels (Katz LE et al 1998). The increased serum IGFBP-I levels during fasting and hypoglycaemia is thought to prevent the hypoglycaemic action of free circulating IGFs (Katz LE et al 1998). Similarly Ghrelin levels increase during episodes of fasting and decrease during feeding (Ariyasu H et al 2001). Although the present study did not explore the role of IGF-I, IGFBP-1 or Ghrelin during spontaneous and ITT induced hypoglycaemia it is possible that these do respond differently to hypoglycaemia induced by the ITT and
hypoglycaemia induced spontaneously. Hence in this context further studies are needed to understand how hypoglycaemia induced spontaneously and by the ITT affects levels of IGFBP1 and Ghrelin.

6.2.5 Effect of physiological changes in serum Non-Estrerified-Fatty Acid (NEFA) concentrations on serum GH and cortisol response to spontaneous hypoglycaemia.

It is known from studies in adults that increases in plasma NEFA levels inhibit GH responses to a variety of pharmacological and physiological stimuli (Casanueva FF et al 1987). Since GH plays an important role in intermediary metabolism a feedback relationship has been postulated between NEFA and GH (Daughaday WH et al 1985). Pharmacological reductions in circulating NEFA cause GH release and NEFA elevations reduce or block GH secretion stimulated by a variety of physiological or pharmacological conditions (Fineberg SE et al 1972, Tsushima T et al 1970). It is thought that NEFA block GH secretion by acting directly at the level of the pituitary gland and block GH releasing hormone-stimulated GH secretion (Casanueva FF et al 1987). The interaction between GH, NEFA and the hormone leptin is an important mechanism by which the adipose tissue may be involved in regulating GH secretion.

There have been no previous studies investigating the effects of spontaneously generated NEFA in response to hypoglycaemia and GH secretion in children. In the present study the plasma NEFA concentrations ranged from 0.05mmol/L to 3.5mmol/L. The lowest plasma NEFA concentration was found in the group with hyperinsulinism due to the dominant anabolic effects of insulin inhibiting the lipolytic response to hypoglycaemia.
The highest plasma NEFA concentration was found in the children with the fatty acid oxidation disorder and in the group of children with ketotic hypoglycaemia. On analysing the effects of plasma NEFA concentration on the serum GH response to spontaneous hypoglycaemia there was no significant correlation between plasma NEFA concentration and serum GH levels. Children with HI have the lowest documented plasma NEFA concentrations and they would be expected to generate the highest serum GH responses. This study found no significant difference in the serum GH responses in children with low or high plasma NEFA. This finding is in contrast with the studies by Casanueva et al (Casanueva FF et al 1987). One reason for this difference could be related to the high pharmacological concentrations of NEFA used in the studies by Casanueva et al to assess the GH response to GHRH. In the study by Casanueva et al (Casanueva FF et al 1987), the plasma NEFA concentrations were increased to a maximum of 11.61 +/- 0.83mmol/L, and then the anterior pituitary stimulated with GHRH. It was shown that the GH response to GHRH was virtually completely blocked at this high plasma NEFA concentration. These plasma NEFA concentrations represent supraphysiological levels and are never observed in the normal physiological response to hypoglycaemia. The highest level of plasma NEFA concentration were found in our group of children was 3.5nmol/L. This is the normal physiological concentration of NEFA observed in this group of children.

It is not known in humans if ketone bodies play any direct role in stimulating or suppressing GH secretion from the hypothalamic-pituitary axis. Quabble et al (Quabble HJ et al 1983) investigated the effect of ketone body infusion on serum GH secretion and
found that the net GH response depended on the concentration of both NEFA and ketone bodies. The effect of ketone bodies on GH secretion was linked to the NEFA concentration. When ketone bodies and NEFA are elevated serum GH levels were suppressed. Studies in the rhesus monkey have shown that intra-ventricular infusions of beta-hydroxybutyrate may modulate the hypothalamic response to GH (Quabble HJ et al. 1996). In our study there was no correlation between total ketone body concentration and serum GH response. Children with HI again had the lowest serum ketone bodies as well as NEFA and hence would be expected to have high serum GH levels. In fact children with HI had the lowest serum GH responses to hypoglycaemia.

6.2.6 Different Isoforms of GH.

Since GH consists of a complex mixture of several structurally related isoforms with the 22 kDa isoform being the most abundant, it is theoretically possible that the predominant GH response to spontaneous hypoglycaemia may be with the 20kDa isoform. This study did not measure the 20 kDa isoform. Again this seems highly unlikely since in the ITT the predominant response was in the 22 kDa isoform. The antibody used for detecting serum GH in this study was specifically directed towards the 22 kDa isoform with no cross reactivity to 20 kDa. The physiological role of the 20 kDa isoform is not clear. There have been no studies looking at the role of the 20 kDa GH isoform in response to hypoglycaemia in childhood.

The precise mechanism responsible for the paradoxical serum GH and cortisol counter-regulatory hormonal responses to spontaneous hypoglycaemia are not clear. In summary
the serum GH response to hypoglycaemia generated by the ITT is different from that found in spontaneous hypoglycaemia. The inappropriately low serum GH response observed in spontaneous hypoglycaemia is not related to the time of sampling, the degree of hypoglycaemia, the plasma NEFA concentration or the serum cortisol concentration. The different serum GH responses to hypoglycaemia observed in the ITT may be related to the rapid decrease in the blood glucose concentration, as well as a rapid change in the prevailing metabolic milieu when insulin is administered. It is conceivable that insulin itself may have a central action. The finding of a poor serum GH response at the time of spontaneous hypoglycaemia does not necessarily imply GH deficiency nor does it imply that the poor GH response is the aetiology of the hypoglycaemia. This finding raises the fundamental question as to whether serum GH should be routinely measured in children with spontaneous hypoglycaemia.

The impact of intermittent and recurrent hypoglycaemia on counter-regulatory hormonal responses is discussed further in the next section.

6.3. Serum GH and cortisol responses to hypoglycaemia in babies with HI.

Infants with HI are subject to recurrent and severe hypoglycaemia, which if untreated can lead to permanent brain damage. Biochemically inappropriate insulin levels are present at the time of hypoglycaemia, with suppression of glycogenolysis, gluconeogenesis and lipolysis. The roles played by the various counter-regulatory hormones including cortisol in this condition are not clear. Morris et al (Morris AAM et al 1996) conducted a review
of all the diagnostic fasts in a tertiary referral centre over a 2.5-year period and highlighted the fact that neonates with hyperinsulinism generated the poorest cortisol responses to hypoglycaemia. In their study they did not explore the possible underlying mechanisms of this finding.

6.3.1 Cortisol response to hyperinsulinaemic hypoglycaemia.

The present study has shown that all thirteen neonates had a poor serum cortisol response to symptomatic hyperinsulinaemic hypoglycaemia. This serum cortisol response was blunted over the period of sampling for fifty minutes in the group of neonates who were not given ACTH at the time of hypoglycaemia. In the neonatal group that were given ACTH at the time of hypoglycaemia there was a significant (P<0.01) increase in the serum cortisol response at the end of the sampling period. In the six neonates in whom serum ACTH was measured at the time of hypoglycaemia, the serum ACTH was inappropriately low for the degree and severity of hypoglycaemia.

Neonates with HI require large amounts of dextrose to maintain normoglycaemia. The normal glucose requirement of 4-6mg/kg/min is significantly increased sometimes up to 20mg/kg/min (Aynsley-Green A et al 2000). During the diagnostic fast these neonates can become hypoglycaemic rapidly (fifteen minutes) when the intravenous fluids are stopped. Hence one possibility why they may be mounting such a poor serum cortisol counter-regulatory response is the rapidity with which the blood glucose concentration drops and thus not allowing the counter-regulatory drive to switch on. There may well be a time lag before serum cortisol is released because serum cortisol release is dependent
on ACTH. The fact that sampling for serum cortisol levels was continued for fifty minutes post hypoglycaemia should detect the delayed serum cortisol response. The inappropriately low serum ACTH at the time of hypoglycaemia suggests that the mechanism for the poor serum cortisol response may well be located at the level of the hypothalamus or pituitary.

Since babies with HI are subject to recurrent hypoglycaemic episodes it could be questioned whether the previous episodes of hypoglycaemia alter the counter-regulatory hormonal responses to subsequent episodes of hypoglycaemia? Studies in adults suggest that there may be some evidence for this. For example Davis et al (Davis MR et al 1991) investigated the counter-regulatory hormonal adaptation to recurrent hypoglycaemia in normal humans, and found that mild acute hypoglycaemia modulates counter-regulatory hormonal responses to a subsequent hypoglycaemic event. Prior hypoglycaemia episodes reduced serum cortisol response compared to a single hypoglycaemic event. None of the patients in the present study were hypoglycaemic for forty eight hours before conducting the controlled diagnostic fast. This implies that the poor serum GH observed in spontaneous hypoglycaemia in the older children and the poor serum cortisol response observed in hyperinsulinaemic hypoglycaemia are not related to depletion of these two hormones from the pituitary and adrenal glands respectively.

The effects of intermittent hypoglycaemia on counter-regulatory hormonal responses were examined in healthy volunteers by Widom et al (Widom B et al 1992). They compared the counter-regulatory hormonal response before and after four episodes of hypoglycaemia. They found that a lower blood glucose concentration was required to
elicit the secretion of counter-regulatory hormones during the fourth of the four daily consecutive episodes of hypoglycaemia compared with the first of such episodes. Possible explanations for this include central nervous system adaptation to hypoglycaemia or hormonal depletion due to repeated episodes of hypoglycaemia. The former hypothesis is supported by studies in which rats rendered either continuously (insulinoma or insulin pump) or intermittently (daily insulin injections) hypoglycaemic for 4 days were found to have an increase in blood brain glucose transport (McCall SL et al 1986). Kerr et al (Kerr D et al 1989) studied the effect of continuous and recurrent hypoglycaemia on the counter-regulatory hormonal responses in six healthy women. They found with respect to serum cortisol that there was a blunted response to recurrent hypoglycaemia in comparison to continuous hypoglycaemia. This is again unlikely to be the explanation in our group of babies since we were able to demonstrate a significant rise in serum cortisol after ACTH administration.

Davis et al (Davis MR et al 1997) studied the effects of specific levels of antecedent hypoglycaemia on subsequent counter-regulatory hormonal responses and found that in normal humans there is a hierarchy of blunted counter-regulatory hormonal responses that are determined by the depth of antecedent hypoglycaemia. In our patients with HI there was no hypoglycaemia in the preceding 48 hours before the fast was commenced. We were not able to assess the effects of specific levels of antecedent hypoglycaemia on subsequent counter-regulatory hormonal responses in our cohort of patients.

Babies with HI have inappropriate high insulin levels in relation to their blood glucose, and hence insulin per se may have a role in modulating the counter-regulatory hormonal
responses to hypoglycaemia. It is now clear that insulin has access to the blood brain barrier and can exert widespread modulatory influences on a number of important neuronal pathways (Baskin DG 1987). Insulin receptors are localised in the hypothalamus and insulin sensitive neurons are present in other parts of the central nervous system as mentioned earlier (Corp ES et al 1986, Havrankova J et al 1983). It has been shown that intrahypothalamic hyperinsulinism in newborn rats causes malformations and morphological alterations in the hypothalamic nuclei especially VMN and the LHA which are thought to play an important role as glucosensors (Plagemenn A et al 1999). It is tantalising to think that HI in the human newborn may also modulate the counter-regulatory hormonal response. The fact that the ACTH is inappropriately low for the degree of hypoglycaemia suggests that there may be a central component to this poor cortisol response.

Further compelling evidence that insulin itself may modulate the counter-regulatory hormonal response is provided by a study by Davis et al (Davis MR et al 1991). In this study an adult patient with an insulinoma showed a 63% reduction in cortisol counter-regulatory hormonal response to hypoglycaemia. After resection of the insulinoma, cortisol counter-regulatory responses normalised.

It is well established that the VMN and LHA areas of the brain are the glucose responsive regions. The specialized neurons in these regions respond to changes in extracellular glucose as a result of K\textsubscript{ATP} channel expression (Ashcroft MJL et al 1990a). As in the pancreatic β-cell these channels link cell excitability with metabolic status of the cell. The K\textsubscript{ATP} channel in the ventromedial hypothalamic region is thought to be similar to the
pancreatic β-cell with SUR-1 and KIR 6.2 components and responsive to diazoxide as well as tolbutamide (Inagaki N et al 1995b). Hence theoretically babies with HI who have mutations in the genes encoding SUR-1 and KIR6.2 may also have impaired function of the glucose responsive neurons in the hypothalamic area. It is speculative to think that the low ACTH found at the time of hypoglycaemia may reflect the abnormal function of the glucose responsive neurons in the hypothalamic region with reduced signal to the corticotrophs in the anterior pituitary. This present study did not investigate the serum cortisol responses in relation to the mutational analysis in babies with HI.

In conclusion babies with HI generate poor cortisol responses at the time of symptomatic hypoglycaemia. This poor cortisol response seems to be related to the inability to generate an adequate ACTH drive to the hypoglycaemic stimulus. The precise reason for the low ACTH is not clear but may be due to the central actions of insulin or a problem with the hypothalamic glucose responsive neurons, especially in those babies with abnormalities in the SUR-1 and KIR6.2 proteins. This study only concentrated on the cortisol responses in babies with HI and not on other causes of hypoglycaemia in the neonatal period. Therefore it is not possible to compare these responses with other causes of hypoglycaemia. The conclusions reached only apply to babies with HI.

6.3.2 GH response to hyperinsulinaemic hypoglycaemia.

Paradoxically all thirteen neonates showed appropriate and in some cases exaggerated growth hormone responses to the stimulus of hypoglycaemia. Term and premature neonates have been reported to have high serum GH concentrations (Ogilvy-Stuart AL et
al 1998). At birth serum GH concentrations in cord blood from term babies are approximately 100-fold higher than that in adults (Adrian TE et al 1983). By the sixth postnatal day basal pre-feed GH levels fall in term neonates by 65%. The higher GH levels in term neonates and especially in premature babies may be related to the decreased negative feedback associated with low IGF-I levels (Miller JD et al 1992). The neonatal somatotrophs may also not fully respond to the GH inhibitory action of somatostatin.

The clinical relevance of this elevated basal GH level in the neonatal period is not clear. In the neonatal period the most abundant form of GH is the 22 kDa isoform with neonates producing the same fractions of other isofoms such as the 20 kDa GH as in adults (Boguszewski CL et al 2000). All our patients had elevated serum GH values even before the time of hypoglycaemia. These elevated serum GH levels persisted during the time of sampling. Although the serum GH level increased at the time of hypoglycaemia this was not significantly different from the level before the time of hypoglycaemia.

6.3.3 Plasma insulin in relation to blood glucose concentration.

On comparing the plasma insulin concentration against the plasma glucose there was no correlation. The plasma insulin concentrations were raised in all infants with HI at the time of hypoglycaemia. The plasma insulin concentrations ranged from 3.7-18.7 μu/l. These levels of insulin are inappropriate for a blood glucose concentration less than 3mmol/l, suggesting a basic defect in mechanisms regulating insulin secretion. It is important to note that the insulin concentrations are not very high and yet these children
had high glucose requirements. Hence it is speculative to think that there may be an element of increased insulin sensitivity in the gluco-responsive tissues such as the muscle or liver. This degree of hyperinsulinaemia was sufficient to suppress fatty acid mobilisation and ketone body formation as shown by the poor plasma NEFA and ketone body response in our group of infants. For a given level of blood glucose concentration plasma insulin can be very variable, for example at a blood glucose concentration of 2.4mmol/L the plasma insulin levels can be either 8μu/L or 18μu/L. Hence for the diagnosis of HI the insulin value at the time of hypoglycaemia need not be necessarily high.

6.3.4 Comparison of plasma Insulin against serum cortisol.
Plasma insulin levels were raised in all babies with HI as noted above. On comparing the plasma insulin levels with serum cortisol at the time of hyperinsulinaemic hypoglycaemia there was no significant correlation (R= 0.16). A raised plasma insulin level did not seem to have any impact on the serum cortisol response generated.

6.3.5 Serum GH levels in comparison to birth weight in babies with HI.
Babies with HI are classically born with macrosomia. Since the serum GH was high in this group of babies, a comparison was made between the birth weight of these babies with the serum GH levels. There was no significant correlation between birth weight and the level of serum GH. All babies with HI had elevated serum GH levels. Again the clinical relevance of this is unclear.
In conclusion babies with HI show poor serum cortisol counter-regulatory hormonal response to symptomatic hypoglycaemia and this response persists for at least 50 minutes. The precise aetiology of this remains unclear but is not related to the depletion of cortisol from the adrenal gland. There is a suggestion that the mechanism may be related to the lack of drive from the central nervous system. Whether this low serum cortisol is associated with increased sensitivity of the peripheral tissues to insulin has not been established, nor is the benefit of supplementing these babies with hydrocortisone. These babies are not cortisol deficient but lack the ability to initiate ACTH release from the anterior pituitary. This may further be related to the lack of stimulus from the hypothalamic glucose responsive neurons. Since the K<sub>ATP</sub> channels are important in glucose sensing further studies will be needed to understand the link between mutations in the K<sub>ATP</sub> channel and serum cortisol counter-regulatory hormonal responses.
6.4 Overview and ideas for future research

In summary this study has shown that children with spontaneous hypoglycaemia have inappropriately low serum GH responses and appropriate serum cortisol responses. The serum GH response to ITT induced hypoglycaemia is different compared to that observed in spontaneous hypoglycaemia. Physiological changes in the serum concentrations of NEFA and ketone bodies do not have any significant effect on the serum GH response at the time of spontaneous hypoglycaemia. In the neonatal group with HI this study has shown that there is an inappropriately low serum cortisol response at the time of hyperinsulinaemic hypoglycaemia. This inappropriately low serum cortisol response is due to a lack of drive from the pituitary corticotrophs as evidenced by the low serum ACTH at the time of hyperinsulinaemic hypoglycaemia. These neonates have sufficient cortisol reserve in the adrenal glands, since ACTH administration at the time of hyperinsulinaemic hypoglycaemia elicits a marked serum cortisol response which persists for fifty minutes.

What are the implications of these finding to clinical practice and how can we try and understand further as to the mechanisms of these findings?

Serum GH has been routinely measured at the time of hypoglycaemia although even prior to this study it has been observed that serum levels of GH correlate poorly with spontaneous hypoglycaemia (Aynsley-Green A et al 1991). The present study has confirmed the finding of Aynsley-Green et al (1991) and further shown that the inappropriately low serum GH response to spontaneous hypoglycaemia is not related to missing peaks of pulsatile GH secretion.

The present study has also shown that a low serum GH response at the time of
spontaneous hypoglycaemia is not the cause of the hypoglycaemia. Since serum GH is routinely measured in children with spontaneous hypoglycaemia this raises the fundamental question as to whether serum GH should be routinely measured in all children with spontaneous hypoglycaemia. If GH deficiency is thought to be the underlying cause of the hypoglycaemia then provocation testing with glucagon or the ITT would be more appropriate. Other clinical data (for example, growth velocity) as well as biochemical (serum IGF I, IGFBP3 measurements) clues may be more important pointers to GH deficiency.

The present study has shown that the serum GH response to spontaneous hypoglycaemia is fundamentally different from that observed in the ITT. The precise mechanism for this still remains unclear. Further studies need to be designed to investigate in detail as to how the ITT causes rapid changes in plasma glucose as well as intermediary metabolites such as serum NEFA and ketone bodies concentrations.

As for the neonatal group with HI the present study has shown that the inappropriately low serum cortisol responses are due to a lack of ACTH from the pituitary corticotrophs. This is clinically extremely relevant since the finding of a low serum cortisol in these babies is sometimes associated with the administration of hydrocortisone replacement therapy. There is no evidence to suggest that hydrocortisone replacement therapy benefits these babies. Hydrocortisone replacement therapy has no effect on the underlying disease process in HI. It is conceivable that by raising serum cortisol levels as a result of administering exogenous hydrocortisone the sensitivity to insulin in the major insulin sensitive tissues such as the liver and muscle may be altered. Hence
glucose turnover studies using stable isotopes can be designed to investigate the impact of hydrocortisone replacement therapy

The mechanism for the low serum ACTH is still unclear. Further studies are required to investigate what role CRF plays in this respect. These babies can be given CRF at the time of hypoglycaemia and the serum ACTH response measured. This will allow a greater insight into whether the low serum ACTH is due to lack of drive from hypothalamic CRF or whether the problem is inherent in the pituitary corticotrophs.
6.5 Appendix 1

Patient Information Sheet

1. The aim of the study

The aim of this project is to investigate the effect of low blood sugar (hypoglycaemia) on the hormones that are involved, under ordinary circumstances, in returning blood sugar levels back to normal values. In adult patients with hypoglycaemia there is a classical response of special hormones known as cortisol and growth hormone, which try to counteract the effects of the low blood sugar. In children we do not fully understand the precise way in which these hormones are released in response to hypoglycaemia. As a result it is sometimes difficult to be sure whether these hormones are working properly or not and we often have to do further and even more complicated tests to answer that question.

2. Why is the study being done?

One of the reasons that we have found it difficult to clarify the role of cortisol and growth hormone is the need to take lots of small blood samples over a long period of time to precisely define the changes in cortisol and growth hormone. Further, the timing of growth hormone and cortisol release may well be different in children compared to adults and there may be a difference between the sexes.

3. How is the study to be done?

If you agree to participate in this study, your child will have some additional blood samples taken before and when they are hypoglycaemic. Small amounts of blood will be taken at approximately 10 minute intervals after the episode of hypoglycaemia for 50 minutes. The extra samples that we take will do no harm to your child as the amounts of
blood we require are extremely small and the total amount we would remove would only fill half an egg-cup. Children rapidly make up that type of reduction in blood.

4. What are the risks and discomfort?

No risk to the child can be foreseen. There is discomfort from the insertion of the cannula but this has to be done anyway and we would normally numb the skin anyway with local anaesthetic cream.

5. Who will have access to the case/research records?

Only the researchers and a representative of the Research Ethics Committee will have access to the data collected during this study.

6. What are the arrangements for compensation?

This research has been approved by an independent Research Ethics Committee who believe that it is of minimal risk to your child. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study.

This research is covered by a no-fault compensation scheme which may apply in the event of any significant harm resulting to your child from involvement in the study. Under this scheme it would not be necessary for you to prove fault. You also have the right to claim damages in a court of law. This would require you to prove fault on the part of the Hospital/Institute and/or any manufacturer involved.

7. What are the potential benefits?

This study will not bring any immediate benefits to your child. However, we believe that the study will provide us with important information on how young children generate cortisol and growth hormone in response to a low blood sugar. It is likely that with this
information we will not need to perform further detailed investigations of cortisol and
growth hormone in your child.

8. Do I have to take part in this study?

If you decide, now or at a later stage, that you do not wish to participate in this research project, that is entirely your right and will not in any way prejudice any present or future treatment.

9. Who do I speak to if problems arise?

If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by post via the Research and Development Office, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, or if urgent, by telephone on 0171 242 9789 ext 2620 and the Committee administration will put you in contact with him.

10. Details of how to contact the Researcher:

If you have any questions about the research project please contact Dr. K.Hussain in the first instance.
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6.7 Publications

Publications arising from this work.


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**Published Abstracts**


Hussain K, Chapman JC, Shepherd RM, Cosgrove KE, Swift SM, Dunne MJ Aynsley-Green A. Defects in ATP-sensitive K⁺ channels are not causality related to
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Lindley KJ, Bitner-Glindzicz M, Glaser B, Dunne M, **Hussain K, Aynsley-Green A.** A 120-kb contiguous gene deletion on chromosome 11p causing severe infantile hyperinsulinism (HI), Sensorineural Hearing Loss (SHL), Inflammatory Enteropathy (IE) and a Renal Tubulopathy (RT) provides new insights into the control of the development and structure of these organ systems. *Hormone Research* 2000; 53(suppl 2): 25.


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### 6.8 Presentations

**European Network for Research into Hyperinsulinism, Workshop, May 1999 in Helsinki, Finland. Insulin secreting adenoma. K.Hussain, A.Aynsley-Green. (Oral).**

**European Network for Research into Hyperinsulinism, Workshop, May 1999 in Helsinki, Finland. Impaired cortisol counter-regulatory response to persistent hyperinsulinaemic hypoglycaemia in infants. K.Hussain, P.Hindmarsh, A.Aynsley-Green. (Oral).**


Society for the Study of Inborn Errors of Metabolism (SSIEM), September 7-10th 1999, Genoa, Italy. Hyperinsulinaemic hypoglycaemia associated with abnormal urine organic


Royal College of Paediatrics and Child Health (RCPCH), 4th Spring Meeting University of York, United Kingdom, 10-13 April, 2000. Lack of growth hormone response to spontaneous hypoglycaemia. K. Hussain, P. Hindmarsh, A. Aynsley-Green. (Oral).

American Academy of Paediatrics/Society for Paediatric Research, 12-16th May 2000, Boston, Massachusetts, USA. *Defects in ATP-sensitive K*⁺* channels are not causality related to hyperinsulinism in an infantile adenoma.* K.Hussain, JC.Chapman, RM.Shepherd, KE.Cosgrove, SM.Swift, MJ.Dunne, A.Aynsley-Green. (Poster).


Neonatal hyperinsulinism (HI) is associated with abnormal cortisol responses to hypoglycaemia (HY). K.Hussain, P.Hindmarsh, A.Aynsley-Green. (Poster).

Spontaneous hypoglycaemia in childhood is accompanied by paradoxical cortisol and growth hormone counter-regulatory hormonal responses. K.Hussain, P.Hindmarsh, A.Aynsley-Green. (Poster).

Hyperinsulinism in infancy is one of the most difficult problems to manage in contemporary paediatric endocrinology. Although the diagnosis can usually be achieved without difficulty, it presents the paediatrician with formidable day to day management problems. Despite recent advances in understanding the pathophysiology of hyperinsulinism, the neurological outcome remains poor, and there is often a choice of unsatisfactory treatments, with life long sequelae for the child and his or her family. This paper presents a state of the art overview on management derived from a consensus workshop held by the European network for research into hyperinsulinism (ENRH). The consensus is presented as an educational aid for paediatricians and children’s nurses. It offers a practical guide to management based on the most up to date knowledge. It presents a proposed management cascade and focuses on the clinical recognition of the disease, the immediate steps that should be taken to stabilise the infant during diagnostic investigations, and the principles of definitive treatment.

Arch Dis Child Fetal Neonatal Ed 2000;82:F98–F107

Keywords: hyperinsulinism; treatment; hypoglycaemia; neurological damage; cation channels

Hyperinsulinism is the most common cause of persistent or recurrent hypoglycaemia in infancy. It is a major cause of neurological damage and life long handicap, and the fact that the incidence of such damage (in up to 20% of survivors) has changed little during the past 20 years2 reflects the major difficulties in managing the disease.

Until recently, the pathogenesis of hyperinsulinism remained an enigma, but during the past five years there has been an explosion of knowledge concerning the molecular genetics and the membrane physiology of the condition, which has given not only new insights into its cause, but has also opened up new concepts of treatment.

It is now clear that the uncontrolled release of insulin is the final manifestation of a number of different processes that either alter intra-cellular biochemical pathways of the pancreatic β-cell (thereby generating abnormal signals for the secretion of insulin), or alter the transport of cations across the cell membrane. These abnormalities perturb the stimulus-secretion coupling mechanisms that normally ensure that the amount of insulin secreted is directly related to the ambient blood glucose concentration.

Therefore, hyperinsulinism is characterised by the presence of insulin concentrations that are inappropriately high for the concentration of blood glucose. A "normal" insulin concentration for normoglycaemia is inappropriate in the presence of hypoglycaemia!

Despite the new knowledge of pathogenesis, the management of the disease still presents the paediatrician with a choice of unsatisfactory treatments that have major long term implications for the child and his or her family.

Because of the urgent need to improve prognosis, the European network for research into hyperinsulinism (ENRH) was created in 1997 through the support of the European Union. The network brings together leading basic and clinical scientists from seven European countries to encourage collaboration, generate new scientific data, and from these, to define a consensus guide to management.

A workshop was held in Finland in May 1999 to consider the latter, and this paper reflects the debate that occurred. As in any other area of medicine, consensus was achieved for many of the components of the management cascade. Nonetheless, controversy still surrounds other aspects, and these are defined and considered below. Further areas of clinical research that need to be developed are also highlighted.

Abstract

Background

Hyperinsulinaemic hypoglycaemia has previously masqueraded under a variety of different descriptive names, including “idiopathic” hypoglycaemia of childhood, leucine sensitive hypoglycaemia, neonatal insulinoma, pancreatic microadenomatosis, nesidioblastosis, persistent hyperinsulinaemic hypoglycaemia of infancy, and congenital hyperinsulinism. Both sporadic and familial forms of the disease are recognised, the former having an estimated incidence in western Europe of one in 5000.
Management of hyperinsulinism in infancy

... In isolated European communities, including parts of Finland, the disease incidence is much higher, the highest incidence is found in societies with high rates of consanguinity. In these cultures, particularly in the Arabian peninsula, the incidence may be as high as one in 2500 births.

Clinical presentation and immediate management

Most infants with hyperinsulinism present during the 1st postnatal days, with others during the 1st year. Rarely, older children present de novo with symptoms of hypoglycaemia. Figure 1 shows the age distribution in one large series of cases.

Hyperinsulinism can be associated with well-defined clinical conditions. Thus, the Beckwith-Wiedemann syndrome manifests a transient hyperinsulinism and these babies have pathognomonic physical signs including exomphalos, macroglossia, and transverse creases of the ear lobes. This disease and the hyperinsulinism caused by maternal diabetes, rhesus incompatibility, perinatal asphyxia, and maternal glucose and drug treatment during delivery will not be considered further in this paper.

Infants with hyperinsulinism might have a characteristic appearance with macrosomia, strongly resembling the infant of a diabetic mother. The appearance suggests the occurrence of prenatal hyperinsulinism. However, not all infants have this appearance, and some of the most difficult management problems arise in infants born of normal or low birth weight, including preterm infants.

The first clinical manifestations of hyperinsulinism include non-specific features such as "floppiness", "jitteriness", poor feeding, and lethargy. More dramatically, the infants may have seizures, coma, and even averted neonatal death.

The importance of accurately measuring blood glucose in the presence of any of these symptoms cannot be overemphasised.

Considerable controversy surrounds the definition of neonatal and infantile hypoglycaemia. Pragmatically, any infant with a persistent measurement of blood glucose less than 2.6 mmol/litre (as measured by an accurate laboratory method and not a bedside screening test), particularly when accompanied by symptoms, should be the focus of particular attention, and the diagnosis of hyperinsulinism considered. Any persistent hypoglycaemia, whether symptomatic or not, needs investigation and treatment.

Blood must be drawn and saved at the time of hypoglycaemia (together with the next urine sample passed) for assay of the substances listed in Table 1.

A baby who cannot maintain normoglycaemia despite frequent enteral feeds (if necessary via a nasogastric feeding tube) needs to have the support of an intravenous glucose infusion to prevent neuroglycopenia. It should commence at the normal neonatal and infantile hepatic production rate of glucose; namely, between 4–6 mg/kg/min. The infusion rate should then be titrated upwards as necessary to maintain the blood glucose value above 2.6–3.0 mmol/litre.

The definition of glucose requirement to maintain normoglycaemia is a key diagnostic as well as therapeutic step. The demonstration of an increased glucose requirement is a powerful indicator of underlying hyperinsulinism.

Many children require infusion rates in excess of 15–20 mg/kg/min. Clearly, it is essential to document that the infant actually needs high infusion rate. Infants are often referred to regional centres with alleged high glucose requirements, but on being challenged by a cautious decrease are found not to be so dependent. It is, of course, possible that the glucose dependency may change dramatically for the better in the 1st postnatal day, particularly in infants who may have experienced mild birth asphyxia.

The need to assess carefully and regularly the glucose infusion requirement without causing severe hypoglycaemia, symptomatic or not, is a key aspect of the initial management process.

In infants who are dependent on high rates of glucose infusion, the insertion of a central venous catheter through the umbilicus or via a peripheral vein is mandatory. This is because the sudden loss of a peripheral venous cannula in a child who has had multiple venepunctures can cause a rapid onset of dramatic symptomatic hypoglycaemia during attempts to re-site the line.

The need for early referral to a specialist centre

Participants at the consensus workshop unanimously criticised the current practice of late...
referral to a centre experienced in the management of hyperinsulinism, and urged earlier transfer.

Practitioners should not underestimate the practical difficulties of managing infants with hyperinsulinism, not least because of the volatility of glucose control and the vulnerability to unexpected and unpredictable severe hypoglycaemic episodes, but also because of the risks of fluid overload and sepsis contributing to the considerable morbidity encountered by these infants.

Of central importance is the need for dedicated nursing support delivered in a unit that has the infrastructure, quality standards, and training for rapid and repeated accurate blood glucose measurement. Protocols for the emergency management of the hypoglycaemic episodes need to be in place, and staff should have the experience and the knowledge to impart confidence to parents at a time of major emotional stress.

Members of ENRHI argue that late referral to a specialist centre after days or even weeks of fruitless attempts to make a diagnosis and establish effective treatment is one of the main reasons why the neurological morbidity remains so high. Indeed, because of the propensity of this disease to cause neural damage, it could be deemed negligent not to transfer early.

The transfer process itself needs to be carefully orchestrated, as outlined in Table 2. Transfer without a secure intravenous line and a trained nursing or medical escort is not an acceptable practice.

Establishing the diagnosis and the cause of hyperinsulinism

Figure 2 outlines the consensus model for the normal control of insulin secretion, and the local abnormalities that can occur. Knowledge of this simple schematic is essential to understand the rationale for the diagnostic and management cascades.

The biochemical diagnosis

There is now a well-established consensus on the primary and secondary diagnostic indicators for hyperinsulinism, as shown in Table 3. In many children, the diagnosis depends on the aggregation of several components, particularly because in the neonate the normal tight association seen in older children and adults between plasma insulin and blood glucose concentrations is not present. Attention is drawn to the following:

- The characteristic metabolic profile of hypoketonacidemic hypoglycaemia arises from the anabolic effects of insulin preventing metabolic counterregulation as the blood glucose concentration falls.
- The presence of any measurable insulin during hypoglycaemia is strongly indicative of a defect in regulating basal insulin secretion in the face of a falling blood glucose concentration. The interpretation of the results depends on the sensitivity and specificity of the local insulin assay.
- Hyperinsulinism can occur in the absence of florid hyperinsulinism—a normal insulin concentration for normoglycaemia is inappropriate in the presence of hyperinsulinism.
- Insulin release is pulsatile and measurement of C peptide might be more helpful than a single measurement of insulin because it reflects overall insulin production; there is little extra value gained from measuring proinsulin concentrations.

Table 2 Recommended guidelines to referring hospitals for transfer of babies with hyperinsulinism (transfer of babies/children with hypoglycaemia, regardless of aetiology)

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Before transfer discuss the case with the endocrine registrar/consultant on call</td>
<td></td>
</tr>
<tr>
<td>2. Must have secure intravenous access at all times before transfer (even if not requiring intravenous fluids at time of transfer)</td>
<td></td>
</tr>
<tr>
<td>3. Baby child must be transferred with nurse and doctor escort</td>
<td></td>
</tr>
<tr>
<td>4. Before transfer ensure you have:</td>
<td></td>
</tr>
<tr>
<td>- 10% dextrose (preferably 500 ml bag)</td>
<td></td>
</tr>
<tr>
<td>- Hypostop/sugary drink</td>
<td></td>
</tr>
<tr>
<td>- Glucagon intramuscular/intravenous 0.1 mg/kg up to 1 mg maximum</td>
<td></td>
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<tr>
<td>5. Monitoring:</td>
<td></td>
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<tr>
<td>- Check blood glucose before leaving, then hourly if stable, or 15-30 minutes if unstable</td>
<td></td>
</tr>
<tr>
<td>- Recheck blood glucose 15 minutes later. If still low increase infusion by 1 mg/kg/min until normoglycaemia attained</td>
<td></td>
</tr>
<tr>
<td>- If intravenous access lost give:</td>
<td></td>
</tr>
<tr>
<td>- (a) Hypostop and repeat blood glucose in children &gt; 2 years</td>
<td></td>
</tr>
<tr>
<td>- (b) Give one dose of glucagon and repeat blood glucose measurement for infants</td>
<td></td>
</tr>
<tr>
<td>6. Hypoglycaemic event during journey (blood glucose &lt; 2.6 mmol/litre)</td>
<td></td>
</tr>
</tbody>
</table>

Glucose requirements > 6–8 mg/kg/min to maintain blood glucose above 2.6–3 mmol/litre
Laboratory blood glucose < 3.6 mmol/litre
Detectable insulin at the point of hypoglycaemia with raised C peptide
Inappropriately low blood free fatty acid and ketone body concentrations at the time of hypoglycaemia
Glycemic response after the administration of glucagon when hypoglycaemia
Absence of ketonuria

Figure 2 The sequence of events leading to the release of insulin from a pancreatic β cell. In brief, SUR1 and Kir6.2 constitute the ATP sensitive potassium (KATP) channel. These two proteins are encoded by genes located on chromosome 11p15.1. Mutations in these genes account for some cases of hyperinsulinism. The KATP channel is normally kept open by ATP concentrations at the time of hypoglycaemia. ATP raises the cytosolic ATP to ADP ratio, which in turn causes the closure of the KATP channel. Once closed, the cell membrane becomes depolarized, allowing the influx of calcium through specific voltage gated calcium channels. This is the trigger for insulin exocytosis. Mutations have also been described in the genes for glucokinase dehydrogenase (GDKH) and in the glucokinase (GK) system. These defects increase the intracellular ATP to ADP ratio, thereby initiating insulin secretion. In these conditions, there is no abnormality in the potassium or calcium channel function. PHHI, persistent hyperinsulinemic hypoglycaemia of infancy; glucose-6-P-glucose-6-phosphate. (Reproduced with permission from Shepherd et al).
• Very high glucose infusions rates (greater than 20 mg/kg/min) might be needed to secure normoglycaemia. Titration of the infusion rate against the blood glucose concentration achieved allows the impact of changes in treatment to be assessed.

• Progressive hepatic enlargement, presumably as a result of glycogen deposition, might occur during high infusion rates of glucose, but also secondary to fluid retention as a result of high fluid infusion rates and diazoxide treatment.

• Babies show a glycaemic response to glucagon injection when hypoglycaemic because of the mobilisation of glycogen deposits from the liver. This phenomenon can be of diagnostic as well as therapeutic benefit. There is a lack of consensus over the dose of glucagon used in different centres, ranging from 0.1 mg/kg to a single overall dose of 1.0 mg. In general, a positive response can be said to occur when there is a clear increment in blood glucose despite severe hypoglycaemia. The injection of glucagon can be useful at times of emergency, such as the re-siting of a venous cannula. Glucagon should be administered with caution because of its insulin stimulatory effect and the propensity to cause rebound hypoglycaemia.

• Other markers of hyperinsulinism including low circulating levels of branched chain amino acids at the time of hypoglycaemia are of research interest only. The measurement of blood ammonia concentration is now mandatory in view of the recent description of the syndrome of hyperammonaemic hyperinsulinism. This condition is likely to be the explanation of the so-called “leucine sensitive” hyperinsulinism in which children show sensitivity to protein ingestion.

• Plasma concentrations of cortisol and growth hormone might not be consistently raised during episodes of even severe symptomatic hypoglycaemia. Nonetheless, congenital hypopituitarism is an important differential diagnosis to consider; in this diagnosis, however, the plasma insulin concentration should be undetectable, and there may be other stigmata of the condition including mid line defect and, in boys, micropenis. If in doubt, specific pituitary stimulation tests should be performed at the tertiary referral centre.

• An increase in the blood glucose concentration after somatostatin injection is suggestive of hyperinsulinism, but it is not recommended as a routine diagnostic criterion.

Establishing the Genotype and Clinical Phenotype

Advances in the molecular genetics of the disease have shown hyperinsulinism to be caused, in about 30–50% of patients, by abnormalities in either genes controlling intracellular metabolic pathways or membrane cation transport. Most abnormalities relate to defects in the genes controlling the sulphonylurea receptor (SUR1) and the inward rectifier potassium channel (Kir6.2), proteins that together form the functional ATP dependent potassium channel (Kir6.2/SUR1) in the β cell membrane. Over 40 different mutations have been reported, most of which are recessively inherited. Domi-

nantly expressed abnormalities have also been described. Although the molecular genetics of some of these cases are now known, there is no information on the pancreatic lesion in these patients because most patients with autosomal dominant hyperinsulinism do not require surgery.

Recently, a further twist has been added to the story of the genetics of hyperinsulinism, with the discovery that “focal” hyperplasia in the endocrine pancreas is the result of a unique combination of events: somatic loss of the maternal allele on the short arm of chromosome 11, in a patient harbouring an SUR1 mutation on the paternal allele. The juxtaposition of SUR1 and several imprinted genes on chromosome 11p15 appears to be responsible for this unique genetic mechanism of disease.

Gene mutations that cause abnormal activation of glucose signalling of insulin secretion by altering the enzyme kinetics of glutamate dehydrogenase or of glucokinase have been described recently. Other loci have yet to be defined in rare patients who present with hyperinsulinism in association with lactic acidosis. Because these abnormalities are not associated with abnormal Kir6.2/SUR1 channels, most patients with these defects respond to diazoxide.

Despite these recent developments, less than 50% of patients studied to date have a definable genetic abnormality. Therefore, it is likely that other abnormalities will be revealed as the search continues for new defects in the secretory apparatus, including defects in the intracellular control of calcium signalling of exocytosis. For a more detailed description of the genetics of hyperinsulinism see Glaser et al elsewhere in this issue.

Unfortunately it is not yet possible to use genetic analysis routinely to screen for mutations except in specific populations with unique and characteristic abnormalities usually associated with consanguinity.

There is considerable variation in the clinical phenotype of severity in hyperinsulinism. In general, children with hyperinsulinism born to consanguineous parents, particularly those of Jewish or Arabic ancestry, have a severe form of the disease that is usually resistant to medical treatment. This also applies to patients from particular regions in Finland. It should be predicted that these patients are likely to require early surgery to control the hypoglycaemia. Siblings are also at risk of the disease and should be screened for the onset of neonatal hypoglycaemia.

In most European and white children, however, there is no family history of intermarriage or afflicted members. Moreover, with the exception of hyperammonaemic hyperinsulinism it is not possible to predict at presentation which infants will respond dramatically to diazoxide treatment, although in general, neonates presenting with severe hypoglycaemia in the 1st
postnatal days have the most unresponsive form. It is equally impossible to predict which infants will have "transient" as opposed to "persistent" hyperinsulinism. In general, children with hyperammonaemic hyperinsulinism and upregulation of glucokinase have a milder disease phenotype, which usually responds rapidly to moderate doses of diazoxide, but a genetic heterozygosity certainly exists.

**IMAGING OF THE PANCREAS**

The need for methods to identify "focal" areas of hyperplasia has been given new impetus through the reports that preoperative percutaneous trans-hepatic pancreatic venous sampling can identify "hot spots" of insulin hypersecretion. In children, preoperative pancreatic venous sampling should be performed under general anaesthesia in an interventional angiographic room. Care should be taken to maintain blood glucose concentrations at between 2.5 and 3 mmol/litre. The right portal vein is punctured with a 21 gauge catheter needle. Specially shaped 3F catheters are then exchanged over a guide wire. Multiple venous samples are taken in the splenic, mesenteric, and portal veins but also in the small pancreatic veins draining the head, the body, and the tail of the pancreas. The concentrations of insulin, glucose, and C peptide are mapped according to the venous anatomy of the pancreas. A focal form is diagnosed when a single or two contiguous veins have high amounts of insulin. The C peptide to insulin ratio is close to one in those veins in that setting. A diffuse form is diagnosed when several non-contiguous veins have high concentrations of insulin. In cases of high concentrations of glucose the mapping is non-contributive. In a few patients, despite a good control of glucose below 3 mmol/litre, all the sampled veins show low or normal concentrations of insulin. These patients correspond to focal forms in which the draining vein of the lesion has not been selectively sampled. When followed by meticulous intraoperative microdissection, aided by immediate histological confirmation of hyperplasia, there is evidence that the long term prognosis is much better than in patients subjected to blind 95% partial pancreatectomy. Furthermore, focal disease might occur only in the head of the pancreas, and this may not be resected with blind partial pancreatectomy.

The ability to identify focal endocrine hyperplasia is a new and major advance in diagnosis and management. However, the logistic aspects of this approach are substantial in terms of the availability of staff experienced in interventional radiology and in pancreatic histology. Moreover, expert nursing care is needed because the infants are at serious risk of hypoglycaemia because medical treatments need to be discontinued for several days before the procedure. In addition, the imaging procedure requires that the infant’s blood glucose be brought as close as possible to hypoglycaemic values for up to several hours. The technique requires further evaluation before it can be recommended for general use. Moreover, because of the logistic problems, the consensus work-

shop recommended that the use of the technique should be restricted to one or two national centres in each country that have the comprehensive resources needed to manage the children competently.

Alternative means have been proposed for identifying focal disease. These include the intra-arterial calcium stimulation test, which is based on the insulin stimulatory effect of rapid selective injection of calcium into the branches of the coeliac arteries supplying the different parts of the pancreas. This technique also requires further evaluation, with members of the consensus workshop expressing concern over the potential for infarction of the coeliac arteries.

Other techniques, including preoperative and intraoperative direct pancreatic ultrasound, computed tomography, magnetic resonance imaging, and coeliac angiography have been used by many investigators. The limited published information has not shown these techniques to be of value, particularly in the neonate. However, imaging should be considered in the older child in whom the possibility of the presence of an isolated adenoma is high.

The consensus workshop participants agreed that currently available imaging techniques were not of value in the routine investigation of neonatal and early infantile hyperinsulinism; they should only be considered in the search for an insulinoma in an older child presenting with recent onset of hypoglycaemia.

**Management**

It is important at the outset to identify the objectives of management. They are to: (1) prevent hypoglycaemic brain damage and allow normal psychomotor development; (2) establish normal feed volume, content, and frequency for the age of the child; (3) ensure normal tolerance to fasting for age with developing hypoglycaemia; and (4) maintain family integrity.

A regimen cannot be said to be successful if it does not fulfil these objectives. However, practice it might be very difficult to achieve them.

**MEDICAL TREATMENT**

**Initial stabilisation**

The mainstay of initial medical treatment is provision of adequate carbohydrate to maintain blood glucose concentrations above 2.3.3 mmol/litre. Very high infusion rates of glucose (> 20 mg/kg/min) in addition to frequent enteral feeds may be required. This may demand the insertion of a central venous catheter to allow the administration of glucose high concentrations, together with a nasogastric feeding tube for regular feeds. Glucose polymer can be added to the enteral feeds to increase the carbohydrate intake. However, it is important not to cause too great an osmotic load in the gastrointestinal tract that might predispose to the onset of necrotising enterocolitis. Because of the immaturity of intestinal amylase during the 1st year of life, the administration of cornstarch might not be effective.
Hyperinsulinism in infancy

With reports ranging from 15% to 60%, or will respond in vitro to higher concentrations. There is little could perhaps reflect the selection of cases with which infants respond fully to these drugs, there is controversy over the frequency overcome the fluid retaining effects of diazoxide by different mechanisms, the diuretic pharmacokinetics of diazoxide in childhood.

A logical progression through the available treatments is proposed (table 4), which allows a clear definition of the clinical phenotype. The essential principle of management is to introduce only one change in dose or treatment at a time and to assess the impact before moving on to the next change.

The drugs of first choice are those that can be given orally, because some children may respond very well, even if they require ongoing treatment for months or years. It is reasonable to argue that it is essential to prove that a child will not respond to such agents before introducing more powerful hormones.

Diazoxide (10–20 mg/kg/day in two to three divided doses) and chlorothiazide (7–10 mg/kg/day in two divided doses) are recommended for initial treatment and should always be given together. Both these agents activate potassium channels by different mechanisms, the diuretic also being given concurrently for its ability to overcome the fluid retaining effects of diazoxide. There is controversy over the frequency with which infants respond fully to these drugs, with reports ranging from 15% to 60%, or more. This difference in responsiveness could perhaps reflect the selection of cases being referred to the centres. There is little information on the pharmacodynamics or pharmacokinetics of diazoxide in childhood. Children who fail to respond to a dose of 15–20 mg/kg/day might have pancreata that will respond in vitro to higher concentrations. In practice, the limit of clinical tolerance is determined by the magnitude of side effects, in particular fluid retention and cardiac failure, and most children who will respond do so at doses up to 15 mg/kg/day.

It is logical, in addition to activating potassium channels by diazoxide and chlorothiazide, to try to decrease voltage dependent calcium channel activity by blocking agents, such as the slow release form of nifedipine at a dose of 0.25–2.5 mg/kg/day. The clinical response to this drug is highly variable, an observation that has now been explained by studies performed in vitro on β cells from patients with persistent hyperinsulinaemic hypoglycaemia of infancy. Thus, some children with KATP channel abnormalities might have a concurrent defect in voltage gated calcium channels. It is not possible to predict which children will respond without trying the drug. The drug has not proved as useful as was first hoped, yet nifedipine may still have a role as an adjuvant, although, the long term effects of continuous treatment are unknown. The drug is light sensitive and there are practical difficulties in its formulation when used in small children. Further research is needed to define the precise value and role of calcium channel blocking drugs; nonetheless, their use is one tangible benefit that has arisen from a better understanding of the pathophysiology of the β cells in this condition.

The side effects of these drugs are important. Diazoxide causes fluid retention that can precipitate cardiac failure. Rarely, it causes a blood dyscrasia. The most distressing effect from the parental viewpoint is the stimulation of generalised hypertrichosis. Nifedipine appears to be remarkably safe in the doses used, but blood pressure monitoring is mandatory. Second line agents are those that need to be given by infusion or injection. Ideally, they should be given when the orally administered drugs have been shown not to be effective, particularly if the child remains glucose infusion dependent. In practice, however, there may be

**Table 4 Drugs used in the medical management of hyperinsulinism**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Mechanism of action</th>
<th>Dose</th>
<th>Response rate</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazoxide</td>
<td>Hyperinsulinemic hypoglycaemia</td>
<td>Increases K&lt;sub&gt;ATP&lt;/sub&gt; channels, increases adrenaline (epinephrine) secretion, increases gluconeo genesis</td>
<td>5–20 mg/kg/day orally 8 hourly</td>
<td>Limited experience</td>
<td>Hypertension, rarely leucopenia, thrombocytopenia</td>
</tr>
<tr>
<td>Drug</td>
<td>Chlorthiazide (act synergistically with diazoxide by activating non-K&lt;sub&gt;ATP&lt;/sub&gt; channels)</td>
<td>Calcium channel antagonist, inhibits insulin release</td>
<td>Dose 0.21–2.5 mg/kg/day orally 8 hourly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Nifedipine (slow release preparation)</td>
<td>Calcium channel antagonist, inhibits insulin release</td>
<td>Dose 0.25–2.5 mg/kg/day orally 8 hourly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Glucagon</td>
<td>Calcium channel antagonist, inhibits insulin release</td>
<td>Dose 1–10 mg/kg/hour intravenous infusion, 1 mg bolus dose intramuscular or intravenous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Octreotide</td>
<td>Hyperinsulinemic hypoglycaemia</td>
<td>Dose 5–20 µg/kg/day intravenous or subcutaneous infusion</td>
<td>Suppression of growth hormone, TSH, ACTH. Steatorrhea, cholelithiasis, abdominal distension, decreases gastric acid/pancreatic enzymes</td>
<td></td>
</tr>
</tbody>
</table>

ACTH, adrenocorticotrophin; K<sub>ATP</sub>, ATP sensitive potassium channel; TSH, thyroid stimulating hormone.
compelling reasons to begin them because of the enormity of the management problems in controlling the hypoglycaemia.

The two most important substances are glucagon and somatostatin. There is a consensus that both hormones are of value, but controversy over the doses used and the need to give them singly or in combination. Both hormones when given in high doses cause tachyphylaxis.

Glucagon has a powerful effect on mobilising glucose from hepatic glycogen. Its administration by means of a continuous intravenous infusion at rates of between 5 and 10 μg/kg/hour can help reduce the infusion rate of glucose needed to maintain normoglycaemia. Some authorities recommend its early use to gain rapid stabilisation of blood glucose concentrations; others argue that it is a powerful insulin secretagogue, and its administration in theory will maintain a drive to insulin secretion. For this reason, they propose using glucagon only with the concurrent administration of somatostatin. The somatostatin analogue, octreotide, activates potassium channels in the β cell membrane, and may also affect the intracellular translocation of calcium. The doses proposed for initiating combination treatment are 1.0 μg/kg/hour of glucagon together with 10 μg/kg/day of octreotide. It should be noted that these doses are less than those advocated for single hormone infusion. Further systematic research is needed to define the effects and role of these powerful hormones.

The use of steroids remains a controversial issue. In general, high doses of steroids have been contraindicated. However, the recent demonstration of cortisol unresponsiveness during hyperinsulinaemic hypoglycaemia raises important questions for further research, not only on the mechanism of this observation, but also on the impact of introducing cortisol substitution. Somatostatin administration leads to the inhibition of a number of other hormone systems, and in some centres such children are given hydrocortisone replacement, particularly during surgical procedures. This approach also needs further investigation and validation.

FURTHER MANAGEMENT IN MEDICALLY RESPONSIVE CHILDREN
The overall aim of medical treatment is to prevent hypoglycaemia while allowing a normal feeding pattern to be established. Children who respond rapidly to diazoxide treatment (as demonstrated by an ability to withdraw glucose support) should be maintained on their initial doses with repeated systematic attempts to withdraw the diazoxide during the next few months. Some children, particularly those with hyperammonaemic hyperinsulinaemia, might require treatment for many years. Others, without hyperammonaemia, might also remain diazoxide dependent until adulthood. Attempts to withdraw diazoxide should only be made in hospital.

In severe early onset hyperinsulinaemia, there is a tendency for gradual improvement in the clinical severity of the defect with the passage of time. This observation led Glaser et al to adopt an aggressive medical treatment strategy comprising long term subcutaneous glucagon and octreotide infusions and continuous overnight gastrostomy feeds. The approach has been used with some success in children with the Ashkenazi Jewish and Palestinian Arab phenotype of hyperinsulinaemia. The part played by this approach should play in treating other populations is still to be defined, and the short and long term effects of this powerful hormonal treatment need further evaluation. Many families find the intensity of the regimen impossible to cope with in their daily lives.

FAILURE OF MEDICAL TREATMENT: SURGICAL TREATMENT
Children who fail to respond to medical treatment should be managed in a centre where there is the closest of collaboration and dialogue between the medical and surgical teams. Some experts recommend that aggressive medical treatment should be continued for at least four to six weeks in early onset hyperinsulinaemia. Hypoglycaemia must be prevented during this period, through a combination of high carbohydrate administration rates and drugs, as described above. The rationale is that an improvement in the severity of the hyperinsulinaemia may be seen during this period; however, there are no published studies that evaluate the change in the need for surgery in infants in whom medical treatment is initially unsuccessful at two to three weeks compared with five to six weeks. The criteria for successful medical management are a feeding regimen acceptable to the family, with normal blood glucose concentrations during reasonable periods of fasting. If an acceptable regimen for home oral feeding cannot be established without hypoglycaemia, a surgical approach to management should be considered.

Because of the risk of major management related complications, including central line sepsis, venous thrombosis, hepatic dysfunction, bleeding diatheses, impaired nutrition, and hypoglycaemic encephalopathy, the consensus view expressed by the workshop was that children should be subjected to surgery sooner rather than later.

The absolute indications for surgery are: (1) demonstration of focal hyperplasia in children unresponsive to medical treatment; and (2) glucose infusion dependency despite maximum doses of diazoxide, chlorothiazide, nifedipine, glucagon, and somatostatin.

Infants with diffuse disease will normally require a 95% pancreatectomy to control the hyperinsulinaemia. After this procedure there is a high incidence of pancreatic endocrine and exocrine insufficiency. In infants with focal forms of the disease, an opportunity exists for partial pancreatectomy, preserving the normal pancreatic tissue. The focal areas of abnormal pancreas are usually not macroscopically apparent at surgery and their resection requires skilled microdissection aided by immediate histological examination of the resected tissue. Confirmation of the histological diagnosis is by frozen section, the distinction between focal...
shown that individuals with frankly diabetic glucose tolerance tests have evidence of persistent inappropriate insulin secretion in the face of hypoglycaemia. This suggests that such individuals are diabetic by virtue of having a reduced mass of functionally abnormal β cells. The diabetic phenotype might be the result of enhanced apoptosis in hyperinsulinaemic β cells.

The Future

The important distinction between focal and diffuse disease has been highlighted through this commentary. Current methods of diagnosis are either too insensitive or too invasive and must be of high priority. The role of the genome in the diagnostic investigations is also areas for further research, not least because so many children at present have not been shown to possess a known mutation.

New pharmacological agents with which to inhibit insulin secretion are urgently needed. Long acting octreotide and insulin specific analogues are available from pharmaceutical companies, but are either not licensed for human use, or only for adults.

Finally, the proof of principle that β cells from some resected pancreata continue to replicate in tissue culture, coupled with the ability to affect gene therapy in vitro, offers a tantalising future prospect of reimplanting the patient's own genetically modified cells that have regained normal glucose dependence of insulin secretion.

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Hyperinsulinism in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency reveals the importance of β-oxidation in insulin secretion

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A female infant of nonconsanguineous Indian parents presented at 4 months with a hypoglycemic convulsion. Further episodes of hypoketotic hypoglycemia were associated with inappropriately elevated plasma insulin concentrations. However, unlike other children with hyperinsulinism, this patient had a persistently elevated blood spot hydroxybutyrylcarnitine concentration when fed, as well as when fasted. Measurement of the activity of L-3-hydroxyacyl-CoA dehydrogenase in cultured skin fibroblasts with acetoacetyl-CoA substrate showed reduced activity. In fibroblast mitochondria, the activity was less than 5% that of controls. Sequencing of the short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) genomic DNA from the fibroblasts showed a homozygous mutation (C773T) changing proline to leucine at amino acid 258. Analysis of blood from the parents showed they were heterozygous for this mutation. Western blot studies showed undetectable levels of immunoreactive SCHAD protein in the child’s fibroblasts. Expression studies showed that the P258L enzyme had no catalytic activity. We conclude that C773T is a disease-causing SCHAD mutation. This is the first defect in fatty acid β-oxidation that has been associated with hyperinsulinism and raises interesting questions about the ways in which changes in fatty acid and ketone body metabolism modulate insulin secretion by the β cell. The patient’s hyperinsulinism was easily controlled with diazoxide and chlorothiazide.

HI can be caused by gain-of-function mutations of glucokinase and glutamate dehydrogenase and defects in the SUR1 or KIR6.2 subunits of the KATP channel in the β cell membrane (12). In these disorders, the pathogenesis of HI can be explained using a simple model of β cell signaling: Increased β cell glucose metabolism leads to increased ATP production from acetyl-CoA and a rise in the ATP/ADP ratio. This closes KATP channels, depolarizing the cell membrane and causing calcium influx through voltage-gated channels, which finally triggers insulin secretion (12, 13). Defects causing HI increase ATP production by increasing glucose or glutamate metabolism, or they cause permanent depolarization. The occurrence of HI in a patient with an FAOD is difficult to explain using this model. Our findings are therefore discussed by reference to the Prentki two-pathway model of β cell signaling (14). This takes account of the fact that a shift from fatty acid oxidation to esterification is a key event in the β cell’s response to glucose. It also recognizes that it is possible to demonstrate the presence of a KATP channel-independent mechanism that augments the β cell’s secretion of insulin in response to high glucose concentrations (14-16).

Methods

The hypoglycemia screen. Investigation of hypoglycemia in infancy entails measurement of insulin, NEFA, and D-3-hydroxybutyrate at a time when the blood glucose is less than 2.6 mM (17, 18). Typical results from our unit are shown in Table 1. HI is diagnosed on the basis of plasma insulin greater than or equal to 3 mU/l at the time of hypoglycemia; in seven recent cases, the range was 5.7-53 mU/l. In HI the plasma NEFA concentration at the time of hypoglycemia is low (<0.7 mM), whereas in fasted children who are not hypoglycemic, the plasma NEFA concentration is greater than 0.9 mM, and, in FAOD and ketotic hypoglycemia, the plasma NEFA concentration at the time of hypoglycemia is greater than 1.4 mM. The blood concentration of D-3-hydroxybutyrate at the time of hypoglycemia is less than 1.4 mM in FAOD and less than 0.4 mM in HI.

Case report. FS is the second child of nonconsanguineous Indian parents. She was born at 38 weeks’ gestation, weighing 3.2 kg. She fed poorly from birth. At 4 months, she had a grand mal convulsion and her blood glucose level was 1.4 mM. Further investigations showed that there were no ketones in her urine after an episode of hypoglycemia and that she had HI (1.3 mM blood glucose, 134 pmol/l (18 mU/l) simultaneous plasma insulin, and 108 pmol/l C-peptide), with normal serum cortisol (381 nmol/l). She was given frequent feeds containing glucose polymer, and her blood glucose remained at 3.0-4.5 mM. However, she had two further convulsions at home; on one occasion her plasma glucose was 2.9 mM and insulin 83 pmol/l (11 mU/l) on arrival in hospital. Nighttime hypoglycemia was managed by the use of uncooked cornstarch.

At 14 months, FS was referred to Great Ormond Street Hospital. She showed evidence of HI, but this was intermittent and unpredictable (Table 1). She required a glucose intake of 8 mg/kg/min to maintain blood glucose levels greater than 2.6 mM; a requirement of more than 6 mg/kg/min indicates HI (18). On one occasion, she tolerated an 18-hour fast without becoming hypoglycemic. On another occasion, after only a 4-hour fast, she had a blood glucose level of 2 mM, insulin level of 668 pmol/l (89 mU/l), NEFA of 0.17 mM, and D-3-hydroxybutyrate of 0.01 mM. The results were consistent with HI; however, on other occasions, hypoglycemia occurred without HI. For example, 3 hours after a glucagon provocation (19), she had a blood glucose of 2.1 mM with an insulin level of 10 mU/l, NEFA of 2.37 mM, and D-3-hydroxybutyrate of 1.38 mM (NEFA/D-3-hydroxybutyrate ratio at upper end of normal range; ref. 17). Urine organic acid analysis showed mildly raised 3-hydroxybutyrate, 3-hydroxyglutarate, and 3,4-dihydroxybutyrate.

FS was readmitted at 19 months because of recurrent hypoglycemia with fits. She had frequent episodes of hypoglycemia in the hospital. Once when the blood glucose was 2.3 mM, the plasma insulin was 2.6 mU/l, demonstrating a failure to switch off insulin release completely. In view of the high normal NEFA/D-3-hydroxybutyrate ratio recorded on one occasion, a series of blood spot acylcarnitine analyses were undertaken; these all showed raised hydroxybutyrylcarnitine.

In view of the hypoglycemic screens suggesting HI, FS commenced on diazoxide (5 mg/kg/d) and chlorothiazide (7 mg/kg/d). This led to an improvement in the blood glucose profile, and she could tolerate a 24-hour fast without becoming hypoglycemic. Feeding remained a major problem, eventually requiring a gastrostomy. She was discharged on diazoxide and chlorothiazide. Her glucose control has remained good with no further seizures or episodes of hypoglycemia.

Acylcarnitine analysis by electrospray ionization tandem mass spectrometry. Blood spots were obtained from FS 1-2 hours after a feed and at times of hypoglycemia. The acylcarnitine profiles were compared with those obtained in random blood spots from 33 normal infants and children and in fasting blood spots from 14 children who were ketogenic at the end of a diagnostic fast, but whose metabolite and endocrine profile was within our normal range (17). They were also compared with fasting and nonfasting blood spot acylcarnitine profiles from six children with HI.

For analysis of free carnitine and acylcarnitines (as butyl esters), 4.7-mm discs were punched from the Guthrie Card (filter paper containing the blood spot) into 96-well polystyrene microtiter plates. The internal standards added to each sample were [9-13C]carnitine and D-3-[1-13C]acetyl, -[1-13C]propionyl, -[1-13C]octanoyl, and -[1-13C]palmitoyl carnitines. The carnitine species in a blood spot were analyzed by sonication with 85% methanol and transferred to
solution in methanol to a 96-well polypropylene microtiter plate. The dried sample was dissolved in butanolic HCl and heated at 45°C for 60 minutes. The butanol/HCl was removed with a stream of oxygen-free nitrogen, and the dried sample was reconstituted in 150 µl of 1:1 acetonitrile/water. A 7-µl injection was made into the electrospray ionization source of a Micromass Quattro LC mass spectrometer (Micromass Ltd., Altrincham, United Kingdom). For analysis of butylicarnitine species (0.8 min), the first quadrupole was set to scan from mass/charge ratio (m/z) 200 to 600, and the second mass filter was set to detect fragment ions of m/z 85. Fragment ions of this mass/charge ratio are produced on collision-induced dissociation of the butyl esters of free carnitine and all acylcarnitines; they are believed to originate from the carbonyl group of the carnitine plus carbons 2, 3, 4, and 5.

The size of the hydroxybutyrylcarnitine peak was compared with that of the 3-octanoylcarnitine internal standard (added in an amount equivalent to a concentration of 10 µM in the blood). The blood spot acetylcarnitine concentration was measured by comparison of the peak size with that of the D3-acetylcarnitine standard. Differences between blood spot acetylcarnitine concentrations in FS and ketotic and hyperinsulinemic children were evaluated using the t test.

**Measurement of 3-hydroxyacyl-CoA dehydrogenase activity.** Short-, medium-, long-chain, and 2-methyl-short-chain 3-hydroxyacyl-CoA dehydrogenase activities were measured in fibroblasts essentially as described by Jackson et al. (20). Fibroblast pellets were resuspended in 25 mM phosphate, 0.2 mM EDTA, and 0.2% vol/vol Triton X-100 (pH 8.0) and incubated on ice for 30 minutes. After centrifugation (11,600 g, for 10 minutes), 3-hydroxyacyl-CoA dehydrogenase activity was measured in the supernatant in the reverse direction by following the disappearance of NADH at 340 nm. The reaction medium consisted of 0.1 M potassium phosphate (pH 7.0)/0.1 mg/ml NADH/0.3 mg/ml BSA (fatty acid free), plus 40 µM ketoacyl-CoA substrate (short chain, acetoacetyl-CoA [Sigma Chemical Company, Poole, United Kingdom]; medium chain, 3-ketoocotanoyl-CoA; long chain, 3-ketohexadecanoyl-CoA). The reaction was carried out at 37°C for 10 minutes.

**DNA sequence analysis.** DNA was isolated from fibroblasts from FS and a control. Exon-specific PCR-amplification of Schad sequences was performed with the appropriate primer pair for each exon (Table 2; optimal experimental conditions for primer pairs available on request). PCR products were analyzed on a 1.3% agarose gel and visualized by ethidium bromide staining.
Established sequences. The forward primers are presented in the middle column, the reverse primers in the right column. The forward primers are presented in the middle column, the reverse primers in the right column.

Table 2
Sequences of primer pairs used to amplify exon 1 to exon 8 of Schad

| Exon 1  | CCGGTCTCTTCTGCTGTC | GCCAGACTTCCACACTGAGC |
| Exon 2  | CATATTTTCTGCTGACCTGG | CCCCCTGCCCTGCAATTGAAAGC |
| Exon 3  | CATATTCCAGTGACCTGG | GACAAATTTTCTGACACAGG |
| Exon 4  | CATCAAGAGGTACCTGTC | AAAGGACAGCTACAGCAGC |
| Exon 5  | CGTGTGTTTGTGGCTCAG | CAGGAAACAGTCTCGG |
| Exon 6  | GCCCATTTTGTGTGACTTC | CTAGAGACCTGAGATTCGAAGA |
| Exon 7  | GTTATAAGGGACAAAGGTC | GGGGACTTTTGAACGTC |
| Exon 8  | CCAGCAGCTTCTACCTCGAAGTCT | GTAGGCGGAAAGCTCAACAGC |

See Vredendaal et al., 1998 (2); sequences derived from AF026856-67, NM_005327, and unpublished sequences. The forward primers are presented in the middle column, the reverse primers in the right column.

an ABI prism 377 DNA sequencer using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California, USA). Sequence data were analyzed with the Lasergene 99 software program (DNA STAR Inc., Madison, Wisconsin, USA).

Restriction fragment length analysis. Genomic DNA of FS, her parents, and 100 controls (50 ethnically matched) was subjected to PCR amplification using exon 7-specific SCHAD primers (Table 2). The PCR products were exposed to Apl Roche Molecular Biochemical, Mannheim, Germany) for 2 hours and analyzed on a 2% agarose gel.

In vitro expression of SCHAD. Total RNA was isolated from fibroblasts of FS and a control. RNA (10 μg) was reverse transcribed using oligo(dT) and MMLV RT (Invitrogen Life Technologies Corporation, Groningen, The Netherlands). The complete coding sequence of SCHAD, 81 nucleotides of the 5'UTR, and 33 nucleotides of the 3'UTR were amplified using primers CAGAGTCTCGGCTTCCCAGGG and AGGTGCTTTCTTCAGACGG, and Advantage cDNA polymerase mix (CLONTECH Laboratories Inc., Palo Alto, California, USA) for 30 cycles. The PCR products were applied to a 1% agarose gel, and the band with the appropriate size was excised, eluted, and reamplified with the same primers for 30 cycles. Amplified cDNAs were cloned in pCR2.1 (Invitrogen), and individual clones were sequenced as described earlier.

In vitro expression. The wild-type and mutant SCHAD coding sequence and subcloned in pBluescript-SK*. To ascertain proper subcloning, the integration was confirmed by EcoR1-BstEII restriction digestion of normal SCHAD sequence and subcloned in pBluescript-SK+. To ascertain proper subcloning, the integration sites of the inserts and the inserts of the obtained plasmids were sequenced completely from both sides. Wild-type and mutant proteins were synthesized in a reticulocyte lysate (Promega Corp., Madison, Wisconsin, USA) by coupled in vitro transcription translation from the T7 promoter according to the manufacturer's instructions. Plasmid containing no insert was used as a control. Enzyme activity was assayed immediately after protein expression, by measuring the disappearance of NADH at 340 nm, using acetoacetyl-CoA (Sigma) as a substrate. Production of SCHAD protein was analyzed by SDS PAGE followed by Western blotting. Proteins recognized by the antibody were visualized by chemiluminescence (ECL+).

Results

Acylcarnitine analysis (electrospray ionization tandem mass spectrometry). A "p" cursors of m/z 85" scan obtained on the patient's blood spot is shown in Figure 1. The spectrum shows a clear peak of m/z ratio of 304, indicating the presence of hydroxybutyrylcarnitine (butyl ester) or an isomeric compound. The concentration was always more than 0.8 μM, even in nonfasting samples (Table 3). An hydroxybutyrylcarnitine peak could be seen in some normal controls, in some of the normal children who were ketogenic at the end of a diagnostic fast, and in some of the hyperinsulinemic children who had hypoketotic hypoglycemia at the end of a fast. However, none of these children had a blood hydroxybutyrylcarnitine concentration greater than 0.5 μM (Table 3). The mean (± 1 SD) acetylcarnitine concentration in blood spots from FS was 27.74 ± 9.8 μM, which was significantly higher (P < 0.0005) than the concentration in blood from normal controls (16.54 ± 3.34) but not significantly different from the blood spot acetylcarnitine concentrations in other hyperinsulinemic children (fasting, hypoglycemic = 26.7 ± 9.7; nonfasting, normoglycemic = 26.4 ± 9.7). Other carnitine species in blood spots from FS were normal apart from occasional mild elevations of C3, C4, and C10 species (≤ 2 of 16 determinations outside normal range; NS): free carnitine 47-68

![Figure 1](attachment:image.png)

Figure 1
Analysis of butyl esters of carnitine and acylcarnitine species in blood spot from FS. Peak identities are: m/z 218, free carnitine; 227, D9-carnitine; 260, acetyl-carnitine; 263, 3-acylcarnitine; 277, 3-propionylcarnitine; 304, hydroxybutyrylcarnitine; 456, O3-octanoylcarnitine; 459, O3-palmitoylcarnitine; and 482, oleoylcarnitine.
Protein C4/C16 HAD ratio and HAD/citrate synthase ratio in control fibroblasts, fibroblasts from patient FS, and from SB, a known LCHAD-deficient patient.

Vicies of 3-hydroxyacyl-CoA dehydrogenases in chc patient and in controls

{C4 HAD}{C8 HAD}{Cl6 HAD} ratio

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Hydroxybutyryl carnitine concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=16)</td>
<td>Mean</td>
</tr>
<tr>
<td>Normal</td>
<td>1.78</td>
</tr>
<tr>
<td>Fasting</td>
<td>0.20</td>
</tr>
<tr>
<td>Non-fasting (n=33)</td>
<td>0.096</td>
</tr>
<tr>
<td>Hypersulinemic hypoglycemia (n=6)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

The concentration of hydroxybutyryl carnitine (μmol/l) in (a) blood spots from FS; (b) blood spots from non-fasting controls; (c) blood spots from non-fasting controls; and (d) blood spots from hypersulinemic children who had hypoketonemia at the end of a diagnostic fast.

Table 3

Comparison of concentrations of hydroxybutyryl carnitine in blood

<table>
<thead>
<tr>
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</tr>
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Activity of 3-hydroxyacyl-CoA dehydrogenases. The short-chain HAD activity measured in fibroblasts from FS was significantly lower than that in control fibroblasts whether the activity was expressed per mg protein, as a ratio to LCHAD, or as a ratio to citrate synthase (Table 4). Residual activity in fibroblasts from FS was 35-40% of the activity of the controls. Long-chain HAD activity in fibroblasts from the known LCHAD-deficient patient was significantly reduced, but long-chain HAD activity in fibroblasts from FS was not significantly different from that in controls. There was no significant difference in medium-chain HAD activity between FS and controls. When SCHAD and LCHAD activity were measured in a mitochondrial fraction from fibroblasts, the values for FS were 4% and 3% in two separate experiments (SCHAD) and 142% (LCHAD) of the simultaneously assayed control fibroblast mitochondrial fraction.

Western blotting. Western blotting of fibroblasts from FS indicated a complete lack of SCHAD immunoreactive protein, whereas SCHAD was readily detectable in both control fibroblasts and fibroblasts from an LCHAD-deficient patient (Figure 2).

In vitro expression of SCHAD. To assess the effect of the P258L substitution on the activity of the protein, the wild-type and the mutated protein were synthesized in vitro from plasmids containing the wild-type SCHAD coding sequence and the SCHAD coding sequence harboring the C773T point mutation, respectively. The absence of amplification artifacts in the wild-type construct and of mutations other than the C773T point mutation in the patient construct was ascertained by sequencing of the SCHAD expression plasmids in two directions. Expression of the wild-type SCHAD protein in a reticulocyte lysate system yielded a protein with an apparent molecular mass of approximately 35 kDa, which is in agreement with the calculated molecular mass of the wild-type protein of 34.3 kDa. The in vitro expressed mutant protein reacted strongly with the anti-SCHAD antibody and had an apparent molecular mass 0.5 kDa less than that of the wild-type protein (Figure 5). Immunoblot analysis showed that comparable amounts

DNA analysis. Sequence analysis of DNA fragments containing nt 773 from patient DNA and from DNA of the parents, followed by Apal digestion and analysis on an agarose gel, confirmed that FS is homozygous and showed that her parents are heterozygous for the C773T mutation (Figure 3). The mutation could not be detected in DNA from 200 control chromosomes. The C773T mutation leads to replacement of Pro258 (Pro246 of the mature SCHAD protein) by Leu. Pro258 is completely conserved in SCHAD sequences from different species (Figure 4).

In vitro expression of SCHAD. To assess the effect of the P258L substitution on the activity of the protein, the wild-type and the mutated protein were synthesized in vitro from plasmids containing the wild-type SCHAD coding sequence and the SCHAD coding sequence harboring the C773T point mutation, respectively. The absence of amplification artifacts in the wild-type construct and of mutations other than the C773T point mutation in the patient construct was ascertained by sequencing of the SCHAD expression plasmids in two directions. Expression of the wild-type SCHAD protein in a reticulocyte lysate system yielded a protein with an apparent molecular mass of approximately 35 kDa, which is in agreement with the calculated molecular mass of the wild-type protein of 34.3 kDa. The in vitro expressed mutant protein reacted strongly with the anti-SCHAD antibody and had an apparent molecular mass 0.5 kDa less than that of the wild-type protein (Figure 5). Immunoblot analysis showed that comparable amounts

Table 4

Activities of 3-hydroxyacyl-CoA dehydrogenases in the patient and in controls

<table>
<thead>
<tr>
<th>Subjects</th>
<th>C4 HAD (mU/mg protein)</th>
<th>C8 HAD (mU/mg protein)</th>
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<th>C4 HAD/C16 HAD ratio</th>
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<th>C16 HAD/citrate synthase (U/UCS)</th>
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<tr>
<td>Controls</td>
<td>0.66 ± 0.15</td>
<td>2.28 ± 0.46</td>
<td>1.22 ± 0.26</td>
<td>0.54 ± 0.07</td>
<td>0.14 ± 0.04</td>
<td>0.36 ± 0.03</td>
<td>0.26 ± 0.06</td>
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<td>(n=16)</td>
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</tr>
<tr>
<td>Patient</td>
<td>0.26 ± 0.16</td>
<td>1.91 ± 0.49</td>
<td>0.97 ± 0.15</td>
<td>0.16 ± 0.13*</td>
<td>0.05 ± 0.02*</td>
<td>0.34 ± 0.07</td>
<td>0.21 ± 0.05</td>
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<td>(n=6)</td>
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<tr>
<td>Immuno in FS</td>
<td>39%</td>
<td>84%</td>
<td>80%</td>
<td>30%</td>
<td>36%</td>
<td>94%</td>
<td>77%</td>
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Immuno in FS % of controls

Table 5

Activities of 3-hydroxyacyl-CoA dehydrogenases in the patient and in controls

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Activities of short-chain (C4 HAD), medium-chain (C8 HAD), and long-chain (C16 HAD) 3-hydroxyacyl-CoA dehydrogenase expressed as HAD activity per milligram protein, C4/C16 HAD ratio, and HAD/citrate synthase ratio in control fibroblasts, fibroblasts from patient FS, and from SB, a known LCHAD-deficient patient. U = 1 μmol/min. U/UCS = units of citrate synthase activity in μmol/min. Significantly different from controls (Student's t test): *P < 0.001, †P < 0.01, ‡P < 0.05.
of protein were obtained for the wild-type and mutated SCHAD protein, whereas the vector alone did not yield a positive band of similar molecular mass (Figure 3). SCHAD activity was assayed in three separate experiments (Figure 3). The wild-type SCHAD exhibited a distinct but variable activity, whereas the mutated SCHAD did not yield activity above background in all experiments performed, indicating that the P258L substitution abolishes enzyme activity of in vitro expressed SCHAD.

Discussion

FS suffered frequent, severe hypoglycemia. Three hypoglycemia screens indicated definite HI (blood glucose <2.6 mM, plasma insulin >3 mU/l; ref. 20) as did the glucose requirement of 8 mg/kg/min (although many hyperinsulinemic infants require more than 15 mg/kg/min; ref. 18). The episode of hypoglycemia that followed the glucagon test was not attributable to HI but rather suggested impaired fatty acid oxidation. (The pathogenesis of hypoglycemia in FAOD is probably multifactorial. NEFA cannot be used and blood concentrations of ketone bodies are low, so glucose utilization is increased; however, in addition, gluconeogenesis is probably impaired because acetyl-CoA concentrations in the liver are low.) Treatment with diazoxide and chlorothiazide led to a dramatic improvement in glucose homeostasis, providing further evidence that HI was the main cause of hypoglycemia.

The possibility of impaired fatty acid oxidation was investigated by examination of the blood acylcarnitine profile. All samples showed an elevated concentration of a carnitine species with an m/z ratio of 304. This could be due to a hydroxybutyrylcarnitine or isomeric carnitine species. A similar peak has been reported in children suffering enzymatic deficiencies in the SCHAD pathway or after exposure to KBUD. The SCHAD hypothesis was consistent with a NEFA/0-3-hydroxybutyrate ratio toward the upper limit of normal. The deacylase hypothesis seemed unlikely upon comparison of FS with the case described previously (25). Plasma concentrations of 0-3-hydroxybutyrate did not suggest a KBUD.

Rather than prove that the compound in the blood was L-3-hydroxybutyrylcarnitine and that the urine contained excess L-3-hydroxybutyrate (which is indistinguishable from 0-3-hydroxybutyrate on simple organic acid analysis), it was decided to move directly to an assay of SCHAD. SCHAD is expressed in fibroblasts. Clear evidence of reduced SCHAD activity was seen in fibroblasts from FS. There was no impairment in hydrogenation of 3-ketoocanoyl-CoA, a medium-chain substrate, in keeping with our organic acid and acylcarnitine analyses but at variance with the evidence of impaired activity in medium-chain 3-hydroxyacyl-CoA dehydrogenase activity in an SCHAD-knockout mouse and in another SCHAD-deficient patient (10, 11).

There was high residual SCHAD activity in the patient’s fibroblasts, which was surprising, as Western blots indicated that the fibroblasts contained no immunoreactive protein. There was no fall in residual enzyme activity in the patient’s fibroblasts after immunoprecipitation of residual SCHAD protein. The residual activity could be due to several enzymes: (a) Tri-functional protein HAD activity. This is unlikely because
this enzyme has low activity toward acetocetoyl-CoA (27) and residual SCHAD activity was detectable after immunoprecipitated of SCHAD protein from an LCHAD-deficient cell line. (b) Peroxisomal l- and o-3-hydroxyacyl-CoA dehydrogenases (28). (c) Presence of type II 3-hydroxyacyl-CoA dehydrogenase (29) or human brain multifunctional dehydrogenase (30) in fibroblasts. (d) Short-chain 2-methyl-3-hydroxyacyl-CoA dehydrogenase (31). Using 2-methylacetoyl-CoA as substrate, activity of this enzyme was low (0.32 mU/mg protein in control fibroblasts, 0.42 mU/mg in FS). Because it is only half as active toward straight chain as toward 2-methylacyl-CoA esters (31), it is unlikely, however, that this enzyme was responsible for the residual activity.

Given that the residual activity was substantially reduced when a mitochondrial preparation was used, it seems likely that it was due to peroxisomal enzyme(s). Sequencing of the Schad gene from fibroblasts showed that FS is homozygous for a C773T mutation that leads to a change from proline to leucine at amino acid 258 (246 in the mature protein after cleavage of the mitochondrial targeting sequence); the parents are heterozygotes. Several lines of evidence indicate that C773T is responsible for the reduction in SCHAD activity and is a disease-causing mutation: (a) No other difference from the wild-type sequence was detected in the cDNA of FS. (b) The C773T mutation was not found in 200 control chromosomes, including 100 from individuals with the same ethnic background, indicating that the mutation is not a common polymorphism. (c) As shown in Figure 4, Pro258 is part of a highly conserved amino acid region, and the equivalent of Pro258 is present in SCHAD of all species investigated. (d) Analysis of the crystal structure of SCHAD reveals that Pro258 is the starting point of one of the a-helices (a12) of the C-terminal domain (32). Barycki et al. argue that the orientation of these a-helices relative to one another is critical for enzyme function. Replacement of the proline at the starting point of an a-helix by leucine is likely to prevent normal protein folding. This could lead to rejection by the chaperonin system (leading to destruction of the nascent protein) or to synthesis of a protein with reduced catalytic activity.

The Western blots indicated reduced immunoreactive SCHAD protein in fibroblasts. This suggests that the P258L substitution alters the tertiary structure of the nascent enzyme to such a degree that it is not recognized by chaperonins and is destroyed. This phenomenon has been described for point mutations in short-chain acyl-CoA dehydrogenase (33). Additional evidence that the C773T mutation in the SCHAD coding sequence is responsible for the patient's disease is provided by the functional assay after in vitro expression of the mutated protein. We included 81 nucleotides of the 5'UTR, in addition to the complete coding sequence, as a template for the expression of the SCHAD protein, to ensure protein formation starting from the translation start site. It has been shown that the sequences upstream of the translation start codon may lead to aberrant choice of translation start sites by the in vitro expression system (34). The SCHAD protein expressed from the wild-type construct had an apparent molecular mass of the expected size. The mutated sequence yielded a comparable amount of protein with a slightly lower apparent molecular mass as determined by SDS-PAGE. This is probably due to the P258L substitution. The protein folding introduced by proline in the wild-type sequence cannot be undone by denaturing the protein, so the wild-type protein may exhibit a different migration pattern in a denaturing gel than the mutant protein. The complete absence of SCHAD activity of the in vitro synthesized protein harboring the P258L substitution further corroborates the link between the mutation and the patient's disease.

The acylcarnitine profile in blood from FS was unique with regard to the presence of more than 0.6 µM of hydroxybutyrylcarnitine. However, it was also abnormal to the extent that the acetylcarnitine concentration was usually above the normal range. Comparison with other hypoinsulinemic children suggested that elevation of blood spot acetylcarnitine is

![Figure 4](image)

Diagram of aligned SCHAD sequences from several evolutionarily distant species. Pro258 (in bold), which is replaced by Leu in the DNA of FS, is completely conserved in the SCHAD coding sequences from different species, including bacteria, Human, NP_005318; Pig, AAD20939; Caenorhabditis elegans, ZV238, Chlamydomonas acetobutylicum, AAA95971.

![Figure 5](image)

Expression of wild-type and C773T mutant SCHAD using a reticulocyte lysate system. (a) Western blot showing no protein with vector alone, immunoreactive SCHAD protein with an apparent molecular weight of 35 kDa with the wild-type SCHAD construct, and immunoreactive SCHAD protein with a slightly lower apparent molecular weight with the C773T mutant construct. (b) SCHAD enzyme activity obtained with expression of the wild-type and C773T mutant SCHAD proteins. Enzyme activity is equal to the nanomole substrate converted per microliter of reticulocyte lysate between 10 and 30 minutes' incubation.
The postulated pathways for \( \beta \) cell signaling (from ref. 14). PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CL, citrate lyase; CPT1, carnitine palmitoyl transferase 1; m, mitochondrial; c, cytosolic; AcCoA, acetyl-CoA; LCF A-CoA, long-chain fatty acyl-CoA.

The diagram illustrates the processes involved in lipid signaling in \( \beta \) cell insulin secretion. The pathway begins with glucose metabolism through the pyruvate dehydrogenase (PDH) complex, leading to the conversion of pyruvate to acetyl-CoA. Acetyl-CoA then enters the Krebs cycle and is converted into acetyl carnitine, which inhibits carnitine palmitoyl transferase (CPT1) and blocks the entry of long chain fatty acyl-CoA (LCFA-CoA) into the mitochondrion. Thus LCFA-CoA is converted instead into diacylglycerol, triglycerides, fatty acids, and acylated proteins. LCFA-CoA or the complex lipids derived from them are potent regulators of enzymes, ion channels, and signal-transducing effectors.

It is proposed that the complex lipids or acylated proteins augment insulin secretion by a \( K_{ATP} \)-independent mechanism. Unequivocal evidence for such a lipid-linked signaling mechanism is lacking, but there is experimental evidence for a \( K_{ATP} \)-independent mechanism that augments insulin secretion in response to glucose (13–15).

What effects would FAOD in general, and SCHAD deficiency in particular, have on the lipid signaling pathway?

Any defect leading to accumulation of cytosolic LCFA-CoA and increased esterification could induce inappropriate insulin secretion. Accumulation of triglyceride, particularly in liver, occurs in LCHAD deficiency, in very long chain acyl-CoA dehydrogenase (VLCAD) deficiency, and in medium chain acyl-CoA dehydrogenase (MCAD) deficiency during fasting-induced decompensation (35). However, our data do not suggest that these FAOD produce HI (Table 1). It is possible that in SCHAD deficiency, accumulation of short chain acyl-CoA esters in the mitochondrial causes insulin secretion by inhibition of CPT1. The isoform of CPT1 in the \( \beta \) cell is the same as that in the liver (36). This enzyme, located in the outer mitochondrial membrane, has one inhibition site that faces the cytosol and is inhibited by dicarboxylic fatty acid esters (physiologically by malonyl-CoA) and a second inhibition site, facing the intermembrane space, that is inhibited by short chain monocarboxylic acid esters (37).

What would be the role of inhibition of CPT1 by L-3-hydroxybutyryl-CoA? We hypothesize that it is the cell’s ketone body-sensing mechanism. High levels of L-3-hydroxybutyrate and acetoacetate in the blood will lead to a rise in intramitochondrial acetoacetate-CoA via the ketone body utilization pathway. In a cell containing abundant SCHAD, operation of the enzyme in the “reverse” direction would lead to accumulation of L-3-hydroxybutyril-CoA. Inhibition of CPT1 by L-3-hydroxybutiryl-CoA would inhibit fatty acid oxidation and, in the \( \beta \) cell, lead to insulin secretion. In muscle, acetoacetate and 3-hydroxybutyrate inhibit fatty acid oxidation by a mechanism independent of malonyl-CoA (38).

Our patient’s hypoglycemia responded to diazoxide, which inhibits insulin secretion by keeping \( K_{ATP} \) channels open. This does not disprove the hypothesis that HI in SCHAD deficiency is due to stimulation of the lipid-signaling pathway; the Prentki model suggests that large amounts of insulin are only secreted if both the \( K_{ATP} \) channel and the lipid-signaling pathway are activated.

There are significant differences between FS and previous putative cases of SCHAD deficiency. In contrast to the child reported by Tein et al. (6), FS had no evidence of skeletal or cardiac myopathy and SCHAD activity was low in her fibroblasts. The cases reported by Bennett et al. in 1996 (7) had a vigorous ketone response to hypoglycemia and medium- and long-chain 3-hydroxydicarboxylic acids in the urine. Neither of these features was
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A recessive contiguous gene deletion causing infantile hyperinsulinism, enteropathy and deafness identifies the Usher type 1C gene

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Usher syndrome type 1 describes the association of profound, congenital sensorineural deafness, vestibular hypofunction and childhood onset retinitis pigmentosa1. It is an autosomal recessive condition and is subdivided on the basis of linkage analysis into types 1A through 1E (refs 2-6). Usher type 1C maps to the region containing the genes ABCC8 and KCNJ11 (encoding components of ATP-sensitive K+ (KATP) channels), which may be mutated in patients with hyperinsulinism7-10. We identified three individuals from two consanguineous families with severe hyperinsulinism, profound congenital sensorineural deafness, enteropathy and renal tubular dysfunction. The molecular basis of the disorder is a homozygous 122-kb deletion of 11p14-15, which includes part of ABCC8 and overlaps with the locus for Usher syndrome type 1C and DFNB18 (ref. 11). The centromeric boundary of this deletion includes part of a gene shown to be mutated in families with type 1C Usher syndrome, and is hence assigned the name USH1C. The pattern of expression of the USH1C protein is consistent with the clinical features exhibited by individuals with the contiguous gene deletion and with isolated Usher type 1C.

Insulin secretion is primarily controlled by pancreatic β-cell membrane potential, which is largely determined by the activity of the Na+-K+ ATPase pump and the rate of K+ 'leak' through open ATP-sensitive potassium (KATP) channels, which are coupled to the metabolic status of the β-cell. KATP closure in response to hyperglycaemia, or a functional absence of KATP channels caused by mutation in ABCC8 (formerly SUR1) or KCNJ11 (formerly Kir6.2), leads to membrane depolarization and constitutive activation of voltage-dependent Ca2+ channels and insulin release9,10,11. Three children, V1 from family 1, and IV2 and IV4 from family 2, presented with severe hyperinsulinism requiring pancreatectomy, enteropathy, profound congenital sensorineural deafness and delayed motor milestones (Fig. 1). In vitro studies of isolated β-cells9,10,11 from resected pancreata of these children revealed that the number of functional KATP channels and the open state probability was reduced compared with control β-cells and that the KATP channels present were insensitive to ATP, ADP and diazoxide. This loss of KATP channel number and function in β-cells from individuals with hyperinsulinism is consistent with a recessive mutation or deletion in either ABCC8 or KCNJ11.

We amplified genomic DNA from these children using PCR primers specific for exons of ABCC8. Only regions 3' of intron 22 were successfully amplified, consistent with the presence of a homozygous deletion 5' of ABCC8 exon 23 (Fig. 2B). P1-derived artificial chromosome (PAC) pDJ398-22 contains the entire ABCC8 gene and overlaps with PAC 6-106F23, which extends approximately 170 kb 5' to ABCC8. FISH analysis of metaphase spreads from families 1 and 2 also suggested the presence of deletion breakpoints in both PACs (data not shown).

Using sequence available in GenBank, we designed STS primers centromeric to intron 22 of ABCC8 and estimated the extent of the deletion (Fig. 2B). We then designed primers to

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Fig. 1 Two extended families with multiple affected members. The families are not known to be related, but share the same common Arabic surname, although they are from different countries. All six affected children suffered from severe hyperinsulinism with congenital deafness. Three affected children, V1 from family 1, V2 from family 2 and IV4 from family 4, were available for study.
amplify across the deletion (Fig. 2b, primers 'a' and 'b'). Sequencing across the deletion indicated that it is 122,815 bp. A multiplex PCR reaction was designed using primers 'a' and 'b' located on either side of the deletion breakpoint, and primer 'c', which is located within the region deleted in affected members of this family and which defines the presence of wild-type sequence (Fig. 2b and Fig. 3). All four parents were shown to be heterozygous for the deleted allele.

BLAST searching of PACs pDJ239B22 and 6-106F23 showed that a gene, hereafter referred to as USHIC (also known as PDZ-73 and AIE-75), mapped to clone 6-106F23 (refs 13,14). Comparison of the clone sequence with the published cDNA sequences of the gene established the intron-exon structure and revealed that 19 of 21 exons were deleted in our patients. This gene was originally identified by immunoscreening of a metastatic colon cancer cDNA expression library and also by using serum from patients with an X-linked variant of autoimmune enteropathy (AIE) to identify possible autoantigens. It is highly expressed in the epithelium of the gut. Immunohistochemistry of gut biopsy tissue from the three affected children (V, from family 1, and IV, and IV', from family 2) using an antibody specific for the USHIC protein confirmed the absence of its expression, in addition to an inflammatory enteropathy. Gut biopsies from patients with other types of congenital and acquired inflammatory enteropathy showed positive staining for USHIC (Fig. 4a–c). Thus, partial deletion of USHIC may be responsible for the enteropathy seen in our patients.

The location of this gene within the previously defined Usher 1C region rendered it a candidate gene for this syndrome (Fig. 2a). We screened for mutations in USHIC in two unrelated families with type 1 Usher syndrome mapping to 11p14–15. We obtained immortalized cell lines from a Louisiana Acadian family from a public repository because the Usher syndrome in this population has been previously linked to this interval. Family 3 is a previously undescribed Pakistani family. Sequencing of USHIC in the two families showed different homozygous mutations in exon 3. In family 3, insertion of a cysteine in a run of six cysteines between positions 233 and 238 of the cDNA predicts a frameshift of the encoded protein (Fig. 5a) and a premature stop signal at codon 148 (cDNA numbered according to ref. 13). The mutation was observed in the three affected siblings. No heterozygotes for this sequence were detected in 80 ethnically matched controls and 96 pan-ethnic controls using DHPLC (data not shown).

In the Acadian cell lines, we found a G—>A change at position 216 of the cDNA (Fig. 5a). The affected individual was homozygous for the substitution and the parents were heterozygous. The sequence variant removed a DraIII site, present in 96 pan-ethnic controls (data not shown). Although this does not change an amino acid, examination of the sequence suggested that it might create a new splice site (Fig. 5b). Analysis of USHIC lymphoblastoid cDNA from the Acadian family showed that the affected individual produced a shortened RT–PCR product (Fig. 5c). Sequencing revealed a 39-bp deletion, consistent with the creation of a new splice site within exon 3 (ref. 15).

We examined expression of USHIC by RT–PCR of human fetal tissues and designed RT–PCR primers to examine the expression of splice forms containing exon 3, as the gene is known to be alternatively spliced. We detected USHIC expression in human fetal tissues between 10 and 13 weeks gestation, including the ear,
eye, gut, kidney, brain, adrenal, muscle and heart (data not shown). Expression in human lymphoblasts was weak. Further RT-PCR studies using sets of primers specific for different isoforms showed that the longest isoform of USH1C (previously called PDZ-73) was detected in all these fetal tissues including fetal ear and fetal eye. A shorter, 45-kD isoform of the protein, which also includes exon 3, was detected by RT-PCR in gut and kidney, but only a faint RT-PCR product was observed in fetal ear and eye (data not shown).

Using the monoclonal antibody to USH1C, we performed immunohistochemistry on sections of human gut, human fetal ear and eye. Antibody staining was strongest in tissues expressing the 45-kD isoform of the protein (that is kidney, gut, brain and testis). We saw strong positive staining in sections of normal gut (Fig. 4a). In the developing eye at 10.5 weeks, we observed lower levels of expression of the USH1C protein in the outermost aspect of the developing outer neuroblastic layer of the retina, part of which gives rise to the development of the rods and cones of the retina (Fig. 4f). In the developing ear at 10.5 weeks gestation, we detected lower levels of positive staining in the apical and basal surfaces of the cells destined to form the sensory areas of the labyrinth of the inner ear (that is, organ of Corti regions, macular regions of saccule and utricle, and crista regions of the ampullae of the lateral and posterior semi-circular canals; data not shown). These results are consistent with weak expression of the 45-kD isoform in the eye and ear, confirmed by RT-PCR, even though expression of the longer 73-kD isoform is detected in these tissues by RT-PCR. Thus USH1C is expressed in the tissues affected in Usher syndrome as well as those affected in the contiguous gene deletion syndrome detailed here.

We have confirmed the presence of an autosomal recessive contiguous gene syndrome consisting of severe hyperinsulinaemic hyperglycaemia, congenital sensorineural deafness, renal tubular dysfunction and severe enteropathy caused by a 122-kb deletion of the short-arm of chromosome 11. The hyperinsulinism can be explained by the deletion of more than half of ABCG8 (ref. 16), whereas the enteropathy may be explained by partial deletion of USH1C, given the expression pattern of this gene and its association with autoimmune enteropathy.

The USH1C mutations found in two families with Usher type 1C suggest that deletion of this gene is also responsible for the eye and ear abnormalities seen in these patients. Deletion of most of USH1C causes severe enteropathy in addition to Usher syndrome in the Arab patients, but a frameshift mutation in exon 3 in family 3 appears to cause Usher syndrome alone without clinical gastrointestinal symptoms. Moreover, the splice mutation in the Acadian cell lines appears to cause a localized in-frame deletion of 39 bases without clinical effect on the gut. The resulting transcript may be unstable and the overall effect of this mutation may also be that of a null mutation. It is therefore difficult to explain why enteropathy has not been reported in association with Usher syndrome type 1C. Possibly, individuals with Usher syndrome type 1C may have a subclinical disorder of gut architecture, only evident on gut biopsy. Alternatively, tissue-specific expression of multiple isoforms of the protein may account for mutations that have very limited clinical effects.

USH1C contains three PDZ domains, which are thought to be important modulators of protein-protein interactions. PDZ domains derive their name from the first proteins recognized to have the common conserved motif of 80–90 amino acids: the post-synaptic density protein PSD95, the Drosophila melanogaster tumour suppressor protein discs large 2 and the yeast protein pr seizure 2.
gene dig A and the tight junction protein ZO-1 (ref. 17). Proteins containing PDZ domains occur in a wide variety of species and tend to be plasma-membrane associated. It is therefore thought that the PDZ domains localize their ligands (receptors, channels, components of signal transduction pathways or other PDZ-domain-containing proteins) to particular subcellular domains and organize and coordinate multiprotein complexes at the plasma membrane. Proteins containing PDZ domains are expressed in the Muller cells of the retina, where they appear to co-localize with KCNklO, an inwardly rectifying potassium channel expressed in the Midler cells of the retina, where they appear to co-localize with KCNklO, an inwardly rectifying potassium channel expressed in the retinal blood vessels of the inner ear.

The locus for DFNB18, a profound congenital non-syndromic deafness without vestibular symptoms, maps to the same region in Usher 1C. These two disorders may be allelic and molecular analysis of families with non-syndromic deafness mapping to the DFNB18 locus will determine this or whether there is a second deafness gene in this region.

Deletion of most of USH1C in affected children of the Arab families with hyperinsulinism and deafness, characterization of different homozygous point mutations in affected individuals from two unrelated families with Usher syndrome, and demonstration of expression in the developing ear and eye provide evidence that USH1C underlies this disorder. Thus, we have identified a class of genes that appear to be important for the development and maintenance of both auditory and phototransduction.

Methods

Patients. Three children with severe hyperinsulinism and sensorineural deafness from two unrelated families were initially identified. Genetic analysis using family 1 (6 months of age) had normal electroretinograms (ERGs), but IV, (aged 2.5 years) and IV, from family 2 (aged 4 months) had attenuated ERGs, which may be observed before the onset of clinical symptoms of failing vision in individuals with Usher syndrome. These three affected children, V, from family 1; IV, from family 2 and IV, from family 2, had severe gastrointestinal symptoms including diarrhea, failure to thrive, intractable vomiting and a feeding disorder, all with focal dysmotility. Small bowel biopsy from V, from family 1; IV, and IV, from family 2, demonstrated crypt hyperplasia and villus atrophy, an inflammatory infiltrate within the lamina propria and disorganization of the surface epithelium. Appearance was indistinguishable from autoimmune enteropathy. Affected individuals IV, from family 1; III, and III, from family 2 did not undergo gastroenterological investigation. All three patients had generalized aminoaciduria and in one, urinary excretion of retinal binding protein and N-acetylglucosamine were found to be elevated, suggesting a proximal renal tubulopathy.

Family 3, a consanguineous Pakistani family, has not been described previously. All three affected individuals suffer from profound congenital sensorineural deafness. The eldest affected was diagnosed as having retinitis pigmentosa on fundoscopy, confirmed by electroretinogram, at the age of 24 y. He reports impairment of balance and also suffers from epilepsy. His brother has retinitis pigmentosa and reduced responses on ERG, consistent with retinal dystrophy and problems with balance. Their sister has confirmed retinitis pigmentosa. DNA samples from their parents were not available for analysis.

Sequencing of ARCC8 as described previously. Absence of amplification following multiple attempts, in the presence of good amplification of controls, was taken as evidence that at least one of the primers was within the deleted region.

Deletion mapping. Three affected children (V, from family 1; IV, and IV, from family 2) had normal karyotypes on high resolution G-banding. We amplified the exons of ARCC8 as described previously. cDNA was obtained by reverse transcription of total RNA from affected individuals and was used to screen the affected and control families. Absence of amplification following multiple attempts, in the presence of good amplification of controls, was taken as evidence that at least one of the primers was within the deleted region.
Sequences of primers used in the multiplex PCR reaction are primer 'a' (located in intron 2 of USH1C), 5'-GCACTGATGTCCTCGATCA-3'; primer 'b' (located in intron 22 of ABCG8), 5'-TACGGACAAAGGCTCTGTG-3'; and primer 'c' (located in intron 21 of ABCG8), 5'-CTTACCCTACCTCTCTTCCC-3'.

DNA sequencing. Primer sequences for exons of USH1C are available upon request. PCR products were purified using microspin columns (Pharmacia) and sequenced on an ABI377 using the fluorescent dideoxy terminator method.

DHPLC analysis. We analysed DNA from controls using the Transgenic Wave DNA analysis system (DHPLC). PCR fragments were generated using touchdown PCR, heteroduplexed with wild-type DNA and analysed according to the manufacturer's instructions.

RT-PCR. Human fetal material was obtained from the MRC Human Embryo Resource at the Institute of Child Health, approved by the Local Ethical Committee. RNA was extracted using TRIZOL (Gibco). We carried out reverse transcription with Superscript reverse transcriptase using oligo dT and random primers. Expression of isoforms of USH1C containing exon 3 was examined using primers in exon 3 (5', 5'-AGTGGGTCTCAA TGAACT-3') and exon 5 (SR, 5'-AGTGAATACCTGATCCG-3'). For lymphoblastoid cell lines, nested RT-PCR reactions used primers in exon 1 and exon 11 (5', 5'-CGGCGCGCTGGTCGCTTTCC-3', and 11R, 5'-GATCCGATTAGAATCGAC-3') and then primers 3F and 5R.

Immunohistochemistry. We carried out immunostaining of paraffin-embedded sections with an antibody in titre of 1:100 in PBS (pH 7.4) for 1 h at RT. Biotinylated anti-mouse antibody (Dako) at 1:50 in PBS for 1 h at RT followed by peroxidase labelled streptavidin (Sigma) at 1:50 in PBS for 1 h at RT was used for detection. Visualization with diaminobenzidine peroxidase and counterstaining with hematoxylin was employed.


GenBank accession numbers. PAC pD323982, AC003969; PAC 6-106F23, AC051137; AIE-75, AB006955 and AB018687; PDZ-73 and PDZ-45, AF039700 and AF039699, respectively.

Note added in proof: While this manuscript was in press, the paper by Verpy et al. was brought to our attention, which details identification of USH1C by an independent approach.

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A child presenting with disordered consciousness, hallucinations, screaming episodes and abdominal pain

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Clinical information

A previously well 2-year-old child presented to an Accident and Emergency department at 3.30 in the morning with disordered consciousness, hallucinations, screaming episodes and abdominal pain. She was seen by the paediatric team and thought to have an intussusception and thus was referred to a regional paediatric surgical unit at another hospital. During this initial assessment no measurement of blood glucose level was made. Four hours later she was reviewed by the surgical team and an intussusception was excluded on clinical and radiological grounds. She was then referred on to the paediatric medical team within the same hospital. At this time she was still hallucinating but otherwise her examination was unremarkable. The child’s mother was questioned about any medications that the child might have been exposed to, but mentioned only the possibility of rat poison. A urine specimen was sent for toxicology. Only during this third assessment was a blood sample drawn for measurement of blood glucose by a reagent strip with a glucometer (BM Stix) and the child noted to be profoundly hypoglycaemic with a BM Stix reading of 0.8 mmol/l (14 mg/dl). The result was not confirmed by laboratory measurement. She was treated with a bolus of 10% dextrose given intravenously and two subsequent BM Stix readings showed values of 6.1 mmol/l (100 mg/dl) and 6.4 mmol/l (115 mg/dl). Her full blood count, routine chemistry, and capillary blood gas values were normal. Her urine did not show the presence of any ketone bodies. She was subjected to an EEG, (which did not suggest any epileptiform activity) and a CT scan of her brain, which was normal.

Sixteen hours after her admission she became unresponsive, began convulsing and was unable to maintain her airway. She required intubation and ventilation and transfer to a paediatric intensive care unit was arranged. Her blood glucose value on the blood gas printout was 1.4 mmol/l (25 mg/dl). She was treated with a further bolus of 50% dextrose. After transfer and initial stabilisation, her glucose requirement was calculated to be 14.5 mg/kg per min in order to maintain a blood glucose concentration > 2.6 mmol/l (normal glucose requirements for her age 4–6 mg/kg per min). Despite continuous intravenous infusion of glucose she had two further hypoglycaemic episodes and appropriate endocrine and metabolic blood samples were taken at the time of the hypoglycaemia (Table 1). These further two episodes of hypoglycaemia may have been due to the miscalculation of glucose requirements as there was confusion about the percentage of dextrose being infused and the glucose requirements in mg/kg per min. This resulted in the child receiving less glucose than required to maintain normoglycaemia. Urine and blood specimens were sent for routine toxicology. She was extubated after 24 h with no complications and subsequently transferred to another tertiary centre for further assessment of the cause of the hypoglycaemia.

At this centre, specific questioning revealed a family history of non-insulin dependent diabetes in the maternal grandmother but any possible exposure to her medicines was strongly denied. The child underwent a 24 h blood glucose profile and was subjected to a diagnostic fast which lasted 18 h without her becoming hypoglycaemic. Ultrasonic imaging of her pancreas was normal. Results from the hypoglycaemic screens taken in intensive care then became available (Table 1) and showed a picture consistent with hyperinsulinaemic hypoglycaemia.

Her previous blood and urine samples (Table 2) were screened specifically for the presence of a sulphonylurea like agent and were found to be strongly positive. Further analysis by high performance liquid chromatography confirmed the drug type as glibenclamide. Despite this evidence, the child’s mother insisted that the child could not have been given this medication. Following a multi-disciplinary meeting the child and her parents were referred back to their local social services for further management. She has experienced no further episodes of hypoglycaemia.

Table 1 Plasma glibenclamide levels and the corresponding plasma insulin levels

<table>
<thead>
<tr>
<th>Blood test</th>
<th>Time of blood Sampling (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>821</td>
</tr>
<tr>
<td>Sulphonylurea (mg/l)</td>
<td>234</td>
</tr>
<tr>
<td>C-peptide (pmol/l)</td>
<td>4034</td>
</tr>
</tbody>
</table>

Table 2 Initial urine analysis consistent with glibenclamide poisoning

<table>
<thead>
<tr>
<th>Time of urine sample after admission</th>
<th>7 Hours</th>
<th>6 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphonylurea level (ug/l)</td>
<td>7.6</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

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Diagnosis: Non-accidentally induced hyperinsulinaemic hypoglycaemia

Discussion

Hypoglycaemia in the paediatric age group occurs most commonly in the neonatal period. In later childhood it occurs most frequently during periods such as fasting and intermittent illness when catabolism is occurring and the counter-regulatory mechanisms are induced. Hyperinsulinaemic hypoglycaemia, however, can occur spontaneously without a stressful stimulus and characteristically presents most commonly during the first post-natal weeks as a result of dysregulation of insulin secretion [3]. Hyperinsulinaemic hypoglycaemia can also be induced pharmacologically, either intentionally as a diagnostic tool, accidentally as a complication of the treatment of diabetes mellitus, or as a consequence of poisoning either with insulin itself [4], or with drugs such as sulphonylureas which stimulate insulin release [11]. Non-accidental poisoning as a form of child abuse was first recognised by Kempe et al. [5].

Hyperinsulinism is the commonest cause of intractable hypoglycaemia beyond the neonatal period [6]. In children under the age of 1 year who need pancreatic surgery to control the hyperinsulinism, the underlying pathology is usually a diffuse pancreatic pathology such as "nesidioblastosis" or focal β-cell hyperplasia, whereas in older children a discrete lesion such as a pancreatic islet cell adenoma is more likely. In the case of an islet cell adenoma, the clinical presentation is usually one of insidious onset, unlike the acute onset seen in this case. The classical biochemical profile of hyperinsulinaemic hypoglycaemia is one in which a low blood glucose level is accompanied by an inappropriately high plasma insulin levels, with low ketone body and fatty acid concentrations [2,3].

Hyperinsulinaemic hypoglycaemia may also be a manifestation of non-accidental poisoning, either by the administration of insulin [4] or from drugs that induce insulin secretion such as the sulphonylureas [11]. The only different biochemical consequence of insulin administration and drugs that cause insulin release is the difference in the plasma C-peptide concentration, with a low C-peptide associated with the administration of insulin, and a high level after sulphonylurea induced insulin release. Kempe et al. [5] first drew attention to the possibility of children suffering abuse by being poisoned with medications and in 1976, Roger et al. [10] reported six cases admitted to Great Ormond Street Hospital in London with persistent non-accidental poisoning by parents. They state in their article that bizarre symptoms and signs without any apparent pathological explanation should lead to the consideration of pharmacological causes. McClure et al. [7] found that the most common drugs used to poison children were anticonvulsants, followed by opiates. They also found that the combined annual incidence of non-accidental poisoning, non-accidental suffocation and Munchausen syndrome by proxy, was 0.5/100,000 for children aged below 16 years.

Glibenclamide is a sulphonylurea anti-diabetic agent. Its mechanism of action is thought to involve the stimulation of the sulphonylurea receptor on the pancreatic β-cell, causing release of preformed insulin and C-peptide. There is no information available on the pharmacokinetics of this drug in children. In adults the starting dose is usually about 5 mg/day. The drug appears in plasma or serum within 15-60 min after oral ingestion and mean peak plasma or serum concentration of approximately 140–350 μg/ml is usually attained within 2–4 h, range 2–8 h [1]. The elimination half-life is estimated to be 1.4–1.8 h (range 0.7–3 h). The elimination half-life and pharmacokinetics may well be very different in the childhood period. Few cases of glibenclamide induced poisoning have been reported in children. Pavone et al. [9] reported glibenclamide induced hypoglycaemia persisting for 15 h in a 11-month-old child, causing brain damage. In our case it was not possible from the information available to determine the exact timing of administration of the drug or how much of the drug was given. However symptoms persisted for at least 60 h.

Our case illustrates several important learning points:

1. First this child presented with non-specific symptoms. The symptoms of hypoglycaemia in the childhood period can be very variable occurring either as a result of autonomic or neuroglycopaenic effects. The neuroglycopaenic effects include the symptoms of hallucinations, abdominal pain, screaming episodes and fits, as demonstrated by this child. We suggest that any child who presents with unexplained symptoms such as these must have a bedside measurement of blood glucose performed. It is imperative that any abnormal BM Stix reading must be checked by laboratory plasma glucose measurement.

2. When confronted with a hypoglycaemic child thought must be given to the underlying aetiology. Before treatment, appropriate metabolic and endocrine blood samples must be taken. We would recommend samples for insulin, C-peptide, growth hormone, cortisol, ketone bodies, fatty acids, lactate, acylcarnitines and serum for toxicology. Although this may appear a daunting list, all substances can be measured at a later date if 2 ml of plasma and four spots on a standard Guthrie card are saved. The next available urine sample should be collected for measurement of urinary organic acids.

3. If the biochemical response to hypoglycaemia is missed, the child will need to be referred to a regional referral centre and subjected to further invasive and potentially hazardous diagnostic tests [8].

4. After treating a hypoglycaemic episode the blood glucose must be monitored frequently preferably hourly at least for the first a few hours. This especially applies in the case of hyperinsulinaemic hypoglycaemia, since delivering a bolus of glucose without a subsequent glucose infusion can cause rebound hypoglycaemia.

5. After giving a bolus of dextrose, an infusion of dextrose must be maintained to ensure a continuous supply of glucose initially at a rate of 4-6 mg/kg per min but subsequently titrated to the blood glucose concentration achieved. Any child who has a glucose requirement of more than 8–10 mg/kg per min of glucose could potentially have hyperinsulinism.

6. A full history of the medications used by all the members in the family must always be obtained in the initial history taking and this should be enquired into repeatedly if there are grounds to suspect poisoning. Sulphonylureas and other anti-diabetic drugs are not included within the standard toxicology screen performed by the National Poisons Unit in London and must be specifically requested if warranted by the clinical circumstances.

We report the case of a child who was non-accidentally poisoned with glibenclamide (a sulphonylurea) causing severe hyperinsulinaemic hypoglycaemia with near tragic consequences. This case illustrates several key learning points.
points regarding the need for the measurement and monitoring of blood glucose levels and to be vigilant to the possibility of drug induced hyperinsulinism.

References