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HANNA HUOPIO

Congenital hyperinsulinism in the Finnish population

Doctoral dissertation

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ABSTRACT

INTRODUCTION: Congenital hyperinsulinism (CHI) is a heterogeneous disorder characterized by a dysregulation of insulin secretion. ATP-sensitive potassium channels (K_{ATP} channels), which consist of a hetero-octameric complex of four sulfonylurea receptor 1 (SUR1) and four Kir6.2 proteins, play a major role in controlling the insulin release from pancreatic beta cells. Mutations in genes encoding these proteins are the most common cause of CHI. In addition, mutations in genes encoding the metabolic enzymes glucokinase and glutamate dehydrogenase have also been shown to cause CHI. The severity of CHI varies from a mild drug-responsive form to a severe drug-resistant form that may necessitate pancreatectomy. Early diagnosis and effective treatment are the cornerstones in avoiding irreversible brain damage due to prolonged hypoglycemia.

AIMS OF THE STUDY: The aims of this thesis were to study the genetic background of Finnish CHI patients and to investigate the correlations between different genotypes and phenotypes.

METHODS: Genetic studies were performed using the candidate gene approach and generally accepted standardized methods (polymerase chain reaction, single-strand conformation polymorphism, haplotype analysis, restriction fragment length polymorphism). The associations between the novel base substitutions and CHI were confirmed by expressing the mutant proteins in xenopus oocytes. The genotype-phenotype characterizations were supplemented by measuring the acute insulin responses to calcium, glucose and tolbutamide in CHI patients with different genotypes. Furthermore, glucose tolerance, insulin sensitivity and insulin secretion in heterozygous relatives of CHI patients with the two Finnish founder mutations SUR1-V187D and SUR1-E1506K were studied by generally accepted, standardized methods (the oral glucose tolerance test, intravenous glucose tolerance test, hyperinsulinemic-euglycemic clamp, hypoglycemic clamp, hyperglycemic clamp and tolbutamide test).

RESULTS: During 1972-2000 altogether 44 patients were diagnosed with CHI, which corresponds to a national incidence of 1 : 42 000 in Finland. The incidence was remarkably higher in two narrow high-risk areas in central and southwestern parts of Finland. Nine novel base substitutions (six in SUR1, two in Kir6.2, one in glucokinase genes) were found, accounting for 60% of all Finnish CHI-patients. The two founder mutations, recessively inherited SUR1-V187D and dominantly inherited SUR1-E1506K, were found in 88% of the genetically characterized patients. All other mutations were detected in single patients. The mutation SUR1-V187D was found in severe cases, whereas SUR1-E1506K was associated with a milder, diazoxide-responsive form of CHI. The acute insulin responses to calcium and tolbutamide proved to be useful in differentiating the distinct forms of diffuse CHI. A positive insulin response to calcium and a negative response to tolbutamide suggested a diffuse form of CHI due to a K_{ATP} channel mutation. The heterozygous carriers of SUR1-V187D had normal glucose tolerance, insulin sensitivity and appropriate insulin secretion. However, the insulin secretion capacity was severely impaired in subjects with the dominant SUR1-E1506K mutation.

CONCLUSIONS: This study showed that mutations in K_{ATP} channel genes are the major cause of CHI in the Finnish population. The detection of the two founder mutations has important clinical implications. In the case of SUR1-E1506K, diazoxide therapy should be the treatment of choice, whereas the patients with SUR1-V187D may need surgical treatment. Heterozygosity for SUR1-V187D does not, however, predispose to disturbances of glucose metabolism. In contrast, SUR1-E1506K causes CHI in early life, but predisposes to later development of insulin deficiency and diabetes mellitus.

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To Jukka, Maija, Joose and Janne

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Hanna Huopio

LIST OF ABBREVIATIONS

AIR	Acute insulin response
ATP	Adenosine triphosphate
BMI	Body mass index
Bp	Base pair
BWS	Beckwith-Wiedemann syndrome
CHI	Congenital hyperinsulinism
FADH ₂	Flavin adenine dinucleotide phosphate
GCK	Glucokinase
GDH	Glutamate dehydrogenase
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide-1
HI	Hyperinsulinism
HIHA	Hyperinsulinemia-hyperammonemia syndrome
HNF	Hepatocyte nuclear factor
IPF-1	Insulin promoter factor 1
IVGTT	Intravenous glucose tolerance test
K _{ATP} channels	ATP-sensitive potassium channels
Kir6.2	Potassium inward rectifying channel 6.2
MBD	Minimal brain dysfunction
MODY	Maturity-onset diabetes of the young
Mt	Mutated
NADH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding domain
NBF	Nucleotide-binding fold
OGTT	Oral glucose tolerance test
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHHI	Persistent hyperinsulinemic hypoglycemia of infancy
RFLP	Restriction fragment length polymorphism
RIA	Radioimmuno assay
SGA	Small for gestational age
SSCP	Single-strand conformation polymorphism
SUR1	Sulfonylurea receptor 1
TCA	Tricarboxylic acid cycle
Wt	Wild-type

LIST OF ORIGINAL PUBLICATIONS

- I Otonkoski T, Ämmälä C, Huopio H, Cote GJ, Chapman J, Cosgrove K, Ashfield R, Huang E, Komulainen J, Ashcroft FM, Dunne MJ, Kere J, Thomas PM. A point mutation inactivating the sulfonylurea receptor causes the severe form of persistent hyperinsulinemic hypoglycemia of infancy in Finland. *Diabetes*. 1999; 48:408-15.
- II Huopio H, Reimann F, Ashfield R, Komulainen J, Lenko HL, Rahier J, Vauhkonen I, Kere J, Laakso M, Ashcroft F, Otonkoski T. Dominantly inherited hyperinsulinism caused by a mutation in the sulfonylurea receptor type 1. *J Clin Invest*. 2000; 106:897-906.
- III Huopio H, Jääskeläinen J, Komulainen J, Miettinen R., Kärkkäinen P., Laakso M., Tapanainen P., Voutilainen R. and Otonkoski T. Acute insulin response tests for the differential diagnosis of congenital hyperinsulinemia. *J Clin Endocrinol Metab*; In press.
- IV Huopio H, Vauhkonen I, Komulainen J, Niskanen L, Otonkoski T, Laakso M. Carriers of an inactivating beta-cell ATP-sensitive K(+) channel mutation have normal glucose tolerance and insulin sensitivity and appropriate insulin secretion. *Diabetes Care* 2002; 25:101-106.
- V Huopio H, Otonkoski T, Vauhkonen I, Reimann F, Ashcroft F, Laakso M. A new subtype of autosomal dominant diabetes due to the E1506K mutation in the sulfonylurea receptor 1 gene. Submitted.

CONTENTS

1 INTRODUCTION	15
2 REVIEW OF THE LITERATURE	16
2.1 Insulin secretion and action	16
2.1.1 Development and structure of endocrine pancreas	16
2.1.2 Biosynthesis of insulin	17
2.1.3 Insulin secretion	18
2.1.4 Insulin action	21
2.2 Congenital hyperinsulinism (CHI)	22
2.2.1 Clinical symptoms and diagnosis	22
2.2.2 Molecular genetics	23
2.2.2.1 Mutations in potassium K_{ATP} channel genes	23
2.2.2.2 Mutations in the glucokinase gene	26
2.2.2.3 Mutations in the glutamate dehydrogenase gene	26
2.2.3 Mouse models	27
2.2.4 Treatment	28
2.2.5 Long-term prognosis	29
2.2.6 Other hyperinsulinemic conditions in newborns	31
2.2.7 CHI and diabetes mellitus	32
3 AIMS OF THE STUDY	34
4 SUBJECTS AND METHODS	35
4.1 Subjects	35
4.1.1 Patients in the genetic studies (Studies I-III)	35
4.1.2 Patients in the acute insulin response tests (Study III)	39
4.1.3 SUR1-V187D heterozygotes (Study IV)	39
4.1.4 SUR1-E1506K heterozygotes (Study V)	40

4.2 Methods	41
4.2.1 Genetic analysis (Studies I-III)	41
4.2.2 Acute insulin response tests (AIRs) (Study III)	44
4.2.3 Studies of glucose metabolism in adults (Studies IV, V)	45
5 RESULTS	49
5.1 Genetic findings (Studies I-III)	49
5.1.1 SUR1-V187D (Study I)	49
5.1.2 SUR1-E1506K (Study II)	52
5.1.3 Other K _{ATP} channel mutations (Study III)	55
5.1.4 GCK-Y214C	56
5.2 Acute insulin responses in CHI patients (Study III)	56
5.3 Glucose metabolism in SUR1-V187D heterozygotes (Study IV)	58
5.4 Glucose metabolism in SUR1-E1506K heterozygotes (Study V)	60
6 DISCUSSION	65
6.1 Genotype-phenotype correlations	65
6.2 Representativeness of the study groups	70
6.3 Methods	72
6.4 Concluding remarks	73
7 SUMMARY	76
8 REFERENCES	78
ORIGINAL PUBLICATIONS	

1 INTRODUCTION

Congenital hyperinsulinism (CHI) is the most common cause of recurrent and persistent hypoglycemia in infancy (1). This condition was first described in 1954 by Mc Quarrie, who reported a follow-up study of 25 otherwise healthy infants with idiopathic spontaneous hypoglycemia (2). In most of these cases multiple generalized convulsions preceded the diagnosis. He further reported that the disorder appeared to vary in severity from time to time, was unaccompanied by any pathological lesions, and tended to recover spontaneously over time. The only etiological factor in this series was strong familial clustering of cases.

During the last five years the molecular genetics and pathophysiological mechanisms underlying CHI have started to become uncovered (3). Recent studies have shown that CHI is a heterogeneous disease characterized by hypoglycemic episodes due to persistent inappropriate insulin secretion. The clinical heterogeneity includes variation in the age of onset, the severity of the disorder, and the responsiveness to medical treatment. Four different genes have been shown to be responsible for this disorder (4). In addition, two distinct pathological forms of CHI have been described, the diffuse form showing involvement of beta cells throughout the pancreas, and focal adenomatous hyperplasia (5, 6). Despite this progress, the molecular etiology of a significant proportion of patients still remains to be established (4, 7).

Although CHI is a rare disorder, it is a major cause of permanent neurological damage and epilepsy in infancy. Understanding the pathophysiology and genetic background of the disease is likely to lead to more accurate diagnosis and effective treatment. Furthermore, studies concerning the regulation of insulin secretion in CHI patients may lead to better understanding not only of mechanisms underlying CHI, but also other conditions associated with inappropriate or insufficient insulin secretion, particularly type 2 diabetes. In this study, we investigated the genetic background of Finnish CHI patients, the correlation of different genotypes and phenotypes, and the glucose metabolism in carriers of different CHI-associated mutations.

2 REVIEW OF THE LITERATURE

2.1 Insulin secretion and action

2.1.1 Development and structure of endocrine pancreas

The fetal pancreas is formed by ventral and dorsal buds originating from the endodermal lining of the duodenum which fuse together to form a single organ (8, 9). The ventral bud forms the posterior part of the head of the pancreas, whereas the remaining part of the pancreas (anterior portion of the head of pancreas, body and tail) arises from the dorsal bud. Fetal pancreatic development is characterized by proliferation, branching and differentiation of the pancreatic epithelial cells to form a ductular tree, exocrine acini and endocrine islets. Ductal, endocrine and exocrine cells are derived from common precursors (10). The development of functionally mature islet cells is controlled by an extensive network of interacting transcription factors, many of which are expressed exclusively in the pancreas (11). The same transcription factors that regulate islet cell differentiation during fetal development also regulate insulin gene transcription later in adults, and mutations in these genes may cause diabetes (12).

The islets of Langerhans are scattered within the glandular substance of exocrine pancreas throughout the gland (10). There are four main types of endocrine cells. The insulin-secreting beta cells represent 70-80% of all endocrine cells in the dorsally derived islets. The glucagon-producing alpha cells and the somatostatin-producing delta cells (10% and 3-5%, respectively) represent the other main endocrine cell types of this area. The fourth cell type, the pancreatic polypeptide-producing PP cells, is found particularly in the ventrally derived islets. Insulin secretion from beta cells begins already before the 12th week of gestation. However, functional maturation of the beta cells occurs postnatally, so that glucose does not acutely stimulate insulin secretion in fetal beta cells (13). The anabolic effect of insulin stimulates growth during fetal life, whereas the blood glucose level of the fetus is regulated maternally.

The endocrine pancreas consists of approximately one million islets of Langerhans, which comprise about 1% of the total mass of pancreas (9). Each islet is a functional unit

comprising of insulin-secreting beta cells in the central part and the three other endocrine cell types in the periphery. The islets are richly vascularized and innervated, receiving information about the nutrient and hormonal status of the blood, as well as about the neural influences of autonomic nervous system. The direction of the blood flow from the central region to peripheral zone is postulated to play a role in paracrine modulation of insulin secretion (14).

2.1.2 Biosynthesis of insulin

Insulin is produced exclusively by the pancreatic beta cells. The insulin gene locates on chromosome 11p15.5 (15). The control of glucose homeostasis by insulin requires a strict regulation and fast-acting mechanisms of insulin biosynthesis. An elevated glucose concentration leads to insulin secretion as well as up-regulation of insulin biosynthesis. Glucose appears to regulate insulin biosynthesis at the levels of transcription and translation (16). Beta cells contain a large pool of cytoplasmic insulin mRNA, which is inactive at hypoglycemic glucose levels, but is recruited in response to higher glucose levels, leading to a 50-fold increase in biosynthesis (17). An increase in the glucose concentration results in insulin gene transcription within minutes through multiple effects on several of the proteins in the transcription activation complex (18, 19). The translational response shows a 20-30 min delay (17). Several studies have demonstrated that physiologically stimulated insulin secretion from pancreatic beta cells promotes insulin biosynthesis by enhancing insulin gene transcription in an autocrine manner that involves the action of insulin receptors on the beta cell membrane (20-22).

A precursor molecule of insulin, preproinsulin, is produced in the rough endoplasmic reticulum of beta cells (23). It is cleaved to proinsulin by microsomal enzymes and transported to Golgi apparatus, where packaging into clathrin coated secretory granules takes place. Proinsulin consists of a single chain of 86 amino acids and has about 7-8% of the biological activity of insulin. Maturation of secretory granules is associated with loss of the clathrin coating and proteolytic cleavage of proinsulin by prohormone convertases 1, 2 and carboxypeptidase H, to insulin and a smaller connecting peptide, C-peptide. The normal secretory granule contains insulin and C-peptide in equimolar amounts and only small quantities of proinsulin and partially cleaved intermediates, split products. Insulin molecule

consists of two peptide chains (A chain with 21 amino acids and B chain with 30 amino acids) which are connected by two disulfide bridges (24). The half-life of endogenous insulin is 3-5 min. Approximately 50% of insulin is removed in a single pass through the liver. C-peptide, a 31-amino-acid residue, has no apparent metabolic activity, and a half-life three to four times that of insulin.

Exocytosis of insulin finally results from the fusion of the secretory granules with the beta cell plasma membrane, which is followed by insulin release into the extracellular space. Only a small proportion of insulin is released even under maximal stimulatory conditions, and the circulating levels of insulin therefore depend on the regulation of secretion rather than rate of biosynthesis (25). The first phase of biphasic glucose-stimulated insulin secretion arises from the release of readily releasable granules which are located immediately below the plasma membrane. The second slower phase results from the time- and ATP-dependent mobilization of granules situated further away from the plasma membrane (26).

2.1.3 Insulin secretion

K_{ATP} channel-dependent pathway. The ATP sensitive potassium (K_{ATP}) channels play a major role in the regulation of insulin secretion in human beta cells (27). These channels consist of two types of protein subunits: the sulfonylurea receptor SUR1 and the inward rectifying potassium channel Kir6.2 (28). The wild-type channel is composed of an octameric complex of four SUR1 and four Kir6.2 subunits (Figure 1a) (29). K_{ATP} channels are not only found in pancreatic beta cells, but also in other tissues. Different combinations of Kir6.1 or Kir6.2 and SUR1 or SUR2 proteins account for the diverse properties of K_{ATP} channels: in brain Kir6.2+SUR1, in cardiac and skeletal muscle Kir6.2+SUR2A, in smooth muscle Kir6.2+SUR2B, and in vascular smooth muscle Kir6.1+SUR2B (30).

The SUR1 protein consists of two groups of transmembrane domains and two intracellular nucleotide-binding folds (NBFs) each containing highly conserved Walker motifs A and B (Figure 1b) (31, 32). SUR1 acts as a regulator of K_{ATP} channel activity, conferring sensitivity to Mg-ADP, diazoxide and sulphonylureas (33). NBF2 of SUR1 seems to play an important role in the modulation of K_{ATP} channel activity by Mg-ADP (34), whereas NBF1

of SUR1 is essential for the channel activation by diazoxide (35). The pore-forming subunit Kir 6.2 is the primary site for ATP binding and ATP-induced channel inhibition. Neither the SUR1 subunit nor Kir6.2 subunit form operational channels alone. However, coexpression of these proteins results in K_{ATP} channel activity that is inhibited by ATP and sulfonylureas and stimulated by diazoxide (30, 36).

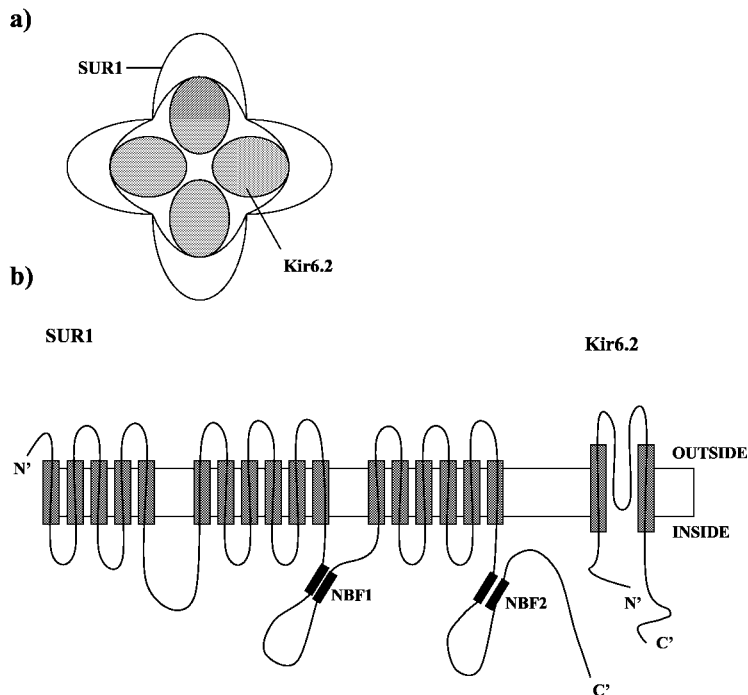


Figure 1. a) The structure of the K_{ATP} channel, which is composed of an octameric complex of four SUR1 and four Kir6.2 subunits. b) SUR1 consists of two groups of transmembrane domains and two nucleotide-binding folds (NBF). Kir6.2 is the pore-forming unit of the K_{ATP} channel.

Glucose enters the beta cell via the glucose transporter GLUT2. Its phosphorylation to glucose-6 phosphate by glucokinase (GCK) determines the rate of glycolysis (37). In beta cells, the main product of glycolysis is pyruvate, which also functions as a substrate for the tricarboxylic acid (TCA) cycle in the respiratory chain in the mitochondrion. (38). As a result of electron transfer from TCA cycle to the respiratory chain by NADH and $FADH_2$, ATP is formed and exported to the cytosol. An increase in intracellular ATP/ADP ratio leads to K_{ATP} channel closure and depolarization of beta cell membrane (34). This in turn,

opens the voltage-gated L-type Ca^{2+} channels and allows an influx of calcium. The sharp rise in cytosolic calcium concentration initiates the release of insulin by the process of exocytosis (Figure 2) (27). This K_{ATP} channel-dependent pathway appears to be responsible for the first phase insulin secretion, i.e. the exocytosis of preprepared insulin granules.

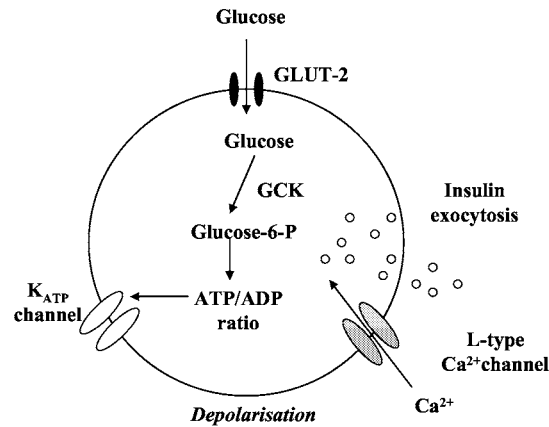


Figure 2. The role of K_{ATP} channel in normal coupling of glucose levels to insulin secretion. An increase in intracellular ATP/ADP ratio leads to a closure of K_{ATP} channels and beta cell membrane depolarization. This, in turn, opens the voltage-gated L-type Ca^{2+} channels and allows an influx of calcium, which finally leads to exocytosis of insulin.

K_{ATP} channel-independent pathway. The other less-known pathway, not depending on K_{ATP} channels, plays a role mainly for the second phase of insulin release. It also requires the elevation of intracellular calcium concentration, which follows the action of K_{ATP} channel-dependent pathway described above (39). The molecular mechanisms of the K_{ATP} channel-independent pathway are complex and poorly understood. The possible mechanisms include the increase in cytosolic long chain acyl CoA esters (40), a putative mitochondrial signal (41) and the pyruvate malate shuttle (42), which all may couple to the exocytosis of insulin. The two pathways, K_{ATP} channel-dependent and -independent, work in synergy. Both are essential to the biphasic glucose-stimulated insulin secretion (43).

Other regulators of insulin secretion. Although glucose is a major regulator of insulin secretion by pancreatic beta cells, its action is modulated by several neural and hormonal stimuli. The insulinotropic hormones, gastric inhibitory peptide (GIP) and glucagon-like peptide (GLP-1) secreted by intestine endocrine K- and L-cells, are the major regulators of postprandial glucose homeostasis (44, 45). Their stimulation of insulin secretion depends on

the presence of specific beta cell receptors and requires normal or elevated glucose concentrations.

Other potentiators of insulin secretion include certain amino acids like leucine and arginine, as well as acetylcholine and cholecystokinin. Inhibition of insulin secretion, as encountered in stress, is induced by neurotransmitters and hormones such as norepinephrine and somatostatin, which act on G-protein coupled receptors and thereby inhibit adenylate cyclase and modify Ca^{2+} and K^+ channel gating (46). In addition to this, paracrine hormone effects of glucagon (stimulatory) and somatostatin (inhibitory) are involved in the regulation of insulin secretion (14).

Insulin secretion has some variability according to age. Studies with human pancreatic islets have demonstrated that fetal pancreas is already responsive to glucose during the first half of gestation, but the maturation of biphasic insulin secretion develops later during the postnatal phase (13). In neonates, insulin secretion in relation to blood glucose concentration is not regulated with the same level of tight control seen in older children and adults (47). Several studies have also presented that beta cell function declines with age (48, 49). The decrease of insulin secretion in the elderly has been found to associate with diminished glucose-responsiveness of beta cells as well as defects in the biphasic insulin secretion (50, 51).

Several therapeutic drugs modulate insulin secretion by interacting directly with K_{ATP} channels. Sulfonylureas, widely used to enhance insulin secretion in patients with type 2 diabetes, act by inhibiting the K_{ATP} channels and thereby increasing insulin exocytosis. On the contrary, K_{ATP} channel openers such as diazoxide, lead to hyperpolarization of beta cell membrane and thus to inhibition of insulin secretion (52).

2.1.4 Insulin action

Insulin action is mediated via insulin receptors, which are widely expressed in human tissues; e.g. in the skeletal muscle, adipose tissue, liver and brain (53). The major function of insulin is to promote storage of ingested nutrients. In muscle cells, insulin promotes protein synthesis by increasing amino acid transport and by stimulating the ribosomal protein synthesis. Furthermore, it promotes glycogen synthesis by increasing glucose

transport and activity of glycogen synthetase as well as inhibiting the activity of glycogen phosphorylase. In adipose tissue insulin promotes triglyceride storage by inducing lipoprotein lipase, by increasing glucose transport to fat cells, and by inhibiting lipolysis. In the liver, insulin action results in a shift of glycogenolysis to glycogen synthesis and inhibition of gluconeogenesis (54). Moreover, insulin effects on pancreatic alpha cells, muscle cells and in adipose tissue indirectly inhibit the hepatic glucose production.

2.2 Congenital hyperinsulinism (CHI)

Congenital hyperinsulinism (CHI), also referred to as persistent hyperinsulinemic hypoglycemia of infancy (PHHI), is a genetic disorder characterized by dysregulated insulin secretion (1, 55). It is the most common cause of persistent and recurrent hypoglycemia in infancy. The worldwide national incidence of the disease is estimated to be 1/50,000 live births, but in some areas of high consanguinity, it is as high as 1/2,500 (56, 57). Both sexes are equally affected (58). Although a rare disease, it is a major cause of severe mental retardation and epilepsy if not treated properly (58-60).

2.2.1 Clinical symptoms and diagnosis

The clinical phenotype of CHI is variable. Due to the anabolic effect of insulin, the newborn with CHI may have a characteristic appearance with macrosomia at birth reflecting the prenatal hyperinsulinism, and thus resembling an infant of a diabetic mother (58). In most cases, the symptoms of hypoglycemia, such as floppiness, jitteriness, poor feeding, and lethargy, appear during the first postnatal hours or days, but in a few cases later during the first year (55). Characteristically, children with CHI have increased need of glucose and fail to maintain normoglycemia with normal enteral feeding. Rarely, the infants may have seizures, coma or may even die (61).

The diagnosis of CHI is based on the detection of hypoglycemia (blood glucose < 2.6 mmol/l) and detectable insulin secretion with raised C-peptide concentration during hypoglycemia. Typically, children with CHI have increased requirement of glucose up to 15-20 mg/kg/min to maintain normoglycemia, i.e. blood glucose above 2.6-3 mmol/l. In a healthy newborn, normoglycemia is maintained with glucose dosage of 6-8 mg/kg/min. Due

to the antilipolytic and anabolic effect of insulin, inappropriately low blood free fatty acid and ketone body concentrations are detected at the time of hypoglycemia. The glycemic response to the administration of glucagon when hypoglycemic due to the mobilization of glycogen deposits from the liver can be used as a diagnostic as well as therapeutic method. The final and more definite diagnosis of CHI is reached by the synthesis of biochemical findings, genetic analysis, and immunohistochemical staining which will be discussed in more detail in the following sections (55).

2.2.2 Molecular genetics

During the last few years there has been a significant increase in knowledge concerning the molecular genetics of CHI. Currently, mutations in four different genes have been identified to cause CHI. The most common known cause are mutations in the K_{ATP} channel gene SUR1 (62). A few single mutations have also been detected in the genes encoding the Kir6.2 protein (28, 63, 64), glucokinase (GCK) (65) and glutamate dehydrogenase (GDH) (66-68). However, the molecular etiology remains to be established in at least 50% of all CHI cases (4).

2.2.2.1 Mutations in potassium K_{ATP} channel genes

The genes encoding the K_{ATP} channel proteins SUR1 and Kir6.2 are clustered on chromosome 11 at position 15.1. The SUR1 gene, a member of the ATP-binding cassette superfamily, contains 39 exons encoding 1581 amino acids (Genbank database Accession no. L78207). The Kir6.2 gene, a member of the inwardly rectifying K^+ channel family, is located approximately 5000 bp downstream of the stop codon of SUR1 (Genbank no. D50582).

In 1995, soon after the localization of CHI to chromosome 11p15.1 and cloning of the SUR1 gene (31, 69, 70), the first CHI-associated SUR1 mutations were identified (62). Further studies of CHI families have shown a large degree of allelic heterogeneity at the SUR1 locus. So far, more than 50 CHI-associating SUR1 mutations have been reported (4, 7, 71). Mutations are spread throughout the coding sequence of SUR1, but most of them are clustered in the second nucleotide-binding fold (NBF2). The most common molecular mechanism underlying CHI is a lack of K_{ATP} channels or reduced response to MgADP

stimulation, which may result from decreased expression level of the channel proteins, expression of non-functional channel proteins, channels with altered regulation by intracellular nucleotides, or combinations of all three (72). In some cases, CHI may result from defective trafficking of K_{ATP} channels to the cell membrane (73, 74). The impaired K_{ATP} channel activity leads to constitutive membrane depolarization, activation of voltage-gated Ca^{2+} channels and inappropriate exocytosis of insulin, regardless of the blood glucose level (75, 76). Most SUR1 mutations appear to be recessive (77), but a dominant negative mutation engineered into the Kir6.2 gene of the mouse suggests that dominant mutations may occur also in humans (78).

The Kir6.2 gene, which locates five kilobases downstream of the SUR1 gene, seems to be a minor cause of CHI. So far, only three recessively inherited mutations (Y12X, L147P, W19R) in the Kir6.2 gene have been reported (28, 63, 64). All three mutant Kir6.2 proteins failed to form active K_{ATP} channels when coexpressed with wild-type SUR1 and led to a severe form of CHI.

Detailed analysis of genotype-phenotype correlations is complicated by the wide range of different mutations and the low allelic frequencies of single mutations. Furthermore, most patients with CHI-associated mutations are compound heterozygotes or have only a single identified mutation. However, in some genetically isolated populations a few mutations may be a major cause of CHI, and therefore these populations make the studies of the genotype-phenotype correlations possible. For example, in the Ashkenazi Jewish population, the splice site mutation 3992-9g->a was found in 70% and the deletion of the codon for F1388 (Δ F1388) in 20% of CHI patients (62, 71). Furthermore, mutations in K_{ATP} channel genes explain CHI to varying degrees in different populations. For example, in the Japanese population SUR1 mutations account for only about 20% of CHI cases whereas in Ashkenazi Jews two single SUR1 mutations account for 90% of all cases (71, 79). A database integrating the clinical, molecular genetic, histopathological and electrophysiological data of all European patients is under development and will make more detailed phenotype-genotype correlation studies possible in the future (<http://umd2.necker.fr:2007/>).

The mutation site modifies significantly its effects on K_{ATP} channel function. For example, a mutation in the NBF2 of SUR1 gene abolishes the stimulatory effect of Mg-nucleotides

(MgADP, MgATP) and generates K_{ATP} channels that could be opened by diazoxide, but not in response to metabolic inhibition (34, 80). Furthermore, Walker A motif of SUR1-NBF1 but not NBF2 plays an essential role in the activation of K_{ATP} currents by diazoxide (35).

Focal form of CHI. In roughly one third of all cases, focal adenomatous hyperplasia is the cause of CHI (81-84). Previous studies have shown that in the imprinted chromosomal region 11p15 the tumor suppressor genes H19 and P57^{KIP2} are maternally expressed, whereas insulin-like growth factor 2 (IGF-2) gene, which plays a central role in pancreatic tumorigenesis, is paternally expressed (85). The crucial mechanism of focal CHI is a somatic loss of maternal alleles within a limited area of pancreas. This leads to the expression of paternal alleles and to imbalance between the tumor suppressor genes and IGF-2 and further to focal adenomatous hyperplasia whereas the rest of pancreas presents entirely normal histology (5). Heterozygous paternally inherited K_{ATP} channel mutations will be then expressed and lead to uncontrolled insulin secretion in the focal area of the pancreas. Focal CHI seems to be genetically more homogenous than diffuse CHI, as it is linked to K_{ATP} channel gene mutations in almost two-thirds of all cases (84). In diffuse CHI, abnormal and enlarged beta cell nuclei are seen throughout the pancreas, and the beta cell nuclear crowding (the number of nuclei per 1000 μm^2 beta cell) index, which reflects the nucleo-cytoplasmic ratio of beta cells, is low. In the focal form, beta cell nuclear crowding index is increased, and abnormal nuclei do not exist (6).

Although the clinical presentations of focal and diffuse forms of CHI are similar, the treatments differ. Therefore, it is worthwhile to aim to distinguish these two conditions (86). Unlike in the case of insulinoma, focal adenomatous hyperplasia is often macroscopically invisible (5). The most efficient methods to localize the focal lesions include preoperative selective pancreatic venous catheterization (86-88) and perioperative microscopic examination of frozen sections (89). Using this strategy, the Paris group was able to successfully identify and resect the focal lesion in 22 of 52 cases of CHI (90, 91).

In beta cells with K_{ATP} channel mutations, the rise in intracellular calcium concentration should theoretically induce insulin secretion due to the constant activation of calcium channels. Therefore, peripheral intravenous calcium stimulation combined with selective arterial calcium stimulation of the pancreas may be useful in distinguishing the two forms of

CHI. In previous studies, patients with K_{ATP} channel mutations responded to peripheral calcium stimulation whereas the normal controls did not (92). The results of selective arterial calcium stimulation of pancreas with hepatic sampling correlated anatomically with the location of focal disease in 1/3 and 2/3 of cases (92, 93).

2.2.2.2 Mutations in the glucokinase gene

Glucokinase (GCK) controls the rate-limiting step of beta cell glucose metabolism and is responsible for glucose-mediated insulin secretion (37). It catalyzes the ATP-dependent phosphorylation of glucose to glucose-6-phosphate in the glycolytic pathway. Mutations affecting the activity or glucose affinity for GCK will displace the threshold for insulin secretion in response to glucose from its normal physiological setting of 5 mmol/l (94). Loss-of-function mutations in GCK that lead to decreased glucose phosphorylation and insulin secretion have been found to be associated with maturity-onset diabetes of the young (MODY) (95, 96). In a recent report, the complete deficiency of GCK has been found to result in neonatal diabetes mellitus (97). In a reverse situation, an autosomal dominant missense mutation, V455M in the GCK gene has been found to result in increased affinity of glucokinase for glucose and to familial hyperinsulinism (65). The mutation was detected in one single family with five affected individuals in three generations. All patients responded well to diazoxide treatment. Interestingly, insulin-deficient diabetes developed later in life in the oldest family member. More recently, further systematic screening of the GCK gene has led to the identification of another activating GCK mutation (A456V) in a family with hyperinsulinism (98).

2.2.2.3. Mutations in the glutamate dehydrogenase gene

Dominantly inherited activating mutations in the glutamate dehydrogenase (GDH) gene are the cause of hyperinsulinism-hyperammonemia (HIHA). Gene defects of GDH simultaneously increase the release of insulin by pancreatic beta cells and impair the detoxification of ammonia in the liver (67). Most mutations are located in the allosteric regulatory domain of the enzyme within the GTP-binding site, but recent studies have described mutations also outside this narrow range (66, 68, 99). In pancreas, excessive activity of the mutated GDH increases the reversible conversion of glutamate to α -ketoglutarate and therefore increases the flux of substrates into the tricarboxylic acid cycle (67, 100). This further results in increased conversion of ADP to ATP, membrane

depolarization and exocytosis of insulin. Leucine stimulates insulin secretion by acting as an activator of GDH to increase glutamate oxidation (101, 102). In the liver, the increased activity of GDH may lead to hyperammonemia through two mechanisms. Firstly, the oxidative deamination of glutamate to α -ketoglutarate leads to increased ammonia formation. Secondly, excessive GDH activity reduces intrahepatic glutamate concentration, which leads to the accumulation of ammonia by the inhibition of the urea cycle (103). Plasma ammonium levels in HIHA patients are persistently three to eight times normal (67). The clinical manifestations of HIHA include normal birth weight, mild and late onset of hypoglycemia, diazoxide responsiveness and protein-sensitive hypoglycemia (67). Therefore, postprandial hypoglycemic response to a protein meal is a sensitive indicator of the HIHA syndrome (104).

2.2.3 Mouse models

Animal models expressing manipulated SUR1 and Kir6.2 combinations have been generated with variable success. The first transgenic mice expressing the dominant-negative mutation *Kir6.2G132S* in pancreatic beta cells exhibited hyperinsulinism despite hypoglycemia, resembling CHI in humans, but developed hyperglycemia with reduced glucose-induced insulin secretion and decreased beta cell mass in adult life (78).

The K_{ATP} channel-deficient *Kir6.2^{-/-}* mice confirm the role of K_{ATP} channels as a key regulator of both glucose- and sulfonylurea-induced insulin secretion (105). In *Kir6.2^{-/-}* mice, the resting beta-cell membrane potential as well as basal intracellular calcium concentrations are significantly elevated. However, neither glucose at high concentrations nor sulfonylureas elicited a rise in intracellular calcium concentration or in insulin secretion. However, *Kir6.2^{-/-}* mice showed only a mild impairment in glucose tolerance, which may be due to the increased insulin-induced glucose disposal *in vivo*, which could be explained at least in part by the disruption of SUR2A/Kir6.2 K_{ATP} channels in skeletal muscle as well as by the hyperglycemia-preventive responses of glucose-dependent insulintropic hormones (GIP, GLP-1). However, older *Kir6.2^{-/-}* mice were obese and glucose-intolerant. In both *Kir6.2G132S* and *Kir6.2^{-/-}* mice, alpha cells, which are normally present in the periphery of islets, appeared in the center of islets, indicating the importance of K_{ATP} channels for islet cell differentiation and structure (106). Furthermore, the high frequency of apoptotic beta

cells before the appearance of hyperglycemia suggested that K_{ATP} channels play a significant role in beta-cell survival. (107, 108).

SUR1 knockout mice ($SUR1^{-/-}$) were expected to provide the most appropriate CHI model (109). Nevertheless, frank hypoglycemia, and abnormally elevated insulin secretion were observed only in the first day of life, and at the age of 5 days, the situation had reversed to hyperglycemia. The mice were normoglycemic unless stressed, but became hyperglycemic when glucose-loaded and hypoglycemic when fasted. $SUR1^{-/-}$ mice showed a loss of first-phase insulin release, but exhibited an attenuated second-phase insulin response, probably arising from K_{ATP} channel-independent mechanisms of insulin secretion.

2.2.4 Treatment

The treatment of CHI aims at preventing hypoglycemic brain damage and allowing normal psychomotor development. It should be adjusted to normal family life, ensure normal tolerance to fasting and enable normal volume and content of feedings for age without developing hypoglycemia (55). Because some children with hyperinsulinism may require treatment for several months and years, orally administered drugs should be favored.

Medical treatment. The K_{ATP} channel agonist diazoxide (5-20 mg/kg/day in two or three divided doses) administered together with chlorothiazide (7-10 mg/kg/day in two divided doses), is the mainstay of medical management of CHI. These agents act synergistically, both activating potassium channels by different mechanisms (110). The diuretic also diminishes the fluid retention caused by diazoxide. CHI patients without K_{ATP} channel mutations are expected to respond well to diazoxide therapy. The mechanism of diazoxide-responsiveness in patients who lack functional K_{ATP} channels has been shown to be associated with the presence of K^+ channels sensitive to diazoxide, whereas these channels were not detected in drug-resistant patients (111). As expected, patients with GCK-related CHI respond well to diazoxide therapy (65). In most cases of HIHA syndrome, diazoxide with a leucine-restricted diet is effective treatment (4). However, recent studies have shown that some patients with HIHA are unresponsive to diazoxide (112). Theoretically, diazoxide therapy, by hyperpolarizing the beta cell and limiting calcium entry, may delay the onset of apoptosis of beta cells. Diazoxide therapy could thus offer long-term benefits in preventing

the decrease in beta cell mass (113).

Attempts to regulate insulin secretion through beta-cell voltage-dependent calcium channels have not proved as useful as hoped. In some cases, there is a clinically beneficial response to the calcium channel antagonist nifedipine (0.25-2.5 mg/kg/day), and it has a role as an adjuvant in the treatment of hyperinsulinism (114-116).

The second line agents, somatostatin and glucagon, are also of proven benefit in the treatment of CHI. Continuous glucagon infusion (5-10 µg/kg/hour) mobilizes glucose from hepatic glycogen stores, but it also may increase insulin secretion. This treatment has been found to be effective in the early stabilization of blood glucose in the severely hyperinsulinemic neonate (55). The somatostatin analogue octreotide (5-20 µg/kg/day intravenously/subcutaneously), activates G protein-coupled rectifier K⁺ channels in the beta-cell membrane and thus inhibits insulin secretion (111). The role of octreotide in long-term management of CHI is controversial. According to several reports somatostatin analogues appear to be useful in short-term management of CHI, but their long-term use has previously been avoided because of the potential adverse effects on linear growth and effects on other hormone systems (117, 118). However, long-term octreotide therapy is often well tolerated and may be the treatment of choice in order to avoid subtotal pancreatectomy (119-123) or when hyperinsulinism has recurred after surgical treatment (124).

Surgical treatment. Patients who fail to respond to medical treatment should be referred for surgery to avoid permanent neurological damage (55, 125). The absolute indications for surgery include focal adenomatous hyperplasia and glucose infusion dependency despite maximal medical therapy (55). In the case of focal CHI, the patient can be cured by a partial resection of the focal lesion (81, 86, 88, 126, 127), whereas in the diffuse form of the disease, 95% subtotal pancreatectomy is often necessary (125, 128).

2.2.5 Long-term prognosis

Insulin secretion. In most CHI patients, the natural course of the disease leads to a slow progressive loss of beta-cell function (129). Recent studies have suggested that increased beta-cell apoptosis may explain this. However, there is direct evidence for an increased

apoptosis rate only within focal pancreatic lesions, and it has not been possible to show this in diffuse CHI (130). The mechanism of apoptosis has not been defined even though there is evidence towards intracellular calcium level as an important determinant of beta cell apoptosis (113, 131). In CHI, mutations in pancreatic beta cell K_{ATP} channels result in continuous beta cell membrane depolarization and in opening of voltage-gated calcium channels. This, in turn, leads to an increase in intracellular calcium concentration, which may start the activation of apoptosis cascade and result in a loss of beta cell mass. Pharmacological closure of K_{ATP} channels using the sulfonylurea-based drugs may increase the frequency of apoptosis by the same mechanism, whereas diazoxide therapy may contribute to beta cell survival (113).

Follow-up studies of CHI patients have shown that diabetes develops in most children who undergo 95% pancreatectomy (129, 132, 133). In most cases diabetes appears in puberty when the reduced beta cell mass fails to compensate the increased insulin resistance (129, 134-136). However, recent studies have suggested that impaired K_{ATP} channel activity may contribute to the late development of diabetes independently of subtotal pancreatectomy (137).

Neurological outcome. CHI is still associated with increased risk of severe brain damage and epilepsy. The reported incidence of such sequelae varies from 0% (138) to up to 30-60% (59, 60, 135, 139-141). In many cases psychomotor retardation is detected after delayed diagnosis of CHI, which emphasizes the importance of early and accurate diagnosis. Recurrent and profound hypoglycemic episodes are extremely dangerous in patients with CHI because there is no alternative fuel available for the brain cells due to the suppressed ketone body formation. To avoid brain damage, blood glucose concentration should be maintained constantly above 2.6 mmol/l because even moderate hypoglycemia may have serious neurodevelopmental consequences (142, 143).

K_{ATP} channels have been found in many regions of the brain, including the substantia nigra (144), neocortex (138), hippocampus (146) and hypothalamus (147). Studies with Kir6.2 knockout mice have shown that K_{ATP} channels play an important role in glucose sensing in ventromedial hypothalamus glucose-responsive neurons and are essential for the maintenance of glucose homeostasis (148). The highest expression region of K_{ATP} channels

in brain is the substantia nigra pars reticulata, which plays the major role in the control of seizures (149, 150). K_{ATP} channels are activated by various metabolic stresses, including hypoxia and hypoglycemia. In normal mice, hypoxia induces inactivation of neurons in substantia nigra. However, in Kir6.2 knockout mice the neuron activity is enhanced, and therefore predisposes to generalized seizures after brief hypoxia (151). These results emphasize the role of normal K_{ATP} channel function in protection against generalized seizures. This could also in part explain the high frequency of epilepsy among CHI patients.

2.2.6 Other hyperinsulinemic conditions in newborns

Hypoglycemia in the neonate of a diabetic mother is related mostly to hyperinsulinism, and partly to diminished glucagon secretion and decreased ability to produce glucose (152). Poorly controlled maternal diabetes causes fetal hyperglycemia, which stimulates fetal insulin secretion. After birth, hyperglycemia persists and leads to suppression of fasting hepatic glucose production. Erythroblastosis fetalis shares many features, like macrosomia, with infants of diabetic mothers (153). In this condition hyperinsulinism may be related to compensatory hypersecretion of insulin as a result of increased hemolysis that provides glutathione, which splits the disulfide bonds of insulin. Hyperinsulinism may also be related to some clinically defined conditions like rhesus incompatibility (154), perinatal asphyxia, and prematurity or to being born small-for-gestational age (155).

In some cases hyperinsulinism is associated with additional clinical symptoms suggesting an underlying syndrome (156). Beckwith-Wiedemann syndrome (BWS) is an overgrowth syndrome related to defects in imprinting on chromosome 11p15.5 (157, 158). BWS is frequently associated with macrosomia, macroglossia, abdominal wall defects, hypoglycemia in the neonatal period and a predisposition for childhood tumours. The frequency of hypoglycemia among BWS patients is about 30 - 50%, and it is transient in most cases (159). In less than 5% of all cases hypoglycemia persists beyond the neonatal period. A homozygous deletion of 11p14-15 associated with infantile hyperinsulinism, enteropathy and deafness leads to Usher syndrome subtype 1C (160). In addition, in recently described exercise-induced hyperinsulinism (EIHI), a short-term anaerobic exercise results in increase of insulin secretion and symptomatic hypoglycemia (161). The first defect in fatty acid beta-oxidation associating with hyperinsulinism has also been reported recently

(162). A recessively inherited mutation in short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) was found in a single homozygous patient with diazoxide-responsive form of CHI.

2.2.7 CHI and diabetes mellitus

The association between CHI and diabetes is mostly unknown. As discussed in chapter 2.2.5, patients who have been treated with subtotal pancreatectomy are at increased risk to develop diabetes in puberty. However, it has been proposed that SUR1 mutations may also directly contribute to the development of diabetes independently of surgical treatment (137).

Type 2 diabetes is a heterogeneous disorder that results from varying degrees of abnormalities in insulin secretion and insulin action (163). Some early studies have suggested a high incidence of type 2 diabetes in families with HI (164, 165). However, the role of CHI mutations in the genetic susceptibility for type 2 diabetes has remained unclear. No specific mutations in Kir6.2 or in SUR1 associated with type 2 diabetes have been detected (52). Some associations between the variants of the SUR1 or Kir6.2 genes and type 2 diabetes have been reported in some (166-170), but not in all studies (171-173).

Maturity-onset diabetes of young (MODY) defines genetically and clinically heterogeneous subtypes of diabetes characterized by early onset, autosomal dominant inheritance and a primary defect in insulin secretion (174). MODY2 results from the loss-of-function mutations in GCK, which lead to defective metabolic regulation of K_{ATP} channels and to mild hyperglycemia. Fewer than 50% of patients present with overt diabetes, and there is a low prevalence of microvascular complications (95). In contrast, the dominantly inherited CHI-associated mutations V455M and A456V in GCK result in higher rates of glycolysis and therefore a higher rate of insulin secretion (65). The long-term effects of this activating mutation are unclear but diabetes developed in the oldest affected family member in one study (65), conceivably due to eventual beta-cell failure.

The other known MODY subtypes are caused by mutations in five transcription factors (175). Mutations in hepatocyte nuclear factor (HNF)-4 α (MODY 1) (176) and HNF-1 α (MODY 3) (177, 178) lead to severe forms of diabetes which often result in microvascular

complications and require insulin treatment. MODY 3 is the most common form of MODY subtypes, accounting for 20-75% of all MODY cases in different populations. Mutations in the HNF-1 β gene lead to a rare subtype of MODY (MODY 5) and severe kidney disease (179). Insulin promotor factor 1 (IPF-1) has a crucial role in the embryonic development of the pancreatic islets and in the regulation of endocrine pancreatic tissue-specific genes in adults (insulin, GLUT2, GCK, somatostatin). Heterozygous deletion of IPF-1 has been found to associate with a MODY phenotype ranging from normal to impaired glucose tolerance and overt non-insulin-dependent diabetes (MODY 4) (180). A homozygous mutation in IPF-1 has been described in one child born with pancreatic agenesis (181). Mutations in NEUROD1 gene, which has a role as a regulator of endocrine pancreatic development, associate with MODY subtype 6 (182).

3 AIMS OF THE STUDY

This study was undertaken to characterize the different forms of congenital hyperinsulinism in the Finnish population.

The specific aims of this thesis were

1. To investigate the genetic background of Finnish CHI patients. (Studies I-III)
2. To determine the correlations between different genotypes and phenotypes. (Studies I-V)
3. To study new methods in differentiating the distinct forms of CHI, and to evaluate insulin secretion in CHI patients with different genotypes. (Studies III,V)
4. To investigate the glucose metabolism of known carriers (parents and siblings of CHI patients) of different CHI-associated mutations. (IV,V)

4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Patients in the genetic studies (Studies I-III)

All patients diagnosed with CHI at the Departments of Pediatrics of the five University Hospitals of Finland during 1972-2000 were included in the study. These patients (n = 44) are likely to represent all affected individuals diagnosed during that time, but the possibility of isolated undiagnosed cases cannot be excluded. Two previously genotyped patients with HIHA syndrome were not included. CHI was diagnosed using the generally accepted criteria, including nonketotic hypoglycemia, inappropriately elevated insulin levels and an increased need for glucose administration to prevent hypoglycemia (55). The details of the patients are shown in Table 1.

The patients of study I (altogether 24 patients of whom 23 were alive, born during 1983-1997) were found by contacting their own physicians. Haplotype analysis was performed on all of them. The SUR1 and Kir6.1 genes of patients 16 and 20 (Table 1) were sequenced. The presence of SUR1-V187D was tested by RFLP-analysis in the other patients included in the study and in 50 normal controls living in Eastern Finland.

In studies II and III the patient group was enlarged due to the efforts to find all Finnish CHI patients and due to novel cases born during that time. Altogether 44 cases born during 1972-2000 were included. SSCP-analysis of the genes studied (see chapter 4.2.1) was performed on all of them except for the SUR1-V187D homozygotes (n = 6). Thus, the whole patient group consisted of 44 patients, of whom 38 were included in SSCP screening. The exon 37 of SUR1 gene of patients 2, 9, 19, 25 (SSCP variants) and patient 18 (control) was sequenced (Study II). The presence of SUR1-E1506K was studied by RFLP-analysis in all other patients and in normal healthy controls from Eastern Finland. The haplotype analysis and linkage study was performed on all E1506K family members.

Table 1. CHI patients. Patients included in AIR tests are shown in italics.

Case	Sex	Age (y)	Age at onset	Genotype	Treatment of hyperinsulinism	Histology	Diabetes	Neurology
1	F	29	1 day	'de novo' GCK-Y214C heterozygous	Subtotal pancreatectomy Diazoxide (no response)		No	Mental retardation Epilepsy
2	M	28	<1 day	<i>Materna SUR1-E1506K</i>	<i>Diazoxide</i> <i>Subtotal pancreatectomy</i>	<i>Diffuse</i>	Yes	<i>Epilepsy</i>
3	F	28	1.2 years	<i>Unknown</i>	<i>Diazoxide</i>		No	<i>Epilepsy</i>
4	F	28	<1 day	Unknown	Diazoxide		No	Mental retardation Epilepsy
5	M	21	4 months	<i>Unknown</i>	<i>Diazoxide</i> <i>Subtotal pancreatectomy</i>		Yes	<i>Impaired verbal expression and fine motor function, MBD</i>
6	F	20	7 months	<i>Materna SUR1-E1506K</i>	<i>Frequent feeds</i>		No	<i>Normal</i>
7	F	18	<1 day	Homozygous SUR1-V187D	Subtotal pancreatectomy	Diffuse	Yes	Mental retardation Epilepsy
8	F	17	5 months	<i>Maternal SUR1-E1506K</i>	<i>Diazoxide</i>		No	<i>Normal</i>
9	F	17	<1 day	<i>Maternal SUR1-E1506K</i>	<i>Frequent feeds</i>		No	<i>Normal</i>
10	M	16	<1 day	Paternal SUR1-V187D	Subtotal pancreatectomy	Focal	Yes	Normal
11	M	16	<1 day	Homozygous SUR1-V187D	Subtotal pancreatectomy		Yes	Mental retardation Epilepsy
12	F	15	1 day	<i>Maternal SUR1-V187D</i>	<i>Subtotal pancreatectomy</i>	<i>Diffuse</i>	Yes	<i>Normal</i>
13	F	14	<1 day	Paternal SUR1-V187D Maternal SUR1-V1550D	Subtotal pancreatectomy	Diffuse	Yes	Normal
14	F	13	7 months	Unknown	Diazoxide		No	Normal

Case	Sex	Age (y)	Age at onset	Genotype	Treatment of hyperinsulinism	Histology	Diabetes	Neurology
15	M	13	<1 day	Unknown	Subtotal pancreatectomy	Focal (susp.)	Yes	Normal
16	M	11	<1 day	Paternal SUR1-V187D Maternal SUR1-A1457T	Subtotal pancreatectomy	Diffuse	Yes	Mild impairment in verbal expression and in motor function
17	M	11	<1 day	Maternal SUR1-V187D	Subtotal pancreatectomy		Yes	Normal
18	F	11	1 day	Paternal SUR1-D1471N Maternal SUR1-V187D	Subtotal pancreatectomy	Diffuse	Yes	Normal
19	F	10	<1 day	Maternal SUR1-E1506K	Diazoxide		No	Normal
20	F	9	<1 day	Homozygous SUR1-V187D	Subtotal pancreatectomy	Diffuse	No	Normal
21	M	9	<1 day	Paternal Kir6.2-K67N Maternal Kir6.2(-54)	Subtotal pancreatectomy		No	Normal
22	M	9	1 day	Paternal SUR1-V187D	Subtotal pancreatectomy	Diffuse	Yes	Normal
23	M	8	<1 day/ 1 month	Paternal SUR1-V187D	Subtotal pancreatectomy	Focal	No	Mental retardation Epilepsy
24	M	7	<1 day	Unknown	Octreotide		No	Normal
25	F	7	<1 day	Maternal SUR1-E1506K	Diazoxide		No	Normal
26	F	7	1 day	Paternal SUR1-D1471N Maternal SUR1-V187D	Subtotal pancreatectomy	Diffuse	Yes	Normal
27	F	7	<1 day	Homozygous SUR1-V187D	Subtotal pancreatectomy	Diffuse	Yes	Mild impairment in motor function
28	M	(7)	<1 day	Homozygous SUR1-V187D	Subtotal pancreatectomy	Diffuse		Died due to a postoperative gut perforation at the age of 16 days
29	M	6	5 months	Unknown	Diazoxide		No	Impairment in verbal expression and in motor function

Case	Sex	Age (y)	Age at onset	Genotype	Treatment of hyperinsulinism	Histology	Diabetes	Neurology
30	F	5	<1 day	Unknown	Octreotide		No	Normal
31	M	5	<1 day	Homozygous SUR1-V187D	Subtotal pancreatectomy	Diffuse	No	Normal
32	M	5	<1 day	Maternal SUR1-E1506K	Diazoxide		No	Normal
33	F	4	<1 day	Unknown	Diazoxide		No	Normal
34	M	4	<1 day	Unknown	Diazoxide	Focal (susp.)	No	Epilepsy
35	M	4	10 months	Unknown	Diazoxide		No	Normal
36	F	3	<1 day	Unknown	Diazoxide		No	Normal
37	M	3	<1 day	Paternal SUR1-L1551V	Diazoxide		No	Normal
38	M	3	1 month	Unknown	Diazoxide		No	Mental retardation
39	M	2	<1 day	Unknown	Diazoxide		No	Normal
40	M	2	<1 day	Unknown	Diazoxide		No	Normal
41	F	1	<1 day	Paternal SUR1-V187D	Octreotide		No	Normal
42	F	1	<1 day	Unknown	Diazoxide		No	Normal
43	F	1	<1 day	Paternal SUR1-L1551V	Diazoxide		No	Normal
44	M	1	1 year	Unknown	Diazoxide		No	Normal

In the case of the other mutations (Study III) the SSCP variants were sequenced. The existence of these mutations among the other patients and 50 healthy controls from Eastern Finland was studied by RFLP-analysis, except for SUR1-V1550D which was studied by sequencing the variant and studying its presence by SSCP.

4.1.2 Patients in the acute insulin response tests (Study III)

All CHI-patients who were able to participate were included in acute insulin response tests (AIRs), except for diabetic patients requiring more than 0.5 U/kg/day insulin. The first study group consisted of six patients in whom no K_{ATP} channel gene mutations had been identified (Table 1, patients 3, 5, 29, 30, 35). The second group composed of one homozygous (Table 1, patient 20) and five patients presumed to be compound heterozygotes (Table 1, patients 12, 18, 22, 26, 41) with the mutation SUR1-V187D. In three SUR1-V187D heterozygotes the other mutation was still pending, and two of them carried the mutation SUR1-D1471N. The third group consisted of six patients (Table 1, patients 2, 6, 8, 9, 19, 25) who carried the dominant SUR1-E1506K mutation. The fourth group consisted of a single 9-year old boy with two different Kir6.2 mutations (Table 1, patient 21).

4.1.3 SUR1-V187D heterozygotes (Study IV)

The subjects for this study were the parents of five CHI patients homozygous for the SUR1 mutation V187D. All ten parents were invited to take part in the study but one pair was unwilling to participate. Genetic analysis confirmed that all eight were heterozygous carriers of the V187D mutation (Table 1, parents of the patients 7, 20, 27 and 28/31). One of them had been diagnosed with gestational glucose intolerance, but none had been diagnosed with diabetes.

The control group consisted of 10 age-, gender- and BMI matched healthy volunteers who had to fulfill the following criteria: 1) age between 30 and 40 years, 2) no diabetes; 3) no first-degree relatives with a history of diabetes; 4) no drug treatment nor any disease that could potentially modify carbohydrate metabolism, and 5) no strenuous physical activity more than three times per week.

4.1.4 SUR1-E1506K heterozygotes (Study V)

Altogether 14 adult relatives of CHI patients carrying the dominant SUR1-E1506K mutation were invited to take part in the study, but two of them were unwilling to participate. All study subjects were heterozygous carriers of SUR1-E1506K. One diabetic patient on glucocorticoid therapy was excluded. Therefore, the study group comprised 11 subjects, of whom two were known to be diabetic and six had been diagnosed with glucose intolerance during pregnancy (Figure 3).

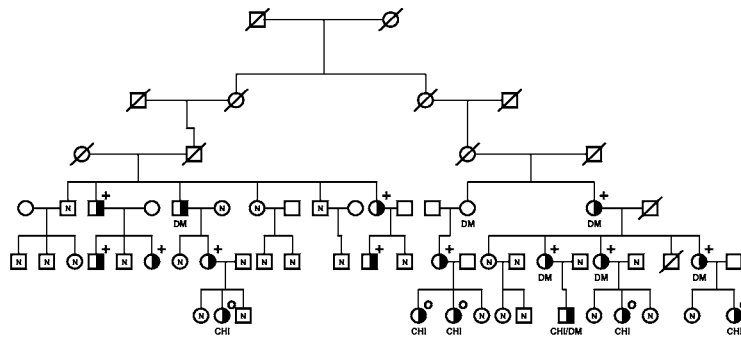


Figure 3. A pedigree of the patients with the SUR1-E1506K mutation. Heterozygous carriers are indicated by half-filled symbols and individuals with normal genotype by 'N'. The symbol '+' indicates the individuals who participated in the study V, and the symbol 'o' indicates the CHI patients with SUR1-E1506K who underwent IVGTT in the study III.

The control group consisted of 19 healthy controls who had been studied previously in our hospital with the same protocol (183) and fulfilled the following criteria: 1) age between 30 and 55 years, 2) no diabetes, 3) no first-degree relatives with a history of diabetes, 4) no drug treatment nor any disease that could potentially modify carbohydrate metabolism, 5) no strenuous physical activity more than three times per week, and 6) normal glucose tolerance according to World Health Organization criteria (184).

4.2 Methods

4.2.1 Genetic analysis (Studies I-III)

All previously described candidate genes of CHI including SUR1 (62), Kir6.2 (63), GCK (65) and GDH (exons 11 and 12) (67) were screened using the standard methods of molecular genetics. Peripheral blood samples were collected from all patients and DNA was prepared from blood leukocytes by proteinase K-phenol-chloroform extraction.

Polymerase chain reaction (PCR). The immediate promoter region of SUR1 (220 bp upstream from the transcriptional start site), all 39 SUR1 exons, the single exon of Kir6.2, the promoter region (334 bp upstream from the transcriptional start site) and the 10 exons of glucokinase (GCK) gene and exons 11 and 12 of glutamate dehydrogenase (GDH) gene were amplified using primers designed by us according to the reported sequences of SUR1 or synthesized as previously reported (71, 172, 185). The PCRs were run with thermo cyclers (PTC-100 Programmable Thermal controller, MJ-Research Inc., Watertown, MA, USA): The exons and exon-intron junctions were amplified with PCR in a volume of 6 μ l that consisted of 50 ng of genomic DNA, 5 pmol of each primer, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM of MgCl₂, 0.1% Triton X-100, 100 μ M dNTP, 0.14 units of DNA polymerase (Dynazyme DNA polymerase, Finnzymes, Espoo, Finland) and 0.55 μ Ci alpha-³³P dCTP (NEN Life Science Products, Boston, MA, USA). PCR reaction included a denaturation step at 94°C for 4 min, followed by 30-35 cycles starting from denaturation step at 94°C for 30-60 sec, annealing at 50-65°C for 30-60 sec, extension at 72°C for 30-60 sec with final extension at 72°C for 4 min. The PCR conditions and primers used in the detection of the novel mutations are shown in Table 2.

Table 2. Novel CHI- associated mutations in the Finnish population.

Substitution	Primers (5' -> 3')	PCR program °C /Cycles	Size of the product (bp)	Restriction endonuclease
SUR1-V187D	CACACGTCTCTGCGATCTTCC CCTGCTTACCTCACCTGAT	94-62-72 / 30	212	<i>Tth111I</i>
SUR1-A1457T	ACCCTGCTCCCTCCTACTG GTCCTTGAGTGCCCAACC	94-64-72 / 30	192	<i>HphI</i>
SUR1-D1471N	ACCCTGCTCCCTCCTACTG GTCCTTGAGTGCCCAACC	94-64-72 / 30	192	<i>BseDI</i>
SUR1-E1506K	ATCCCATCTGCTCCACTCAC ATCCCACTAAACCCTTCCAG	94-60-72 / 30	254	<i>MnlI</i>
SUR1-V1550D	GGGTGGTATTCCCACCATC GTATGGGCAGGGTCCGAAT	94-65-72 / 30	230	
SUR1-L1551V	GGGTGGTATTCCCACCATC GTATGGGCAGGGTCCGAAT	94-65-72 / 30	230	<i>BseLI</i>
Kir6.2-(-54)	ACCGAGAGGACTCTGCAGTGA GTTGCAGTTGCCTTCTTGGGA	94-65-72 / 35	216	<i>NlaIII</i>
Kir6.2- K67N	GAAAGGCAACTGCAACGTGG TAGTCACTTGGACCTCAATG	94-58-72 / 30	278	<i>BseNI</i>
GCK-Y214C	TCCAGCACTGCAGCTTCTGTG GAGCCTCGGCAGTCTGGAAG	94-64-72 / 30	177	<i>BseXI</i>

Single strand conformation polymorphism analysis (SSCP). SSCP was performed essentially according to the method of Orita et al. (186) (Studies II-III). Separation of different variants of the gene is achieved by the mobility shift of the unique tertiary structures that are formed dependent on the primary sequences of single-stranded DNA. The PCR products were first diluted 2-20-fold with 0.1% sodium dodecyl sulphate (SDS), 10 mM EDTA and then diluted (1:1) with the loading mix (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). After denaturation at 98°C for 3 min, samples were immediately cooled on ice. 3µl of each sample were loaded onto a 6% nondenaturing polyacrylamide gel of 0.4 mm in thickness (acrylamide/N,N-methylene-bis-acrylamide ratio 49:1) containing 10% of glycerol and was run with a perpendicular electrophoresis. The runs were performed at two different gel temperatures: 38°C for approximately 4 h and 29°C for 5 h. Autoradiography of the gel dried on filter paper was carried out overnight at -70°C with intensifying screens.

Direct sequencing. Variant forms of DNA detected by SSCP analysis were identified by direct sequencing of both DNA strands (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit, USB corporation, Cleveland, OH, USA).

Restriction fragment length polymorphism (RFLP). The sequence variants were confirmed by RFLP analysis. The PCR-product amplified from 50 ng of genomic DNA was digested with the specific restriction enzyme for 2-16 h at 37-65°C and separated by electrophoresis on 9% PAGE gel.

Haplotype analysis. Three (study II) to six (study I) microsatellite markers were chosen for haplotype analysis: D11S1791, D11S1981, D11S1890, D11S921, D11S1888, and D11S4138. Both SUR1 and Kir6.2 genes map between the markers D11S1890 and D11S1888 and on either side of the marker D11S921. Based on the population history of Finland and previous molecular genetic studies, it was expected that any founder mutations in SUR1 and Kir6.2 genes present in the Finnish population should be traceable using these haplotypes as markers for mutation-bearing chromosome (187). One of the primers was labelled with fluorescent dye during synthesis. The polymorphic DNA fragments were amplified by PCR using optimized conditions. Electrophoresis was carried out to separate dinucleotide alleles of different sizes, using an automatic laser fluorescence DNA sequencer (ALFexpress, Pharmacia, Uppsala, Sweden) with 6% Hydrolink gels. Two internal standards were included in each line. Two-point linkage analysis was calculated using the LINKAGE package (Study III) (188).

Electrophysiological studies

Human beta cell studies. Pancreatic tissue was obtained after subtotal pancreatectomy from one patient homozygous for the SUR1-V187D mutation (Table 1, patient number 31). Islet-like cell clusters were cultured after collagenase digestion of the tissue (189) and insulin positive cells were studied by patch-clamp techniques (190). Control experiments were performed on human islets from heart-beating cadaver organ donors.

Recombinant K_{ATP} channel studies. Site-directed mutagenesis of rat SUR1 was performed and the mutant sequences were expressed in oocytes isolated from xenopus laevis frogs. Oocytes were co-injected with a mixture containing mRNAs encoding SUR1 (wild type or

mutant) and Kir6.2 (wild-type or mutant) (191). Control oocytes were injected with water. Both whole-cell currents using a two-electrode voltage-clamp and macroscopic currents using the excised patch studies were recorded. The basis of the patch clamp method is the formation of a high resistance seal between the cell membrane and the glass wall of a micropipette placed against the surface of the cell (192). The currents flowing through the ion channels in the patch of the membrane spanning the tip of the pipette can be recorded, and the potential across the membrane patch can be altered by applying a variable voltage to the pipette. To produce the inside-out patch the pipette is withdrawn from the cell surface after forming a cell-attached patch. An isolated membrane patch is then produced that has its intracellular membrane surface facing the bath solution.

4.2.2 Acute insulin response tests (AIRs) (Study III)

The patients who participated in the AIR tests were admitted to the pediatric ward of the Kuopio University Hospital. Patients on diazoxide treatment came to hospital six days and the patient on octreotide treatment two days before the tests for the discontinuation of the medication. Patients with diabetes (daily dose of insulin < 0.5 units), had their last subcutaneous insulin injection in the morning of the day before the tests. A stable insulin infusion was started (Actrapid 0.5 units/ml in NaCl 0.9 % at the rate of 0.025 U/kg/h) in the same evening and was maintained until the end of the calcium stimulation test.

The study protocol consisted of an intravenous calcium stimulation test in all patients (n=18) and was followed by intravenous glucose tolerance and tolbutamide tests in non-diabetic subjects (n=12) (92, 193). An intravenous catheter was placed in the antecubital vein for the infusion of calcium, glucose, and tolbutamide. Another cannula was inserted in the contralateral antecubital vein for the blood sampling. All patients were monitored carefully for 24 h after the tests.

Calcium stimulation test. After baseline blood collection, a bolus of elemental calcium (Calcium Sandoz® 2 mg/kg, maximal dose 90 mg) was infused within 60 seconds to evaluate the insulin response to an acute elevation of plasma calcium level. Samples for the measurement of blood glucose, plasma insulin, plasma C-peptide and plasma calcium were

drawn at -5, 0, 1, 3 and 5 min. The blood glucose level was measured at 15 and 30 minutes after the calcium bolus.

Intravenous glucose tolerance test (IVGTT). One hour after the calcium stimulation test, an IVGTT was performed on patients who were not on insulin treatment to determine the first phase insulin secretory capacity. After baseline blood collection (-5 and 0 min) a bolus of glucose (0.5 g/kg max 20 g in a 20 % solution) was injected within 2 min to acutely increase the blood glucose level. Samples for the measurement of blood glucose, plasma insulin and plasma C-peptide were drawn at 1, 3, and 5 min after the glucose infusion.

Tolbutamide test. The tolbutamide test was performed on non-diabetic patients in order to investigate the beta cell response to the K_{ATP} channel antagonist tolbutamide. Sixty minutes after the IVGTT, a bolus of tolbutamide (25 mg/kg, max dose 1000 mg) was injected within one minute. Venous blood was collected at -5, 0, 1, 3 and 5 min. After this, a maintenance glucose infusion was started to avoid hypoglycemia.

4.2.3 Studies of glucose metabolism in adults (Studies IV, V)

The subjects were admitted to the metabolic ward of the Department of Medicine, Kuopio University Hospital for 2 days. An intravenous catheter was placed in the antecubital vein for the infusion of glucose. Another cannula for blood sampling was inserted into a wrist vein, and the hand was surrounded by a heated box (50°C). On the first day, after 12 hours fasting, basal clinical and biochemical data were collected. Two different study protocols were used: 1) In the SUR1-V187D carrier study (Study IV), on the first day a 4-hour oral glucose tolerance test (OGTT) was performed. On the second day, an IVGTT was performed, followed immediately by the hyperinsulinemic euglycemic (2-hour) and hypoglycemic clamp (2-hour) studies. Afterwards, four carriers of the V187D mutation and six control persons participated in the tolbutamide test. 2) In the SUR1-E1506K carrier study (Study V), a 2-hour OGTT was performed, followed by a hyperglycemic clamp on the first day. On the second day, an IVGTT was performed, followed by the 3-hour hyperinsulinemic euglycemic clamp. The results of the IVGTT performed in five CHI patients during AIR tests described in chapter 4.2.2.2 were included in study V. The validation of measuring insulin sensitivity by the hyperinsulinemic euglycemic clamp after

an IVGTT has been previously reported (194).

Oral glucose tolerance test. OGTT was performed to evaluate the degree of glucose tolerance and insulin and C-peptide responses to an oral glucose load. A glucose dose of 75 g per person was administered during two minutes and samples for blood glucose, plasma insulin, and plasma C-peptide were drawn in the Study IV at 0, 30, 60, 90, 120, 150, 180, 210 and 240 minutes and in the Study V at 0, 30, 60, 90 and 120 minutes.

Hyperglycemic clamp. To evaluate the maximal glucose-induced beta cell insulin response the hyperglycemic clamp study was performed (study V). Immediately after the 2-hour OGTT (0-120 min), blood glucose was acutely increased to 20 mmol/l by a constant 20% glucose infusion and clamped at 20 mmol/l for the next 60 min by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals. At 150, 165 and 180 min, samples were taken for the measurement of plasma insulin and C-peptide.

Intravenous Glucose Tolerance Test. An IVGTT was performed to determine the first phase insulin secretory capacity. After baseline blood collection, a bolus of glucose (300 mg/kg in a 50% solution) was given (within 30 seconds) into the antecubital vein to acutely increase the blood glucose level. Samples for the measurement of blood glucose and plasma insulin were drawn at -5, 0, 2, 4, 6, 8 and 10 min.

Hyperinsulinemic euglycemic clamp. Tissue sensitivity to insulin was evaluated with the hyperinsulinemic euglycemic clamp technique (195). After IVGTT, a priming dose of insulin infusion (Actrapid 100 IU/ml, Novo Nordisk, Gentofte, Denmark 160 mU/ m² body surface area / min) was administered during the initial 10 minutes to acutely raise plasma insulin level followed by a continuous insulin infusion at the rate of 80 mU/ m² body surface area. Blood glucose was clamped at 5.0 mmol/l for the next 120 min in the Study IV and for the next 180 min in the Study V by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals. During the euglycemic clamp the glucose levels were averaged over each 20-min interval. The mean value of the last hour was used to calculate the rates of whole body glucose uptake (M-value). The insulin sensitivity index was calculated by dividing the M-value with the mean value of insulin

concentrations taken at 140, 160 and 180 min during the hyperinsulinemic-euglycemic clamp.

Hyperinsulinemic hypoglycemic clamp. The hypoglycemic clamp was performed immediately after the euglycemic clamp (0-120 min) in order to investigate C-peptide secretion and counterregulatory hormone responses during hypoglycemia (study IV). Blood glucose was clamped at 3.0 mmol/l for the next 120 min (120-240 min) by infusing 20 % glucose at varying rates according to blood glucose measurements performed at 5-min intervals. The measurements of plasma insulin, plasma C-peptide, serum glucagon, serum epinephrine, serum norepinephrine, serum cortisol and serum growth hormone were drawn at 90 min during the euglycemic clamp (normoglycemic state) and again during hypoglycemia at 225 and at 240 min during the hypoglycemic clamp.

The symptoms of hypoglycemia were recorded at 0, 30, 60 and 120 min from the beginning of the hypoglycemic clamp, using a questionnaire described previously (196, 197). The study subjects were asked to evaluate the severity of autonomic symptoms (such as sweating, shaking, nervousness and pounding of the heart) and the neuroglycopenic symptoms (such as blurred vision, weakness, hunger, tiredness, dizziness, difficulty in thinking, faintness and tingling) on a visual scale from 0 (absent) to 10 (severe). The sums of these scales at each four time points constituted the hypoglycemia symptom score.

Tolbutamide test (Study IV). To investigate the beta cell response to a sulfonylurea drug the tolbutamide test was performed in four V187D heterozygotes and in six controls. It is known that the sulfonylureas induce insulin secretion through direct interaction with SUR1. Thus it was hypothesized that in the carriers of V187D, the increment in insulin secretion would be lower in the case of defective channel function.

After a 12-h overnight fast, the baseline values of blood glucose, plasma insulin and plasma C-peptide were measured in duplicate at 5-min intervals. A glucose bolus (300 mg/kg in a 50 % solution) was injected into the antecubital vein within 30 seconds. At 20 min following the end of the glucose injection, a bolus of tolbutamide (3 mg/kg) (Orinase Diagnostic^R, Pharmacia & Upjohn, Kalamazoo, Michigan, USA) was injected. Venous blood was collected at 19, 22 and 29 min after the glucose bolus for the measurement of blood glucose, plasma insulin and plasma C-peptide. Plasma insulin and plasma C-peptide

responses to tolbutamide injection (0-3 min) were calculated as the difference between measurements obtained at the two time points. The tolbutamide-stimulated plasma insulin and plasma C-peptide responses during 0-10 min after the tolbutamide bolus were calculated by means of the trapezoidal method as the incremental area under the curve.

Assays and Calculations. Blood glucose was measured with the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus, Yellow Springs Instrument Co., Inc, Ohio, USA). For the determination of plasma insulin and C-peptide, blood was collected into EDTA tubes. After centrifugation, the plasma was stored at -20°C until analysis. Plasma insulin and C-peptide were determined by radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden, and C-peptide by 125J RIA kit, Inestar Co., Stillwater, Minn., USA). This insulin assay also detects proinsulin and proinsulin conversion products, with a crossreactivity of 47 %. The incremental insulin and glucose areas under the curve were calculated by the trapezoidal method. The counterregulatory hormone responses were analysed by high-pressure liquid chromatography (HPLC-EC, in-house method) (epinephrine, norepinephrine), chemiluminometric enzyme immunoassay (EIA / Immulite 2000Cortisol/Growth Hormone (hGH), Diagnostic Products Corporation, Los Angeles, USA) (cortisol, growth hormone) and radioimmunoassay (RIA / Glucagon Double Antibody RIA, Diagnostic Products Corporation) (glucagon).

Statistical Analysis. All calculations were performed with the SPSS for Windows software (SPSS Inc., Chicago, USA.). Data are shown as the median or mean \pm SEM or SD. Differences in the variables between groups were analyzed using the nonparametric Kruskal-Wallis and Mann-Whitney tests.

5 RESULTS

5.1 Genetic findings (Studies I-III)

Screening of the candidate genes for CHI led to the detection of nine novel mutations (six in SUR1, two in Kir6.2, one in GCK) which altogether associate with 60% of all Finnish CHI patients in this study. The two founder mutations V187D and E1506K in the SUR1 gene associate with 88% of the genetically characterized cases. All Finnish K_{ATP} channel mutations identified are shown in Figure 4. Several polymorphisms, most of them previously described, were also detected (166, 198).

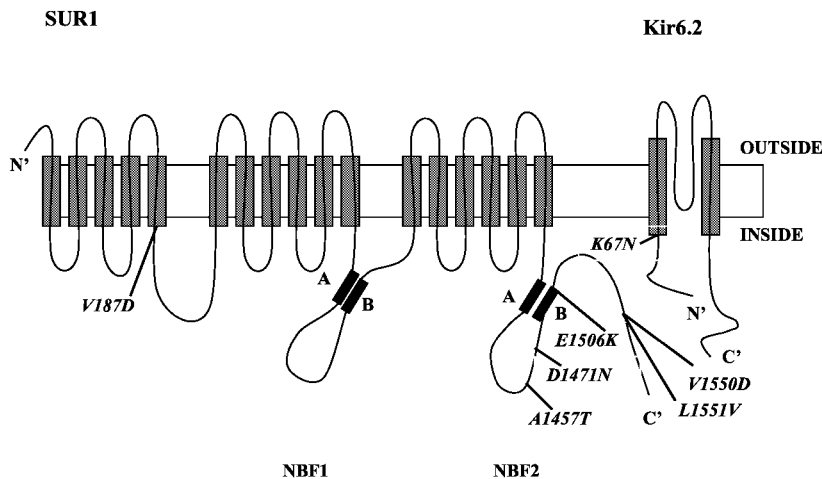


Figure 4. The Finnish K_{ATP} channel mutations.

5.1.1. SUR1-V187D (Study I)

Detection of the mutation. Sequencing of the SUR1 exon 4 of the patient 20 (Table 1) revealed a homozygous T → A point mutation which resulted in the amino acid change from valine to aspartic acid (Figure 5). This mutation V187D locates in the transmembrane area of the SUR1 protein. The mutation leads to the creation of a *Tth111I* restriction endonuclease site, thus giving means to test for its presence. The mutation was detected in 16 Finnish patients of whom six were homozygous and ten were heterozygous. In all cases chromosomes carrying the mutation were associated with the same conserved haplotype.

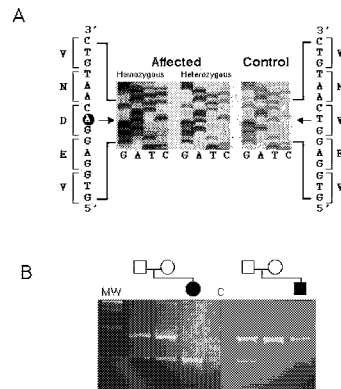


Figure 5. The mutation SUR1-V187D. a) T→A point mutation leads to the amino acid change from valine to aspartic acid. b) Detection of the mutation by RFLP with the *Tth111* restriction endonuclease (the upper 279-bp product represents the wild-type allele and the lower 208-bp product represents the mutant allele, MW=molecular weight marker).

Clinical findings. All cases with the V187D mutation were geographically clustered in Central Finland (Figure 6). The calculated incidence in that high-risk area was as high as 1 : 3 200 whereas the national-wide incidence in Finland was 1 : 40 400 during the years 1983-1997 when 1 to 2 new cases were diagnosed annually (birth cohort ~60 000 per year).

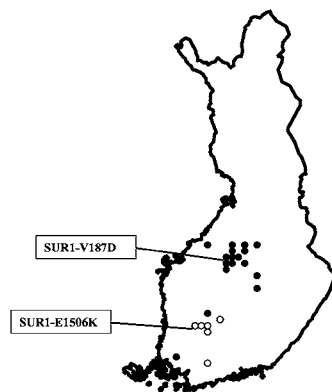


Figure 6. Birthplaces of the parents of the patients with the SUR1-V187D (●) and the SUR1-E1506K (○) mutations.

All patients presented with hypoglycemia during the first postnatal day. One patient (Table 1, patient 23) was treated for transient hypoglycemia after the birth but returned to hospital due to severe recurrent hypoglycemia at the age of one month. None of the patients treated

with diazoxide showed any response. However, a partial response to octreotide treatment was achieved in heterozygous V187D carriers but not in patients who were homozygous for V187D. Subtotal pancreatectomy was performed in all except the youngest case, who was treated successfully with octreotide. In two cases with paternally inherited SUR1-V187D (Table 1, patients 10, 23) focal adenomatous hyperplasia was detected after the surgery, and thus, the somatic loss of maternal alleles may be the mechanism underlying their disease. In all other cases histological examination revealed the diffuse form of the disease.

Electrophysiology. The recordings from beta cells isolated from a patient homozygous for the V187D mutation after subtotal pancreatectomy (Table 1, patient 31) showed no spontaneous K_{ATP} channel activity, and both diazoxide and somatostatin were without effect on channel activity (Figure 7). The results of recombinant K_{ATP} channel studies were consistent with these results. Kir6.2/SUR1-V187D-injected oocytes were insensitive to metabolic regulation due to the nonfunctional K_{ATP} channels.

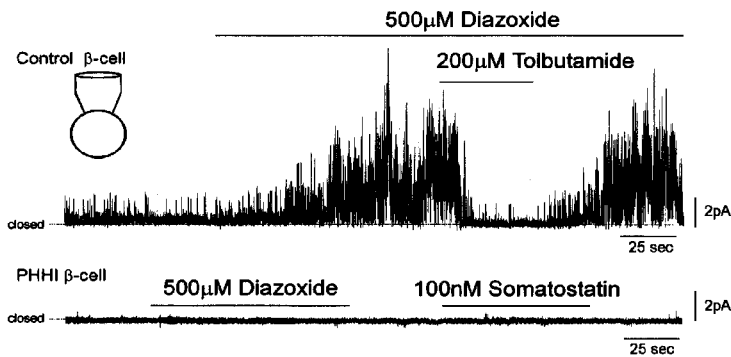


Figure 7. Ion channel recordings from control beta cells and beta cells of a patient with a homozygous SUR1-V187D mutation (Table 1, patient 30). In control cells, spontaneously active K_{ATP} channels are further activated by the addition of diazoxide and inhibited by tolbutamide. In contrast, there are no effects of diazoxide or tolbutamide on the beta cells of the patient 30.

5.1.2. SUR1-E1506K (Study II)

Detection of the mutation. The E1506K mutation, which results in the heterozygous GAG to AAG substitution and the amino acid change from glutamic acid to lysine, was found in SUR1 exon 37 in seven patients with CHI (Figure 8). This missense mutation is located in the second nucleotide binding fold of SUR1. Its presence leads to the disappearance of an *MnlI* restriction endonuclease site, which provides a means to confirm its presence. All seven patients were heterozygous for the mutation, and no other mutations in K_{ATP} channel genes were detected. Mutation E1506K was not found in 100 normal healthy Finnish control chromosomes, nor in the chromosomes of 80 type 2 diabetic patients.

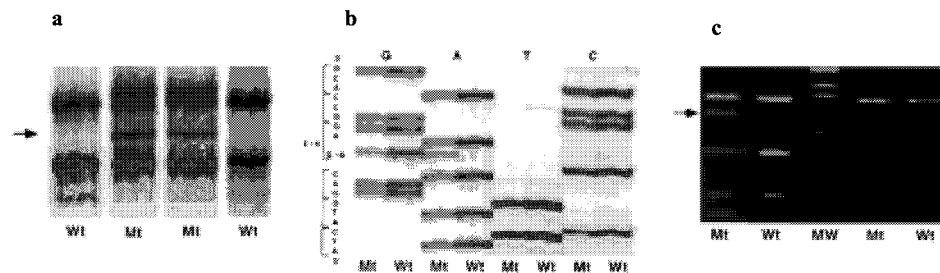


Figure 8. Mutation SUR1-E1506K. a) SSCP analysis of SUR1 exon 37 ('wt'= wild-type, 'mt'=mutated. b) G->A point mutation results in the substitution of glutamic acid to lysine. c) Detection of the mutation by RFLP with the *MnlI* restriction endonuclease (the SUR1-E1506K mutation causes the disappearance of *MnlI* restriction site and a formation of a new 89-bp product).

In all CHI cases the mutation was maternally inherited and shared a distinct haplotype. The paternally inherited haplotypes were all very different. All the cases were geographically clustered in the Southwestern part of Finland (Figure 6). The large six-pedigree family (Figure 9) with seven patients was analyzed for genetic linkage using both nonparametric and parametric approaches. The nonparametric analysis tested the significance of the observation that a haplotype including a rare single nucleotide polymorphism, E1506K, was inherited by all of the affected individuals, but none of those unaffected in the previous generation. The result of this analysis strongly supported the hypothesis that the segregation of CHI in this pedigree was dependent on the segregation of the haplotype. The likelihood of obtaining the observed haplotype-sharing pattern by chance was 1.2×10^{-7} . In the parametric

analysis the linkage of the mutant allele to the phenotype gave a LOD score of 6-6.28 when calculated using a dominant model and 3.83-4.1 when calculated using a recessive model.

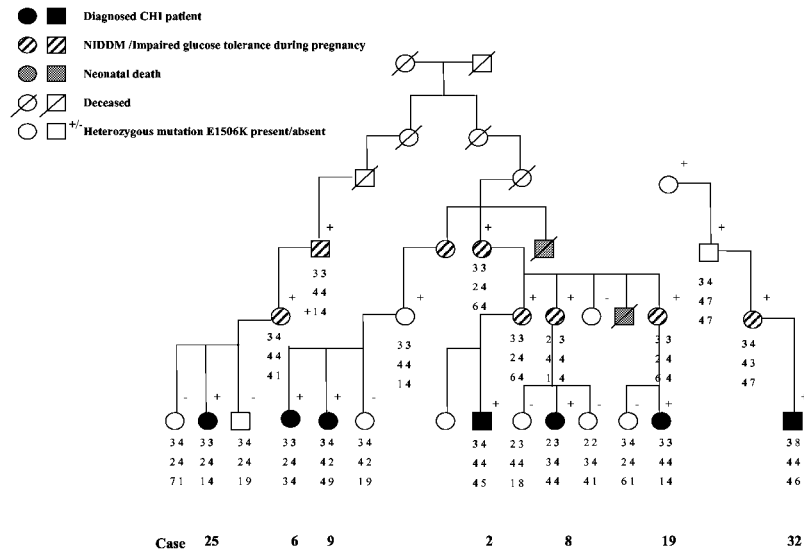


Figure 9. The SUR1-E1506K pedigree and the haplotype analysis. The haplotype 3-4-4 associates with SUR1-E1506K (the order of the markers: D11S1890, D11S921, D11S1888).

Clinical findings. Five of seven CHI patients with SUR1-E1506K presented with hypoglycemia during the first few hours after birth. In two cases, the symptoms of hypoglycemia appeared later, at the age of five and seven months. All patients who were treated with diazoxide showed a good response (Table 1, patients 8, 19, 25, 32). In the two mildest cases (Table 1, patients 6, 9) hypoglycemia was treated with extra glucose administration and frequent feeds without the need for medication. The oldest patient of this group was treated with subtotal pancreatectomy at the age of 3.5 years (Table 1, patient 2). The histological examination of the resected pancreas revealed features typical for the diffuse form of CHI, with abnormally large beta cell nuclei distributed throughout the pancreas.

At least two of the mothers (Figure 9, the mothers of cases 2 and 19; generation II) had had symptoms that could be classified as hypoglycemic during the first 2 years of life. The symptoms always manifested in the morning and included irritability, convulsions, deviation of eyes, and even unconsciousness. Mild and non-specific symptoms including trembling and sweating during fasting were detected also in the other mutation E1506K carriers in

three generations. All except one of the mothers had impaired glucose tolerance or diabetes during pregnancy. Two of them as well as the grandmother of the patients 2, 8 and 19 (Table 1) were diabetic. The studies of glucose metabolism performed on two of the mothers (mothers of cases 2 and 8, Table 1), which revealed a severely reduced insulin secretory capacity, are discussed more widely in study V.

Electrophysiology. The results of recombinant K_{ATP} channel experiments suggested that SUR1-E1506K is capable of forming functional channels with Kir6.2, and that these channels are insensitive to metabolic inhibition but can be opened with diazoxide. The lack of MgADP activation may contribute to the failure of metabolic inhibition to stimulate Kir6.2/SUR1-E1506K currents in oocytes.

Unlike all previously reported SUR1 gene mutations, heterozygosity for E1506K is sufficient to cause the disease. To determine whether this arises from a gene dosage or dominant negative effect, Kir6.2 was coexpressed together with a 1:1 mixture of wild-type and mutant CHI. The result of this test showed that the amplitude of K_{ATP} current activated by metabolic inhibition in these oocytes was approximately half that found for oocytes expressing Kir6.2/SUR1 channels (Figure 10).

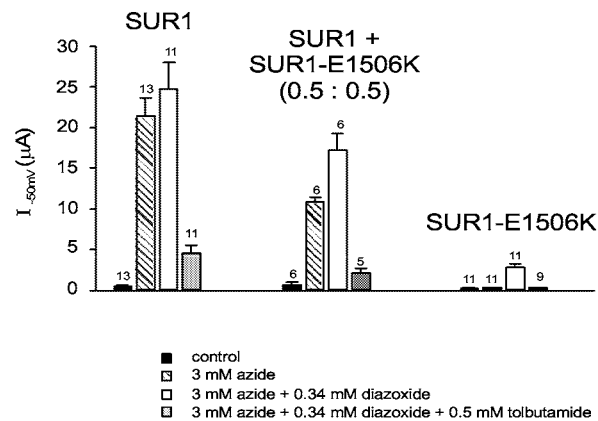


Figure 10. Effects of metabolic inhibition on Kir6.2/SUR1 (wt) and Kir6.2/SUR1-E1506K (mt). Oocytes were coinjected with mRNA encoding Kir6.2 and either SUR1, SUR1-E1506K, or 1:1 mixture of SUR1 plus SUR1-E1506K. Mean whole-cell currents in control solution (black bars), after exposure to 3mM azide (hatched bars), in the continued presence of azide plus diazoxide (white bars), and, finally, after the addition of tolbutamide (shaded bars).

5.1.3. Other K_{ATP} channel mutations (Study III)

Detection of the mutations. All of the other four mutations of SUR1 located in the second NBF area (Figure 4). These substitutions were detected in single families with one or two affected children. None of these novel mutations were detected in 100 normal Finnish control chromosomes.

The substitution A1457T was found to be maternally inherited in one compound heterozygote patient with paternally inherited SUR1-V187D (Table 1, patient 16). The clinical phenotype of this patient was very severe, and he was pancreatectomized at the age of 23 days. The mutation SUR1-A1457T locates in exon 36 of the SUR1 gene and leads to amino acid change from alanine to threonine and to a formation of a new HphI endonuclease restriction site. The studies of electrophysiology suggested that SUR1-A1457T, when expressed with wild type Kir6.2, did not form functional channels.

The mutation D1471N in SUR1 gene was detected just recently in two sisters with maternal SUR1-V187D. It located in exon 37 and leads to amino acid change from aspartic acid to asparagine. The electrophysiological analysis of this substitution will be performed in the near future.

The maternally inherited substitution V1550D in exon 39 of the SUR1 gene was detected in one individual with paternally inherited SUR1-V187D (Table 1, patient 13). The clinical phenotype of this patient was severe, necessitating subtotal pancreatectomy at the age of 3 weeks. Like in the case of the mutation SUR1-V187D, this mutation results in the amino acid change of valine to aspartic acid and to a loss of functional K_{ATP} channels.

Heterozygous SUR1 mutation L1551V in exon 39 which leads to amino acid change from leucine to valine was detected in two sisters with no other identified SUR1 mutations (Table 1, patients 37, 43). They both had a diazoxide-responsive form of CHI. The recombinant K_{ATP} channel studies showed that L1551V forms functional channels but shows reduced sensitivity to metabolic inhibition. The formation of a new *Bse*LI endonuclease restriction site gives the means to test for its presence.

Two different Kir6.2 mutations were identified in a single patient (Table 1 patient 21) with a severe form of CHI treated by subtotal pancreatectomy at the age of 11 days. The maternally inherited substitution located 54 bases proximal of the translation initiation site formed a new start codon and a frame shift. The paternally inherited K67N substitution causes amino acid change from lysine to asparagine. The functional effects of these Kir6.2 mutations are still under investigation.

5.1.4 GCK-Y214C

The 'de novo' substitution Y214C in the GCK gene, which leads to amino acid change from tyrosine to cysteine, was detected in a single CHI patient with severe form of the disease (Table 1, patient 1). Despite a subtotal pancreatectomy in early childhood, this patient still presents with hypoglycemia at the age of 31 years. The association between this mutation and the clinical CHI has been uncertain until now.

5.2 Acute insulin responses in CHI patients (Study III)

Clinical and Biochemical Characteristics of the patients. The major clinical characteristics of the patients studied with AIR tests are shown in italics in Table 1.

Calcium stimulation test. The acute plasma insulin and C-peptide responses to calcium were significantly increased in patients with SUR1-E1506K as compared with either patients without K_{ATP} channel mutations, or with those carrying the SUR1-V187D mutation. The response to calcium was not significantly different between the SUR1-V187D carriers and patients without mutations. The results of the calcium stimulation test are shown in Figure 11 a and b.

Tolbutamide stimulation test. The plasma insulin and C-peptide responses to tolbutamide appeared to be lower in patients with SUR1-V187D and SUR1-E1506K mutations as compared with patients without K_{ATP} channel mutations. One of 4 patients with SUR1-E1506K showed a clear response to tolbutamide but the response was absent in all other cases. Interestingly, the patient with Kir6.2 mutations had a high normal response to tolbutamide (Figure 11 c and d).

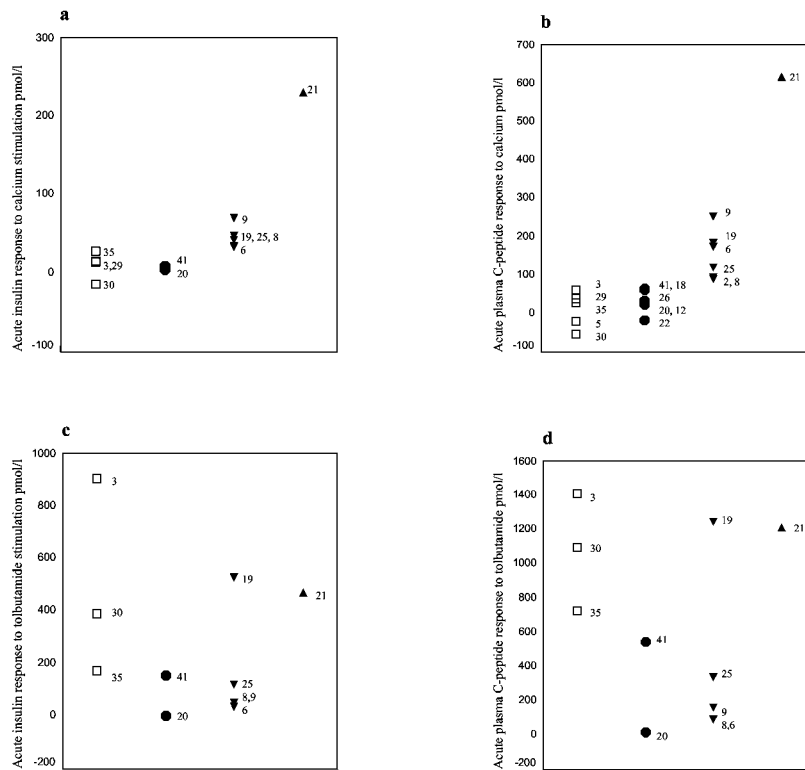


Figure 11. Acute plasma insulin and C-peptide responses to calcium (a,b) and tolbutamide (c,d) expressed as the mean of the increment of measurements at 1, 3 and 5 min after stimulation in patients without K_{ATP} channel mutations (□), in SUR1-V187D homozygous and heterozygous patients (●), in SUR1-E1506K heterozygous patients (▼), and in the patient with two Kir6.2 mutations (▲). The number of each patient is shown beside the symbol (Table 1).

Intravenous glucose tolerance test. The first-phase insulin secretion, calculated as a sum of plasma insulin measured at 1 and 3 min during the IVGTT (199), was abnormally low in the prepubertal SUR1-V187D homozygous patient (Table 1, patient 20) and also in the

postpubertal SUR1-E1506K heterozygotes (Table 1, patients 6, 8 and 9). This result is discussed in more detail in context of the SUR1-E1506K heterozygote study.

5.3 Glucose metabolism in SUR1-V187D heterozygotes (Study IV)

Clinical and Biochemical Characteristics of the Study Groups. Table 3 shows the clinical and biochemical characteristics of the study groups. The groups were comparable with respect to age, gender and BMI. Furthermore, there were no significant differences in fasting glucose, insulin, C-peptide, HbA1c, or blood pressure.

Table 3. Clinical and Biochemical Characteristics of the Study Groups (Study IV)

	Controls n=10	V187D-heterozygotes n=8
Age (years)	35.6 ± 1.1	35.3 ± 0.9
Gender (M/F)	5/5	4 / 4
Body mass index (kg/m ²)	23.2 ± 0.6	24.0 ± 1.2
Fasting blood glucose (mmol/l)	4.3 ± 0.2	4.7 ± 0.2
Fasting insulin (pmol/l)	49.2 ± 6.6	42 .0± 5.4
Fasting C-peptide (pmol/l)	530 ± 35	450 ± 51
GHbA1c (%)	5.4 ± 0.1	5.4 ± 0.1
Fasting hepatic insulin extraction (pmol C-peptide/pmol P-insulin)	10.7 ± 0.3	10.7 ± 0.4

The results are given as means ± SEM

Oral glucose tolerance test (OGTT). All individuals in both study groups had normal glucose tolerance according to the World Health Organization criteria (184) determined by OGTT. The blood glucose levels were similar in both groups (V187D-heterozygotes and controls) at all time points measured after the oral glucose load. Furthermore, there were no significant differences in plasma insulin nor plasma C-peptide responses at any time point. Similarly, the incremental plasma insulin and C-peptide areas under the curve were comparable between the study groups.

Intravenous glucose tolerance test (IVGTT). Figure 12 shows the acute blood glucose (a) and plasma insulin (b) responses in the IVGTT. There were no differences in plasma insulin levels, nor in the incremental plasma insulin areas under the curve. Furthermore, the incremental glucose area under the curve was similar in both study groups.

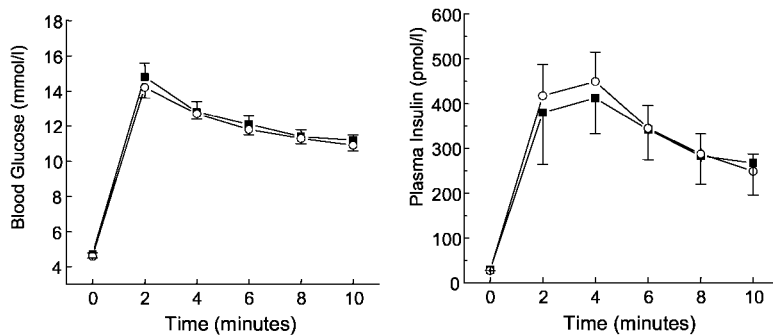


Figure 12. Blood glucose and plasma insulin concentrations during the IVGTT in control subjects (○) and in SUR1-V187D heterozygotes (■).

Hyperinsulinemic euglycemic and hypoglycemic clamp. The rates of whole body glucose uptake did not differ significantly between the groups (11.1 ± 0.9 vs. 10.2 ± 0.8 mg/kg/min). Furthermore, there were no differences in the plasma insulin and C-peptide levels measured at the steady state during the euglycemic clamp or during the hypoglycemic clamp. Finally, neither the counterregulatory hormone responses (serum glucagon, epinephrine, norepinephrine, cortisol and growth hormone) in normoglycemia and in hypoglycemia nor the symptoms of hypoglycemia evaluated during the hypoglycemic clamp differed significantly between the groups.

Tolbutamide stimulation test. Figure 13 shows that plasma insulin and C-peptide responses to tolbutamide injection were similar in V187D-heterozygotes and controls when expressed as a difference between the hormone levels measured at 0 and 3 min after the tolbutamide bolus. The incremental areas under the curve (0-10 min) did not differ either (P-insulin 1744 ± 338 versus 2226 ± 491 pmol/l·min; C-peptide 5950 ± 1266 vs. 6607 ± 870 pmol/l·min, V187D-carriers and controls, respectively).

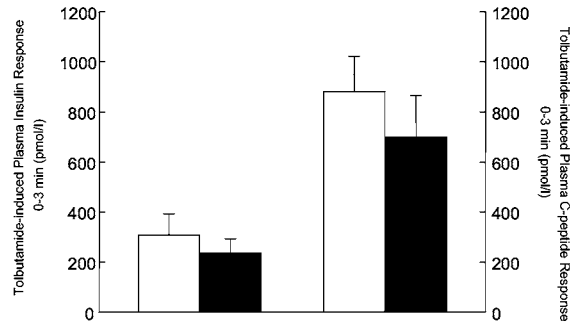


Figure 13. Tolbutamide-induced plasma insulin and plasma C-peptide responses in control subjects (□) and SUR1-V187D heterozygotes (■), expressed as the difference between measurements obtained at 2 min after and 1 min before the tolbutamide injection.

5.4 Glucose metabolism in SUR1-E1506K heterozygotes (Study V)

Clinical and biochemical characteristics of the study groups. Table 4 shows the clinical and biochemical characteristics of the three study groups (controls, non-diabetic SUR1-E1506K heterozygotes and diabetic SUR1-E1506K heterozygotes). Diabetic subjects were somewhat older and heavier than the other subjects. There were no significant differences in fasting C-peptide or blood pressure levels. However, fasting blood (fB) glucose levels were significantly elevated in SUR1-E1506K heterozygotes with diabetes, and fasting plasma insulin was reduced in non-diabetic SUR1-E1506K heterozygotes compared to control subjects. Glycated hemoglobin A1c was 6.8 ± 0.3 % in the diabetic patients (n=4).

Oral glucose tolerance test. All nineteen individuals in the control group had normal glucose tolerance. One person in the SUR1-E1506K heterozygote group had normal glucose tolerance, one person increased fasting glucose and 5 subjects impaired glucose tolerance (IGT). Four people fulfilled the criteria for diabetes mellitus (184). The age at diagnosis of diabetes was 39, 42, 44, and 60 years in the diabetic patients.

The blood glucose, plasma insulin and C-peptide at all time points measured after the oral glucose load are shown in Figure 2/ Publication V.

Table 4. Clinical and Biochemical Characteristics of the Study Groups (range in parenthesis) (Study V)

	Controls (n=19)	SUR1-E1506K heterozygotes No diabetes (n=7)	SUR1-E1506K heterozygotes Diabetes (n=4)
Gender (M/F)	10/9	3/4	0/4
Age	41 ± 2 (31-55)	45 ± 6 (22-68)	50 ± 7 (39-71)
Body mass index (kg/m ²)	25.8 ± 0.9 (20.2-32.0)	24.2 ± 1.0 (20.9-27.9)	28.8 ± 1.9 (24.8-33.3)
Fasting blood glucose (mmol/l)	4.6 ± 0.1	5.0 ± 0.3	8.3 ± 1.3**
Fasting plasma insulin (pmol/l)	55.8 ± 4.6 (23.4-90.0)	34.3 ± 3.7* (22.2-46.2)	57.5 ± 10.8 (36.0-82.8)
Fasting C-peptide (pmol/l)	514 ± 36	444 ± 40	578 ± 54

The results are given as means ± SEM. P < 0.05 versus controls, P** < 0.01 versus controls.*

Intravenous glucose tolerance test. Figure 14 shows blood glucose (a and b) and plasma insulin (c and d) responses in the IVGTT during the first 10 min. Fasting plasma insulin levels were similar in all three study groups. Plasma insulin levels at all time points measured after the intravenous glucose administration, as well as the incremental plasma insulin areas under the curve, were significantly reduced in both groups with the E1506K mutation ($P < 0.001$ and $P < 0.001-0.01$ in non-diabetic and diabetic groups, respectively). The incremental glucose areas under the curve were comparable in all study groups.

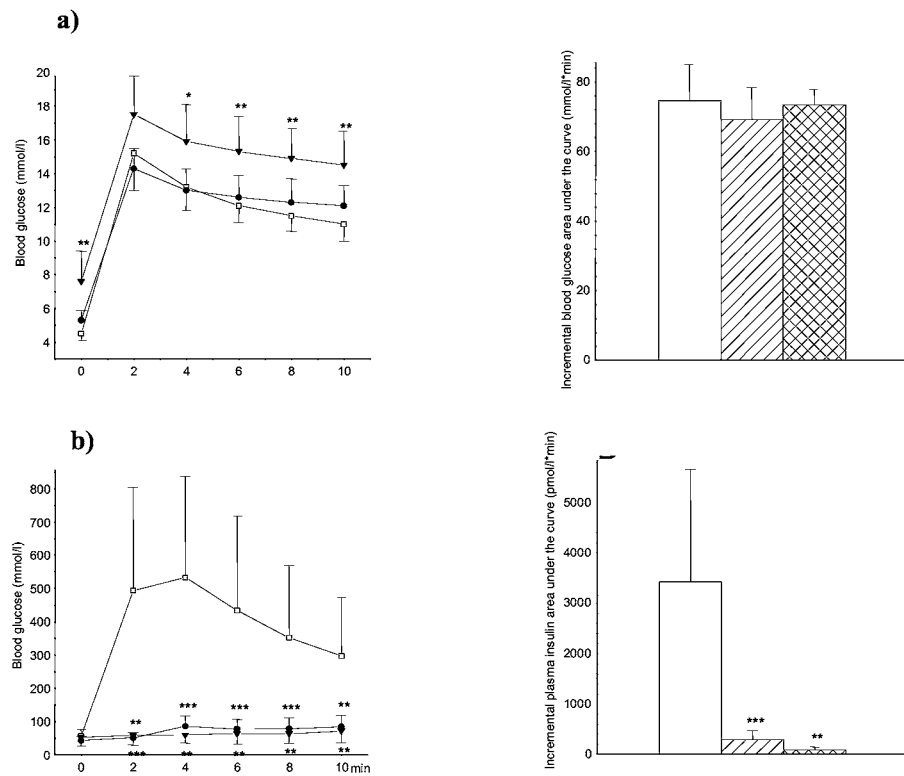


Figure 14. Blood glucose (a) and plasma insulin (b) responses during the IVGTT in control subjects (□; white bars), in SUR1-E1506K heterozygotes without (●; lined bars) and with diabetes (▼; hatched bars). * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ (control vs. other groups).

Figure 15 depicts plasma insulin values during the IVGTT in patients with CHI and in subjects belonging to the different study groups, according to age. The plasma insulin was calculated as the sum of the insulin concentrations at 1 and 3 min (199). The 1-min insulin concentration was calculated as a mean of 0 and 2 min insulin levels, and the 3 min insulin concentration was calculated as a mean of 2 and 4 min insulin levels. The first-phase insulin secretion was normal or high in the youngest CHI patients, but CHI patients aged > 10 years had impaired first-phase insulin secretion. Insulin secretion further decreased linearly with age in the E1506K carriers, independently of their glucose tolerance status. All control subjects had higher insulin levels than the adult E1506K carriers.

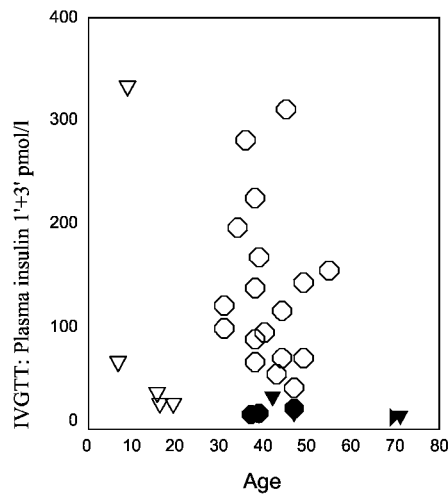


Figure 15. The sum of the 1- and 3- min insulin concentration during an IVGTT in patients with CHI (∇), in control subjects (\circ), and in the SUR1-E1506K heterozygotes with (\blacktriangledown) or without (\bullet) diabetes.

Hyperglycemic clamp. The maximal glucose-stimulated plasma insulin and C-peptide responses expressed as the mean values of plasma insulin and C-peptide determined at 150, 165 and 180 min during the hyperglycemic clamp, were markedly reduced in both study groups with the SUR1-E1506K mutation ($P < 0.05$ and $P < 0.01$ in non-diabetic and diabetic carriers, respectively)(Figure 16).

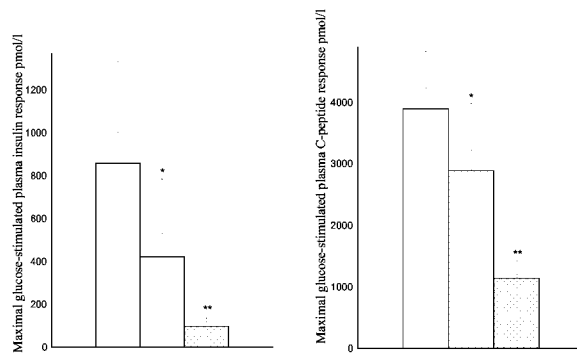


Figure 16. Maximal glucose-stimulated plasma insulin (a) and plasma C-peptide responses during the hyperglycemic clamp in control subjects (white bars), and in SUR1-E1506K heterozygotes without (lined bars) or with diabetes (hatched bars). * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ (control vs. other groups).

Hyperinsulinemic euglycemic clamp. The rates of whole body glucose uptake (M-value) did not differ significantly between the groups (60.5 ± 3.9 vs. 64.4 ± 6.1 vs. 51.7 ± 8.8 $\mu\text{mol/kg/min}$ in controls, non-diabetic and diabetic SUR1-E1506K heterozygotes,

respectively). However, compared to control subjects the rate of whole body glucose uptake was reduced by 15% in diabetic SUR1-E1506K heterozygotes. The insulin sensitivity indexes did not significantly differ among the study groups. Figure 17 plots the insulin sensitivity index against the incremental insulin area under the curve during the IVGTT, for all subjects. SUR1-E1506K heterozygotes had as wide a range of insulin sensitivity as control subjects, but their acute phase insulin secretion was considerably lower.

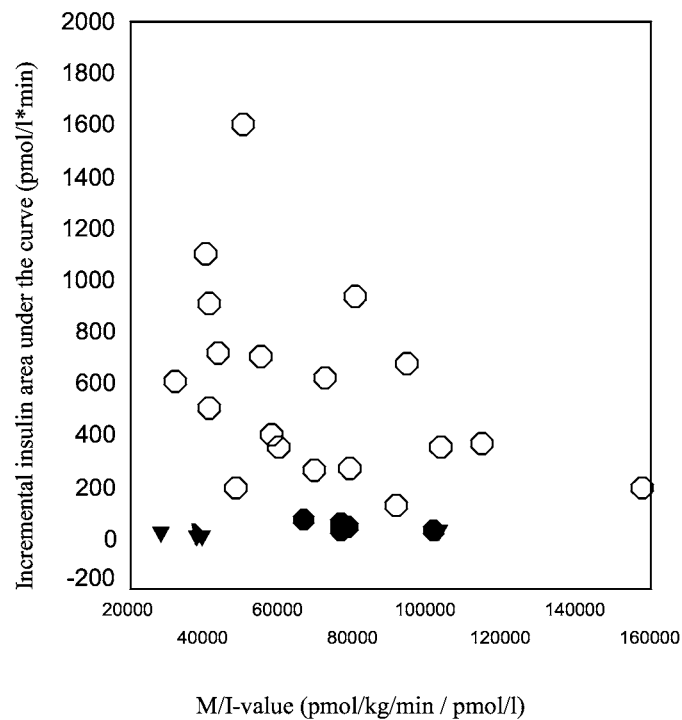


Figure 17. Incremental insulin area under the curve during an intravenous glucose tolerance test, according to the rates of whole body glucose uptake (M) divided by steady state insulin concentration (I) during the hyperinsulinemic euglycemic clamp in control subjects (o), and in carriers of the SUR1-E1506K mutation with (▼) or without (●) diabetes

6 DISCUSSION

6.1 Genotype-phenotype correlations

Mutations in genes encoding the K_{ATP} channel subunits SUR1 and Kir6.2 were detected in 60% of all Finnish CHI cases. As it was assumed, considering the specific features of Finnish population genetics (187, 200), two founder mutations (SUR1-V187D and SUR1-E1506K) were associated with the major proportion of all genetically characterized cases. Our studies have shown that these mutations lead to distinct functional abnormalities and different clinical phenotypes.

SUR-V187D. The recessively inherited missense mutation V187D is located in the transmembrane area of the SUR1 protein. It was detected in 16 out of 44 Finnish CHI patients, of whom six were homozygous and ten were heterozygous for the mutation. The same conserved haplotype was detected in all disease-associated chromosomes, which reflects the founder effect of this mutation. Furthermore, all cases were geographically clustered in Central Finland, where the incidence is as high as 1: 3,200 births.

In all cases the onset of the disease was soon after birth. In one case (Table 1, patient 23) the diagnosis was delayed, and the patient returned to the hospital with severe hypoglycemia at the age of one month. The clinical course of patients with the SUR1-V187D mutation has been very severe, and most patients have undergone subtotal pancreatectomy. None of the patients responded to diazoxide treatment, but a partial response to octreotide was seen in some heterozygous patients. Problems in neurological development were seen in about 30% of SUR1-V187D patients. This ranged from mild impairment of motor development to severe mental retardation and epilepsy (Table 1). One patient (patient 28 in table 1, the brother of case 31), who was born prematurely at the gestational age of 28 weeks died after subtotal pancreatectomy at the age of 16 days due to necrotizing enterocolitis and gut perforation.

The electrophysiological studies of beta cells isolated from a CHI patient homozygous for the V187D mutation (case 31), as well as the results of recombinant K_{ATP} channel experiments were consistent with the phenotype and showed that the mutation led to a loss

of functional K_{ATP} channels, with no activation by diazoxide. Interestingly, the disease phenotype is almost equally severe in patients homozygous or heterozygous for the V187D mutation. Thus, even a single allele of the V187D mutation will lead to a severe, drug-unresponsive form of CHI in compound heterozygotes. As it was expected, the acute insulin response to the K_{ATP} channel antagonist tolbutamide was severely decreased in the SUR1-V187D patients. However, unexpectedly, these patients did not show any response to calcium stimulation. A possible explanation for this finding could be that the subjects with SUR1-V187D have very little remaining beta cell function, which is maximally stimulated even under basal conditions.

Many parents who are heterozygous carriers of SUR1-V187D have reported symptoms that could be classified as hypoglycemic during fasting. However, our studies of their glucose metabolism indicated that despite the heterozygosity of this severe mutation, the carriers had normal insulin secretion, normal tissue-sensitivity to insulin and no inappropriate insulin secretion during hypoglycemia. These results confirmed the totally recessive nature of CHI caused by SUR1-V187D and also support the idea of compound heterozygosity or focal disease with maternal loss of alleles in patients with heterozygous SUR1-V187D mutation.

SUR1-E1506K. The mutation E1506K, located in the second NBD of SUR1, associates with a very different phenotype. It was detected in a heterozygous form in seven Finnish CHI patients in whom no other mutations in SUR1, Kir6.2, GCK or GDH genes were detected. Figure 6 shows that all these patients come from a limited area in the Southwestern part of Finland. The onset of this form of CHI is variable. The symptoms of hypoglycemia appeared soon after birth in five cases, but not until the age of five and seven months in the two other cases. All patients who were treated with diazoxide responded well. The two mildest cases (Table 1, patients 6 and 9) could be managed by a few weeks of intravenous glucose administration and frequent feedings. The oldest patient of this group (Table 1, case 2) was treated with subtotal pancreatectomy at the age of 3.5 years. It seems likely, however, that he could have been managed with effective diazoxide treatment. The clinical phenotype is in agreement with the results of the electrophysiological analysis. The SUR1-E1506K mutation leads to a reduction of K_{ATP} channels, but not a complete loss. The mutant channels were insensitive to metabolic inhibition, but a partial response to diazoxide was retained.

Unlike the other mutations described in the SUR1 gene, the E1506K mutation is inherited in a dominant manner. At least two of the mothers in generation two (Figure 9) had severe symptoms suggestive of hypoglycemia in their childhood. Almost all SUR1-E1506K heterozygotes reported non-specific symptoms like trembling and sweating during fasting. Furthermore, two newborn babies in two previous generations had died during the early neonatal period for an unknown reason.

The results of linkage analysis supported the idea of dominant inheritance and showed unambiguously that CHI in these patients depended on the inheritance of the E1506K allele of the SUR1 gene. The parametric linkage analysis strongly supported a dominant rather than recessive mode of inheritance. Furthermore, the inspection of the haplotypes surrounding the SUR1 gene also supported this observation. In all patients, the haplotype harboring the E1506K allele was similar, whereas the other haplotype was different in all cases. If the inheritance were recessive, the fact that six different haplotypes were observed among the non-E1506K chromosomes would imply that at least six different mutations segregated within this subpopulation, which is unlikely considering the rarity of the disease. Despite the dominant nature of SUR1-E1506K, it did not exert a dominant negative effect when expressed together with the wild type gene in xenopus oocytes. The molecular mechanism responsible for the dominant inheritance thus remains obscure at this point.

As expected, all patients with E1506K showed a significant response to calcium stimulation in AIR tests. Theoretically, this can be explained by a consequence of constant depolarization of the beta cell membrane. The response to tolbutamide varied according to the age of the patient. The youngest patient showed a good response to tolbutamide, whereas it was severely decreased in other patients with SUR1-E1506K.

Interestingly, all but one of the mothers, heterozygous for SUR1-E1506K, had impaired glucose tolerance during pregnancy. Further studies of glucose metabolism of SUR1-E1506K heterozygotes indicated that this mutation predisposes to insulin deficiency and to the development of diabetes mellitus in later life. Glucose tolerance was impaired in 10 of the 11 SUR1-E1506K heterozygotes studied (increased fasting glucose 1/11, impaired glucose tolerance 5/11, and diabetes 4/11). The severely blunted first-phase glucose-stimulated insulin secretion, and reduced maximal glucose-stimulated insulin secretory capacity detected in SUR1-E1506K heterozygotes indicated that these individuals had a

severe defect in insulin secretion, which developed after puberty. In contrast, these subjects had quite normal insulin sensitivity. Thus, the hyperbolic relation between insulin sensitivity and beta-cell function seen in control subjects was lost in the SUR1-E1506K heterozygotes.

Interestingly, a situation resembling the SUR1-E1506K heterozygotes has been described in transgenic mice expressing a dominant negative Kir6.2 mutation in their beta cells (78): In these mice, hyperinsulinism is evident in the neonatal period, but insulin deficiency, mainly attributed to increased beta-cell apoptosis, develops later. A similar increase in beta-cell apoptosis, could be one possible explanation for the gradually progressing deficiency of insulin secretory capacity seen in SUR1-E1506K heterozygotes. Theoretically, the increased apoptosis could be explained by the continuously elevated intracellular calcium concentration as a consequence of sustained beta-cell depolarization and opening of the voltage-dependent calcium channels

MODY is a genetically and clinically heterogeneous subtype of type 2 diabetes, which is characterized by early onset, autosomal dominant inheritance and a primary defect in insulin secretion (201). Mutations in six different genes (hepatocyte nuclear factor (HNF)-4, GCK, HNF-1, insulin promotor factor1, HNF-1, and NEUROD1) are known to be responsible for MODY. Our results indicate that SUR1-E1506K results in a rare subtype of diabetes that also fulfils the criteria of MODY. Furthermore, abnormal glucose tolerance during pregnancy, which was seen in almost all SUR1-E1506K heterozygote women, is a typical finding of MODY (194).

Other K_{ATP} channel mutations. In addition to the two major founder mutations, six different heterozygous substitutions in K_{ATP} channel genes were detected. The mutations SUR1-A1457T and SUR1-V1550D were detected in single patients with SUR1-V187D in the other allele. Both of these patients were treated with pancreatectomy and developed diabetes in puberty. The results of electrophysiology were congruent with the clinical phenotype and showed that all these mutations (A1457T, V1550D and V187D) resulted in the total loss of functional channels in the plasma membrane. The AIR test were not performed in these two cases because of the severely reduced endogenous insulin secretion capacity. The heterozygous mutation SUR1-D1471N was detected in two sisters with maternal V187D.

They both had a severe early onset form of CHI and were treated with subtotal pancreatectomy. Both of them developed diabetes prepubertally.

The substitution L1551V in the SUR1 gene of two sisters led to a diazoxide-responsive form of CHI. In vitro studies indicated that this mutation resulted in K_{ATP} channels that were less sensitive to metabolic inhibition and were not activated by diazoxide. In the case of these patients, the diazoxide responsiveness may be due to the effect of another, yet unidentified SUR1 mutation in the maternal allele.

One compound heterozygote patient was identified with two novel mutations in the Kir6.2 gene, K67N and G to A substitution 54 bases proximal to the translation initiation site. He presented with a clinically severe, drug-unresponsive form of CHI and was treated with subtotal pancreatectomy at the early age of 11 days. In the nine years since pancreatectomy, he has not subsequently required treatment, either for insulin deficiency or excess. The other mutation, located in the 5' untranslated region, is predicted to result in the formation of a novel start codon and thus, in a scrambled protein. However, the positive insulin response to calcium stimulation in this patient suggested that the beta cells were at least partially depolarized at fasting plasma glucose levels, suggesting abnormalities in K_{ATP} channel function. He also showed significant responses to glucose and tolbutamide stimulation, which may reflect that the beta cells were only partially depolarized or that these agents acted through K_{ATP} channel-independent pathways.

GCK gene variant. A single *de novo* substitution of the glucokinase gene (Y214C) was detected in a CHI patient with a severe diazoxide-unresponsive form of the disease. Unlike most patients treated with subtotal pancreatectomy, she still had a tendency for hypoglycemia at the age of 26 years. Previously, two activating GCK mutations have been described in a single patient with CHI (65, 98). It is difficult to explain how the observed GCK mutation could explain the phenotype of our patient, including diazoxide-unresponsive severe hyperinsulinism. Nevertheless, these observations are potentially important. Further enzyme kinetic studies are warranted to clarify the association between the GCK-Y214C mutation and CHI in this case.

6.2 Representativeness of the study groups

The Finnish population as a genetic isolate offers a good premiss for the molecular genetic studies of recessive monogenic disorders (200). The high incidence of some autosomal recessive disorders in Finland, which are rare in other populations, can be explained by Finnish population genetics (187). The small breeding units and little external gene flow have favoured the occurrence of a mutation in the small founding population that has expanded while isolated. The major expansion that has led to the present population started some 2000 to 2500 years (i.e. 80 to 100 generations) ago mainly with a small number of founders of Baltic Finnic and German origin. The population rapidly spread over the southern and western parts of Finland. The main expansion to the northern and northeastern regions started only some 300 to 500 years ago. Finnish people have lived quite isolated in the northern district of the populated world and in the borderline area between the east and west. Differences in the culture, religion and language have also discouraged the immigration into the area.

CHI-patients. The subjects for this thesis were the CHI patients diagnosed at the Departments of Pediatrics of the five University Hospitals of Finland since the year 1972. These patients (n = 44) are likely to represent all severely affected individuals diagnosed during that time, but the possibility of isolated undiagnosed cases especially during the earliest phase cannot be excluded. Two patients with previously diagnosed HIHA-syndrome were not included. They were both diagnosed with hyperinsulinism and hyperammonemia at the age of six months and have managed well with a protein-restricted diet and low-dose diazoxide administration. Mutations in GDH were detected in both of them (C1492A and C1506T).

The calculated incidence of CHI in Finland during 1972-2000 was 1 : 40 000 (birth cohort ~60 000 per year) which corresponds well with the international incidence rates. In all cases included, CHI was diagnosed using generally accepted criteria including nonketotic hypoglycemia, inappropriately elevated insulin levels, and an increased need for glucose administration to prevent hypoglycemia (1, 55). All the new cases with CHI during the study were included in the ongoing part of the genetic study. All the studies of this thesis were based on the genetic characterization of the CHI-patients and their relatives. The existence

of the two founder mutations SUR1-V187D and SUR1-E1506K allowed the study of genotype-phenotype correlations.

Because the AIR tests are still experimental, and no clinical benefit was guaranteed for the patients, voluntary participation was emphasized. All CHI-patients who were willing to participate were included in these tests, except that diabetic patients with a daily insulin dose more than 0.5 U/kg were estimated to have very little endogenous insulin secretion and were therefore excluded. The patients who were willing to participate (n = 18) were divided into four groups according to the previously detected genotype. Patients without mutations in K_{ATP} channel genes were used as a control group. Due to the rarity of the specific mutations all study groups were relatively small. Moreover, in the case of presumed SUR1-V187D compound heterozygotes, the other half of the genotype of three patients was still pending, and this may have unknown implications on the results. The age of the patients was also highly variable. Furthermore, some patients had undergone pancreatic surgery while others had not, which may significantly affect especially the results of glucose and tolbutamide stimulations. However, the results of patients with the same genotype were quite congruent, thus indicating that the main features of specific mutations have been preserved during the course of the disease.

Relatives of CHI patients. In both studies performed on the family members and relatives of CHI patients (studies IV and V) all possible subjects with specific genotypes were invited to take part in the study. In the SUR1-V187D carrier study (study IV), eight out of ten parents of the homozygous SUR1-V187D patients were willing to participate. The control group consisted of 10 age-, gender- and BMI matched healthy volunteers (hospital personnel).

In the SUR1-E1506K heterozygote study (study V) the mutation analysis was performed on all known family members of seven patients with SUR1-E1506K. Eleven out of 14 adult heterozygous carriers of E1506K were able to participate. The control group of the SUR1-E1506K carrier study was made up of 19 healthy controls who were previously studied in our hospital. There was more variability in the age and body weight in the E1506K heterozygotes when compared with the control persons. However, the results were similar when analyzed in smaller groups matched by BMI (case vs. control). The SUR1-E1506K mutation was screened among 80 diabetic patients and no carriers were detected. This

indicates that SUR1-E1506K does not predispose to the common form of type 2 diabetes. However, it should be emphasized that these diabetic controls in the mutation analysis represented patients from eastern Finland, whereas the mutation E1506K was detected in patients from western Finland. Further studies are needed to determine the real incidence of this mutation among diabetic patients in the western part of Finland.

6.3 Methods

The genetic studies and the studies of glucose metabolism in adults were performed by using generally accepted and standardized methods. SSCP analysis is a frequently used method for screening of unknown variations in gene sequence with a relatively high sensitivity (80-90%) and specificity (186, 202). Therefore, it is possible that we have missed some variants. Especially in the case of recessive mutations of SUR1 it is presumable that patients with only one known maternal mutation may have another yet undetected mutation in the other allele. Sequencing of the whole SUR1 and Kir6.2 genes might reveal the mutation in these selected cases. Although we have aimed to include the exon-intron boundaries in PCR products in SSCP analysis, it is possible that we have missed some intronic variants that could have an impact on the CHI phenotype. However, to increase the probability of detecting different substitutions we used standardized running conditions with two different temperatures and a gel matrix that has performed well in previous studies (203). Furthermore, our SSCP conditions have been previously validated against the known variants of the lipoprotein lipase gene (204). In addition, we were able to detect several previously described polymorphisms.

The interpretation of the AIR test results (study III) proved to be complicated. The AIR tests aim to differentiate the patients with K_{ATP} channel mutations from patients with intact K_{ATP} channels. To determine optimal therapy for each patient it would be valuable to find simple and reliable means to distinguish patients with different genotypes. Previous reports of CHI-patients have assumed that patients with K_{ATP} channel mutations have an impaired response to tolbutamide, whereas the elevation in extracellular calcium directly stimulates insulin release due to a constant activity of voltage-gated calcium channels (92, 137). However, in focal CHI, the response to tolbutamide stimulation is maintained. Our results support these previous findings of the usefulness of the calcium and tolbutamide stimulation tests in the

differential diagnosis of CHI, but also demonstrate the complexity of these responses and the difficulties in their interpretation. According to our results, a negative response to calcium stimulation does not exclude all patients with K_{ATP} channel mutation, as was clearly demonstrated by the SUR1-V187D mutation. On the other hand, a positive insulin response to calcium was only seen in patients with K_{ATP} channel mutations. Therefore, the K_{ATP} channel gene mutation is very likely the cause of CHI in patients with positive response to calcium and thus, all efforts should be made to find out the specific gene defect in these cases. As was expected, an impaired response in the tolbutamide test was seen only in patients with SUR1 mutations.

6.4 Concluding remarks

During the last five years there has been a significant increase in the knowledge of the genetic basis of CHI. CHI includes a group of genetically heterogeneous disorders that lead to persistent or recurrent hypoglycemia. Mutations in the K_{ATP} channel genes are the major known cause of CHI, although the proportion of the cases they explain differs between populations. In the Finnish population they associate with 60% of CHI cases, whereas in the Japanese population they account for only 20%, and in the Jewish population for as many as 90% of cases. Mutations in the GCK and GDH genes seem to explain only a minor proportion of CHI. On the whole, the molecular etiology remains to be established in almost 40% of Finnish cases and in at least 50% all CHI cases. Therefore, further efforts are needed to find novel candidate genes and genetic mechanisms for CHI.

Correlation analyses between different genotypes and phenotypes are complicated by the wide range of mutations in the K_{ATP} channel genes and the low allelic frequencies of single mutations. Furthermore, most patients with CHI-associated mutations are compound heterozygotes or have only a single identified mutation. However, studies of patients coming from genetically isolated populations (like Finns or Ashkenazi Jews) make it possible to study the genotype-phenotype correlations more reliably. A database integrating the clinical, molecular genetic, histopathological and electrophysiological data of all European patients is under development and will enable more detailed phenotype-genotype correlation studies in the future (<http://umd2.necker.fr:2007/>).

The identification of genotype-phenotype correlations has led to novel treatment strategies. According to previous studies and the results of this thesis, patients who have been treated with subtotal pancreatectomy are at increased risk of developing diabetes due to the decreased beta cell mass and increased insulin resistance in puberty. On the other hand, hyperinsulinism seems to recover spontaneously with time when treated conservatively. Diazoxide therapy may even have a protective effect on beta cells, and should therefore be favored in long-term treatment of CHI (113). Development of beta cell failure as a direct consequence of K_{ATP} channel mutations was shown for the first time in our study of the SUR1-E1506K heterozygotes. In these individuals, the persistent beta cell depolarization due to a K_{ATP} channel mutation leads to progressive beta cell failure. Therefore, in the case of diffuse CHI, all efforts should be made to treat these patients conservatively. However, this aim should not override the most important goal of the management of CHI, which is to prevent hypoglycemic brain damage and to ensure normal psychomotor development. For example, in the case of the homozygous SUR1-V187D, despite the maximal efforts to treat patients medically, hypoglycemia persisted until surgical resection of the pancreas.

The treatment strategy of focal CHI is different. Histological studies have revealed increased beta cell apoptosis of focal lesions. It could be hypothesized that these lesions will disappear spontaneously when treated conservatively. However, if medical treatment fails to maintain normoglycemia, selective excision of the abnormal region results in complete cure because the remaining pancreas is functionally normal. Therefore, it would be valuable to find simple means to separate focal and diffuse conditions. AIR tests performed together with selective arterial calcium stimulations might provide a means for that. This requires the early referral to a center experienced in the management of CHI. In the future, it would be ideal to have some uninvasive method, like positron emission tomography (PET), that could localize the functionally overactive areas of the pancreas.

In this thesis, we have described a family with the E1506K mutation in the SUR1 gene that causes beta cell failure due to a continuous overstimulation of insulin secretion. 'Pancreatic exhaustion' has often been postulated as a cause of type 2 diabetes. According to this concept basal and postprandial insulin secretion are increased to compensate for insulin resistance in peripheral tissues. When the beta cells are no longer able to compensate for insulin resistance, the insulin secretory response becomes progressively impaired, gradually

leading to deficiency in both early and late phases of insulin secretion and to abnormal glucose tolerance. Further studies with transgenic animals may resolve mechanisms of impaired insulin secretion in SUR1-E1506K heterozygotes. These results, however, show that studies with CHI may have some implications for the understanding the pathophysiology of impaired insulin secretion in the development of type 2 diabetes.

7 SUMMARY

1. Mutations in the K_{ATP} channel genes SUR1 and Kir6.2 are associated with about 60% of all CHI cases in Finland. Altogether six mutations in SUR1 and two mutations in Kir6.2 were detected. The founder mutations V187D and E1506K in the SUR1 gene explained the major part of CHI in Finland and were associated with 88% of the genetically characterized cases. Other SUR1 mutations were detected in single families. The two Kir6.2 mutations were found in a single compound heterozygous patient. In addition to this, the substitution Y214C in the GCK gene was detected in a single patient, but the association between this mutation and clinical CHI remains unclear.
2. The two Finnish founder mutations in the SUR1 gene led to very distinct phenotypes. The recessively inherited mutation SUR1-V187D resulted in the loss of functional K_{ATP} channels in pancreatic beta cells. The SUR1-V187D phenotype was severe, diazoxide unresponsive, and in most cases necessitated pancreatectomy. The dominantly inherited mutation SUR1-E1506K impaired the function of K_{ATP} channels and led to a milder, diazoxide-responsive form of CHI. In long-term follow up, it predisposed to the development of insulin deficiency and diabetes mellitus. The other mutations associated with CHI were detected in single families with variable phenotypes.
3. The AIR tests, including the calcium stimulation test, intravenous glucose tolerance test and tolbutamide test were found to be useful in differentiating the distinct forms of diffuse CHI. On the other hand, our studies also demonstrated the complexity of these responses and difficulties in their interpretation. A positive insulin response to calcium stimulation was seen only in patients with K_{ATP} channel mutations. However, the negative result did not exclude the possibility of a K_{ATP} channel mutation, as it was in the case of SUR1-V187D. The unresponsiveness to tolbutamide suggested a diffuse form of CHI due to K_{ATP} channel mutation. It should be noted, however, that according to the electrophysiological studies, not all mutations led to a complete loss of K_{ATP} channel activity and therefore some channel activity and responsiveness to tolbutamide may be left. According to our

results, we recommend that AIR tests accompany other diagnostic strategies in CHI.

4. Heterozygous carriers of the V187D mutation had normal insulin secretion, normal tissue sensitivity to glucose and no inappropriate insulin secretion. Therefore, our results showed that the V187D carriers were not at increased risk of hypoglycemia or other disturbances of glucose metabolism. This result has important clinical implications, especially in the families with healthy children carrying recessive CHI associated SUR1 mutations. Furthermore, these results confirmed the recessive nature of CHI caused by this, and probably most other SUR1 mutations.

The studies of the SUR1-E1506K heterozygotes indicated that this substitution leads to insulin deficiency after puberty and predisposes to the development of diabetes later in life. It resulted in a rare subtype of autosomal dominantly inherited, early-onset insulin deficiency diabetes that fulfills the criteria of MODY. It also may have some implications for the understanding of the pathophysiology of the common form of type 2 diabetes due to ‘pancreatic exhaustion’ following the continuous overstimulation of pancreatic beta cells (Figure 18).

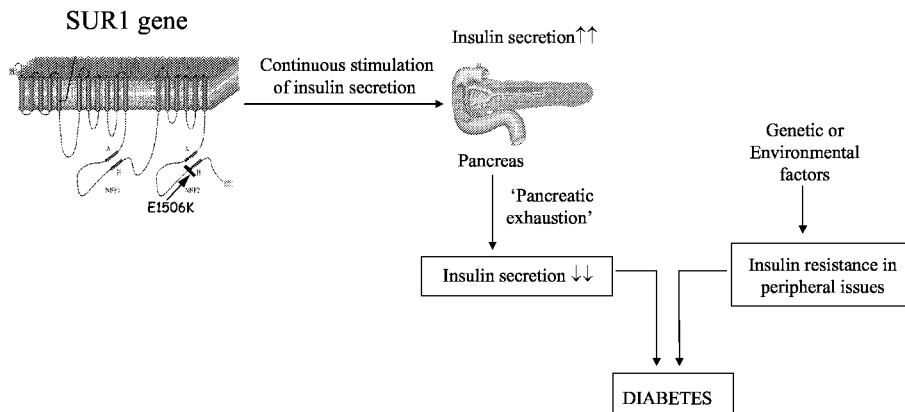


Figure 18

The suggested mechanism of diabetes due to the SUR1-E1506K mutation. Continuous overstimulation of insulin secretion in pancreatic beta cells due to the SUR1-E1506K leads to the ‘pancreatic exhaustion’ and insulin deficiency. The failure of insulin sensitivity to compensate for impaired secretion triggers the development of diabetes.

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