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Coordinator: Prof. Giancarlo Vecchio

"NOVEL MECHANISMS RESPONSIBLE FOR CONTROLLING GLUCOSE-INDUCED INSULIN SECRETION IN PANCREATIC BETA-CELLS"

CHIARA ROMANO

University of Naples Federico II

Dipartimento di Biologia e Patologia Cellulare e Molecolare

"L. Califano"

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Dipartimento di Biologia e Patologia Cellulare e Molecolare “L. Califano”
Università degli Studi di Napoli Federico II

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Polo delle Scienze e delle Tecnologie, Università di Napoli “Federico II”
Terry Fox Foundation
Istituto di Endocrinologia ed Oncologia Sperimentale “G. Salvatore”, CNR, Naples, Italy
Centro Regionale di Competenza in Genomica (GEAR)

Faculty

Italian Faculty

Giancarlo Vecchio, MD, Co-ordinator
Francesco Beguinot, MD
Angelo Raffaele Bianco, MD
Francesca Carlomagno, MD
Angela Celetti, MD
Gabriella Castoria, MD
Fortunato Ciardiello, MD
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Bianca Maria Veneziani, MD

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National Institutes of Health (USA)

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Stephen Marx, MD
Ira Pastan, MD

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Vincenzo Casolaro, MD
Pierre Coulombe, PhD
James G. Herman MD
Robert Schleimer, PhD

Ohio State University, Columbus (USA)

Carlo M. Croce, MD

Université Paris Sud XI, Paris, Francia

Martin Schlumberger, MD

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LIST OF PUBLICATIONS

1) Miele C, Riboulet A, Maitan MA, Oriente F, **Romano C,** Formisano P, Giudicelli J, Beguinot F, Van Obberghen E. Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism. J Biol Chem. 2003; 278(48):47376-87.

2) Fiory F, Oriente F, Miele C, **Romano C**, Trencia A, Alberobello AT, Esposito I, Valentino R, Beguinot F, Formisano P. Protein kinase C zeta and protein kinase B regulate distinct steps of insulin endocytosis and intracellular sorting. J Biol Chem. 2004; 279(12):11137-45.

3) Vigliotta G, Miele C, Santopietro S, Portella G, Perfetti A, Maitan MA, Cassese A, Oriente F, Trencia A, Fiory F, **Romano C**, Tiveron C, Tatangelo L, Troncone G, Formisano P, Beguinot F. Overexpression of the ped/pea-15 gene causes diabetes by impairing glucose-stimulated insulin secretion in addition to insulin action. *Mol Cell Biol* 2004; 24:5005-5015.

4) Oriente F, Andreozzi F, **Romano C,** Perruolo G, Perfetti A, Fiory F, Miele C, Beguinot F, Formisano P. Protein kinase C-alpha regulates insulin action and degradation by interacting with insulin receptor substrate-1 and 14-3-3epsilon. J Biol Chem. 2005; 280(49):40642-9.

5) Miele C, Raciti GA, Cassese A, **Romano C**, Giacco F, Oriente F, Paturzo F, Andreozzi F, Zabatta A, Troncone G, Bosch F, Pujol A, Chneiweiss H, Formisano P, and Beguinot F. Ped/pea-15 regulates glucose-induced insulin secretion by restraining potassium channel expression in pancreatic beta-cells. 2006 (Accepted for publication in Diabetes)

ABSTRACT

Novel mechanisms responsible for controlling glucose-induced insulin secretion in pancreatic beta-cells

Glucose homeostasis is mainly regulated by the pancreatic hormone insulin. Pancreatic beta cells secrete insulin in response to changes in glucose concentrations through a complex and finely regulated mechanism. A large number of genetic alterations may affect the response of beta cells to glucose contributing to the onset of type 2 Diabetes. Our group has identified a gene overexpressed in type 2 diebetic individuals, *ped/pea-15*. A transgenic mouse model ($Tg_{ped/pea-15}$) ubiquitously overexpressing *ped/pea-15* has been recently developed. Further studies have shown that $Tg_{ped/pea-15}$ displayed insulin resistance. I sought to analyze the role of *ped/pea-15* in glucose-induced insulin secretion. Studies *in vivo* showed that $Tg_{ped/pea-15}$ mice have a 70% reduction of the first and second phase of insulin secretory response to glucose, in addition to fasting iperinsulinemia. Furthermore, Real Time PCR experiments performed on isolated islets from $Tg_{ped/pea-15}$ have shown that insulin secretion impairment is associated to a reduction of the expression of genes essential for insulin secretion: Sur1, Kir6.2 and HNF3β, by 30, 40 and 45% respectively. To investigate the molecular mechanisms underlying this alteration, a model of beta-cells stably overexpressing PED/PEA-15, $MIN_{ped/pea-15}$ was generated. In these cells the lack of glucose-induced activation of the Protein kinase C zeta (PKCζ) is responsible for the reduced expression of Sur1, Kir6.2 e HNF3β. Overexpression of PKCζ in $MIN_{ped/pea-15,}$ indeed, restored glucose-induced insulin secretion and the expression of Sur1, Kir6.2 e HNF3β. Thus, PED/PEA-15 impairs glucose-induced insulin secretion by controlling the expression of the ATP-sensitive potassium channels in the beta-cell.

BACKGROUND

§ 1.0 GLUCOSE HOMEOSTASIS

Glucose homeostasis depends on its hepatic production and its utilization by insulin-dependent tissues such as adipose and muscle tissues. After a meal, glucose is absorbed into the hepatic portal vein, increasing the blood glucose concentration. This situation is detected by the pancreas, which secretes insulin from its beta-cells. Insulin causes glucose to be taken up by muscle, adipose tissues and the liver and converted into glycogen, reducing blood glucose, and in turn, causing the pancreas to stop secreting insulin (Pontieri 1996) (fig.1).

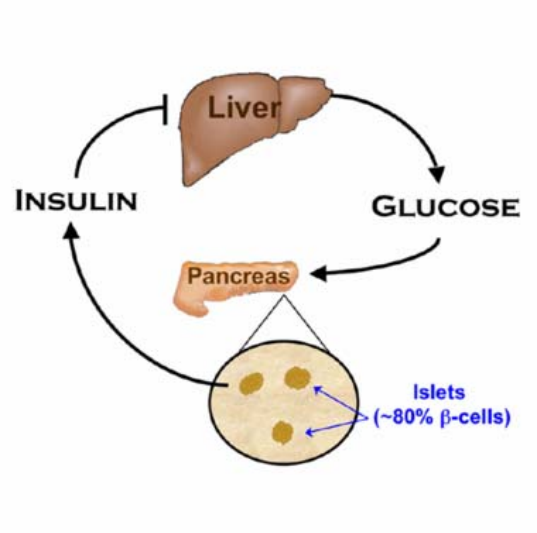


Figure 1. Glucose Homeostasis

§ 1.1 PANCREATIC BETA-CELLS

Beta-cells represent the 70-80% of the Langherans islets of the pancreas and occupy a central position in them (Orci and Unger 1975) (fig.2).The mammalian beta-cells synthesize, store, and secrete insulin in quantities that are matched to the physiological demands of the organism. To achieve this task, beta-cells are regulated both acutely and chronically by the extracellular glucose concentration (Schuit et al 2002). Fundamental property of beta-cells is, indeed, their capacity to secrete insulin in response to changes in glucose concentrations (Newgard et al. 2002) . Insulin concentrations are normally determined by a feed-back control system that is responsive to the prevailing level of plasma glucose (Polonsky and O'Meara 2001). The overall sensitivity of the pancreatic beta-cells to glucose is determined by the sensitivity of peripheral tissues to the action of insulin with insulin-resistant subjects

having higher insulin levels and insulin secretion rates than insulin-sensitive subjects (Bell and Polonsky 2001).

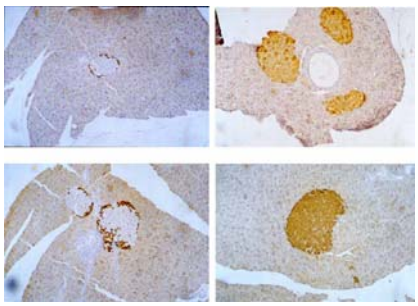


Figure 2. Representation of Langherans islets structure: alpha-cells are in peripheral position and beta-cells are in the centre (*see also publication n°3 appended*).

§ 1.2 INSULIN

Insulin is a quite small protein, having a molecular weight of about 6000 Daltons. It is composed of two chains (A and B) held together by disulfide bonds. Insulin is synthesized in significant quantities only in β-cells of the pancreas. The insulin mRNA is translated as a single chain precursor called preproinsulin and removal of its signal peptide during cotraslational insertion into the endoplasmic reticulum generates proinsulin. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide, thereby generating the mature form of insulin. Insulin and free C peptide are packaged in the Golgi into secretory granules which accumulate in the cytoplasm (fig.3). When the beta-cell is appropriately stimulated, insulin is secreted from the cell by exocytosis and diffuses into islet capillary blood. C peptide is also secreted into blood, but has no known biological activity (Bowen 1999).

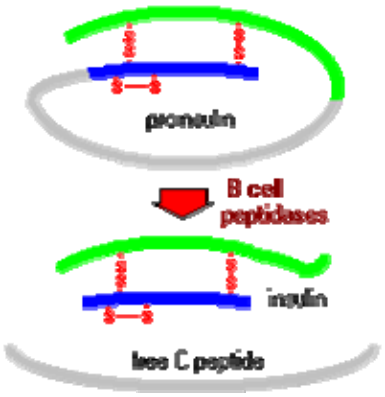


Figure 3. Biosynthesis of insulin.

§ 2.0 GLUCOSE-INDUCED INSULIN SECRETION

Normal insulin secretion shows a rapid response to glucose and a complex pulsatile profile. The increase in insulin secretion that occurs after the intravenous administration of glucose is virtually instantaneous; even after oral glucose ingestion, increases in insulin secretion occur within minutes. The temporal profile of insulin secretion consists of small-amplitude pulses of insulin occurring every 5-10 minutes, superimposed on slower, larger-amplitude oscillations that occur within 1 hour (Bell and Polonsky 2001).

Glucose-induced insulin secretion is a complex mechanism made up of differents steps. Glucose enters the beta-cells by facilitated diffusion; a rise in extracellular glucose concentration increases its phosphorylation to glucose-6-phosphate by glucokinase, the rate-limiting enzyme for glucose metabolism in the beta-cells. Glucose phosphorylation by glucokinase determines the rate of glycolysis and the rate of pyruvate generation (Newgard and McGarry 1995, Matschinsky 1996). Thus, the rate of glycolysis will increase with increasing blood glucose levels. In β-cells, pyruvate is the main product of glycolysis (Ishihara et al. 1999) and, compared to other cell types, an unusually high proportion of glucose-derived pyruvate enters the mitochondrial tricarboxylic acid cycle (Newgard and McGarry 1995) (fig.4A). Subsequent oxidative metabolism generates the trigger for insulin secretion (Wollheim 2000). Electron transfer from the tricarboxylic acid cycle to the respiratory chain by NADH and reduced flavin adenine dinucleotide ($FADH_2$) initiates the production of ATP, which is delivered to the cytosol (fig.4B). Here the rise of the ATP-to-ADP ratio causes a reduction in plasma membrane K^+ conductance, resulting in depolarization of the membrane (Rorsman 1997). Hence voltage-sensitive Ca^{2+} channels, similar to those expressed in other excitable cells, are opened (fig.4C). This is the

critical step by which glucose stimulates insulin secretion, as the increase in cytosolic Ca^{2+} is the main trigger for exocytosis (Rorsman 1997, Lang 1999) (fig.4D).
Insulin is also secreted in response to amino acids and fatty acids and the magnitude of this response is modulated by a variety of neural (for example, sympathetic and parasympathetic autonomic tone) and hormonal factors (for example, glucagons, glucagons-like peptide 1, gastric inhibitory polypeptide and somatostatin). Glucose, however, is the overriding influence (Bell and Polonsky 2001).
Other inductors of insulin secretion are sulfonylureas. Their principal target is the ATP-sensitive potassium (K_{ATP}) channel, which plays a major role in controlling the beta-cell membrane potential. Inhibition of K_{ATP} channels by glucose or sulfonylurea, that binds to the Sur1 subunit, causes depolarization of the beta-cell membrane; in turn, this triggers the opening of voltage-gated Ca^{2+} channels, eliciting Ca^{2+} influx and a rise in intracellular Ca^{2+} which stimulates the exocytosis of insulin-containing secretory granules (Ashcroft et al. 1989).

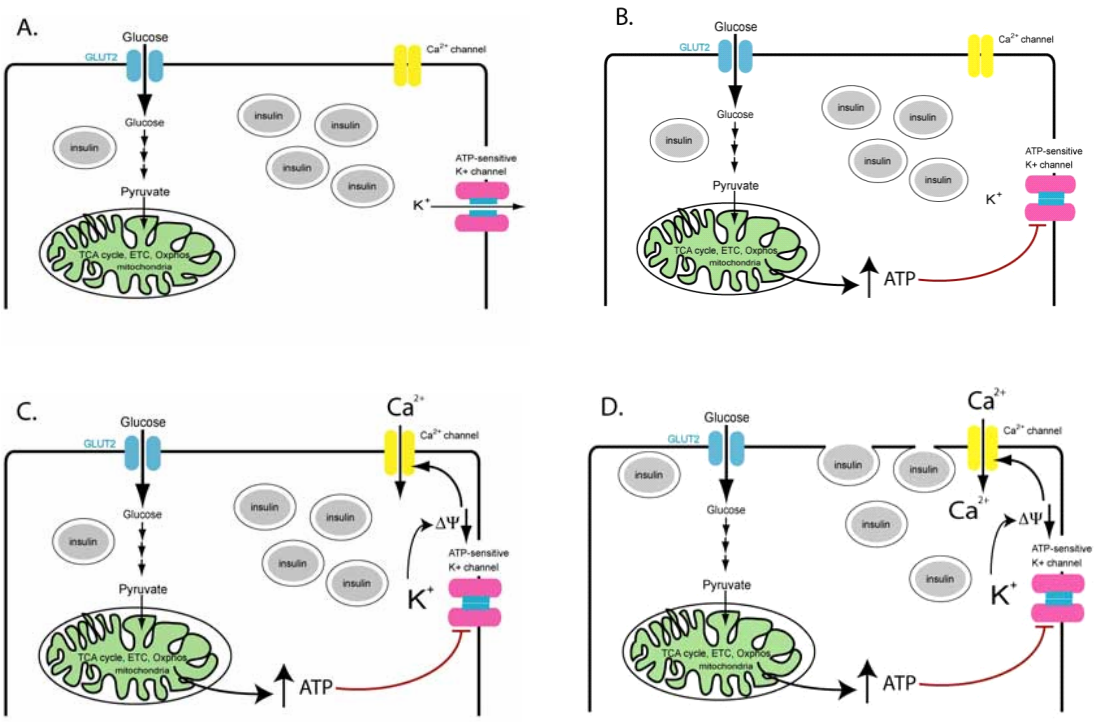


Figure 4. Glucose-induced insulin secretion.

§ 2.1 REGULATION OF INSULIN SECRETION: ATP-sensitive potassium (K_{ATP}) channels

The K_{ATP} channel is a hetero-octameric complex of two different types of protein subunits: an inwardly rectifying K^+ channel, Kir 6.x, and a sulfonylurea receptor, Sur (Ashcroft and Griblle 1999, Aguilar-Bryan and Bryan 1999). Kir6.x belongs to the family of inwardly rectifying K^+ (Kir) channels and assembles as a tetramer to form the channel pore. Binding of ATP to the intracellular domains of this subunit produces channel inhibition (Tucker et al. 1997). Sur is a member of the ABC transporter family, with 17 transmembrane helices, arranged as one group of 5 transmembrane helices, and two repeats each of 6 transmembrane helices followed by a large cytosolic loop containing consensus sequences for nucleotide binding and hydrolysis (fig.5). Interaction of Mg nucleotides with the nucleotide binding domains mediates activation of the K_{ATP} channel (Nichols et al. 1996, Gribble et al 1998, Gribble et al 1997, Shyng et al. 1997). SUR also endows Kir6.2 with sensitivity to certains drugs, such as the inhibitory sulfonyureas and the stimulatory K^+ channel openers (Ashcroft and Gribble 1999, Aguilar Bryan and Bryan 1999). More than one isoform exists for both Kir6.x (Kir 6.1, Kir 6.2) and SUR (SUR1, SUR2A, SUR2B).

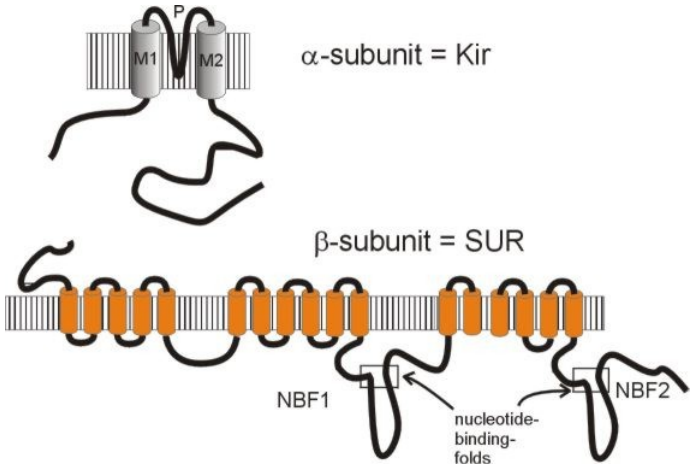


Figure 5. ATP-sensitive potassium (K_{ATP}) channels.

In most tissues, Kir 6.2 serves as the pore forming subunit, but it associates with different SUR subunits; for example, it associates with SUR1 in the pancreas and brain, with SUR2A in heart and skeletal muscle, with SUR2B in brain and smooth muscle (Aguilar Bryan et al 1995, Inagaki et al 1996, Isomoto et al 1996). In vascular smooth

muscle, the K_{ATP} channel is composed of Kir6.1 in association with SUR2B (fig.6). Variation in the subunit composition of the K_{ATP} channel accounts for the different metabolic and drug sensitivities of K_{ATP} channels in different cells. Inhibitor of K_{ATP} channel activity fall into two groups: those that interact with Kir6.2 and those that interact with SUR. Imidazolines (phentolamine, cibenzoline) and antimalarials (quinine, mefloquine) block K_{ATP} channels by binding to Kir6.2 (Mukai et al. 1998, Proks and Ashcroft 1997, Gribble and Ashcroft 1999). In contrast, sulfoniylureas (tolbutamide, glicazide, glimeripide), and benzamino derivates (meglitinide) close K_{ATP} channels by binding with high affinity to SUR (Gribble and Ashcroft 1999, Gribble et al. 1997, Gribble et al. 1998). All drugs that block K_{ATP} channels stimulate insulin secretion, but only those that interact with SUR subunit are used therapeutically.

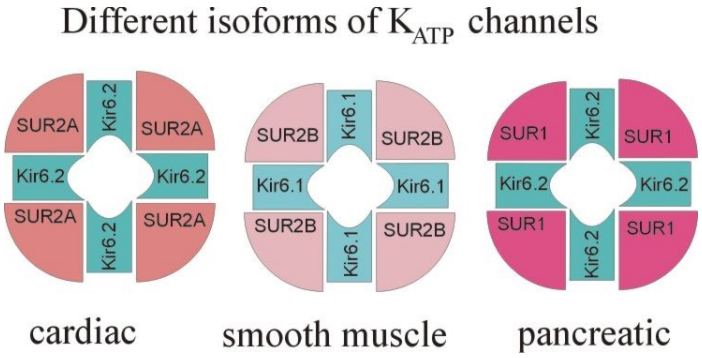


Figure 6. Isoforms of the ATP-sensitive potassium (K_{ATP}) channels.

§ 2.2 REGULATION OF INSULIN SECRETION: The role of Protein Kinase C

The Protein kinase C (PKC) family can be divided in three subgroups based on cofactors requirements: classical PKCs (α, β, and γ), which are dependent on Ca^{2+} and diacylglycerol (DAG) for activity; novel PKCs (δ, ε, η, and θ), which are activated by DAG; and atypical PKCs (ζ, λ, and ι), which are not dependent on Ca^{2+} and are not stimulated by DAG (Hug and Sarre 1993) (Table1). PKCs play an important role in controlling a great number of cellular functions among which insulin secretion. The role of classical PKCs as mediators of glucose-induced insulin secretion has been a subject of controversy, but it was demonstrated that glucose induces PKCα translocation in beta cells and that inhibition of PKCα blocks its translocation and the secretory response of beta-cells (Ganesan et al. 1990, Ganesan et al. 1992). Novel PKCs, in particular PKCε, play a role in cholinergic potentiation of

insulin secretion (Ishikawa et al. 2005) Atypical PKC isoforms are expressed in pancreatic beta-cells (Selbie et al. 1993) and play a role in the regulation of beta-cell growth and insulin secretion, as suggested by several reports on the role of atypical PKCs in insulin secretion (Furukawa et al. 1999, Harris et al. 1996), insulin synthesis (Furukawa et al. 1999, Harris et al. 1996), and cell proliferation (Buteau et al. 2001) in pancreatic beta-cells. PKCζ and PKCλ have been reported to control the function of a number of transcription factors (Furukawa N et al. 1999, Matsumoto M et al. 2003, Hashimoto N et al. 2005). PKCλ has recently been shown to play a major role in regulating glucose-induced insulin secretion through the by modulating the expression of genes involved in this process (Hashimoto et al. 2005). Islets isolated from mice that lack the lambda isoform of PKC in pancreatic beta-cells show an impaired insulin secretion in response to high concentrations of glucose whereas the basal rate of insulin release is increased. The results indicate that PKCλ regulates glucose-induced insulin secretion by modulating the expression of genes important for beta cell function such as Glut2, HNF3β, Sur1, Kir6.2, Hexokinase 1 and Hexokinase 2 (Hashimoto et al. 2005).

CLASSICAL	NOVEL	ATYPICAL
dependent on Ca^{2+} and diacylglycerol (DAG)	dependent on diacylglycerol (DAG)	not dependent on Ca^{2+} and diacylglycerol (DAG)
α	δ	ζ
β1	η	λ
β2	ε	ι
γ	θ	

Table 1. Classification of PKCs.

§ 3.0 GENETIC ALTERATION IN INSULIN SECRETION

Alteration of genes involved in insulin secretion may cause several disorders. Maturity-onset diabetes of the young (MODY) is a clinically heterogeneous group of disorders characterized by non-ketotic diabetes mellitus, an autosomal dominant mode of inheritance, onset usually before 25 years of age and frequently in childhood or adolescence, and a primary defect in pancreatic beta-cell function (Fajans et al. 2001, Owen and Hattersley 2001). MODY can result from mutation in any of

at least six different genes that encode the glycolytic enzyme glucokinase and five transcription factors: hepatocyte nuclear factor HNF-4α, HNF-1α, HNF-1β, insulin promoter factor-1 (IPF-1) and neurogenic differentiation 1/ beta-cell E-box transactivator2 (NeuroD1/BETA2). This all are expressed in pancreatic β-cell and their mutations lead to beta-cell dysfunction and diabetes mellitus in heterozygous state.

Glucokinase is expressed at high levels in the pancreatic β-cells and in the liver. Heterozygous mutations leading to partial deficiency of glucokinase are associated with MODY (Fajans et al. 2001, Owen and Hattersley 2001). The transcription factors HNF-1α, HNF-1β and HNF-4α are involved in the tissue-specific regulation of gene expression in pancreatic beta-cells and other tissues (Cereghini 1996, Ryffel 2001). They belong to a network of transcription factors that controls gene expression during embryonic development and in the adult tissue in which they are co-expressed. In the pancreatic beta-cells, they regulate the expression of insulin as well as proteins involved in glucose transport, glycolysis and mitochondrial methabolism, all of which are important in the regulation of insulin secretion (Ryffel 2001, Emens et al.1992, Okita et al. 1999, Wang et al. 2000, Wang et al. 2000, Shih et al. 2001). Mutations in these genes produce defects in insulin secretory responses to a variety of factors, in particular glucose, which are present before the onset of hyperglycemia (Fajans et al. 2001, Owen and Hattersley 2001). IPF-1 is a homeodomain-containing transcription factor involved in pancreatic development (Jonsson et al. 1994, Edlun 1998, Stoffers et al.1997), transcriptional regulation of a number of β-cell genes including insulin, glucokinase, islet amyloid polypeptide and glucose transporter 2 (GLUT-2) (Edlun 1998), and mediation of glucose-stimulated insulin gene transcription (Marshak et al. 1996). Mutations in the heterozygous state are associated with MODY (Stoffers et al.1997) whereas mouse and human homozygotes fail to develop the pancreas and suffer congenital diabetes mellitus (Stoffers et al.1997). IPF-1 mutation have also been discovered in a small fraction of patients with typical adult-onset type 2 diabetes (Macfarlane et al. 1999, Hani et al. 1999). Subjects with heterozygous mutation in IPF-1 featured reduced insulin secretory responses to glucose and glucagons-like-pepide-1, consistent with an effect in the signalling pathways that regulate secretion in the beta-cell and/or defect in β-cell mass (Cloquet et al. 2000). The transcription factor NeuroD1/BETA2 is involved in the regulation of transcription of the insulin gene and is required for normal pancreatic islet development (Chu et al. 2001). Mutations in NeuroD1 are a rare cause of MODY and result in reduced serum insulin concentrations (Malecki et al. 1999), either due to a signalling defect in the beta-cell, or to a reduction in beta-cell mass, or

both. Mutations in other beta-cell transcription factors may also contribute to the development of MODY or a MODY-like disorder. A nonsense mutation has been found in the LIM-homeodomain transcription factor islet-1 in a Japanese family (Shimomura et al. 2000). This mutation leads to a decreased activity and thus may be the cause of diabetes in this family. In addition to mutations in the nuclear genome, abnormal mitochondrial function resulting from mutations in the mithocondrial genome can lead to diabetes (Maassen et al. 1996). The most common diabetes-associated mutations is an A-to-G transition in the mitochondrial tRNAleu (UUR) gene at base pair 3,243. This results in defects in insulin secretion including failure of glucose to prime the insulin secretory response and abnormal insulin secretory oscillations (Velho et al. 1996). Mice with beta-cell specific disruption of mithocondria develop diabetes as a result of reduced beta-cell stimulus-secretion coupling, followed by reduction in beta-cell mass (Silva et al. 2000).

§ 3.1 TYPE 2 DIABETES: GENETIC ALTERATION IN BETA-CELL DYSFUNCTION

Type 2 diabetes is made up of different forms each of which is characterized by variable degree of insulin resistance and beta-cell dysfunction, and which together lead to hyperglycaemia (American Diabetes Association 2001). At each end of this spectrum are single-gene disorders that affect the ability of the pancreatic beta-cell to secrete insulin (Fajans et al. 2001, Owen and Hattersley 2001) or the ability of muscle, fat and liver cells to respond to insulin (Taylor and Arioglu 1999, Barroso et al. 1999).

In type 2 diabetes, more moderate abnormalities of insulin secretion are present that cause glucose intolerance only in the presence of insulin-resistance. The genetic basis of beta-cell dysfunction in this form of diabetes is more complex, involving both multiple interacting genes and environmental factors, which determine whether and when diabetes will develop and at what age. Several genes involved in type 2 diabetes have been identified and their alterations has been characterized. A common amino-acid polymorphism (Pro12Ala) in peroxisome proliferators-activated receptor-γ (PPARγ) has been associated with type 2 diabetes (Altshuler et al. 2000). Patients homozygous for the Pro12 allele are more insulin resistant than those having one Ala12 allele and have a 1,25-fold increased risk of developing diabetes (Luan et al. 2001). The expression of PPARγ in insulin-responsive tissues (fat and muscle) (Olefsky 2000) and pancreatic beta-cells (Kulkarni et al. 1999) provides a link between insulin resistance and insulin secretion.

Genetic variation in the gene encoding calpain-10, a ubiquitously expressed cysteine
protease, has also been associated with type 2 diabetes, increasing the risk as much as 3-fold (Horikawa et al 2000) by altering both the normal function of the beta-cells and insulin action in muscle and fat. Recently it has been discovered that IRS1 KO-/- mice are insulin resistant but they do not become diabetic probably because of a beta-cell's compensation (Cavaghan et al. 2000). By contrast, IRS2 KO-/- mice show an alteration in insulin-secretion in addition to pheripheric insulin resistance and they develop diabetes (Cavaghan et al. 2000).

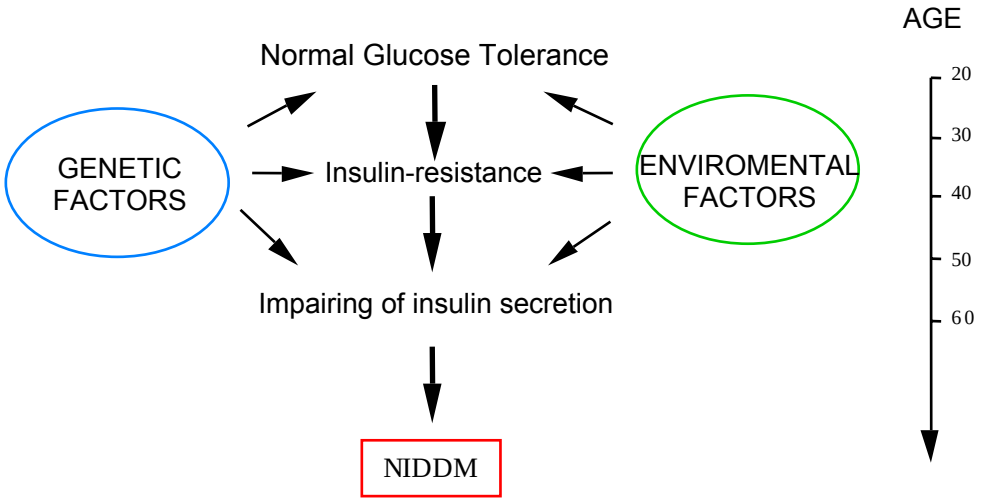


Figure 7. Pathogenesis of Type 2 Diabetes.

§ 4.0 PED/PEA-15 protein

PED/PEA-15 (Phoshoprotein Enriched in Diabetes/ Phosphoprotein Enriched in Astrocytes) is a small scaffold multifunctional protein widely expressed in different tissues and highly conserved among mammals, whose gene maps on human chromosome 1q21-22 (Condorelli et al 1998). PED/PEA-15 is characterized by a nuclear export sequence (NES) and a Death Effector Domain (DED) at the N-terminus side and two different sites of phosphorylation at the C-terminus side: the serine 104 phosphorylated by PKC and the serine 116 phosphorylated by AKT/PKB. (fig.8)
Several studies in cultured cells and in rodent tissues have revealed that PED/PEA-15 regulates multiple cellular functions by binding components of major intracellular transduction pathways (Zhang

2000,Formstecher et al. 2001, Trencia et al 2003, Vaidyanathan et al. 2003, Vigliotta et al. 2004 *see also publication n°3 appended*,). These include ERK1/2, Akt, RSK2 and a number of molecules conveying apoptotic signals to the nucleus (Formstecher et al. 2001, Trencia et al 2003, Vaidyanathan et al. 2003). PED/PEA-15 controls mitogenic signalling by binding extracellular signal-regulated kinases and anchoring them to the cytoplasm (Formstecher et al. 2001). It also inhibits several apoptotic pathways through a number of different mechanisms and plays an important role in tumor development and sensitivity to antineoplastic agents (Condorelli et al. 2002, Condorelli et al. 1999, Estelles et al. 1999, Hao et al. 2001, Trencia et al. 2003, Xiao et al. 2002, Formisano et al. 2005). PED/PEA-15 also binds to and increase the stability of phospholipase D, enhancing its activity and controlling important mechanisms in cell metabolism (Zhang et al. 2000).

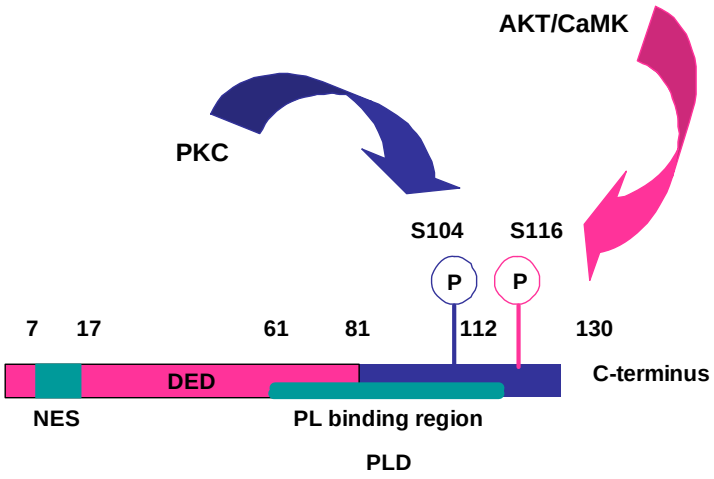


Figure 8. Structure of PED/PEA-15 protein.

§ 4.1 PED/PEA-15 AND GLUCOSE HOMEOSTASIS

Gene profile studies showed that overexpression of the *ped/pea-15* gene is a common defect in individuals with Type 2 diabetes as well as in non-diabetic individuals at high risk to develop diabetes (as the first-degree relatives of diabetic subjects) (Condorelli et al. 1998, Valentino et al. 2006). To investigate the relevance of *ped/pea-15* overexpression to glucose tolerance, transgenic mice ubiquitously overexpressing human *ped/pea-15* were generated ($Tg_{ped/pea-15}$). These mice exhibited mildly elevated random-fed blood glucose levels and decreased glucose tolerance (fig. 9A). In cultured muscle and adipose cells and in

peripheral tissues from $Tg_{ped/pea-15}$, high levels of PED/PEA-15 impair insulin-stimulated GLUT4 translocation and glucose transport, suggesting that PED/PEA-15 overexpression may contribute to insulin resistance in Type 2 diabetes (Condorelli et al. 2001, Vigliotta et al. 2004 *see also publication n°3 appended*). In transgenic mice, indeed, insulin administration reduced glucose levels by only 35% after 45 min, compared to 70% in control mice (fig 9B). In vivo, insulin-stimulated glucose uptake was decreased by almost 50% in fat and muscle tissues from $Tg_{ped/pea-15}$ accompanied by a constitutively activated protein kinase Cα and lack of protein kinase Cζ induction by insulin. These changes persisted in isolated adipocytes from the transgenic mice and were rescued by the protein kinase C inhibitor bisindolylmaleimide.

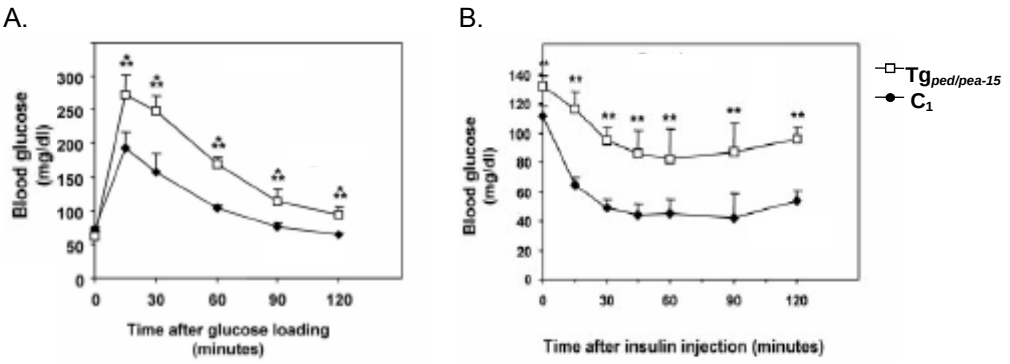


Figure 9. A. Glucose Tolerance Test and **B.** Insulin Tolerance test in **$Tg_{ped/pea-15}$** (*see also publication n°3 appended*).

This point has been further confirmed by other studies where PED/PEA-15-induced insulin resistance is accompanied by PLD-dependent activation of PKCα (Vigliotta et al. 2004 *see also publication n°3 appended*), leading to the failure of PKCζ activation by insulin (Condorelli et al. 2001, et al. 2004 *see also publication n°3 appended*) (fig.10).

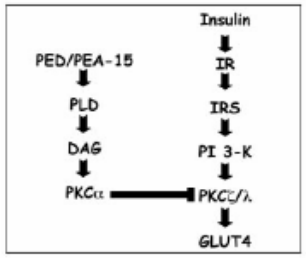


Figure 10. Proposed mechanism of PED action on insulin-stimulated glucose transport **(***see also publication n°3 appended*).

Rescue of PKCζ activity in cells overexpressing PED/PEA-15 restores normal sensitivity to insulin of the glucose transport machinery (Condorelli et al. 2001). Thus, in muscle and adipose cells, PED/PEA-15 generates resistance to insulin action on glucose disposal by impairing normal regulation of PKCζ (Condorelli et al. 2001). Furthermore, more recent studies revealed that, in first-degree relatives of Type 2 diabetic subjects, PED/PEA-15 levels negatively correlate with insulin sensitivity (Valentino et al. 2006).
Other studies (Vigliotta et al. 2004 *see also publication n°3 appended*) have demonstrated that after a treatment with a 60% fat diet nontransgenic mice showed little change in glucose tolerance, while $Tg_{ped/pea-15}$ developed diabetes (fig.11). This additional finding suggests an important interaction of environmental modifiers with *ped/pea-15* gene function, leading to a further derangement in glucose tolerance. Based on insulin tolerance tests, $Tg_{ped/pea-15}$ developed only a further 20% increase in insulin resistance after the high-fat diet while nontransgenic mice undergo a 60% reduction in their sensitivity to insulin becoming as insulin resistant as their transgenic littermates. We have therefore hypothesized that resistance to insulin action alone is not sufficient to cause the alteration in glucose tolerance of the $Tg_{ped/pea-15}$. To verify this hypothesis we started to study glucose-induced insulin secretion.

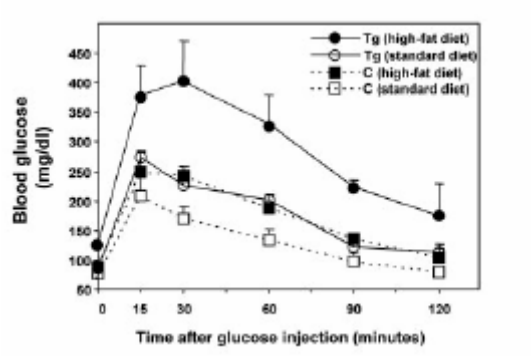


Figure 11. Effect of high-fat feeding on glucose tolerance in $Tg_{ped/pea-15}$. (*see also publication n°3 appended*).

AIM OF THE STUDY

Ped/Pea-15, a gene overexpressed in type 2 diabetics, causes peripheral insulin-resistance and reduces glucose tolerance in transgenic mice overexpressing the protein. Furthermore, these mice were hyperinsulinemic in fasted conditions. Treatment with a 60% fat diet led $Tg_{ped/pea-15}$ to develop diabetes. Since insulin resistance was only slightly worse by the high fat diet in $Tg_{ped/pea-15}$, it was hypothesized that resistance to insulin action alone is not sufficient to cause the alteration in glucose tolerance of the $Tg_{ped/pea-15}$.
To this aim I sought to analyze the effect of PED/PEA-15 on glucose-induced insulin secretion and to investigate the molecular mechanism underlying this process.

MATERIALS AND METHODS

MEASUREMENT OF INSULIN SECRETION *IN VIVO*.
For analysing insulin secretion, mice were fasted overnight and then injected with glucose (3 g.kg of body weight-1) intraperitoneally. Venous blood was drawn at 0, 3, 10 and 30 min after glucose injection by retro-orbital bleeding, and plasma insulin concentrations were measured by radioimmunoassay (RIA) (Kulkarni et al. 1999) with rat insulin as standard (Insulin Rat RIA KIT, LINCO Research, MO).

IMMUNOHISTOCHEMICAL ANALYSIS.
Pancreas from transgenic and control animals were fixed in 4% paraformaldehyde–0.1 M sodium phosphate buffer at 4°C overnight and placed in 30% sucrose at 4°C overnight. After being embedded in Tissue-Tek OTC compound and frozen at < 20°C, tissue sections were prepared. Immunohistochemical analysis was carried out with the H38 rabbit insulin antibody (Santa Cruz Biotechnology, Inc.; 1:200 dilution), the N-17 goat glucagon antibody (Santa Cruz Biotechnology, Inc.; 1:200 dilution), or PED/PEA-15 antiserum (Condorelli 1998) (1:20,000 dilution). Incubation with the primary antibody was followed by incubation with biotinylated anti-rabbit or anti-goat immunoglobulin G and peroxidase-labeled streptavidin. Analysis of serial consecutive islet sections stained with either insulin or the PED/PEA-15 antibodies was used to confirm *ped/pea-15* expression in insulin-immunopositive beta cells.

ISLET ISOLATION, *EX VIVO* INSULIN SECRETION ASSESSMENT AND ESTIMATION PANCREATIC INSULIN CONTENT.
Islets were isolated from 6-month-old mice by collagenase digestion and subsequent centrifugation on a Histopaque (Sigma-Aldrich, MO) gradient as previously described (Kitamura et al. 2001). For assaying of insulin release, 20 islets were manually selected, cultured for 24h, incubated in Krebs-Ringer solution (120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 10 mM $NaHCO_3$, 1,3 mM $CaCl_2$, 1,2 mM KH_2PO_4) for 30 min, and then stimulated at 37°C with various concentrations of either glucose for 1 hour, KCl for 30 minutes, or glyburide (Sigma-Aldrich, MO) for 1 hour. The islets were subsequently collected by

centrifugation, and supernatants were assayed for insulin content by RIA as described above. For measuring total pancreatic insulin, pancreases were solubilized in acid-ethanol solution (75% ethanol, 1.5% HCl) overnight at 4°C and lysates centrifuged for 15 min. at 800g. Pancreatic insulin was measured in the supernatants by RIA.

REAL-TIME RT-PCR ANALYSIS.

Total cellular RNA was isolated from pancreatic islets and cells by the use of RNeasy kit (QIAGEN Sciences, Germany), according to the manufacturer's instructions. For Real-time RT-PCR analysis, 1 μg of islet or cell RNA was reverse-trascribed using Superscript II Reverse Trascriptase (Invitrogen, CA). PCR reaction mixes were analyzed using SYBR Green mix (Invitrogen, CA). Reactions were performed using Platinum SYBR Green qPCR Super-UDG using an iCycler IQ multicolor Real Time PCR Detection System (Biorad, CA). All reactions were performed in triplicate and β-actin was used as an internal standard.

CELL CULTURE PROCEDURES AND TRANSFECTION.

MIN-6 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Germany) containing 25 mM glucose, 50 μM 2-mercaptoethanol, and 10% fetal calf serum (Biochrom, Germany) at 37°C in a 5% CO_2 atmosphere as in (Miyazaki et al. 1990). Transfections of the Ped/pea-15 and PKCζ cDNAs were performed as described previously (Condorelli et al. 2001).

WESTERN BLOT ANALYSIS.

For these studies cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 mg/ml aprotinin) for 1 h at 4°C and lysates were centrifuged at 5,000*g* for 20 min. Homogenates and cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gels, as described by Laemmli 1970. Proteins separated on the gels were electroblotted on 0.45-mm Immobilon-P membranes (Millipore Corporation, MA) as previously described (Condorelli et al. 2001) and probed with antibodies to PED/PEA-15 (Condorelli et al. 1998), PKCζ (Santa Cruz Biotechnology, Inc., CA), or tubulin (Santa Cruz Biotechnology, Inc., CA).

PKC ASSAY.

PKC activity was measured as previously described (Condorelli et al. 2001). Briefly, for these assays, cells were solubilized in lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 2 mmol/l Na_3VO_4, 100 mmol/l NaF, 10% glycerol, 1% Triton X-100, 1 mmol/l PMSF, 10 mg/ml aprotinin) for 1 h at 4°C. Lysates were centrifuged at 5,000g for 20 min and activity of the specific PKC isoforms was assayed after immunoprecipitation with specific antibodies PKCζ (Santa Cruz Biotechnology, Inc., CA) (Condorelli et al. 2001). Soluble pellets were supplemented with the lipid activators (10 mmol/l phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidyl serine, and 4 mg/ml dioleine, final concentrations) and phosphorylation reactions initiated by addition of the substrate solution (50 mmol/l Ac-MBP [4 – 14], 20 mmol/l ATP, 1 mmol/l $CaCl_2$, 20 mmol/l $MgCl_2$, 4 mmol/l Tris, pH 7.5, and 10 μCi/ml [3,000 Ci/mmol] [γ-^{32}P]ATP, final concentrations). The reaction mixtures were further incubated for 30 min at room temperature, rapidly cooled on ice, and spotted on phosphocellulose discs, according to the kit manufacturer's instructions (Promega, Wi). Disc-bound radioactivity was quantitated by liquid scintillation counting.

STATISTICAL PROCEDURES.

Data were analysed with Statview software (Abacus-concepts) by one-factor analysis of variance. *P* values of less than 0,05 were considered statistically significant.

RESULTS AND DISCUSSION

To evaluate the role of PED/PEA-15 overexpression on glucose-induced insulin secretion we started studing the *in vivo* glucose induced insulin secretion in *ped/pea-15* transgenic mice ($Tg_{ped/pea-15}$ mice). Firstly we analyzed the expression of PED/PEA-15 in pancreatic sections of $Tg_{ped/pea-15}$ mice. Staining of pancreas sections with specific PED/PEA-15 antibodies revealed a significant increase in the immunoreactivity of the islet cores, where beta-cells are localized, in $Tg_{ped/pea-15}$ mice compared to their relative wild-type littermates (C_1) (Fig.12A). At variance, both insulin and glucagon immunoreactivities were comparable in $Tg_{ped/pea-15}$ mice and C_1 mice. We next compared insulin secretion following a glucose load in $Tg_{ped/pea-15}$ and in C_1 mice. In C_1 mice, a six fold increase in insulin secretion was observed 3 min after intraperitoneal glucose injection, and the levels remained higher than the baseline values for up to 30 min, indicating a second-phase response (Fig.12B). Based on insulin area under the curve quantitation, the acute first-phase of insulin secretory response to glucose was reduced by almost 70% in $Tg_{ped/pea-15}$ mice ($p< 0.001$). The second-phase was also significantly impaired in $Tg_{ped/pea-15}$ mice compared to C_1 ($p< 0.01$).

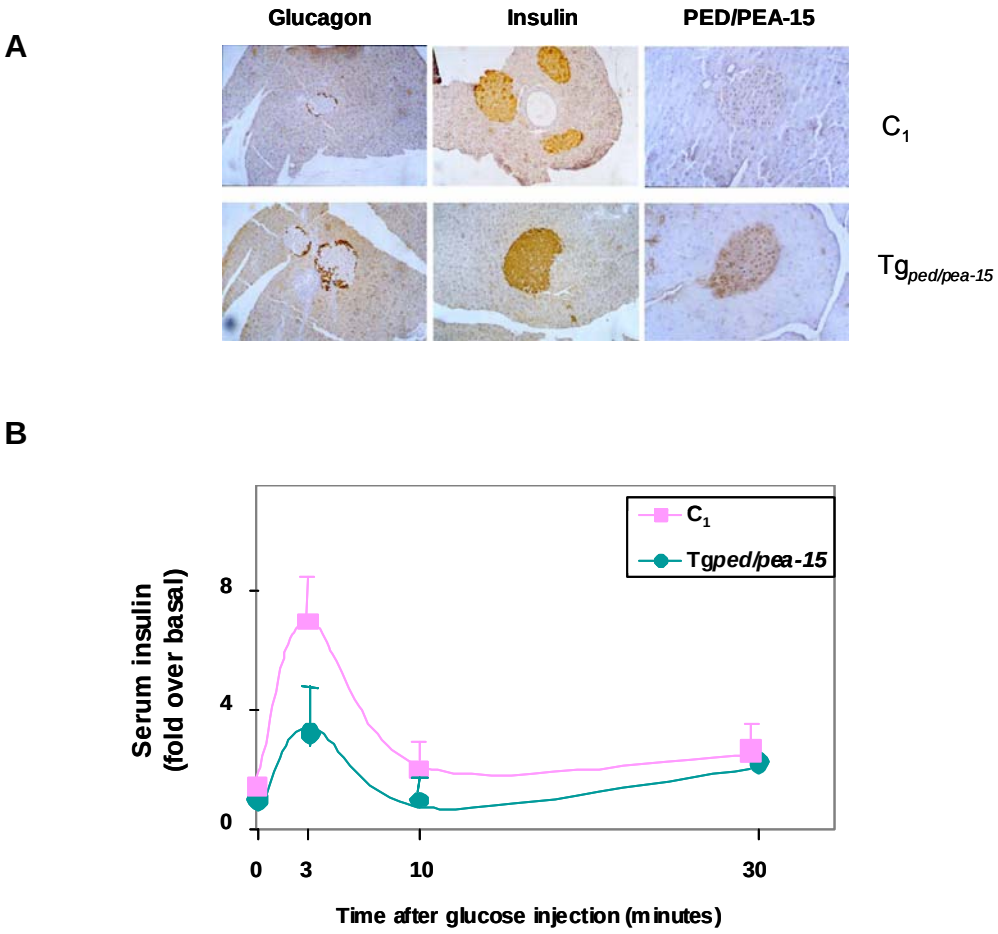


FIG.12. Insulin secretion in *ped/pea-15* transgenic mice.
A. Pancreas from *ped/pea-15* transgenic mice ($Tg_{ped/pea-15}$ mice) and their nontransgenic littermates (C_1) were fixed and embedded in Tissue-Tek OTC, and sections were prepared as described under Materials and Methods. Immunohistochemical analysis of the islets was carried out with PED/PEA-15, insulin, and glucagon antibodies as indicated. Anti-rabbit or anti-goat immunoglobulin G was used as the secondary antibody. Immunoreactivity was revealed by peroxidase-labeled streptavidin. The microphotographs shown are representative of images obtained from eight transgenic (four male and four female) and seven nontransgenic mice (three male and four female). **B.** $Tg_{ped/pea-15}$ mice and C_1 were subjected to intraperitoneal glucose loading, followed by determination of plasma insulin levels at the indicated times. Data points represent the mean ± standard deviation of determinations in 14 $Tg_{ped/pea-15}$ (seven female and seven male) and 16 C_1 (eight female and eight male).

To test the hypothesis that overexpression of PED/PEA-15 in beta-cells is sufficient to impair hyperglycemia-induced insulin secretion, we isolated islets from $Tg_{ped/pea-15}$ and C_1 mice pancreas. First we examined the expression of PED/PEA-15 by immunoblot analysis (Figure 13). The amount of PED/PEA-15 in islets of $Tg_{ped/pea-15}$ was increased by about 3-4 folds ($p<0,001$) compared to that in islets of C_1 mice (fig.13A). Then we measured insulin secretion in islets from $Tg_{ped/pea-15}$ mice and C_1. Islets isolated from $Tg_{ped/pea-15}$ showed 3-fold increased insulin release when exposed to 2.8 mM glucose ($p< 0.001$) and no further release upon exposure to 16.7 mM glucose (fig.13B). At variance with glucose, exposure to the membrane depolarising agent potassium chloride (33 mM) caused a comparable release of insulin by the $Tg_{ped/pea-15}$ mice and C_1 islets. This last result indicates that the site of PED/PEA-15 action on glucose-induced secretion is upstream the membrane depolarization step.

Total insulin content of pancreatic islets from $Tg_{ped/pea-15}$ mice was not increased compared with that in islets from C_1 mice ($Tg_{ped/pea-15}$=110±20pg/mg protein and C1=120±20pg/mg protein) and it may not account for the difference in basal insulin secretion. These findings led us to focus on the possibility that the overexpression of PED/PEA-15 mainly impairs the glucose sensing rather than the insulin biosynthetic machinery of the beta-cell.

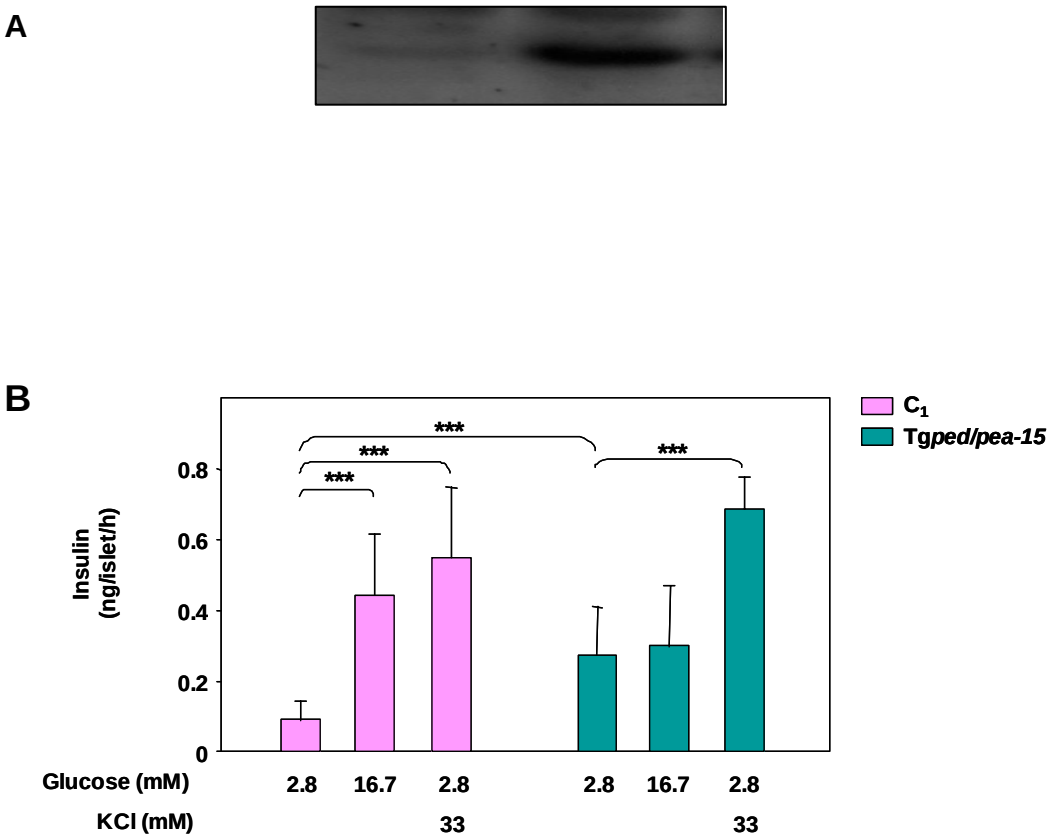


Fig.13. PED/PEA-15 overexpression and insulin secretion in $Tg_{ped/pea-15}$ islets
A. Islets were isolated and solubilized in lysis buffer as described under Materials and Methods and PED/PEA-15 expression was evaluated by immunoblot analysis.
B. Islets were isolated from 6-month old mice from $Tg_{ped/pea-15}$ mice and C_1 mice. Islets were incubated in KRB solution for 30 min and insulin release was determined upon exposure to the indicated concentrations of glucose or potassium chloride, respectively for 60 and 30 further min, as described under Materials and Methods. Bars represent the means ± SD of determinations 11 independent experiments in duplicate. Asterisks denote statistically significant differences (***, $P < 0.001$).

To gain further insight into the mechanisms leading to dysfunction of beta-cell overexpressing PED/PEA-15, we have profiled the expression of several genes relevant to the glucose sensory apparatus by real-time RT-PCR analysis of total RNA isolated from islets of $Tg_{ped/pea-15}$ mice (Figure 14). The amounts of mRNAs for the *Sur1* subunit of the ATP-sensitive K^+ channel was significantly reduced by 30% in these islets compared with those in control mice ($p<0.001$). RNAs encoding the *Kir6.2* potassium channel subunit and the *Sur1/Kir6.2* upstream regulator *Foxa2* were also reduced, respectively, by 40 and 45% in the islets from the $Tg_{ped/pea-15}$ mice ($p<0.01$) (fig.14A). Consistently, islets from $Tg_{ped/pea-15}$ mice exhibited no secretory response to the K^+ channel locking agent gliburyde (fig.14B). At variance, the abundance of *glucokinase, HK1, HK2,* and *GLUT2* mRNAs did not differ between $Tg_{ped/pea-15}$ and C_1 mice. Thus, beta-cell expression profiling revealed that mRNA abundance of both the *Kir6.2* and the *Sur1* transcripts are significantly depressed by the overexpression of PED/PEA-15. PED/PEA-15 action on the Kir6.2 and the Sur1 genes represents a specific abnormality as other genes encoding key components of the glucose-sensitive insulin secretion machinery were unaffected by PED/PEA-15. Consistent with the functional consequence of this defect, the potassium channel locking agent glyburide showed impaired action on insulin secretion in PED/PEA-15 overexpressing islets. Previous studies in mice evidenced that ablation of either the *Sur1* or the *Kir6.2* genes results in a phenotype reminiscent of that characterizing the $Tg_{ped/pea-15}$ mice (Miki et al. 1998, Nenquin et al. 2004, Seghers et al. 2000). Indeed, both the $Sur1^{-/-}$ and the $Kir6.2^{-/-}$ mice feature blunted insulin secretion response to sulfanylureas. The defective insulin response to the sulfanylurea glyburide seems even more pronounced in the $Tg_{ped/pea-15}$ mice as, in these animals, less than 40% decrease in *Sur1* and *Kir6.2* gene expression blocks glyburide action. At variance with the $Sur1^{-/-}$ and the $Kir6.2^{-/-}$ mice however, in the $Tg_{ped/pea-15}$ mice PED/PEA-15 simultaneously depresses the expression of both the *Sur1* and the *Kir6.2* genes. The simultaneous impairment in their function may result in an additive effect on glyburide action. Similar to the $Tg_{ped/pea-15}$ mice, fasted $Sur1^{-/-}$ mice are more hyperinsulinemic than control animals due to persistent activation of voltage-gated calcium channels. Furthermore, both the $Sur1^{-/-}$ and the $Kir6.2^{-/-}$ mice feature impaired glucose tolerance and glucose-induced first and second phase insulin secretion. Thus, at least in part, the loss of potassium channels caused by beta-cell overexpression of PED/PEA-15 may account for the abnormalities in basal and glucose-stimulated insulin secretion and in glucose tolerance observed in the $Tg_{ped/pea-15}$ mice.

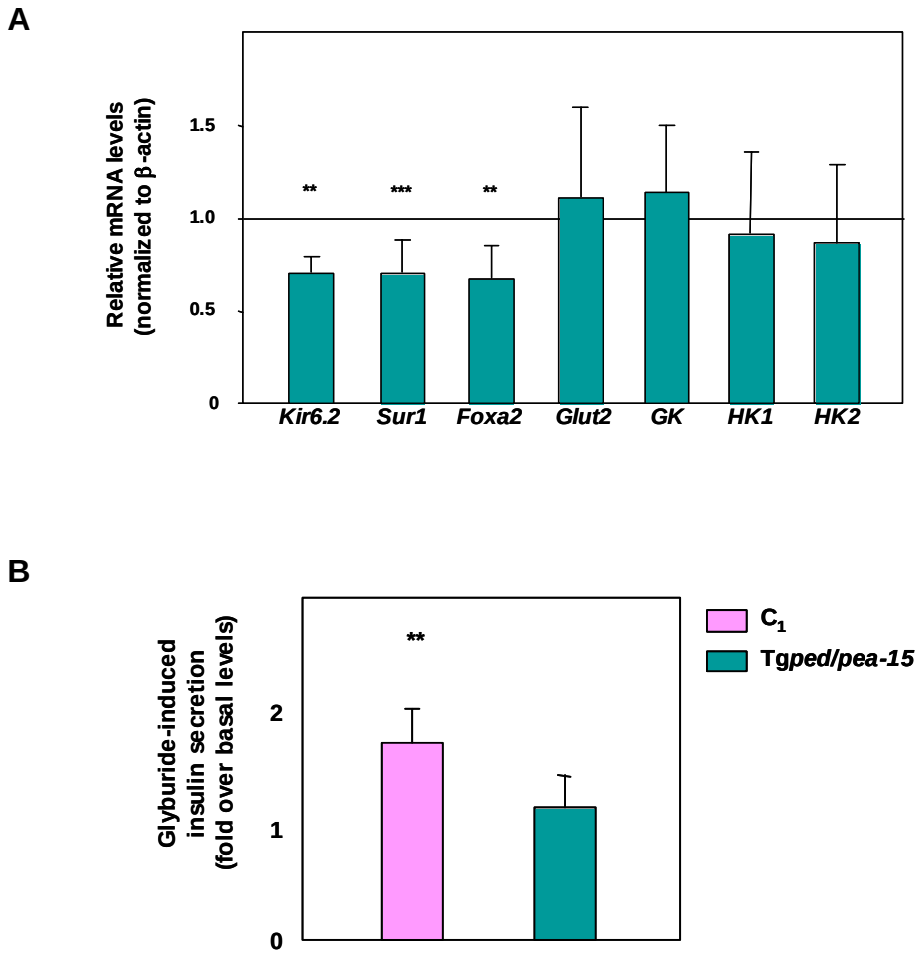
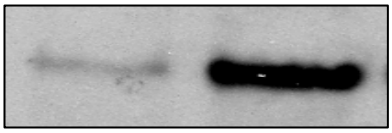


Fig.14. Gene expression profile in isolated islets from $Tg_{ped/pea-15}$ mice.
A. The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from islets of $Tg_{ped/pea-15}$ mice and their non transgenic littermates (C_1). All reactions were performed using beta-actin as internal standard as described under Materials and Methods. The mRNA levels in $Tg_{ped/pea-15}$ mice islets are relative to those in C_1. Each bar represents the mean ± SD of five independent experiments in each of whom reactions were performed in triplicate using the pooled total RNAs from 6 mice/genotype. **B.** Islets were isolated from 6-month $Tg_{ped/pea-15}$ mice and from C_1. Islets were incubated in KRB solution for 30 min. and insulin release was determined after exposure to 10 μM glyburide for 60 further min, as described under Materials and Methods. Bars represent the means ± SD of data from 4 independent experiments each with at least 3 mice/group. Asterisks denote statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

To elucidate the mechanism by which Ped/Pea-15 impairs the expression of Sur1, Kir6,2 and HNF3β (*Foxa2*) we generated MIN6 beta-cell lines stably overexpressing PED/PEA-15 by 10-fold above endogenous levels (MIN-$6_{ped/pea-15}$) (Fig.15A). We then compared insulin secretion in these cells and in cells expressing only endogenous PED/PEA-15, the MIN6 cells. MIN6 cells responded with about 4 folds ($p< 0.001$) induction in insulin secretion when subjected to physiological increases of glucose concentration in the medium (Fig.15B). In these same cells, the overexpression of PED/PEA-15 increased basal insulin secretion of about 2,5 folds ($p< 0.001$) and reduced glucose-induced insulin secretion of about 90%. Treatment with a specific antisense oligonucleotide effectively blocked PED/PEA-15 expression in MIN6 and in MIN-$6_{ped/pea-15}$ cells and almost completely rescued the abnormal insulin secretion in the MIN-$6_{ped/pea-15}$ cells (Fig.15C) ($p< 0.01$). In addition, antisense transfection of MIN-6 control cells enhanced glucose-induced insulin secretion by about 2- fold (Fig.12D) ($p< 0.01$), indicating that this gene has a physiological role in controlling beta-cell secretion in response to glucose. Gene expression profile show a close to 40% decrease in the abundance of the *Sur1*, *Kir6.2* and *Foxa2* mRNAs (Fig.14E) in MIN-$6_{ped/pea-15}$ cells compared to MIN-6 cells ($p<0.001$). At variance, the abundance of *glucokinase, HK1, HK2,* and *GLUT2* mRNAs did not differ between MIN6 and MIN-$6_{ped/pea-15}$ cells. Data obtained in MIN-6 cells further suggest an important role for ped/pea-15 in the regulation of both insulin secretion and gene expression.

A



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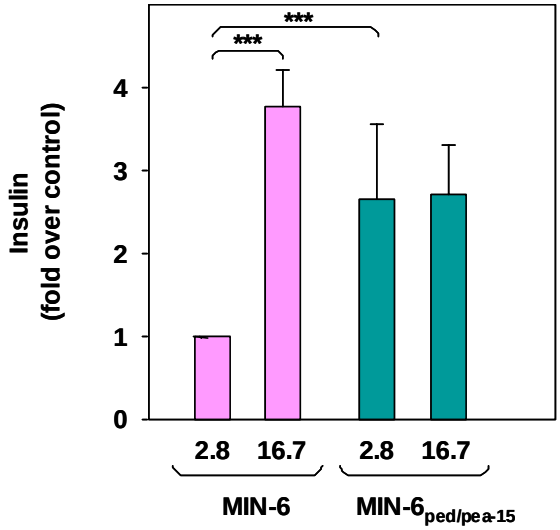
***
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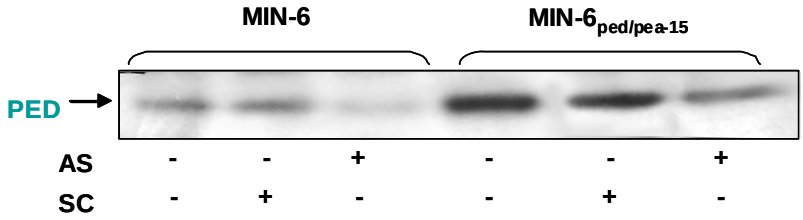
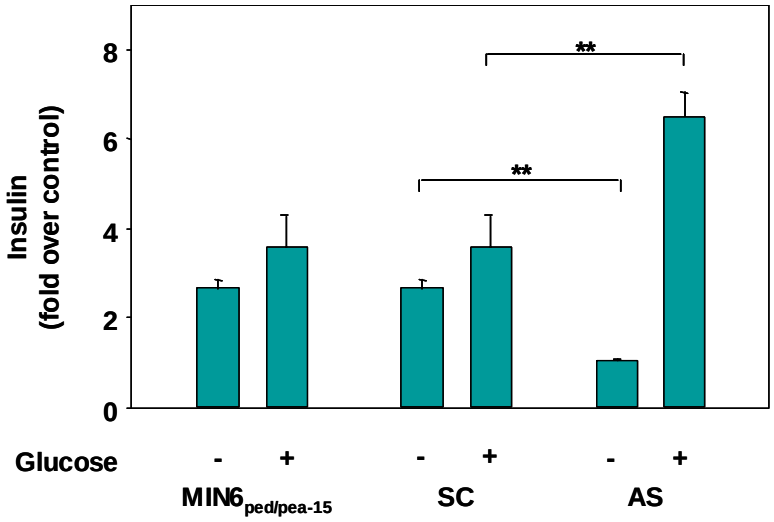
Insulin
(fold over control)
0
1
2
3
4
2.8
16.7
2.8
16.7
MIN-6
MIN-6ped/pea-15

C


MIN-6
MIN-6ped/pea-15
PED
AS - - + - - +
SC - + - - + -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Insulin
(fold over control)
0
2
4
6
8
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MIN6ped/pea-15
SC
AS

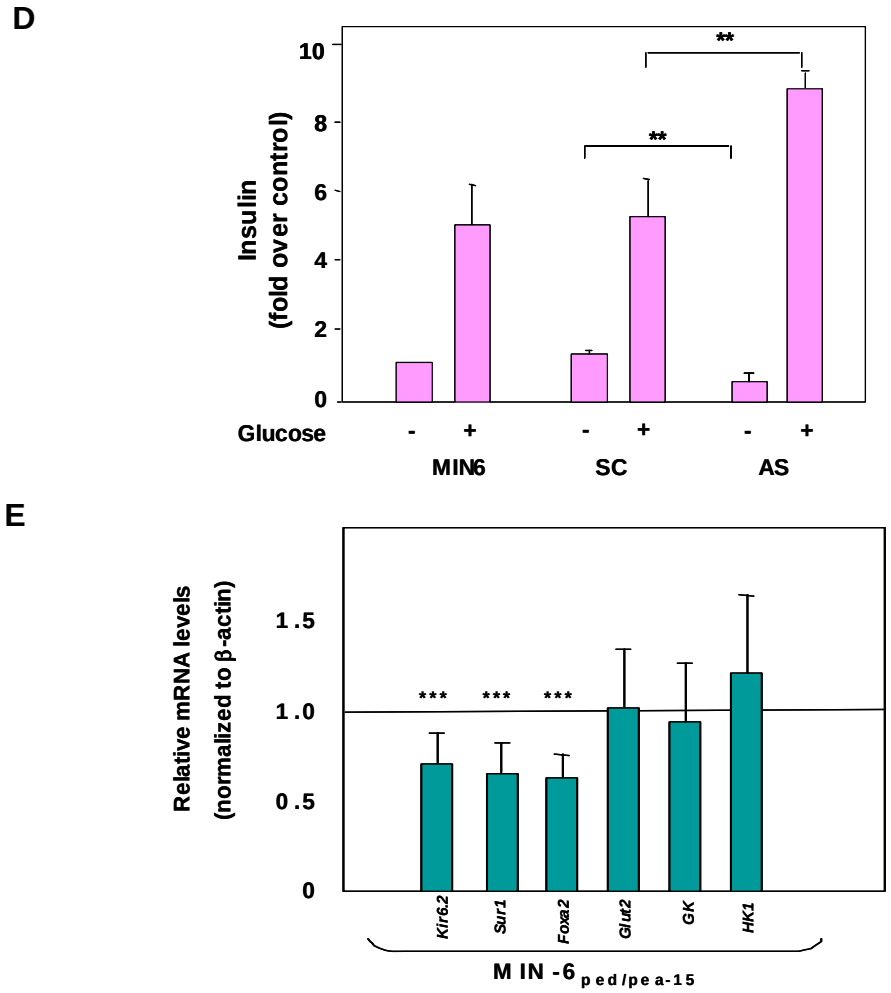

Fig.15. Characterization of *ped/pea-15*-overexpressing MIN6 cells. A. MIN6 beta cells were stably transfected with *ped/pea-15* cDNA and a clone overexpressing *ped/pea-15* by 10-fold was selected MIN-$6_{ped/pea-15}$. **B.** Insulin release was assayed by radioimmunoassay in the culture medium of MIN-6 and of MIN-$6_{ped/pea-15}$ cells upon 60 min incubation with the indicated glucose concentrations. Bars represent mean values ± SD from 6 independent experiments each in duplicate. Asterisks indicate statistically significant differences (***, $P < 0.001$). **C** and **D**. MIN-6 and MIN-$6_{ped/pea-15}$ were transfected either with *ped/pea-15* antisense (AS) or with scrambled (SC) oligonucleotides. Glucose-stimulated insulin secretion was assayed as outlined above. Bars represent values ± standard deviation of triplicate measurements in four independent experiments. Asterisks indicate statistically significant differences (***, $P < 0.01$). **E.** The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from MIN-6 and MIN-$6_{ped/pea-15}$ cells. Bars represent the mRNA levels in the transfected cells and are relative to those in *wild-type* (control) cells. Data are expressed as means ± SD of triplicate reactions for total RNAs from each cell type in five independent experiments. Asterisks indicate statistically significant differences (***, $p < 0.001$).

Recent evidences in skeletal muscle and fat indicated that high cellular levels of PED/PEA-15 impair the function of the atypical PKC isoform PKC zeta (Vigliotta et al. 2004 *see also publication n°3 appended*, Zhang et al. 2000). In addition, the atypical PKC lambda has recently been shown to play a major role in regulating glucose-induced insulin secretion through the modulation of the expression of multiple genes in pancreatic beta-cells (Seghers et al. 2000). These include *Sur1* and *Kir6.2*. In MIN-6, the effect of glucose on insulin release was paralleled by a 2-fold increase in immunoprecipitated PKC zeta activity (fig.16B), with no measurable change in its expression (fig.16A). Furthermore, the effect of glucose was almost completely abolished in Min-$6_{ped/pea-15}$ cells. These data could suggest an important role of atypical PKCs in regulating genes of the insulin secretion pathway. Down-regulation of PKC zeta observed in Min-$6_{ped/pea-15}$ may cause impaired glucose-induced insulin response in islets from $Tg_{ped/pea-15}$ mice by reducing *Sur1* and *Kir6.2* gene expression

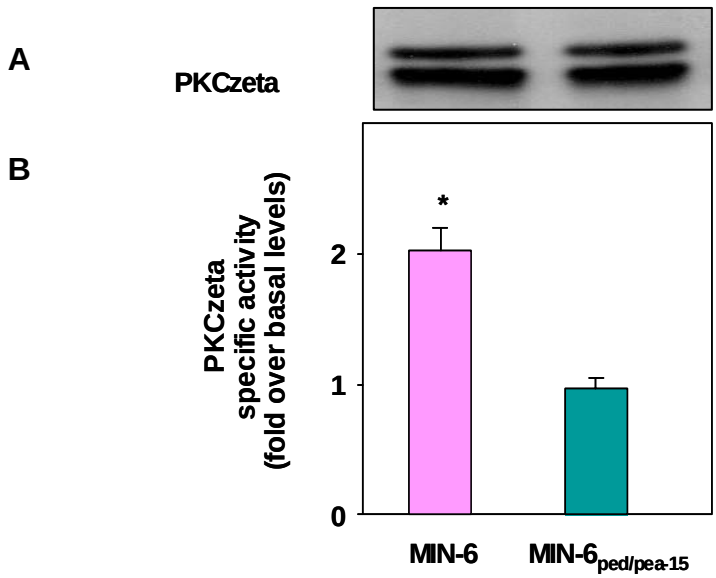

Fig.16. B. MIN-6 cells and MIN-$6_{ped/pea-15}$ were exposed to 16.7 mM glucose for 60 min and then solubilized. Lysates were then precipitated with PKC zeta antibody and PKC activity was assayed in the precipitates as described under Materials and Methods. PKC zeta activity is expressed as fold-increase over basal activity (measured in the presence of 2.8 mM glucose). Bars represent the mean ± SD of data from 4 independent experiments. Asterisks indicate statistically significant differences (*, $P < 0.05$). **A.** For control, aliquots of the cell lysates were normalized for protein and directly blotted with PKC zeta antibodies. Blots were revealed by ECL and autoradiography. The blot shown in the inset is representative of those in the other 3 experiments.

To further address this issue, we forced the expression of PKCζ in MIN-$6_{ped/pea-15}$ cells. As shown in Fig. 17A, overexpression of PKCζ to high levels largely rescued PKC activity in the PKCζ precipitates from glucose-exposed MIN-6 and MIN-$6_{ped/pea-15}$ cells. Importantly, this rescue was accompanied in MIN-$6_{ped/pea-15}$ cells by recovery in *Sur1, Kir6.2* and *Foxa2* gene expression (Fig. 17B). In part, changes in insulin release caused by PED/PEA-15 were also reverted, determining recovery of glucose-induced insulin secretion (Fig. 17C). These data indicate that the changes in atypical PKC function caused by PED/PEA-15 overexpression represent upstream abnormalities impairing the function of multiple genes involved in the regulation of insulin secretion by the beta-cell. PKCζ/λ have been reported to control the function of a number of transcription factors (Furukawa et al. 1999, Matsumoto et al. 2003). Very recent data evidenced that, in PKC lambda null mice, the expression of the *Foxa2* mRNA in addition to that of *Sur1* and *Kir6.2*, was significantly reduced in pancreatic beta-cells (Hashimoto et al. 2005). In addition, in pancreatic beta-cells, the expression of the *Sur1* and *Kir6.2* genes is dependent on *Foxa2* activity (Wang et al. 2002, Lee et al. 2002, Sund et al. 2001). Similar to the PKC$\lambda^{-/-}$ mice, we now report that *Foxa2* mRNA abundance is also reduced in islets from $Tg_{ped/pea-15}$ mice. Thus, impaired activity of atypical PKCs in beta-cells overexpressing PED/PEA-15 may prevent normal potassium channel generation and glucose-regulated insulin secretion by depressing *Foxa2* expression. Supporting this conclusion, we evidenced that forcing the expression of PKCζ, in PED/PEA-15 overexpressing beta-cells, rescued *Foxa2* levels in addition to those of *Kir6.2* and *Sur1* and to glucose-induced insulin secretion.

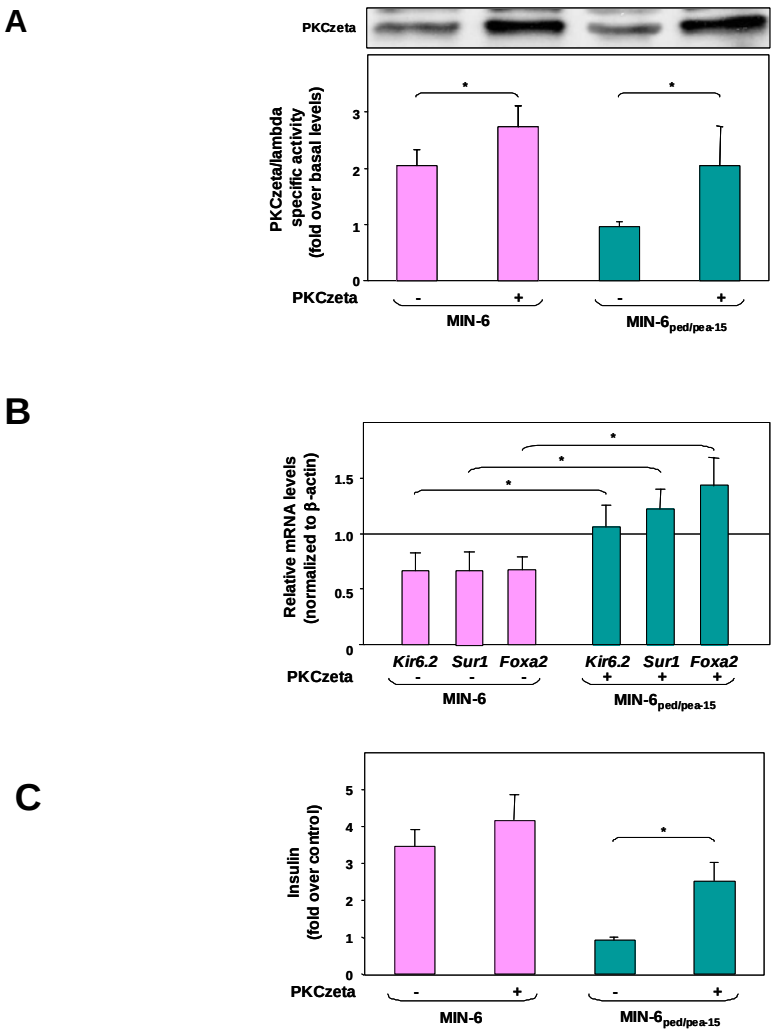

Fig.17. Rescue of PKCzeta activity, gene expression profile and glucose-stimulated insulin secretion in MINped/pea-15 cells. A. MIN-6 and MIN-$6_{ped/pea-15}$ cells were stably transfected with a PKCzeta cDNA, solubilized and lysates immunoprecipitated with a PKCζ antibody. PKCζ activity was assayed in the immunoprecipitates as described under Materials and Methods. For control, aliquots of the lysates were blotted with the PKCζ antibody and filters revealed by ECL and autoradiography. Bars represent the means ± SD of five independent experiments in duplicate. The autoradiograph shown in the inset is representative of blots from five experiments. **B.** The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from MIN-6 and MIN-$6_{ped/pea-15}$ cells. Bars represent the mRNA levels in the MIN-$6_{ped/pea-15}$ cells and are relative to those in the untransfected cells (MIN-6). Data are expressed as means ± SD of triplicate reactions for total RNAs from each cell type in four independent experiments. **C.** The MIN-6 cells, either those transfected with the PKCζ cDNA and the untransfected cells, were exposed to 16.7 mM glucose for 60 min and insulin release in the culture medium was assayed by radioimmunoassay as described under Materials and Methods. Bars represent the means ± standard deviations of duplicate determinations in three independent experiments. Asterisks indicate statistically significant differences (*, $p<0.05$).

CONCLUSIONS

Ped/pea-15, is a novel gene shown be overexpressed in type 2 diabetics and involved in peripheral insulin resistance. In this thesis I have shown that, in addition to fasting iperinsulinemia, $Tg_{ped/pea-15}$ mice have a reduction of insulin secretory response to glucose due to the lack of glucose-induced activation of the Protein kinase C zeta (PKCζ). This alteration results in the downregulation of the expression of the two subunits of the ATP-dependent K+ channel Sur1 and Kir6.2 and their regulator Foxa2. In addition, the control of glucose-regulated insulin secretion appears to represent a physiological function of *ped/pea-15* in beta cells. Indeed, transfection of a specific antisense oligonucleotide into MIN6 cells expressing only endogenous *ped/pea-15* enhanced glucose-induced insulin secretion by almost twofold. Thus, the overexpression of PED/PEA-15 causes simultaneously insulin resistance and impaired insulin secretion. Under appropriate environmental conditions, *ped/pea-15* overexpression leads to diabetes in mice, thus indicating that PED-PEA-15 could contribute to genetic susceptibility to type 2 diabetes in humans. Thus, decreasing *ped/pea-15* levels might be an innovative therapeutic approach for the treatment of type 2 diabetes.

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