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Coupling mechanisms of insulin secretion Roles of mitochondrial metabolism and cAMP

Ulrika Fransson



LUND UNIVERSITY

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| the onset of type 2 diabetes. When insulin resistan increasing their secretion of insulin, causing hyper in fact, many people develop severe insulin resista However, beta-cells in some individuals, perhaps sufficiently to meet the new requirements. This let able to treat diabetes, the defects in the beta-cells of The aim of this thesis was to investigate the mech- glucose stimulation of the beta-cell to insulin secre- We have found that anaplerosis via pyruvate carbo insulin secretion, presumably via generation of an expression of PDK1 is upregulated in INS-1 832/1 knock-down of PDK1 enhances insulin secretion. important for insulin secretion, and points to the in PKA signaling is stimulated by glucose, and that i | ice develops, the pancreatic b rinsulinemia. This state may r ance and hyperinsulinemia wi genetically predisposed, are u ads to hyperglycemia, the ma causing impaired insulin secre- anisms of insulin secretion, are etion. oxylase is essential for both p increased ATP/ADP ratio. Fi 13 cells cultured at high conce Both these findings prove than nvolvement of pyruvate cyclin inhibition of PKA decreases C | eta-cells compensate by not be a major risk factor per se; thout ever acquiring diabetes. inable to increase their secretion in hallmark of diabetes. To be etion must be elucidated. and especially what couples hases of glucose-stimulated arther, we discovered that entrations of glucose, and that it mitochondrial metabolism is ag. We have also showed that 3SIS in INS-1 832/13 cells. |
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Coupling mechanisms of insulin secretion Roles of mitochondrial metabolism and cAMP

Ulrika Fransson



LUND UNIVERSITY

Department of Experimental Medical Science 2006

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ABBREVIATIONS

| ADP | adenosine diphosphate |
|---------------------------|---|
| ATP | adenosine triphosphate |
| BCH | 2-amino-2-norbornane-carboxylic acid |
| cAMP | cyclic adenosine monophosphate |
| CAR | coxsackie and adenovirus receptor |
| CPT1 | carnitine palmitoyl-transferase 1 |
| GLUT | glucose transporter |
| GSIS | glucose-stimulated insulin secretion |
| GDH | glutamate dehydrogenase |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GIP | glucose-dependent insulinotropic peptide |
| GLP-1 | glucagon-like peptide 1 |
| GTP | guanosine triphosphate |
| HSL | hormone sensitive lipase |
| HIF | hypoxia inducible factor |
| HPRT | hypoxanthine-guanine phosphoribosyl transferase |
| IRS | insulin receptor substrate |
| kDa | kilo dalton |
| Km | Michaelis-Menten constant |
| K _{ATP} -channel | ATP-sensitive potassium channel |
| KIC | ketoisocaproic acid |
| KIR | inward rectifying potassium channel |
| LDA | low density array |
| MCT | monocarboxylate transporter |
| NADH | nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| PAA | phenylacetic acid |
| PCR | polymerase chain reaction |
| PHHI | persistent hyperinsulinemia and hyperammonemia in infancy |
| PC | pyruvate carboxylase |
| | |

| PDE | phosphodiesterase |
|------------|---|
| PDH | pyruvate dehydrogenase |
| PDC | pyruvate dehydrogenase complex |
| PDK | pyruvate dehydrogenase kinase |
| PDP | pyruvate dehydrogenase phosphatase |
| PI3-kinase | phosphoinositide 3 kinase |
| РКА | protein kinase A |
| PTGS | post-transcriptional gene silencing |
| RISC | RNA-induced silencing complex |
| RIM | Rab3-interacting molecule |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RRP | readily releasable pool |
| ROS | reactive oxygen species |
| siRNA | small interfering RNA |
| SNAP | soluble N-ethylmalemimide sensitive factor attachment protein |
| SNARE | SNAP receptor |
| SUR1 | sulfonylurea receptor 1 |
| TCA | tricarboxylic acid |
| VAMP | vesicle associated membrane protein |

INTRODUCTION

Diabetes Mellitus

Glucose is one of the main energy sources in the human body, and to maintain normal glucose homeostasis is essential for survival. The balance between glucose appearance in the circulation and glucose uptake is tightly regulated by secretion of insulin and glucagon. These pancreatic hormones control glucose uptake by the tissues and hepatic glucose production. In diabetes, this regulation is disturbed. This leads to elevated fasting plasma glucose levels, which is the major characteristic of diabetes (1). There are two main forms of diabetes: type 1 and type 2 (2). Type 1 is caused by an autoimmune destruction of the pancreatic beta-cells, whereas type 2 is characterized by insulin resistance and defects in insulin secretion. Type 2 diabetes is the most common form and accounts for more than 90% of the 194 million individuals affected worldwide (2003) (3). Despite a tremendous scientific effort, the causes of type 2 diabetes have not yet been fully elucidated.

Insulin resistance

Insulin resistance is tightly linked to obesity, and usually precedes the onset of type 2 diabetes (4). It is characterized by a reduced response to insulin in skeletal muscle, liver and adipose tissue. Insulin lowers the blood glucose by stimulating glucose uptake into skeletal muscle and adipose tissue, and by inhibiting endogenous glucose production by the liver. The actions of insulin are exerted via binding to the insulin receptor. This causes phosphorylation of insulin receptor substrates (IRS), which in turn activate other signaling proteins, where PI3 kinase (phosphoinositide 3 kinase) is one of the most important. The activation initiates a signaling cascade which mediates many effects in the target cells. Glucose uptake is stimulated by translocation of glucose transporter 4 (GLUT-4), the glucose transporter mainly responsible for glucose uptake in target tissues, to the plasma membrane (5). The major site for insulin resistance is skeletal muscle, since muscle glycogen synthesis accounts for most of the total-body glucose uptake, and it has been shown that glycogen synthesis is substantially decreased in subjects with insulin resistance (6). Defects in several steps have been suggested to be involved in the reduced rate of glycogen synthesis, however glucose transport is now thought to be rate-limiting (7). This has been attributed to the increased intramyocellular triglyceride storage seen in obesity (8) which is thought to give rise to elevated intracellular levels of acyl-CoAs. Indeed, fatty acids have been found to abolish insulin activation of PI3 kinase, which decreases glucose uptake via GLUT-4 (9). When the normal responses to insulin fail, blood glucose increases which demands elevated insulin secretion by the pancreatic beta-cells (10).



Figure 1. Blood glucose lowering effects of insulin. The green arrows symbolize stimulation, whereas the red arrow symbolizes inhibition. Modified from Taniguchi *et al.* 2006.

Beta-cell dysfunction

When insulin resistance develops, the pancreatic beta-cells compensate by increasing their secretion of insulin, causing hyperinsulinemia. This state may not be a major risk factor per se; in fact, many people develop severe insulin resistance and hyperinsulinemia without ever acquiring diabetes. However, beta-cells in some individuals, perhaps genetically predisposed, are unable to increase their secretion sufficiently to meet the new requirements. This leads to hyperglycemia, which could worsen the situation further.

In the pathophysiological state called glucose toxicity, chronically elevated glucose levels are believed to severely damage the beta-cells. Glucose toxicity induces changes in gene expression, where decreased insulin expression is the most prominent, and decreases insulin content and insulin secretion (11). Among pathogenic mechanisms is the generation of reactive oxygen species (ROS), which cause oxidative stress. It has been shown that elevated glucose concentrations increase intracellular peroxide levels in islets (12), and administration of antioxidants to Zucker diabetic fatty rats fully restored their impairment of glucose-stimulated insulin secretion (GSIS) (13). Similar to glucose, fatty acids can also be toxic when chronically elevated. Lipotoxicity may be more adverse when also the glucose levels are increased. Prolonged exposure of betacells to fatty acids can e.g. increase basal insulin secretion but inhibit GSIS (14) and induce beta-cell apoptosis (15).

Islets of Langerhans

The islets of Langerhans constitute the endocrine part of the pancreas, and are scattered throughout the pancreas, most abundantly in the pancreatic tail. The adult human pancreas contain $250\ 000 - 1000\ 000$ islets, and the islets can vary in size from just a few cells to about 5000 cells. The mean mass of the endocrine pancreas corresponds to approximately 2% of the total pancreatic weight. The islet consists of four main cell types: the most abundant insulin-secreting beta-cells (70-90%), glucagon-secreting alpha-cells, somatostatin-secreting delta-cells, and the pancreatic polypeptide-secreting PP-cells (16).

Insulin and glucagon are the major regulators of blood glucose levels. Insulin is a polypeptide consisting of an A and a B chain of 21 and 30 amino acids respectively. It mediates its actions via a plasma membrane receptor, which contains a tyrosine kinase domain. When insulin binds to the receptor it becomes autophosphorylated, and subsequently phosphorylates multiple intracellular proteins. Insulin is released in response to elevated glucose levels and promotes glucose uptake, glycogen synthesis and lipid synthesis. Glucagon is a single-chain polypeptide of 29 amino acids, which binds to a seven-transmembrane G-protein linked receptor. This results in activation of adenylate cyclase and generation of cyclic AMP (cAMP). Glucagon is the antagonist of insulin, and is released when blood glucose is low. It stimulates hepatic glucose production via glycogenolysis and gluconeogenesis, and promotes lipolysis from adipose tissue (17). The two hormones also control secretion of each other: insulin restrains glucagon secretion, whereas insulin release is potentiated by glucagon.



Figure 2. The islet of Langerhans. A shows that the islet is readily distinguished from the surrounding exocrine parenchyma. B schematically shows the major islet cell types. Beta-cells are typically found in the core of the islets, while alpha-cells are found in its periphery. Picture adapted from Lee Crompton, University of Manchester.

Glucose-stimulated insulin secretion

To stimulate insulin secretion, glucose must be metabolized in the pancreatic betacells. Glucose enters the beta-cell via glucose transporter 2 (GLUT-2). It has a low affinity for glucose with a K_m of 40 mM, with the consequence that the rate through the transporter is directly proportional to the changes in the blood glucose concentration (18). Inside the cell, glucose is phosphorylated by a low affinity form of hexokinase, glucokinase. Glucokinase is the beta-cell glucose sensor and sets the threshold for stimulation of insulin release by regulating the flux of glucose through glycolysis. Its K_m of 8 mM causes glucose levels below 5 mM not to stimulate insulin secretion, as reviewed by Matschinsky (19). The end product of glycolysis is pyruvate, which then enters the mitochondria and the tricarboxylic acid (TCA) cycle. Mitochondrial metabolism of glucose generates among other things ATP, which leads to an increase in the ATP/ADP ratio.

Triggering pathway

Insulin secretion involves at least two different pathways. In the well defined triggering or KATP-dependent pathway, glucose metabolism increases the ATP/ADP ratio, which closes the ATP-sensitive K^+ -(K_{ATP}) channels (20). This depolarizes the plasma membrane, voltage-dependent Ca2+-channels open, and cytosolic Ca2+concentrations increase, which is the main trigger of exocytosis (21). The opening of the Ca^{2+} -channels is intermittent, which results in oscillations of Ca^{2+} , which may account for oscillations in insulin secretion (22). The KATP-channel consists of two subunits: SUR1, a high affinity sulfonylurea receptor, and KIR6.2, the pore-forming potassium channel. Both subunits are homotetramers, forming a complex of approximately 950 kDa (23). ATP closes the KATP-channel by binding to KIR6.2, whereas MgADP activates the channel by binding to SUR1 (24). It is debated whether it is the absolute levels of nucleotides or the ATP/ADP ratio that is important for regulation of the channel. Hopkins et al propose that the total levels of nucleotides are critical, since in inside-out patches a stronger inhibition of the channel at high nucleotide levels is observed when compared to low levels, when the ATP/ADP ratio is the same (25). However, in intact or permeabilized cells, stimulation with glucose does not change the levels of total nucleotides to a great extent. Rather, the ATP levels increase with a maximum of $\sim 20\%$ (26; 27), whereas the ADP levels decrease substantially (27; 28) thus creating a significant increase in the ATP/ADP ratio.

Amplifying pathway

In 1992, it was discovered that glucose can control insulin release independent from its actions on the K_{ATP} -channels. By adding the drug diazoxide, which selectively opens K_{ATP} -channels (29), the effect of glucose on these channels were prevented. In

the presence of high concentrations of K^+ , which depolarizes the cells, and diazoxide, glucose can still stimulate insulin secretion in a dose-dependent manner (30; 31). This mechanism requires the triggering pathway; it remains functionally silent until Ca²⁺ has been elevated. The amplifying pathway does not increase the levels of Ca²⁺ further, but serves to amplify the secretory response to glucose. Glucose metabolism is essential, and once Ca²⁺ is elevated, the threshold for stimulation of secretion mediated by the amplifying pathway is much lower, leading to insulin secretion at relatively low glucose levels. The pathways responsible for this mechanism remain unresolved. Nevertheless, as reviewed below, numerous metabolites has been the subject of an intense scientific effort to find the regulatory coupling factors in insulin secretion.



Figure 3. Triggering and amplifying pathways of insulin secretion.

Potential stimulus-secretion coupling factors

Nucleotides

The role of adenine nucleotides in the triggering pathway is well established, but an increased ATP/ADP ratio has also been proposed to mediate effects of the amplifying pathway. Under conditions where the K_{ATP}-channels are bypassed, insulin secretion correlates with the increase in ATP/ADP ratio in presence of stimulatory Ca²⁺-levels (32), whereas in the absence of Ca²⁺, glucose-induced ATP elevation does not stimulate insulin secretion. Also, lowering the ATP/ADP levels with azide, when the Ca²⁺-level remains high, decreases insulin secretion (33). Glucose also increases the GTP/GDP ratio under K_{ATP}-independent conditions, which raises the possibility that these nucleotides might act as coupling factors (34). The reducing equivalent NADPH is another candidate. In beta-cells, it is mostly produced by the mitochondrial shuttles, which are much more active than the pentose phosphate

pathway in these cells. In 1977 it was found that NADPH is taken up by the secretory granules and stimulates insulin secretion in toadfish (35). Later, increases in the NADPH/NADP⁺ ratio in response to glucose (36; 37), and stimulation of exocytosis by addition of intracellular NADPH (37) have been observed in rat islets. This suggests that redox state is important for insulin secretion. A possible mechanism of the NADPH-mediated effect might involve the reductants thioredoxin and glutaredoxin, which use electrons from NADPH to reduce protein disulphide bonds. Several enzymes are suggested to be regulated by the redox state of their thiol groups, including many of the glycolytic enzymes (38). Direct evidence of the involvement of these compounds has recently been presented, where co-administration of glutaredoxin together with NADPH further stimulated the NADPH-induced insulin release. On the contrary, addition of thioredoxin had an inhibitory effect (37).

Glutamate

Glutamate is an amino acid which can be generated from the TCA-cycle intermediate α -ketoglutarate, by the enzyme glutamate dehydrogenase (GDH). Glutamate is proposed to couple mitochondrial metabolism to secretion, since stimulatory glucose concentrations increase the levels of intracellular glutamate, and glutamate potentiates insulin secretion at intermediate glucose levels (39). In capacitance measurements it has also been shown that glutamate stimulates Ca^{2+} -dependent exocytosis (40). Glutamate is thought to mediate a signal downstream of mitochondrial metabolism, and act directly on the secretory vesicles. It is taken up by the granules and primes them for exocytosis. The mechanisms are still unknown, but might involve acidification of the granules (41). The glutamate hypothesis is, however, controversial, since elevation of glutamate levels has also been shown not be coupled to insulin secretion (42; 43). Furthermore, it is debated whether activation of GDH will result in production of glutamate or α -ketoglutarate, the latter being a product of an anaplerotic reaction. In patients with persistent hyperinsulinemia and hyperammonemia in infancy (PHHI), an activating mutation in GDH is associated with enhanced insulin secretion (44). Hyperammonemia suggests that the mutated enzyme produces α -ketoglutarate rather than glutamate.

Malonyl-CoA

Stimulation with glucose decreases fatty acid oxidation and causes a switch from fatty acids to glucose as oxidative fuel. This occurs via increased levels of malonyl-CoA, which inhibits carnitine palmitoyl-transferase 1 (CPT1). Blockage of CPT1 leads to reduced transport of long chain acyl-CoAs into the mitochondria, and the rate of fatty acid oxidation is reduced. Malonyl-CoA and long chain acyl-CoA have been suggested as coupling factors based on the fact that glucose and other secretagogues cause an increase in malonyl-CoA and long chain acyl-CoA potentiates insulin secretion (45;

46). However, this hypothesis has been challenged in recent years. In a study by Mulder et al, it has been shown that overexpression of the enzyme malonyl-CoA decarboxylase, which decarboxylates malonyl-CoA to acetyl-CoA, causes a decrease in malonyl-CoA levels and increased fatty acid oxidation, but has no effect on insulin secretion in clonal INS-1-derived beta-cells (47). Also, inhibition of long chain acyl-CoA synthetase by triacsin C causes a reduction of long chain acyl-CoA, but does not influence insulin secretion (48). Again, similar experiments with overexpression of malonyl-CoA decarboxylase have been performed by Prentki and colleagues (49); they observe negative effects on insulin secretion when malonyl-CoA decarboxylase is overexpressed in the presence of increased concentrations of fatty acids in the medium. Thus, the role of malonyl-CoA in insulin secretion is unresolved, but may depend on the conditions and cell types used.

Signals derived from mitochondrial metabolism

Since it has been proven that metabolism of glucose is essential for the amplifying pathway, mitochondrial metabolism has been the focus of the search for coupling signals. Pyruvate, the end product of glycolysis, is transported into the mitochondria and can enter the TCA-cycle in two ways. Either it enters via carboxylation by pyruvate carboxylase (PC) to oxaloacetate or via decarboxylation by pyruvate dehydrogenase (PDH) to acetyl-CoA. Flux via pyruvate carboxylase generates anaplerosis, which is Greek for filling up, since it fills up the TCA-cycle with carbon intermediates. PC is highly expressed in beta-cells as compared to islet non-beta-cells (50), and ~40% of pyruvate entering the TCA-cycle upon glucose stimulation is carboxylated by PC (51), indicating a critical role of the enzyme. As a matter of fact, inhibition of PC with phenylacetic acid (52) decreases insulin secretion in INS-1 cells and rat islets (53-55). The role of anaplerosis is not fully clarified; however, TCA-cycle intermediates cannot accumulate in the mitochondria without altering mitochondrial function, and this suggests that one purpose of anaplerosis in beta-cells is to export intermediates into the cytosol, where they can provide signaling roles for insulin secretion. In other words: anaplerosis is in equilibrium with cataplerosis, i.e. exit of intermediates from the TCA-cycle. Pyruvate cycling is a proposed model, which is based on the existence of two pools of pyruvate in beta-cells, one feeding acetyl-CoA into the TCA-cycle through PDH, and one exchanging with TCA-cycle intermediates via PC. In clonal beta-cells, it has been shown that only cycling via PC is correlated with insulin secretion (55). Pyruvate cycling is proposed to occur via a pyruvate-malate shuttle (56; 57), a pyruvate-citrate shuttle (53) or a pyruvate-isocitrate shuttle (58). These shuttles all generate NADPH, which might function as a coupling factor (see above). The export of malate increases substantially upon stimulation with glucose (57; 59), and although the levels of citrate do not increase to the same extent, there are several pieces of evidence suggesting that export of citrate is necessary for insulin secretion (45; 53; 60). In a recent study, isocitrate dehydrogenase was inhibited by

RNAi, which caused decreases in insulin secretion, pyruvate cycling and NADPH/NADP⁺-ratio (58). This indicates that the pyruvate-isocitrate shuttle is important for the generation of NADPH and regulates insulin secretion. This is further supported by the finding that inhibition of the mitochondrial citrate/isocitrate carrier also inhibits GSIS (61). Conceivably, cycling through these pathways, and perhaps others yet to be identified, may contribute to the metabolic coupling processes in beta-cells.



Figure 4. Mitochondrial metabolism and cycling pathways in pancreatic beta-cells.

A few fuels other than glucose can also stimulate insulin secretion and their properties can shed some light over the involved pathways.

Leucine: Leucine is the only amino acid which acts as a fuel secretagogue and is about one-third as potent as glucose. It is thought to stimulate insulin secretion in two ways. First, it allosterically activates GDH, hereby enhancing the conversion of glutamate to α -ketoglutarate which fuels the TCA-cycle. Second, it can be transaminated to α -ketoisocaproic acid (KIC), which can be metabolized to acetyl-CoA, hereby providing carbons to the TCA cycle. High concentrations of glucose inhibit the stimulatory effect of leucine, since glucose increases the levels of GTP, an allosteric inhibitor of GDH (62; 63).

 α -ketoisocaproic acid (KIC): KIC is a metabolite of leucine and, interestingly, is considerably more potent as a secretagogue than the amino acid, with a stimulatory effect that equals glucose. It has at least two metabolic fates in beta-cells: Either it is

oxidized to acetyl-CoA, or it generates leucine through transamination with glutamate, a reaction that also produces α -ketoglutarate. The combination of these two pathways might explain the strong potency of this secretagogue (63).

2-amino-2-norbornane-carboxylic acid (BCH): BCH is a non-metabolizable analog of leucine. It is not as potent a secretagogue as leucine, which can be explained by the fact that it only acts allosterically on GDH (64). However, under K_{ATP} -independent conditions, BCH elicits an insulin response stronger than that of glucose, which indicates that stimulation of GDH is sufficient to activate the amplifying pathway (65).

Methyl esters of succinate: The methyl group enables the compound to enter the cell. It is then converted to succinate intracellularly. Succinate is the only TCA-cycle intermediate that stimulates insulin secretion, and the reason for that is not known. A suggestion is that site II of the respiratory chain is of importance (64) since succinate dehydrogenase is part of both the TCA cycle and the respiratory chain. It has also been proposed that succinate generates mevalonate, which could be a trigger of insulin release (66).

Pyruvate/methyl pyruvate: The effects of pyruvate on insulin secretion have been thoroughly investigated for a long time. Addition of pyruvate to islets does not stimulate insulin secretion (67), whereas it acts as a secretagogue in the clonal beta-cell line INS-1 (68). A proposed explanation is that primary beta-cells have a low expression of the plasma membrane monocarboxylate transporter (MCT), and therefore are unable to take up pyruvate. Indeed, overexpression of MCT in islets sensitized them to pyruvate (69). INS-1 cells on the other hand, have a higher expression of MCT, which could explain their responsiveness (70). Stimulation with methyl pyruvate elicits insulin secretion in both islets and clonal beta-cells, probably since the methyl group enables pyruvate to enter the cell independently of MCT.

Glutamine: Glutamine does not stimulate insulin secretion by itself, but potentiates leucine-stimulated secretion. This is most probably due to the activation of GDH by leucine which leads to an increased insulin secretion in excess of glutamine. Interestingly, Fex et al recently found that the amino acid can become a secretagogue on its own; this was observed under K_{ATP} -independent conditions in islets from insulin-resistant C57BL/6J mice which exhibit enhanced mitochondrial metabolism, presumably as an adaptation to overcome insulin resistance (71).

Fatty acids: Fatty acids potentiate GSIS but do not stimulate secretion by themselves (72). While fatty acids are metabolized to acetyl-CoA, they do not provide an anaplerotic input. Since addition of this two-carbon moiety is balanced by the production of two molecules of CO_2 during a round in the TCA cycle; this might explain why fatty acids need glucose to stimulate secretion.

Additionally to these mitochondrial fuels, the glycolytic intermediates/fuels mannose and glyceraldehyde stimulate insulin secretion. Surprisingly, succinate is

the only TCA-cycle intermediate that stimulates insulin secretion. Why the other intermediates are unable to elicit insulin secretion is not clarified, but a possibility is that any secretagogue must be able to generate anaplerosis, via pyruvate or α -ketoglutarate (57).

Biphasic insulin secretion and exocytosis

Insulin secretion stimulated by glucose, and some other secretagogues, exhibits a biphasic pattern, with a rapid first phase that peaks after about five minutes, followed by a nadir and then a sustained second phase, which gradually increases the rate of secretion until it reaches a plateau after 25-30 minutes. Only fuel secretagogues are capable of eliciting the second phase: when non-metabolizable secretagogues, such as high K⁺, are used, only the first phase is seen. Type 2 diabetes is associated with a selective loss of first phase secretion, which develops long before other manifestations of the disease are evident (73). The underlying mechanisms for biphasic insulin secretion are still not known, but it is thought to reflect the release of different pools of secretory granules. Only a small fraction of the granules (~40 granules) belongs to a readily releasable pool (RRP). These granules are primed and can undergo exocytosis without further modifications and independently of ATP. The RRP is proposed to be released during the first phase of insulin secretion (74). The transient property of the first phase would then be explained by the depletion of the RRP. The majority of the granules belongs to a reserve pool that has to go through a series of ATP-, Ca^{2+} -, and temperature-dependent reactions, collectively called priming, to be releasable (74; 75). Priming might also involve physical translocation of the granules to the plasma membrane (76). Since these events are energy-demanding, it could explain the slower onset of the second phase.

When exocytosis is studied in single cells, triggered by application of voltage-clamp depolarization, a biphasic pattern is also seen. However, the time frame of this process is very different from that in intact islets. The first phase lasts for a few hundred milliseconds in a beta-cell, compared with a few minutes in islets. The explanations might be that there is no synchronization of exocytosis in the individual cells within the islet, and that stimulation with exogenous Ca2+ creates a signal that is much stronger than what can be achieved in vivo. The fact that the number of vesicles secreted per cell during the first phase seems to be similar in single cells compared to intact islets, argues that the mechanism is the same (77). Exocytosis can be divided in three phases: docking, priming, and fusion. Docking refers to the attachment of granules to the plasma membrane, where a group of proteins called SNARE proteins are involved. When the granules approach the plasma membrane, the protein VAMP expressed on the surface of the granules forms a complex with the plasma membrane protein SNAP-25. This brings the granules in close contact with the plasma membrane. The granules are then primed, and fusion of the two membranes occurs when cytosolic Ca^{2+} increases (78).

Role of cAMP in insulin secretion

It has long been recognized that cAMP potentiates insulin secretion but its role in glucose-stimulated insulin secretion is debated. cAMP has been shown to increase in response to glucose (79; 80), whereas other studies suggest that cAMP is not necessary for GSIS (81). Recent data support the hypothesis that glucose might amplify its own action by elevating cAMP levels, since overexpression of the cAMP-hydrolyzing enzyme PDE3B decreases GSIS (82). The mechanism by which glucose potentially could regulate the levels of cAMP remains to be elucidated. The main pathway for generation of cAMP is via adenylate cyclase, which in turn is activated by G-protein linked receptors. The ligands for cAMP-mediated signaling in beta-cells are glucagon and the incretins glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1). Incretins are peptide hormones released from the gastrointestinal tract during a meal. They cause the incretin effect: an enhancement of insulin secretion seen after oral glucose intake compared with that elicited by intravenous infusion of glucose. Interestingly, the incretin effect is decreased in type 2 diabetes patients (83), and administration of incretins in excess can partly restore the disturbed first phase (84).

Until recently, the only known signaling pathway for cAMP was via protein kinase A (PKA). PKA is activated by cAMP, which releases the catalytic subunits from the regulatory subunits. In capacitance measurements, it has been shown that cAMP stimulates exocytosis, both dependent (85; 86) and independent of PKA. It is suggested that cAMP increases the release probability for the readily releasable pool, a mechanism independent of PKA, and accelerates the refilling of the pool, which is PKA-dependent (86). The targets for PKA in exocytosis are still unknown. However, PKA is known to phosphorylate L-type calcium channels (87), and its most wellstudied target, hormone-sensitive lipase (HSL), which might be involved in insulin secretion by generating a lipid signal (88; 89). In fact, it was recently shown that HSL is localized to, or in close vicinity of, the secretory granules in beta-cells (90), and mice with a beta-cell-specific knock out of the lipase exhibit a selective loss of first phase insulin secretion (Fex et al, manuscript in preparation). In 2000, a new mediator of cAMP signaling was found, cAMP-GEFII (guanine nucleotide exchange factor) (91). cAMP-GEFII binds to cAMP and Rim2, (Rab3-interacting molecule), which is a putative regulator of vesicle fusion to the plasma membrane. It was shown to be responsible for cAMP-dependent, PKA-independent exocytosis in pancreatic betacells. cAMP-GEFII also binds to SUR1, (91) and interestingly, Eliasson and colleagues report that SUR1-deficient mice lack the PKA-independent component of cAMP-dependent exocytosis (92). They hypothesize that this effect is caused by the inability to acidify the granules, a process that might involve the generation of a protein complex consisting of SUR1, ClC3 chloride channels, Rim2 and cAMP-GEFII.

Enzymes

Pyruvate carboxylase

Pyruvate carboxylase (PC) is a biotin-dependent enzyme localized in the mitochondrial matrix, close to the mitochondrial inner membrane. It catalyzes the reaction:

Pyruvate + HCO₃ + ATP $\leftarrow \rightarrow$ oxaloacetate + ADP + P_i

PC is allosterically activated by acetyl-CoA; the reaction is also dependent on Mg²⁺. PC consists of four identical subunits, of approximately 120 kDa (93), and has a halflife of 4.6 days (94). PC can be inhibited by phenylacetic acid, which is metabolized to phenylacetyl-CoA intracellularly and inhibits PC by competition with acetyl-CoA. PC is expressed in a tissue-specific manner, with the highest expression in the gluconeogenic tissues liver and kidney, in adipose tissue and lactating mammary glands which are all lipogenic tissues, and pancreatic islets. In gluconeogenic tissues, PC constitutes the first step in gluconeogenesis. In lipogenic tissues, its primary role is to generate oxaloacetate and NADPH necessary for lipogenesis (95). Pancreatic islets do not express the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), and is hence unable to synthesize glucose. Their low level of lipogenic activity cannot explain the high expression of PC (0.4% of total protein (96)) and hence there must be another mechanism requiring PC. This is thought to be the coupling of glucose to insulin secretion as previously mentioned.

Pyruvate dehydrogenase complex

Pyruvate dehydrogenase complex (PDC) is a mitochondrial enzyme complex, located in the mitochondrial matrix. It catalyzes the reaction:

Pyruvate + CoA+ NAD+ \rightarrow acetyl-CoA + CO₂ + NADH + H+

PDC consists of three catalytic components: pyruvate dehydrogenase (E1) catalyzing the decarboxylation of pyruvate, dihydrolipoamide acetyltransferase (E2) catalyzing the formation of acetyl-CoA, and dihydrolipoamide dehydrogenase reoxidizing E2 with the consequent reduction of NAD⁺ to NADH (97). PDC is regulated by reversible phosphorylation by pyruvate dehydrogenase phosphatases and pyruvate dehydrogenase kinases. Three serine residues on E1 α can be phosphorylated: serine-264 (site 1), serine-271 (site 2) and serine-203 (site 3) (98). The three sites can be phosphorylated independently, and phosphorylation of each site results in inactivation of PDC.

Pyruvate dehydrogenase kinases

Pyruvate dehydrogenase kinases (PDK) phosphorylate the pyruvate dehydrogenase complex (PDC), which inactivates the enzyme. They are inhibited by pyruvate, the substrate for PDC, and activated by the products of both PDC and fatty acid oxidation, acetyl-CoA and NADH. PDKs consist of two different subunits: α , which has kinase activity, and β , which is a regulatory subunit. There are four isoforms of PDK: 1-4, which are tissue-specifically expressed. The molecular weight of PDK1 is 48 kDa whereas PDK2, PDK3 and PDK4 are 45 kDa proteins. PDK1 has been found in heart (99), pancreatic islets (100), and skeletal muscle (101). PDK2 is ubiquitously expressed (102), PDK3 has only been detected in testis, kidney and brain (102), and PDK4 is expressed in heart, skeletal muscle, liver, kidney and pancreatic islets (99-101). PDKs phosphorylate three serine sites on PDC E1 α . All PDKs can phosphorylate site 1 and site 2, whereas PDK1 uniquely phosphorylates site 3 (98; 103).

Pyruvate dehydrogenase phosphatases

Pyruvate dehydrogenase phosphatatases (PDP) dephosphorylates PDC which activates the complex. The reaction is dependent on Mg²⁺ and stimulated by Ca²⁺ (104). PDP is a heterodimer consisting of a regulatory and a catalytic subunit. Two isoforms of PDP has been identified, PDP1 and PDP2. PDP1 is the dominant isoform in heart, and is also found in skeletal muscle. PDP2 is found in liver, kidney, heart and adipose tissue (105). Both PDPs dephosphorylate all three phosphorylation sites. Interestingly, insulin increases the activity of PDPs through protein kinase C δ (106).

Protein kinase A

Protein kinase A (PKA) is also known as cAMP-dependent kinase (cAPK). PKA is a heterotetramer, which consists of two subunits: the 49 kDa regulatory subunit which has high affinity for cAMP, and the 38 kDa catalytic subunit. In the inactive state, the two subunits form a complex of two catalytic and two regulatory subunits. When cAMP binds to the regulatory subunits, it results in dissociation of the complex, and the free catalytic subunits then become enzymatically active. There are at least four types of regulatory subunits, RI α , RI β , RII α and RII β , and three catalytic subunits: C α , C β and C γ , and they are expressed in a tissue-specific manner. All three catalytic isoforms are expressed in beta-cells. C α translocates from the cytosol to the nucleus upon glucose stimulation, whereas C β translocates from the cytosol to the plasma membrane, suggesting different substrates and roles for the individual isoforms (107); the role of C γ is less clear.



Figure 5. Regulation of the PDC complex. Adapted from Sugden et al 2003 (104).

GENERAL METHODS

RNA interference

RNA interference (RNAi) or RNA silencing is a process whereby small doublestranded RNA oligonucleotides induce homology-dependent degradation of target mRNA, causing a knockdown of the translated protein. These RNAs are fragments of 21-23 nucleotides and referred to as small interfering RNA (siRNA). The process was first discovered in nematodes in 1998 by Fire at al (108), but similar phenomena, e.g., post-transcriptional gene silencing (PTGS) of viral genes, have been observed in plants and fungi for many years. RNAi was subsequently observed in insects and in mammals, such as mice and humans (109-111). In nematodes, a full-length dsRNA was introduced into the cells, and provoked RNAi. Although this procedure causes knockdown also in mammals, it concurrently activates an anti-viral interferon response that leads to inhibition of all protein synthesis. Therefore, siRNAs owing to their small size which does not cause this side effect is used in mammals (109). The natural function of RNAi is probably protection of the genome against transposable elements, e.g. virus or transposons, which produce foreign RNA or dsRNA in the host cell. Upon the discovery that RNAi also occurs in mammals, it has been the focus of intense research, which has led to a more detailed knowledge of the mechanism.

The first step in the RNAi mechanism is the processing of long double-stranded RNA into the small 21-23 nucleotide long siRNAs. An RNase III enzyme called dicer cleaves the RNA in a specific way, generating a 3' overhang of two nucleotides on each strand (112). The siRNA then binds to a multiprotein complex called RISC (RNA-induced silencing complex) (113). RISC, or a protein associated with it, unwinds the siRNA, and one strand then guides RISC to the target mRNA which is cleaved by RISC nuclease activity.

siRNA can be transfected into cells using lipid-mediated transfection, electroporation or microinjection. The advantages with this method are that one can precisely choose the amount of siRNA added, and the siRNAs can be introduced without requiring any modifications. The drawbacks are that many cell types are difficult to transfect efficiently. A low transfection efficiency will prevent detectable knock-down even when an effective siRNA has been designed. In the infancy of the technique, choosing an efficacious sequence for RNAi was somewhat of a gamble. Now, a number of very efficient algorithms exist, most of which ensure that 2 out 3 designed sequences will confer 80-95% knockdown of the chosen protein.



Figure 6. Mechanism of RNAi. The scissors symbolize enzymatic cleavage of RNA.

siRNA can also be expressed from plasmid vectors. This allows both transient and stable transfection, which permit analysis over shorter and longer time periods. The plasmid then contains a short hairpin sequence, with the sense and antisense strand of an siRNA, linked with a loop sequence. When transcribed in the cell, the RNA base pairs to form a short hairpin RNA (114). The loop is then cleaved off by dicer, creating a proper siRNA. Instead of placing the short hairpin sequence in a plasmid, it can be expressed in viral vectors, which offer the possibility to transfect virtually all mammalian cell types. The drawbacks are that it is more complicated to regulate the amount of RNA transcribed in the cell and that it is a time-consuming process to create a virus.

Adenoviral vectors

Recombinant adenoviruses are very useful tools to express proteins in mammalian cells. Adenoviral vectors have the advantages that they infect both dividing and nondividing cells, and can express relatively large DNA inserts (up to 7 kb). It is feasible to propagate high titer viral stocks, and the virus has a broad range of infectivity in mammalian cells.

Wild-type adenovirus is an icosahedral particle with a double-stranded DNA genome of 30-40 kb (115). There are several different serotypes, but the human types 2 and 5 (Ad2 and Ad5) have been most extensively used as cell biological tools. The primary virus attachment to the target cell is mediated via protruding fibers from the virus capsid, which bind to the coxsackie and adenovirus receptor (CAR) on the cell membrane (116). The virus is then taken up by clathrin-dependent receptor-mediated endocytosis. The virus life-cycle can be divided into an early and a late phase, which are defined as before and after onset of viral DNA replication. The genes expressed in the early phase trigger the DNA replication.

Generation of adenoviral vectors include deletion of parts of the early genes to allow packing of large DNA inserts, normally the gene regions E1 or E3 (117). If E1 is deleted, the virus is unable to replicate, and the function of E1 must be provided *in trans.* This is accomplished by culturing the virus in the human HEK 293 cell line, which has been transformed with the Ad5 and contains 14% of the adenoviral genome, including the E1 region, incorporated in the nuclear DNA.

The method used in this thesis to create adenoviral vectors is based on the viral plasmid pJM17 (118). It consists of a full-length Ad5 genome where the E1 region is interrupted by the insertion of the bacterial plasmid pBRX. Thereby, the packaging limit for an adenovirus is exceeded, and pJM17 can not give rise to any viral particles. To insert a gene into pJM17, we used the shuttle plasmid pACCMV (119), which contains Ad5 sequences on both sides of the multiple cloning site, into which the gene of interest is cloned. Homologous recombination of pACCMV and pJM17 in 293 cells generates a viral genome with packageable size, where the E1 region is replaced by the same reason, the replication-defective recombinant adenovirus can be amplified in 293 cells, and then used to transfect other cell lines, in which the virus cannot replicate, just express the recombinant gene.



Recombinant adenovirus

Figure 7. Creation of a recombinant adenovirus using pJM17 and pACCMV.

Clonal beta-cells

The cells I have used for my thesis are INS-1 derived cell lines called 832. INS-1 cells are clonal beta-cells, originating from an x-ray-induced rat insulinoma. INS-1 cells have a high insulin content and are responsive to glucose in the physiological range. Their secretory response is about 2- to 4-fold when stimulated with glucose (120). However, this fold response is much lower than what is seen in isolated islets, which

exhibit a more than 10-fold secretory response to increased concentrations of glucose. In order to optimize the INS-1 cells, and investigate whether the cell line might have subpopulations with a stronger secretory response, Newgard and colleagues performed a stable transfection of INS-1 cells with a plasmid containing neomycin and the human insulin gene (121). Different cell clones were obtained and screened by measuring fold increase in insulin secretion at 3 and 15 mM glucose. Both poorly responsive and strongly responsive clones were identified. The clone 832/2 was poorly responsive, and is used for studies of defective beta-cells. The clone 832/13 secreted the greatest quantities of insulin, with a secretory response of 8- to 11-fold and exhibited a more prominent amplifying pathway than the parental INS-1 cells. Therefore this clone was further studied. I have used 832/13 cells for all my secretory experiments except in paper III, where I have compared the properties of 832/13 and 832/2.

There are several other beta-cell lines used, and among them, the INS-1-derived INS-1E has the most physiological properties. INS-1E is an isolated clone from INS-1, selected for its insulin content and secretory response to glucose which is 4- to 6-fold. This cell line also exhibits the amplifying pathway and is more stable than the parental INS-1 (122).

Patch-clamp

Patch-clamp, where the electrophysiologic properties of a single cell can be studied, was invented in 1978 by Neher (123). The patch-clamp technique can measure three different conditions: membrane potential (current-clamp mode), currents passing over the cell membrane and exocytosis monitored as increases in membrane capacitance (voltage-clamp mode). There are four different configurations in patch-clamp and in this thesis, two of them have been used, the standard whole-cell configuration and perforated-patch. In the whole-cell configuration, a heat-polished glass pipette is pressed against the cell membrane. By applying gentle suction, a high-resistance seal called giga seal is formed. The plasma membrane enclosed by the pipette is then ruptured, and the pipette solution infuses the cell replacing the cytosol. This allows control over the intracellular environment, and substances of interest can easily be added to the cytosol. The wash-out of the cytosol can however lead to removal of important factors. This problem is circumvented in the **perforated-patch**, where the pore-forming antibiotic amphotericine B is added to the pipette solution and electrical contact is achieved through small pores in the patch membrane. In this configuration, measurements of metabolically intact cells are made possible, since the pores only allow monovalent ions and very small molecules to pass through.

Low density array

Low density array (LDA) is a 384-well micro fluidic card that makes it possible to perform 384 simultaneous real-time PCR reactions. There are several different configurations of the micro fluidic card, which allow 11 to 380 different genes preloaded on the card, depending on how many replicates are required. This array is very useful as a screening tool, when one has a limited amount of candidate genes and do not need the overwhelming amount of data generated by a micro array. It is also very reproducible compared to micro array, which makes it more reliable, and the quantification is very robust. However, in contrast to micro arrays, novel genes and changes in genes not anticipated will not be revealed. I have designed an LDA which contains almost all genes involved in glucose metabolism, altogether 46 different genes, and analyzed the effect of high concentrations of glucose on their expression pattern.

AIMS

- To investigate the role of anaplerosis and pyruvate carboxylase in insulin secretion
- To identify genes responsible for the impaired insulin secretion seen at high glucose concentrations
- To characterize the molecular mechanisms underlying the insulin response in the highly glucose-responsive cell line INS-1 832/13 compared to the poorly responsive cell line INS-1 832/2

PRESENT INVESTIGATIONS

Role of pyruvate carboxylase in insulin secretion

In paper I, we aimed to investigate the role of anaplerosis via pyruvate carboxylase (PC) in insulin secretion by inhibiting the enzyme in rat pancreatic islets with the pharmacological inhibitor phenylacetic acid (PAA). It has previously been shown that inhibition of PC with PAA decreases insulin secretion (53). To study in more detail how inhibition of PC influences insulin secretion, we analyzed insulin secretion under both K_{ATP}-dependent and -independent conditions, and found that PAA decreased secretion under both circumstances. This shows that the actions of PC and anaplerosis involve K_{ATP}-independent processes. We also performed perifusions of islets to study the dynamics of insulin secretion, and found that both the first and the second phase were equally inhibited by PAA. For these studies, we used glucose as secretagogue. Other secretagogues might exert their actions via other pathways, and to examine that, we stimulated with two other fuel secretagogues, α -ketoisocaproic acid and methyl succinate. Interestingly, insulin secretion stimulated by α -ketoisocaproic acid was inhibited to the same extent as was seen with glucose, whereas secretion stimulated by methyl succinate was unaffected by the inhibition of PC.



Figure 8. PAA inhibits insulin secretion during both first and second phase of insulin secretion. Islets were perifused for 10 min with 2.8 mM, followed by 30 min with 16.7 mM glucose, in the presence (open squares) or absence (filled diamonds) of PAA. Then glucose concentration was lowered to 2.8 mM.

To investigate the underlying mechanisms for the decrease in insulin secretion, we analyzed the effect on several metabolic parameters, such as glucose oxidation, oxygen consumption and mitochondrial membrane potential, and they were all unaffected by inhibition of PC. Since the ATP/ADP ratio is thought to trigger the initiation of the first phase, which we saw was decreased by PAA, we also measured adenine nucleotide levels. The glucose-induced rise in the ATP/ADP ratio significantly decreased in presence of PAA. We also studied the effect of PAA on exocytosis, and found that it had no direct effect. K_{ATP}-channel activity was increased in the presence

of PAA in metabolically active cells, which can be attributed to the decreased ATP/ADP ratio.

The reduction in ATP/ADP ratio was mainly caused by elevated ADP levels at high glucose in the presence of PAA. It is debated whether the ratio or levels of ATP or ADP are most important for regulation of the K_{ATP}-channels. Our results point to the importance of ADP, rather than ATP, which did not change to the same extent. Recently, pyruvate carboxylase was knocked down with RNAi, and surprisingly, this did not affect insulin secretion (124). This was explained by compensatory mechanisms, but the question is whether PC was actually knocked down to the extent that its activity was reduced. I have tried to knock down PC, and even though I achieved more than a 70% reduction at mRNA level, the protein levels were unaffected. Maybe a reduction of protein levels with 50% still leaves enough enzyme for normal function. In fact, PC has been estimated to constitute as much as 0.4% of the total protein content in beta-cells (96).

Our results indicate that anaplerosis via pyruvate carboxylase is important for both the first and the second phase of insulin secretion, and has implications for the triggering as well as the amplifying pathway. We propose that this is mediated via an increased ATP/ADP ratio, which previously has been suggested to be involved in both pathways (32).

Knock-down of PDK1 and its role for insulin secretion

In paper II, we first established a model for hyperglycemia, where we cultured 832/13 cells in high or low concentrations of glucose for 48 h. This should resemble the conditions in diabetes, where blood glucose is high. The cells cultured at high glucose decreased their insulin response to glucose with approximately 50% compared to cells cultured at low glucose concentrations. We then wanted to identify genes whose expression pattern was influenced by culture in high glucose, and for that purpose, we designed a low density array. We chose to analyze the expression of almost all genes involved in glucose metabolism, since this is pivotal for insulin secretion. We analyzed the expression of 46 genes, and found six that were differently expressed following culture in high glucose compared to low glucose. Of these six, we found the pyruvate dehydrogenase kinases (PDKs) to be of particular interest, since PDK1 was upregulated in high glucose whereas PDK2 was downregulated. We hypothesized that the enhanced expression of PDK1 at high glucose levels might be involved in the metabolic disturbance underlying impaired GSIS. In fact, a mutation causing enhanced activity of the transcription factor HIF-1a, which induces PDK1 expression (125; 126), has been found to be a risk factor for type 2 diabetes (127). Therefore we investigated the effect of knock-down of PDK1 with RNAi. Treatment with siRNA decreased the PDK1 mRNA levels with 80%, and a clear decrease was also seen in PDK protein expression. To our satisfaction, we found that insulin secretion after

| TCA cycle | Uncoupling protein-2 | Glycerol-3-phosphate dehydrogenase 2 |
|--|--------------------------------------|---|
| Citrate synthase | Electron transport | Hexokinase 1 |
| Fumarate hydratase | ATP citrate lyase | Lactate dehydrogenase A |
| Isocitrate dehydrogenase 3 (NAD+) | ATP synthase epsilon | Malic enzyme 1 |
| malate dehydrogenase 1 NAD | ATP synthase subunit d | Phosphofructo kinase |
| malate dehydrogenase <i>mitochondrial</i> | Cytochrome c <i>somatic</i> | Phosphoglycerate kinase 1 |
| Succinate-CoA ligase | Cytochrome c oxidase subunit IV | Phosphoglycerate mutase 1 |
| succinate dehydrogenase subunit A | NAD(P)H dehydrogenase quionone 1 | Phosphoglycerate mutase 2 |
| Miscellaneous | NADH dehydrogenase 1 alpha | Pyruvate carboxylase |
| Acetyl-CoA carboxylase | Glycolysis and related | Pyruvate dehydrogenase kinase 1 |
| Acetyl-CoA oxidase | Aconitase2 | Pyruvate dehydrogenase kinase 2 |
| AMP-activated protein kinase | Aldolase b | Pyruvate dehydrogenase kinase 4 |
| Aquaporin 3 | Enolase | Pyruvate kinase |
| Carnitine palmitoyltransferase 1 | Glucokinase | Triose phosphate isomerase |
| Diacylglycerol O-acyltransferase 1 | Glucose transporter 2 | Controls |
| Fatty acid synthase | Glucose-6 phosphatase | HPRT |
| Glutamate dehydrogenase | Glucose-6-phosphate dehydrogenase | 185 |
| | | |

Table 1. List of genes analyzed in the LDA. Out of these, six genes were differently expressed in high versus low glucose (green in the list). Eight genes were not detected (red in the list).

knock-down of PDK1 increased by 35% when stimulated with 16.7 mM glucose. In contrast, insulin secretion provoked by other fuels, such as leucine and succinate, was not potentiated by knock-down of PDK1.

To investigate the mechanisms for the increased secretion, we examined glucose oxidation, since an activation of PDC might result in an increased TCA-cycle flux. This was not the case, since glucose oxidation was unchanged after knock-down of PDK1. We also analyzed the ATP/ADP ratio, which was unchanged as well. We therefore suggest that the increased secretion is due to increased cataplerosis and pyruvate cycling, where citrate leaves the mitochondria. Such a cycling pathway has previously been proposed by Prentki and colleagues (53). This would neither increase glucose oxidation, nor give rise to ATP production. Instead, pyruvate cycling would

increase the generation of NADPH, which is a potential coupling factor. The fact that insulin secretion stimulated by leucine or succinate was unaffected by knock-down of PDK1 supports this hypothesis, since these two fuels would enter the TCA-cycle distal to the presumed exit of citrate, and not be contributing to enhanced cycling.

In conclusion, we have found that knock-down of PDK1 increases insulin secretion, and our data suggest that this is due to increased cycling from citrate to pyruvate. Considering that impaired insulin secretion is a major cause of type 2 diabetes, an inhibitor of PDK1, which would stimulate insulin secretion, could be a potential diabetic drug.



Figure 9. Knock-down of PDK1. A shows the levels of PDK1 mRNA relative to the control gene HPRT in control and siRNA-treated cells. B shows insulin secretion after knock-down of PDK1 when stimulated with glucose, leucine and succinate. Black bars = siRNA-treated cells, grey bars = control.

PKA signaling in 832 cells

In paper III, we have taken advantage of the fact that different subclones of 832 lines exhibit a remarkable difference in terms of secretory performance (121). This raised the possibility that differences between two subclones could be accounted for by one or a few distinct genetic and molecular alterations. To identify such changes, we investigated differences between a highly and a poorly responsive beta-cell line (832/13 and 832/2, respectively). Indeed, glucose increased insulin secretion 8-fold in 832/13 cells, whereas 832/2 cells responded less than 1.5-fold. However, insulin content was equal in both cell lines, suggesting that the difference in secretion was due to altered stimulus-secretion coupling. We found that exocytosis was less efficient in 832/2 cells, and that 832/13 cells were much more sensitive to stimulation with cAMP-elevating drugs. Therefore we further investigated the role of cAMP and analyzed the levels of the nucleotide. Interestingly, the levels of cAMP did not change much in 832/13 cells when stimulating with glucose or forskolin, an agent that increases cAMP levels by stimulating adenylate cyclase. On the contrary, cAMP levels increased dramatically in 832/2 cells in response to forskolin. This suggests that

832/13 cells are more sensitive to cAMP, which was further supported in single-cell exocytosis experiments with caged cAMP. When cAMP was caged, exocytosis was equal in both cell lines, but after release of cAMP, 832/13 cells increased exocytosis by 60%, whereas 832/2 cells did not respond to cAMP.

Since cAMP exerts many of its actions via PKA, we analyzed the expression of PKA in the two cell lines. The catalytic subunit of PKA was 2-fold lower expressed in 832/2 cells, which might explain their poor responsiveness to cAMP. To further confirm the importance of PKA, we created an adenovirus expressing the regulatory subunit of PKA (PKA_{reg}). When the regulatory subunits bind to the catalytic subunits, the enzyme is inactive. Overexpression of PKAreg would then hinder the release of the catalytic subunits and thereby abrogate PKA activity. We saw that transduction of 832/13 cells with the PKA_{reg}-expressing adenovirus increased the expression of the protein, and decreased PKA activity. Also, decreased PKA activity lead to impaired insulin secretion in 832/13 cells, both during stimulation with glucose and forskolin. This was further corroborated by use of Rp-8-Br-cAMPS, an inhibitor of PKA, which strongly inhibited both glucose- and forskolin-induced insulin secretion. However, even though we saw a clear decrease of insulin secretion when cAMP/PKA signaling was inhibited, we could not detect any effect of glucose on cAMP levels. There are previous studies that have detected effects of glucose on cAMP (80), and those who have not seen any difference (81; 128). A possible explanation for these variable data might be that cAMP levels only increase in distinct compartments, an increase that is not detected when measurements of a whole-cell extract is performed (82; 129).

Our data show that one defect in the poorly insulin responsive beta-cell line 832/2is caused by inappropriate PKA signaling, and by decreasing the activity of PKA in highly responsive cells, secretion is decreased. The study also points out the importance of cAMP for exocytosis, and adds new evidence supporting the hypothesis of glucose-regulated cAMP-mediated insulin secretion. We further show that activation of PKA by glucose is important for insulin secretion. The mechanisms for PKA-induced insulin secretion are not clear. However, recent data suggest that PKA stimulates insulin secretion via phosphorylation of IP3-receptors on the endoplasmatic reticulum, causing an increase of intracellular Ca²⁺. The increase would then activate ATP production and cause closure of the KATP-channels (130). This goes well with the hypothesis that PKA mainly regulates the KATP-dependent pathway, proposed by Thams et al (131). It is however contradictory to the findings of Eliasson et al (92), where they show that PKA-dependent insulin secretion accounts for the later part of exocytosis, claimed to be regulated by the amplifying pathway. Whether the effect we have seen on insulin secretion when PKA activity was lowered was due to decreases in the triggering or amplifying pathways needs to be further elucidated. The brief triggering pathway can be difficult to detect during a prolonged 1 h incubation, which allows us to speculate that the inhibition we have found mainly affects the amplifying pathway.



Figure 10. Effects of cAMP/PKA on insulin secretion. A shows exocytosis with caged cAMP. The arrows indicate where cAMP is released. B shows insulin secretion in 832/13 cells transfected with the PKA_{reg} -overexpressing adenovirus.

MAJOR CONCLUSIONS

- Anaplerosis via pyruvate carboxylase is important for both the first and second phase of insulin secretion, most likely by increasing the ATP/ADP ratio
- PDK1 is upregulated in cells cultured in high concentrations of glucose when insulin secretion is impaired, and knock-down of the protein increases insulin secretion
- Insulin secretion in the poorly responsive cell line INS-1 832/2 is at least partly caused by defective PKA signaling, and decreased PKA activity impairs insulin secretion in the highly responsive cell line INS-1 832/13

POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en sjukdom som kännetecknas av för hög blodsockerhalt. Blodsockret regleras av hormonet insulin som utsöndras från de Langerhanska öarna i bukspottkörteln. Insulin sänker blodsockerhalten genom att påverka muskler och fett att ta upp socker, och hindra levern från att producera socker.

Det finns två typer av diabetes; typ 1 och typ 2. Typ 1 diabetes beror på att kroppens immunförsvar bryter ner de celler som producerar insulin (betacellerna), så att det till slut inte kan produceras något insulin. Typ 2 diabetes har två huvudorsaker: insulinresistens och defekta betaceller. Insulinresistens innebär att framför allt musklerna inte reagerar lika bra på insulin som normalt; de tar inte längre upp socker så effektivt. Det beror oftast på att man är överviktig, och kan bero på att det har samlats fett i musklerna. Fettet hindrar signalen från insulin att gå vidare i muskelcellen, och då krävs det mycket mer insulin för att sockerupptaget ska fungera. För att kompensera för insulinresistens producerar betacellerna mer insulin, och om man har normala betaceller fungerar det tillfredsställande. Det är därför många överviktiga aldrig drabbas av diabetes. Om man däremot har betaceller som har någon defekt, som kanske bara märks när cellerna går på högvarv, klarar betacellerna till slut inte av att producera så mycket insulin som krävs för att kompensera för insulinresistensen. Då höjs blodsockret och man får diabetes.

Orsaken till att betaceller hos vissa individer inte fungerar optimalt vet man oftast inte. För att kunna förstå vilka mekanismer som kan gå fel, måste man först veta hur betacellen och insulinutsöndringen fungerar i normala fall. När blodsockerhalten är hög tar betacellen upp socker och bryter ned det. Därigenom bildas många ämnen som kan fungera som signaler som får betacellen att utsöndra insulin. En del ämnen vet man är viktiga, till exempel den energirika molekylen ATP och metalljonen Ca²⁺, medan man är osäker på funktionen av många andra, och vissa signaler vet man väldigt lite om. I min avhandling har jag försökt identifiera några av dessa signaler, hur de produceras och regleras, samt vilken roll de spelar i insulinutsöndringen.

I delarbete I har vi tittat på ett specifikt sätt som socker kan brytas ner på. När socker brutits ner till pyruvat, finns det två olika vägar för fortsatt nedbrytning. Den första vägen är den vanligaste i de flesta celler, medan den andra bara förekommer i vissa celltyper. Denna specifika andra väg kallas anapleros, vilket är grekiska och betyder "fylla på". I de flesta celltyper där anapleros finns används den för att bilda fett eller socker, men betaceller har inte den förmågan, och därför tror man att anapleros har en annan betydelse i betaceller, förslagsvis att påverka insulinsekretion. För att studera anapleros har vi blockerat den, och sedan undersökt vilka konsekvenser det får för insulinutsöndringen. Den halverades, vilket visar att anapleros har stor betydelse för insulinutsöndringen. Vi ville också försöka förstå på vilket sätt anapleros påverkar insulinutsöndringen. Därför undersökte vi bland annat slutsteget där insulin lämnar betacellen från cellytan, och det var inte påverkat av blockeringen. Alltså måste det vara något inne i cellen som påverkas. Vi mätte också mängden av den energirika molekylen ATP, och dess inte lika energirika föregångare ADP, och såg att förhållandet mellan dessa två var förändrat. Detta förhållande har man länge vetat är viktigt för insulinutsöndringen, men att anapleros påverkar det är vi de första som visar.

I delarbete II började vi med att studera betaceller som odlats i höga sockerkoncentrationer. Man kan se experimentet som ett sätt att efterlikna diabetes, där celler också utsätts för höga sockernivåer. Vi såg att betaceller som odlats i hög sockerkoncentration utsöndrade mycket mindre insulin än betaceller som var odlade i låg sockerkoncentration. Orsaken till detta kan vara att socker påverkar produktionen av vissa proteiner: en del proteiner ökar i mängd i närvaro av socker och andra minskar. För att försöka hitta svaret på varför socker påverkar insulinutsöndringen negativt, ville vi undersöka dess påverkan på proteiner. Vi upptäckte ett protein som fanns i mycket större mängd i cellerna som odlats i hög sockerkoncentration. Proteinet är ett enzym, dvs. ett protein som kan driva reaktioner i cellen utan att själv påverkas, och det reglerar den första vägen som socker kan brytas ner på. Proteinet kallas PDK1, och minskar flödet genom den första vägen. Eftersom det fanns i stor mängd när insulinutsöndringen fungerade dåligt, undrade vi vad som skulle hända med insulinutsöndringen om man sänkte nivåerna av PDK1. Vi upptäckte att om man minskar mängden PDK1 så ökar insulinutsöndringen, inte bara i celler som odlats i höga sockerkoncentrationer, utan också i celler som odlats helt normalt. På lång sikt när mekanismen är mer utredd skulle detta kunna användas som ett läkemedel mot diabetes, eftersom det kan öka insulinutsöndringen, som ju inte är tillräcklig i typ 2 diabetes.

I delarbete III har vi jämfört två olika varianter av betaceller, en sort som utsöndrar mycket insulin när man stimulerar dem med socker, och en sort som nästan inte utsöndrar något insulin alls. Genom att jämföra dem kan man hitta mekanismer som kan förklara varför en del betaceller fungerar sämre än andra. Vi såg att mängden av ett annat enzym, PKA, var större i de välfungerande cellerna. PKA är inblandat i en signalväg som har varit mycket debatterad. Vissa påstår att den är viktig för insulinutsöndring, medan andra hävdar att den inte har någon betydelse. Vi sänkte aktiviteten av PKA i de välfungerande cellerna och såg att insulinutsöndringen minskade när vi stimulerade med socker. Den minskade nivån av PKA skulle kunna vara en av anledningarna till att de dåliga cellerna inte fungerar. Vi visade också att PKA verkar ha betydelse för insulinutsöndringen.

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