Persistent hyperinsulinaemic hyperglycaemia of infancy-derived cells; implications for β-cells that replicate in vitro

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INTRODUCTION

Insulin is synthesised, stored and secreted from the pancreatic β-cells. These are located within the islets of Langerhans, which are distributed throughout the pancreas. Less that 2% of the total pancreas is devoted to an endocrine function. When the mechanisms that control insulin release are compromised, potentially lethal diseases such as diabetes and hyperinsulinism are manifest. Studies of a rare neonatal disorder, persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), have now led to the development of a panel of novel human insulin-secreting cell lines. These cells have potential uses in many aspects of diabetes-related research, including cell-based therapies for diabetes and PHHI. Here, we review the origins of PHHI-derived β-cell lines from clinical disease to in vitro gene therapy.

THE IONIC CONTROL OF INSULIN RELEASE

Insulin release from β-cells is mainly dependent on depolarisation of the plasma membrane which activates voltage-dependent Ca2+ channels. Exocytosis of insulin results as the rise in intracellular Ca2+ promotes secretory granule fusion with the plasma membrane and release of the hormone. The resting β-cell membrane potential is determined by the maintenance of a high K+ permeability across the cell membrane by the activity of the Na+/K+-ATPase and the open state of ATP-sensitive K+ (KATP) channels. Glucose, the major physiological stimulus, initiates insulin secretion at concentrations greater than 5·5 mM and promotes a slow depolarisation of the membrane from between −70 to −60 mV to a threshold potential (~ −50 to −40 mV) at which electrical activity in the form of action potentials is initiated with the opening of voltage-dependent Ca2+ channels (Fig. 1). It is the metabolism of glucose, following its uptake into the β-cells, by glucokinase and in the mitochondria, which increases the intracellular ATP/ADP ratio which in turn closes KATP channels, causing the cell depolarisation. KATP channels are therefore the foremost K+ channels governing the cell membrane potential in β-cells. Selective inhibition of KATP channels with drugs such as the sulphonylureas (e.g. glibenclamide, tolbutamide, etc.) will thus mimic the actions of glucose and promote insulin release. Conversely, KATP channel ‘openers’, such as the hyperglycaemia-inducing compound diazoxide, and voltage-gated Ca2+ channel blockers, such as nifedipine, exert the opposite effect and inhibit secretion by preventing Ca2+ entry. The significance of KATP channels in the control of insulin release under normal conditions and in disease states has been greatly enhanced by recent studies which have resolved the structure of the KATP channels and identified defects in these K+ channels which give rise to insulin hypersecretion.
PERSISTENT HYPERINSULINAEMIC HYPOGLYCAEMIA OF INFANCY

Hypoglycaemia is a relatively common childhood metabolic abnormality and when persistent or recurrent is most frequently a consequence of hyperinsulinism; neonatal hyperinsulinism (On-line Mendelian Inheritance in Man:256450). PHHI is a rare neonatal disorder with devastating consequences for the newborn. Sporadic cases of PHHI are thought to be rare in the general population (~1/45 000), but the disease has an incidence in communities with high rates of consanguinity which approaches that of cystic fibrosis in European Caucasians, i.e. approximately 1/2500 live births (see Glaser et al. 1999). Failure to recognise and treat hypoglycaemia promptly carries a substantial risk of severe brain damage and mental retardation because of a lack of fuels to sustain normal brain metabolism. Severe forms usually present within the first few hours or days following birth as the result of extreme and sustained low blood glucose levels due to unregulated secretion of insulin. The clinical characteristics of the condition are, however, heterogeneous and, whilst most cases present symptoms shortly after birth, some cases present at several months to 1 year of age, and even adult cases have also been described. Drug responsiveness in PHHI patients is highly variable and in the majority of cases medical therapy is of limited use (Dunne et al. 1999, Glaser et al. 1999).

**FIGURE 1.** Ionic control of insulin release. In normal β-cells, at non-stimulatory glucose concentrations, the resting membrane potential (~ −70 mV) is determined by open K\textsubscript{ATP} channels. When the extracellular glucose concentration is elevated, glucose is taken up by the β-cell and glucose metabolism is initiated. The rate-limiting step in this process is dependent upon the activity of glucokinase and the formation of glucose-6-phosphate (glucose-6-P). Subsequent metabolic events lead to an increase in the cytosolic ATP/ADP ratio and the closure of K\textsubscript{ATP} channels. This leads to membrane depolarisation and the opening of voltage-dependent Ca\textsuperscript{2+} channels. The increased Ca\textsuperscript{2+} influx then initiates the release of insulin through exocytosis of secretory granules. These events account for first phase insulin release in response to glucose stimulation. Second phase insulin release occurs ‘independently’ of K\textsubscript{ATP} channel function, through glucose ‘augmentation’ pathways which are dependent upon the elevation of cytosolic Ca\textsuperscript{2+} levels, and other glucose-derived signalling factors. PHHI arises from defects (*) in either the Kir6.2 or SUR1 genes. Since these β-cells lack operational K\textsubscript{ATP} channels, the membrane potential remains depolarised (~ −30 mV) in the absence of glucose metabolism. This leads to the persistent activation of voltage-dependent Ca\textsuperscript{2+} channels causing unregulated entry of Ca\textsuperscript{2+} and persistent release of insulin as a consequence.
The molecular basis of PHHI is also heterogeneous. In 1994, familial disease was linked to chromosome (ch) 11p15-1, and later confirmed in multiplex Saudi-Arabian families (Glaser et al. 1994). This region of Ch.11 encodes both subunits of K\textsubscript{ATP} channels, Kir6-2 and SUR1 (see Aguilar-Bryan et al. 1998). In those cases where mutations have been linked to the disease, defects in the SUR1 and Kir6-2 genes are mainly, but not exclusively, inherited in an autosomally recessive manner (see Table 1). Thus, there are reports of non-Mendelian inheritance amongst discordant identical twins, and data to suggest that PHHI can be inherited in an apparent autosomal dominant way (Glaser et al. 1999). Loss of maternally imprinted genes in PHHI has recently been described. In these cases, the patient inherits a single paternal recessive SUR1 gene mutation, and a portion of the pancreas is reduced to hemizygosity because of the loss of maternal imprinted genes (Verkarre et al. 1998). Loss of heterozygosity in the affected β-cells results in expression of the paternal gene defect and insulin hypersecretion. Loss of other maternally expressed imprinted genes (such as the tumour-suppresser genes H19 and p57\textsuperscript{KIP2}) causes the morphologically distinct appearance of focal regions of β-cell hyperproliferation in the pancreata of patients with this condition. This has given rise to the terminology of ‘focal PHHI’ as distinct from ‘diffuse PHHI’ which is not thought to be associated with loss of imprinted genes (de Lonlay et al. 1999). In addition, spontaneous SUR1 gene mutations also give rise to PHHI, and each of these factors has hindered attempts to provide a concise genotype-phenotype relationship in the field, and to identify the genetic lesions in many patients. An estimated 60% of all patients with PHHI remain anonymous in terms of the genetic basis of the condition (Glaser & Aguilar-Bryan 2000).

**COMPONENTS OF β-CELL K\textsubscript{ATP} CHANNELS**

β-Cells express a K\textsubscript{ATP} channel complex formed by subunits belonging to at least two distinct families of proteins (Fig. 2). The K\textsuperscript{+} selective pore is formed by the Kir6-2 subunit, a member of the inward rectifier K\textsuperscript{+} channel family. The other subunit, a larger protein, is a receptor with high affinity for sulphonylureas, designated SUR1. Two closely related genes encode two sulphonylurea receptors, SUR1 and SUR2. Three splice variants of the SUR2 gene have been described and designated SUR2A, SUR2B and SUR2C (Aguilar-Bryan & Bryan 1999). Human SUR1 is 1581 amino acids in length and, like the cystic fibrosis transmembrane conductance regulator and the multi drug resistance protein, it is a member of the superfamily of ATP-binding cassette proteins. Neither the native Kir6-2 subunit nor the SUR1 subunit will form operational K\textsuperscript{+} channels independently. However, when co-expressed, K\textsuperscript{+} channel currents that closely resemble those of the native β-cell K\textsubscript{ATP} channel complex are generated (Inagaki et al. 1995, Sakura et al. 1995). The proposed topological organisation of the K\textsubscript{ATP} channel suggests an obligatory octameric complex formed by four Kir6-2 subunits lining the pore coupled to four SUR1 subunits (Fig. 2). SUR1 affects the trafficking and distribution of Kir6-2 (Sharma et al. 1999). Kir6-2 determines biophysical properties such as ion selectivity, rectification and gating of the complex (Ashcroft & Gribble 1998). However, channel sensitivity to physiological regulators, such as the adenine and guanosine nucleotides, is a complex process involving both subunits. Kir6-2 confers the ATP sensitivity of the complex, whilst ADP (and GDP) binds to one of the nucleotide-binding folds of SUR1 and antagonises the effects of ATP. SUR1 therefore serves as a transmembrane conductance regulator of Kir6-2 by conferring high sensitivity to metabolically derived signals and to key pharmacological agents such as diazoxide and the sulphonylureas. SUR1 is also important for the correct location at the plasma membrane and assembly of the component parts of K\textsubscript{ATP} channels that constitute the molecular couples (Aguilar-Bryan & Bryan 1999, Sharma et al. 1999).

The gene encoding SUR1 comprises 39 exon boundaries and is clustered with the KIR6-2 gene. Their location in the human genome corresponds to the genetic locus of PHHI. The evidence is now convincing that gene defects encoded within SUR1 or KIR6-2 can be correlated with the functional loss of K\textsubscript{ATP} channels in β-cells, and that this leads to the loss of regulated insulin secretion and the pathogenesis of this disease.

**PHYSIOLOGY OF THE PHHI β-CELL**

In isolated PHHI β-cells, the resting cell membrane potential is close to the threshold for the activation of voltage-dependent Ca\textsuperscript{2+} channels, and it is activity of these channels that leads to the appearance of spontaneous, regenerating action potentials in the unstimulated PHHI β-cell (Kane et al. 1996, Dunne et al. 1997). Since Ca\textsuperscript{2+} influx is a key determinant of insulin secretion under normal conditions, inappropriate Ca\textsuperscript{2+} channel activity readily accounts for the unregulated secretion of insulin (Fig. 1). Furthermore, PHHI appears to be caused by the selective loss of K\textsubscript{ATP} channels,
<table>
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<th>Domain</th>
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Tm, putative transmembrane domain (according to Swiss-Prot Q09428); NBF, nucleotide-binding fold; n.d., no data available; M2, one of two potential transmembrane spanning domains.

References: 1, Nestorowicz et al. (1998); 2, Thomas et al. (1996); 3, Thomas et al. (1995); 4, Nestorowicz et al. (1996); 5, Verkarre et al. (1998); 6, Dunne et al. (1997); 7, Meissner et al. (1997); 8, Nichols et al. (1996); 9, Glaser & Aguilar-Bryan (1999); 10, Nestorowicz et al. (1997); 11, Otonkoski et al. (1999); 12, Aguilar-Bryan & Bryan (1999); 13, Huopio et al. (1999); 14, Kentrup et al. (1999).
since the major regulatory and pharmacological properties of delayed-rectifier K⁺ channels and Ca²⁺- and voltage-gated K⁺ channels in PHHI β-cells are similar to control human β-cells (Cosgrove et al. 1998). A transgenic animal model of PHHI has been developed in which transgenic mice were engineered to express a ‘dominant-negative’ version of Kir6·2 in β-cells (Miki et al. 1997, 1999). Affected mice developed hyperinsulinaemic hypoglycaemia during the neonatal period and, as the animals developed, this was followed by hypoinsulinaemia and hyperglycaemia, i.e. the onset of diabetes. A high frequency of apoptotic β-cells in the prediabetic period suggests that unregulated Ca²⁺ influx carries a compound pathology – underpinning inappropriate insulin secretion and then leading to the induction of premature apoptosis (Miki et al. 1997). This has a clinical correlation. In a recent review, Glaser and colleagues (1999) have discussed how long-term, intensive treatment of PHHI patients with somatostatin leads to remission of symptoms without the need for surgery. In such cases, it seems likely that this is caused by destruction of β-cells through programmed cell death. Whether this is a result of unregulated Ca²⁺ entry, or a direct consequence of somatostatin, which is known to be apoptogenic (Patel & Srikant 1997), remains to be determined. In separate transgenic animal studies, a severe loss of β-cell function was also found in tissue isolated from ‘Kir6·2 knock-out’ animals (Miki et al. 1998). However, quite surprisingly, these β-cells were less reminiscent of human PHHI β-cells than the insulin-secreting cells isolated from ‘dominant-negative’ mice. Another interesting feature of the knock-out model mice is that, despite having impaired glucose-dependent insulin release both in vitro and in vivo, they present normal post-prandial blood glucose values. This may implicate a negative correlation between Kir6·2 operation and insulin receptor expression in skeletal muscle tissue, which will tend to improve peripheral glucose clearance despite impaired insulin secretion (Miki et al. 1999). SUR1 ‘knock-out’ mice have also been created (Seghers et al. 1999). In these animals, the β-cell membrane potential is depolarised with

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**Figure 2.** Components of K<sub>ATP</sub> channels in pancreatic β-cells. (A) The predicted membrane topology of the K⁺ channel subunits SUR1 and Kir6·2. SUR1 is an ATP-binding cassette protein. Note the characteristically high number of transmembrane spanning domains 1–17, and the presence of two intracellularly disposed nucleotide-binding domains (NBD). Kir6·2 has two transmembrane domains and an inner loop that controls K⁺ flux. (B) The heteromultimeric K<sub>ATP</sub> channel complex is an obligatory octameric structure composed of (Kir6·2/SUR1)₄. Kir6·2 is proposed to be located towards the inner core of the complex.
With respect to normal, and spontaneous Ca\(^{2+}\) action potentials, inhabitable by nifedipine, are present in 2-8 mM glucose. These mice, like the Kir6.2 knock-out animals, also have normal glucose levels with respect to controls but fail to increase insulin in response to an increased glucose load. The mechanism by which SUR1\(^{-/-}\) mice maintain glycaemia is currently unknown.

### NES2Y: A NOVEL HUMAN β-CELL LINE

Whilst studying the physiology of PHHI β-cells, we recently discovered that these cells will proliferate in vitro. Although a number of rodent and hamster β-cell-like lines have been generated through viral transformation (HIT-T15) (Santerre et al. 1981), X-irradiation (RIN/Ins-1) (Gazdar et al. 1980), transgenic expression of tumour-promoting proteins such as SV40 large T antigen in β-cells (βTC and MIN6) (Efrat 1988) or electrofusion (BRIN BD11) (McClenaghan et al. 1996), attempts to generate human β-cell lines have proved unsuccessful.

We have studied one cell line – designated NES2Y β-cells – in detail. NES2Y cells reproduce the properties and key features of acutely isolated insulin-secreting cells from patients with PHHI. Thus, these cells lack operational K\(_{ATP}\) channels, have markedly impaired cytosolic Ca\(^{2+}\) signalling mechanisms, constitutively release insulin at an elevated rate in the absence of stimuli and do not respond to depolarisation-dependent agonists through increasing the release of insulin (Macfarlane et al. 1999). As PHHI is a rare condition, the availability of the NES2Y β-cell is an important asset to ongoing studies of the molecular pathophysiology of the condition. NES2Y cells do, however, retain some capacity to secrete insulin in response to elevated glucose, and this can be explained through the K\(_{ATP}\) channel-independent pathway of glucose-induced secretion. Glucose 'augmentation' routes have been described in rodent (for review see Aizawa et al. 1998) and human insulin-secreting cells (Straub et al. 1998a,b). These pathways, uncovered in normal β-cells by eliminating the contribution of K\(_{ATP}\) channels to the operation of β-cells, are now recognised as accounting for the second phase of insulin release (Fig. 1). As second phase secretion is dependent upon glucose metabolism and the concomitant entry of Ca\(^{2+}\), the lack of operational K\(_{ATP}\) channels in NES2Y cells (and acutely isolated PHHI β-cells) coupled with the unregulated influx of Ca\(^{2+}\) will promote and fuel glucose-induced secretion. The second messengers involved in governing K\(_{ATP}\) channel-independent signalling events have yet to be completely elucidated, but may include an elevation in cytosolic long-chain acyl CoA, GTP, cAMP, etc. (see Aizawa et al. 1998).

### THE CREATION OF A GLUCOSE-RESPONSIVE HUMAN β-CELL LINE

In addition to loss of K\(_{ATP}\) channel operation, NES2Y β-cells also have impaired expression of the homeodomain transcription factor PDX1 (Macfarlane et al. 1997), a major islet cell differentiation and lineage determination factor (Jonsson et al. 1994). In more recent studies, we have documented how defects in the functional operation of NES2Y β-cells can be overcome following a triple transfection with cDNAs encoding SUR1, Kir6.2 and PDX1. This has led to the generation of a fully glucose-responsive human β-cell line – designated NISK9 β-cells (Macfarlane et al. 1999). The properties of K\(_{ATP}\) channels expressed in the NISK9 β-cells are strikingly similar to those reported in native tissue (Dunne & Petersen 1991). The recombinant channels were inwardly rectifying, inhibited by cytosolic ATP in a concentration-dependent manner, activated by ADP in the presence of ATP, underwent spontaneous run-down in isolated patches, and were modulated by diazoxide, tolbutamide and efaroxan. The operation of these channels has clear functional consequences. Not only did the transfection event underpin the development of glucose responsiveness within a physiologically relevant concentration range, but it also governed both KCl- and tolbutamide-induced increases in the cytosolic Ca\(^{2+}\) concentration and insulin release, and controlled the inhibition of glucose-induced rises in cytosolic Ca\(^{2+}\) by diazoxide. Experiments are currently in progress to determine the contribution of each of the transfected cDNAs to these properties. In cells stably transfected with PDX1 alone, NES-PDX1 cells, insulin mRNA levels are increased following incubation in high glucose, whereas in NES2Y cells they are not (W M Macfarlane, M J Dunne & K Docherty, unpublished observations). Cells transfected with SUR and Kir6.2 (but not PDX1) also express fully operational K\(_{ATP}\) channels and secrete insulin in response to glucose stimulation. However, unlike NISK9 β-cells they fail to exhibit glucose-dependent insulin gene promoter activity because of impaired PDX1 function (W M Macfarlane, M J Dunne & K Docherty, unpublished observations).

### THE PROLIFERATIVE CAPACITY OF NES2Y β-CELLS

It is currently unclear as to why the NES2Y cells (and other PHHI-derived β-cells) proliferate in
culture, and a number of key questions remain unanswered. As we have described previously (Macfarlane et al. 1997), it may be a consequence of the impaired expression of the homeodomain transcription factor PDX1, and its role in pancreatic β-cell lineage determination. NES2Y cells may, therefore, represent a stage in islet cell development at which the cells have retained the ability to replicate while attaining a β-cell-like phenotype. However, stable transfection of NES2Y β-cells with PDX1 does not prevent proliferation of the cells. Alternatively, the ability to proliferate may be a general property of neonatal human islet tissue, which has not been well studied because of the paucity of available tissue. It is also possible that unregulated Ca$^{2+}$ influx – or indeed other second messenger events, is having a permissive role on cell-cycle events. These possibilities are currently under investigation.

**IN SUMMARY**

Our recent work upon a rare disease has established proof of the concept that in vitro gene therapy could be used to successfully reverse a metabolically related disorder. Our results allude to the possibility that in future, following pancreatectomy, acutely isolated β-cells from PHHI patients could be similarly engineered for subsequent autotransplantation. By transgenic manipulation of the NES2Y cells, we have generated the first fully glucose-responsive human insulin-secreting cell line. We believe that these, and other PHHI-derived islet cell lines, will be of major importance for in vitro studies of human β-cell function and potentially valuable in transplantation-based therapies for both diabetes mellitus and PHHI.

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