Uncontrolled insulin secretion from a childhood pancreatic β-cell adenoma is not due to the functional loss of ATP-sensitive potassium channels

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K Hussain and K E Cosgrove made an equal contribution to the study

Abstract

We report the case of an 8-year-old child who presented with severe hyperinsulinaemic hypoglycaemia due to a pancreatic islet cell adenoma. In vivo, there was no beneficial response to the hyperglycaemia-inducing agent diazoxide and as a consequence the child underwent a subtotal pancreatectomy. In vitro studies of adenomatous β-cells revealed no operational defects in ATP-sensitive potassium channel activity and appropriate responses to diazoxide. In comparison with patients with focal adenomatous hyperplasia, genetic analysis of the isolated adenoma showed no loss of heterozygosity for chromosome 11p15 and expression of the cyclin-dependent kinase inhibitor p57kip2. This case illustrates that the excess insulin secretion from an infantile adenoma has an aetiology different from that observed in hyperinsulinism in infancy.

Introduction

Hyperinsulinism in infancy (HI) is the most common cause of recurrent or persistent hypoglycaemia in early childhood (Aynsley-Green et al. 2000). In children under the age of 1 year the disease manifests as either diffuse abnormalities of pancreatic β-cell function (Di-HI), or focal adenomatous hyperplasia of β-cells (Fo-HI) (Rahier et al. 2000), whereas in older children a pancreatic islet cell adenoma is more likely. Pancreatic islet cell adenomas are rare in childhood and, in contrast to the adult insulinomas, are usually benign (Grosfeld et al. 1990). Recent advances in the molecular physiology of β-cells have revealed insights into the pathogenesis of hyperinsulinism in infancy and the clinicopharmacology of therapeutically advantageous agents such as diazoxide and octreotide (an analogue of somatostatin) (Shepherd et al. 2000). In normal β-cells, control of insulin release is functionally coupled to ATP-sensitive potassium (K_{ATP}) channels in the plasma membrane. These channels are open in resting cells and (along with the Na⁺-K⁺ ATPase) maintain the resting membrane potential close to the equilibrium potential for K⁺—that is, approximately −65 mV (Dunne 2000). Glucose uptake and mitochondrial metabolism increase the ratio of ATP/ADP within the cell, which then acts as a signal for closure of K_{ATP} channels. Once the channels are closed, the membrane depolarizes (to around −35 to −40 mV), causing the activation of voltage-gated Ca²⁺ channels, increasing the rate of Ca²⁺ influx and thereby increasing the cytosolic concentration of Ca²⁺ in the vicinity of the plasma membrane. This sequence of events then triggers the release of insulin by the process of exocytosis (Dunne 2000, Henquin 2000). Defects in K_{ATP} channels are responsible for hyperinsulinism in infancy.
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(Kane et al. 1996, Shyng et al. 1998, Otonkoski et al. 1999, Bitner-Glindzicz et al. 2000, Glaser et al. 2000, Cartier et al. 2001, Straub et al. 2001) and this explains why patients with the condition are resistant to medical treatment with diazoxide – an agonist of K\textsubscript{ATP} channels. In the absence of functional K\textsubscript{ATP} channels, β-cells of hyperinsulinism in infancy are depolarized and this causes inappropriate electroactivity and increased basal concentrations of cytosolic Ca\textsuperscript{2+} for the corresponding level of glycaemia (Kane et al. 1996, Shepherd et al. 2000). In patients with Fo-HI – which probably accounts for at least 30% of all patients – expression of paternally-derived mutation(s) in \textit{ABCC8} or \textit{KCNJ11} occurs as a result of the somatic loss of maternal heterozygosity (Verkarre et al. 1998). β-Cell hyperplasia arises because a number of genes that are normally responsible for controlled cell proliferation, including \textit{H19} and \textit{p57kip2} (\textit{CDKN1C}), are also lost from this imprinted region (Verkarre et al. 1998, Rahier et al. 2000). In contrast to our understanding of early-onset hyperinsulinism in infancy, there have been no previous reports of the mechanism of uncontrolled insulin release from children with an isolated β-cell adenoma.

**Patient, methods and materials**

**Clinical details**

The patient was an 8-year-old girl who had been born at term after a normal delivery. There were no neonatal problems and her early development was normal until 7 months before referral, when she showed marked behavioural changes, became aggressive, and was uncooperative. She developed ‘drop attacks’, mainly in the morning, which subsequently became tonic–clonic seizures. During one of the seizure episodes, she was found to have a laboratory blood glucose concentration of 0.6 mmol/l. At her local hospital she failed to respond to maximum doses of diazoxide (20 mg/kg per day) and was referred to Great Ormond Street Hospital NHS Trust, London for further investigations of hypoglycaemia. On admission, she weighed 36 kg (97th centile), her height was 128 cm (50th centile) and she was found to be obese with mild hypertrichosis as a consequence of the commencement of diazoxide. The results of the biochemical investigations of the patient during episodes of hypoglycaemia are summarized in Table 1. These data are consistent with the diagnosis of hyperinsulinaemia-induced hypoglycaemia, with inappropriate suppression of lipolysis and ketogenesis in the face of prevailing hypoglycaemia. This was confirmed by the fact that the patient’s glucose requirement was 10 mg/kg per min (normal 4–6 mg/kg per min) after the diazoxide treatment was stopped. Further endocrine tests showed: (1) normal thyroid function with thyroid stimulating hormone (TSH) 1.1 mU/l (normal reference 0.5–5.0 mU/l) and plasma free thyroxine 20.5 pmol/l (normal reference 9.1–23.8 pmol/l); (2) appropriate growth hormone (GH) responses to glucagon (peak GH > 30 mU/l at 30 min (normal reference > 15 mU/l)); (3) an appropriate cortisol response to a test dose of adrenocorticotropic hormone (peak cortisol 600 nmol/l at 30 min (normal reference > 500 nmol/l)). Plasma ammonia concentration was 40 μmol/l (normal reference 40–50 μmol/l). Nuclear magnetic resonance imaging of the abdomen failed to localize the tumour, and the child was subsequently referred for surgery, when a subtotal (95%) pancreatectomy was performed. Postoperatively there were no surgical complications and the patient experienced no subsequent episodes of hypoglycaemia. Macroscopic examination of the pancreas postoperatively revealed the localization of a circumscribed nodule (~1.5 cm in diameter) at the junction between the head and the body of the pancreas.

**Isolation and preparation of tissue for \textit{in vitro} studies**

Control studies were carried out using intact islets of Langerhans isolated (with permission) from transplantable adult

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biochemical analysis during three episodes of hypoglycaemia</th>
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<tr>
<td><strong>Metabolite</strong></td>
<td><strong>Typical values at time of hypoglycaemia</strong></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>1.9</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>37.2</td>
</tr>
<tr>
<td>C-peptide (pmol/l)</td>
<td>126.3</td>
</tr>
<tr>
<td>βOB (mmol/l)</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>Branched chain amino acids</td>
<td></td>
</tr>
<tr>
<td>Leucine (μmol/l)</td>
<td>189</td>
</tr>
<tr>
<td>Isoleucine (μmol/l)</td>
<td>68</td>
</tr>
<tr>
<td>Valine (μmol/l)</td>
<td>280</td>
</tr>
<tr>
<td>Total carnitine (nmol/l)</td>
<td>69</td>
</tr>
<tr>
<td>Cortisol (nmol/l)</td>
<td>71</td>
</tr>
<tr>
<td>Lactate (nmol/l)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

βOB, β-hydroxybutyrate; NEFA, non-esterified fatty acids. Note that the low cortisol values may reflect the impact of recurrent hypoglycaemia.
Results

Histological characterization of the adenoma

Upon examination, the nodule was seen to be composed of ribbons and trabeculae of cells, with open nuclei with stippled chromatin and granular cytoplasm. The cells were uniform in size, with no nuclear pleomorphism, and there were no mitoses. The nuclei were of normal size and there were no differences in the sizes of nuclei in cells from within the nodule compared with those from the control region of the pancreas. There were no necrotic areas within the nodule. Immunostaining showed the cells to be positive for insulin and proinsulin, and negative for glucagon and somatostatin. The rest of the pancreas was entirely normal, with normal proportions of endocrine tissue.

Functional β-cell data

In β-cells from patients with Di-HI, KATP channels fail to operate and there are no effects of diazoxide on KATP channel activity (Kane et al. 1996, Straub et al. 2001). A markedly different pattern of events was seen in the adenoma β-cells. Figure 1A shows data from an intact β-cell and illustrates the presence of KATP channels in resting cells, and the subsequent activation of these channels by diazoxide (0.5 mM, n = 4/4 cells). KATP channels were consistently recorded when inside-out patches of membrane were formed, and responded to ATP (0.5 mM), diazoxide (0.2 mM) and tolbutamide (0.25 mM) (Fig. 1C, n = 4), as well as internally applied ADP and GDP (n = 4). These data imply that KATP channel function was preserved in adenoma β-cells, and as a consequence there were no spontaneous action potentials in intact cells (n = 6) and basal values of cytosolic Ca2+ concentrations were comparable to those obtained from control human islets: 83 ± 7 nM (n = 11) in adenoma cells compared with 90 ± 9 nM (n = 11) in non-adenoma islets and 80 ± 9 nM (n = 677) in control islets.

Genotype–phenotype investigations of ABCC8

Because adenomatous hyperplasia of β-cells causes Fo-HI through loss of heterozygosity (LOH) of chromosome 11p15, DNA was extracted from both the lesion and the normal portion of the pancreas and was screened for heterozygosity for the R1274R polymorphism in the ABCC8 gene. Both portions of the pancreas were heterozygous at this locus, thereby suggesting that the adenoma was not associated with LOH of this region of chromosome 11. This was further confirmed by p57kip2immunohistochemical staining. The p57kip2 gene is expressed only on the maternal allele and expression is lost in Fo-HI lesions with LOH (Kassem et al. 2001). Figure 2A and B show positively-stained β-cell nuclei within the

donor tissue as described elsewhere (Kane et al. 1996, Straub et al. 2001). A modification of this procedure was used to isolate islets from the pancreas of the patient after surgery. In brief, the tissue was first distended by injection of 5 ml Hanks’ balanced salt solution, containing 6 mg/ml Serva collagenase at 37 °C, directly into the parenchyma using a standard needle (25 gauge). Each sample of pancreas was then cut into small pieces and incubated with 30 ml warm collagenase solution in a water bath at 37 °C for 5 min. The suspension was then transferred through a 1000 µm mesh and then a 750 µm mesh and collected in newborn calf serum on ice. The non-transferable suspension was again incubated with warm collagenase and the process repeated. Digests from each sample now contained islets or single β-cells, or both, which were then washed in minimal essential medium before being placed into standard tissue culture conditions and cultured using RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 in air at 37 °C.

Experiments to record KATP channel currents were carried out using the cell-attached and the inside-out patch configuration of the patch-clamp technique as described elsewhere (Hamill et al. 1981, Kane et al. 1996, Straub et al. 2001). All illustrated records are displayed according to convention, with upward deflections representing outward currents.

Measurements of the cytosolic Ca2+ concentration were estimated in adenoma, non-adenoma and control human islets using microfluorimetry procedures with fura-2 as previously described (Grynkiewicz et al. 1985, Kane et al. 1996, Straub et al. 2001). Heterozygosity on chromosome 11p was studied by extracting DNA from paraffin-embedded blocks of normal and adenoma tissue using standard techniques. A single nucleotide polymorphism, R1274R, located in exon 31 of the SUR1 gene (ABCC8) was then evaluated by polymerized chain reaction amplification and restriction fragment length polymorphism analysis after digestion with Bsil1 as previously described (Nestorowicz et al. 1998, Glaser et al. 1999). For immunohistochemistry, sections 5 µm thick were prepared from archival paraffin-wax-embedded tissue, placed on SuperFrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany), and left to dry at 37 °C overnight. Slides were deparaffinized in xylene, and rehydrated in serial dilutions of alcohol (100%, 90% and 80%) and double-distilled water. Antigen retrieval was carried out as described elsewhere (Cattoretti et al. 1992). Slides were double stained for p57kip2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and insulin (Dako, Glostrup, Denmark) and antibodies were detected with diaminobenzidine tetrachloride-black and fast-red respectively. To prevent cross reactivity, an avidin-biotin blocking kit was used before the incubation with an anti-insulin antibody. As negative control, slides underwent the same procedure but were incubated with PBS without anti-p57kip2 antibody.

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**Figure 1** K<sub>ATP</sub> channels in adenoma β-cells. (A) Intact-cell data of KATP channels and activation by diazoxide (Diaz; 0.5 mM, n = 4). (B) Spontaneous KATP channel activity in an isolated inside-out patch (indicated by the arrow). (C) The pharmacological regulation of KATP channels to illustrate the reversible activation of KATP channels by diazoxide (0.2 mM), and inhibition of diazoxide-sensitive KATP channels by tolbutamide (Tol; 0.25 mM). Similar effects were seen in four other experiments.

**Figure 2** p57<sup>kip2</sup> and insulin staining of the adenoma (A), normal tissue outside the adenoma (B) and a Fo-HI lesion from a different patient (C). All sections were stained for p57<sup>kip2</sup> (brown) and for insulin (red). Definitive p57<sup>kip2</sup> staining is seen in the β-cell nuclei of the adenoma and the normal tissue (arrows); for comparison, in Fo-HI, where the maternal allele of chromosome 11p15 is lost, p57<sup>kip2</sup> staining is absent (C).

adenoma and in the normal adjacent pancreas respectively. For comparison, sections of a lesion from a Fo-HI patient are shown with no p57<sup>kip2</sup>-positive β-cell nuclei (Fig. 2C), confirming loss of the maternal allele in this lesion.

**Discussion**

Hyperinsulinism in infancy is the most common cause of severe hypoglycaemia in infancy and early childhood, and
can cause profound brain damage with long-term handicap (Aynsley-Green et al. 2000, Menni et al. 2001). The diffuse form of this condition is caused by mutations in the genes encoding SUR1 and Kir6.2 of the ATP-sensitive K⁺ channel in β-cells (Aynsley-Green et al. 2000, Glaser et al. 2000). Hyperinsulinaemia in infancy as a consequence of histologically distinct β-cell abnormalities can manifest as either focal adenomatous hyperplasia – Fo-HI – or adenoma (Verkarre et al. 1998). Fo-HI is usually diagnosed at birth or within the first few days after birth and is clinically indistinguishable from Di-HI (De Lonlay-Debeney et al. 1999, Aynsley-Green et al. 2000). As abnormal insulin secretion is caused by defective K<sub>ATP</sub> channels, patients who do not respond to diazoxide treatment require a sub-total pancreatectomy to alleviate the hypoglycaemia. True β-cell adenomas (insulinomas) are rare in childhood and typically present symptoms of recurrent hyperinsulinaemic hypoglycaemia in a previously healthy child. Eighty to ninety percent of insulinomas are benign solitary tumours, and in exceptional cases multiple discrete insulinomas may also occur as part of the multiple endocrine neoplasia type 1 (MEN-1) syndrome (Oliver et al. 1983). As the molecular defects of insulinoma-induced hypoglycaemia are not known, we have described a patient with a single β-cell adenoma, diagnosed in childhood, in whom we have shown that the functional characteristics of β-cells and the molecular aetiology of disease are distinct from those of both Di- and Fo-HI. Using patch-clamp techniques, we have documented that the adenoma β-cells possessed operational K<sub>ATP</sub> channels in intact cells and did not spontaneously generate calcium action potentials. K<sub>ATP</sub> channels were spontaneously active in isolated cell membranes and were modulated by ATP, nucleotide diphosphates and diazoxide in the presence of ATP. These findings suggested that the pathophysiology of insulin release in adenoma was unrelated to defects on chromosome 11p15 and this was confirmed by immunohistochemical data showing normal expression of p57<sup>KIP2</sup> in cells within and outside the discrete lesion.

As the index patient in this study was unresponsive to diazoxide treatment in vivo, the actions of diazoxide on K<sub>ATP</sub> channels in vitro are particularly interesting to the clinical management of these cases, and have implications for the mechanisms of insulin hypersecretion in adenoma. In patients with early-onset hyperinsulinism in infancy, we have previously documented that insensitivity to diazoxide treatment is directly correlated with defects in K<sub>ATP</sub> channels (Kane et al. 1996). However, such a correlation cannot be made for this adenoma patient, because K<sub>ATP</sub> channels were found and were activated by diazoxide in a manner consistent with that seen in normal β-cells. One likely explanation for these findings is that the pathogenesis of inappropriate insulin secretion is unrelated to regulated insulin secretion and arises as a result of defects in the constitutive secretory pathway. All proteins are synthesized on the rough endoplasmic reticulum and then sorted by the various elements of the golgi apparatus. Vesicles bud from the golgi structures, and are targeted to different areas of the cell, depending on their contents. In β-cells, proinsulin is packaged in secretory granules and targeted to the ‘regulated release pathway’ (Rivas & Moore 1989). This pathway ensures that secretory granules are stored close to the cell membrane and that, upon the generation of intracellular signals leading to the initiation of Ca<sup>2+</sup>-dependent exocytosis, insulin is secreted. Other proteins such as collagen and amylin are released from cells via the ‘constitutive pathway’ – a continuous process that is not dependent upon granule storage (Verchere et al. 2000). To co-ordinate these independent processes, newly-synthesized proteins must be critically sorted from within the trans-golgi network and accurately targeted for membrane trafficking. Because, in this study, we have shown no significant defects in the process that governs regulated insulin release (i.e. K<sub>ATP</sub> channels and cytosolic Ca<sup>2+</sup> concentrations), it seems possible that insulin release occurred via the constitutive pathway, which by definition will be diazoxide-insensitive. Support for this hypothesis comes from studies involving rodent insulinoma cell lines, which have shown that insulin and proinsulin are secreted predominantly via a constitutive pathway, rather than a regulated pathway as in normal human β-cells (Nagamatsu & Steiner 1992).

In summary, these studies have shown that insulin secretion in a childhood adenoma is not associated with genetic or functional abnormalities in β-cell K<sub>ATP</sub> channels. Our data support the hypothetical importance of constitutive insulin secretion, and further studies will be needed in other patients to dissect the precise abnormalities that underpin the dysregulation of insulin secretion.

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