Mitochondrial signals and protein phosphorylation in nutrient-stimulated insulin secretion

AKHMEDOV, Dmitry

Abstract
Les cellules β pancréatiques synthétisent l'insuline et sécrètent cette hormone en réponse à des sécrétagogues qui sont ou ne sont pas des nutriments. Dans la cellule β, la fonction mitochondriale est cruciale pour la sécrétion d'insuline dépendante des nutriments. La stimulation due aux nutriments active le métabolisme oxidatif mitochondrial. La production et l'exportation accrues de métabolites mitochondriaux est nécessaire pour la génération de facteurs couplés cytosoliques requis pour le maintien de la sécrétion d'insuline. Les mécanismes induisant l'activation mitochondriale de la cellule β restent inconnus. Cette thèse se focalise sur la régulation de la fonction mitochondriale durant la sécrétion d'insuline stimulée par les nutriments dans la cellule β. Il y est décrit quatre projets sur l'étude de différents aspects de la régulation mitochondriale: la phosphorylation de protéines mitochondriales, le signal calcique mitochondrial et le pH de la matrice mitochondriale.

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Mitochondrial signals and protein phosphorylation in nutrient-stimulated insulin secretion

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SUMMARY

Pancreatic β-cells synthesize insulin and secrete the hormone in response to nutrient and non-nutrient secretagogues. In the β-cell mitochondrial function is crucial for nutrient-stimulated insulin secretion. Nutrient stimulation activates mitochondrial oxidative metabolism. The enhanced production and export of mitochondrial metabolites is necessary for the generation of cytosolic coupling factors required for sustained insulin secretion. The mechanisms mediating mitochondrial activation in the β-cell remain elusive.

This thesis focuses on the regulation of mitochondrial function during nutrient-stimulated insulin secretion in the β-cell. It describes four projects for the study of different aspects of mitochondrial regulation: mitochondrial protein phosphorylation, mitochondrial calcium signaling and mitochondrial matrix pH.

Project I was designed to detect and identify mitochondrial proteins regulated by phosphorylation. We observed a large number of potential mitochondrial phosphoproteins following separation of mitochondrial proteins on 1D and 2D gels. Importantly, many of these phosphoproteins are likely to be resident mitochondrial proteins. One of the putative phosphoproteins was identified as subunit α of ATP synthase by mass spectrometry. Taken together, these findings suggest that protein phosphorylation likely plays a critical role in the regulation of mitochondrial function in the β-cell.

Project II focused on the regulation of pyruvate dehydrogenase (PDH) by phosphorylation during acute and chronic exposure of INS-1E cells to glucose. We find that glucose stimulation increases phosphorylation of the PDH E1α subunit in a time- and concentration-dependent manner decreasing enzyme activity. The observed phosphorylation of PDH E1α was mainly mediated by pyruvate dehydrogenase kinases PDK1 and PDK3. PDH E1α phosphorylation did neither affect glucose-stimulated insulin secretion nor cell survival following culture in either standard or high glucose conditions.

Project III addressed the role of the calcium-binding protein S100A1 in ATP production and mitochondrial calcium handling in INS-1E cells. We demonstrate that S100A1 is localized to the cytosol in this cell type. S100A1 overexpression in the cytosol or mitochondrial matrix did neither affect mitochondrial calcium signaling nor glucose-induced ATP production. These results demonstrate that the S100A1 protein does not play a role as a mitochondrial calcium sensor in INS-1E cells.
Project IV was aimed at studying the role of mitochondrial matrix pH alkalinization in metabolism-secretion coupling in the β-cell. We find that alkalinization of the matrix pH is required for the nutrient-dependent increase of mitochondrial respiration, ATP production and glucose-stimulated insulin secretion in INS-1E cells. These results show that mitochondrial matrix alkalinization is an important signal mediating mitochondria activation during nutrient stimulation.

Taken together, the overall results obtained further strengthen the crucial role of mitochondrial signals in β-cell metabolism-secretion coupling.
RESUME

Les cellules β pancréatiques synthétisent l’insuline et secrètent cette hormone en réponse à des sécrétagogues qui sont ou ne sont pas des nutriments. Dans la cellule β, la fonction mitochondriale est cruciale pour la sécrétion d’insuline dépendante des nutriments. La stimulation due aux nutriments active le métabolisme oxidatif mitochondrial. La production et l’exportation accrues de métabolites mitochondriaux est nécessaire pour la génération de facteurs couplés cytosoliques requis pour le maintien de la sécrétion d’insuline. Les mécanismes induisant l’activation mitochondriale de la cellule β restent inconnus.

Cette thèse se focalise sur la régulation de la fonction mitochondriale durant la sécrétion d’insuline stimulée par les nutriments dans la cellule β. Il y est décrit quatre projets sur l’étude de différents aspects de la régulation mitochondriale: la phosphorylation de protéines mitochondriales, le signal calcique mitochondrial et le pH de la matrice mitochondriale.

Le projet I fut prévu pour détecter et identifier les protéines mitochondriales régulées par la phosphorylation. Nous avons observé un large nombre de phosphoprotéines mitochondriales potentielles après séparation des protéines mitochondriales en gels 1D et 2D. Il est à noter que beaucoup de ces phosphoprotéines sont vraisemblablement des protéines mitochondriales à demeure. Une des phosphoprotéines potentielles fut identifiée comme la sous-unité α de l’ATP synthase par spectrométrie de masse. Ces découvertes suggèrent que la phosphorylation protéique joue probablement un rôle clé dans la régulation de la fonction mitochondriale au sein de la cellule β.

Le projet II se focalise sur la régulation de pyruvate déshydrogénase (PDH) par la phosphorylation durant l’exposition aiguë et chronique des cellules INS-1E au glucose. Nous avons découvert que la stimulation glucidique augmente la phosphorylation de la sous-unité PDH E1α en dépendant du temps et de la concentration, ce qui diminue l’activité enzymatique. La phosphorylation observée de PDH E1α est principalement modulée par pyruvate déshydrogénase kinases PDK1 et PDK3. La phosphorylation de PDH E1α n’affecte ni la sécrétion d’insuline stimulée par le glucose, ni la survie cellulaire après culture dans des conditions standards ou élevées de glucose.

Le projet III s’est concentré sur le rôle de la protéine liée au calcium S100A1 dans la production d’ATP et l’utilisation du calcium mitochondrial dans les cellules INS-1E. Nous avons démontré que S100A1 est localisée dans le cytosol en ce qui concerne cette lignée
La surexpression de S100A1 dans le cytosol ou la matrice mitochondriale n’affecte ni le signal calcique mitochondrial ni la production d’ATP induite par le glucose. Ces résultats démontrent que la protéine S100A1 ne joue pas de rôle comme senseur calcique mitochondrial dans les cellules INS-1E.

Le projet IV cibla l’étude du rôle de l’alcalinisation du pH de la matrice mitochondriale dans le couplage métabolisme-sécrétion au sein de la cellule β. Nous avons trouvé que l’alcalinisation du pH de la matrice est requise pour l’augmentation dépendante des nutriments de la respiration mitochondriale, de la production d’ATP et de la sécrétion d’insuline stimulée par le glucose dans les cellules INS-1E. Ces résultats montrent que l’alcalinisation de la matrice mitochondriale est un signal important de modulation de l’activation mitochondriale durant la stimulation par les nutriments.

L’ensemble des résultats obtenus renforce le rôle essentiel des signaux mitochondriaux dans le couplage métabolisme-sécrétion de la cellule β.
ABBREVIATIONS

ADP – adenosine diphosphate
AKAP – A-kinase anchoring protein
ATP – adenosine triphosphate
BAD – Bcl-2-associated death promoter protein
BCH – 2-aminobicyclo-[2,2,1] heptane-2-carboxylic acid
BCKDH – branched-chain α-ketoacid dehydrogenase
Bcl-2 – B-cell lymphoma 2 protein
cAMP – cyclic adenosine monophosphate
CBP – CREB-binding protein
CIC – citrate-isocitrate carrier
cJNK – c-Jun N-terminal kinase
CoA – coenzyme A
COS-1 – CV-1 (simian) in Origin, and carrying the SV40 genetic material, cell line
COX – cytochrome c oxidase
CPT-1 – carnitine palmitoyl-CoA transferase-1
Csk – C-terminal Src kinase
DIC – dicarboxylate carrier
DMEM – Dulbecco’s Modified Eagle Medium
E3BP – E3 subunit binding protein
EDTA – ethylenediaminetetraacetic acid
EGTA – ethylene glycol tetraacetic acid
ERK – extracellular-signal-regulated kinases
FAD – flavin adenine dinucleotide
FADH₂ – flavin adenine dinucleotide, reduced form
FoxO – Forkhead box protein
GAD65 – glutamate decarboxylase
GC1 – glutamate carrier 1
GDH – glutamate dehydrogenase
GDP – guanosine diphosphate
GRX-1 – glutaredoxin-1
GSIS – glucose-stimulated insulin secretion
GSK3β – glycogen synthase kinase 3 β
GTP – guanosine triphosphate
HEK293 – Human Embryonic Kidney 293 cells
HIT – clonal β-cell line
Hsp75 – heat shock protein 75
K_{ATP} channel – ATP-sensitive potassium channel
Kv – voltage-dependent potassium channel
MAPK – mitogen-activated protein kinases
MICU1 – mitochondrial calcium uptake 1 (mitochondrial inner membrane protein)
MIDD – maternally inherited diabetes and deafness
MPTP – mitochondrial permeability transition pore
mtDNA – mitochondrial DNA
NADH – nicotinamide adenine dinucleotide
NADPH – nicotinamide adenine dinucleotide phosphate
OGC – 2-oxoglutarate carrier
PC – pyruvate carboxylase
PCR – polymerase chain reaction
PDC – pyruvate dehydrogenase complex
PDH – pyruvate dehydrogenase
PDK – pyruvate dehydrogenase kinase
PDP – pyruvate dehydrogenase phosphatase
PEP – phosphoenolpyruvate
PEPCK – phosphoenolpyruvate carboxykinase
PEPCK-M – mitochondrial phosphoenolpyruvate carboxykinase
PINK1 – PTEN-induced kinase 1
PKA – protein kinase A
PKB – protein kinase B
PKC – protein kinase C
PMSF – phenylmethylsulfonyl fluoride
PP2A – protein phosphatase 2A
PP2Cm – mitochondrial protein phosphatase 2C
PP2C-γ – protein phosphatase 2C γ
PPARα – peroxisome proliferator-activated receptor α
PTPDI – protein tyrosine phosphatase D1
PTPMT1 – Protein Tyrosine Phosphatase localized to the Mitochondrion 1
RPMI – Roswell Park Memorial Institute medium
SCS – succinyl-CoA synthetase
SDS – sodium dodecyl sulfate
siRNA – small interfering RNA
shRNA – short hairpin RNA
TCA – tricarboxylic acid cycle
TFAM – mitochondrial transcription factor A
Tim50 – Transport across the inner membrane 50
Tim23 – Transport across the inner membrane 23
TPP – thiamin pyrophosphate
tRNA – transport RNA
INTRODUCTION

1. Glucose homeostasis

All living organisms need an exogenous source of energy to sustain their lives. Humans absorb energy with their nutrition mainly consisting of proteins, carbohydrates and lipids. These constituents are broken down to amino acids, monosaccharides and fatty acids, respectively. Their levels in the bloodstream vary between the fasted and fed states. Variations in glucose concentrations are especially important because glucose is the main energy source for cells in the human body. The brain is particularly sensitive to changes in glucose levels because neurons almost exclusively use glucose as a nutrient in the well-fed state. The glucose concentration in the blood decreases during fasting and increases after a meal. In healthy individuals fasting blood glucose level is precisely controlled to stay within the range of 3.9 to 6.7 mM (70 to 120 mg/dl). Fasting glucose concentrations below or above this range are known as hypoglycemia and hyperglycemia, respectively.

Following a meal, glucose is taken up by the tissues, mainly skeletal muscle, adipose tissue and liver. Glucose uptake, storage and utilization are controlled by hormones produced by the endocrine cells of the pancreas. Insulin is released in response to an elevation of blood glucose. In the liver insulin stimulates glucose phosphorylation by glucokinase, resulting in glucose retention inside hepatocytes. Insulin stimulates glycogen synthesis, glycolysis, production of acetyl-CoA, and subsequent steps of synthesis of fatty acids and triglycerides. At the same time, insulin inhibits glucose production by the liver. This is accomplished by insulin-dependent decrease of glycogenolysis (breakdown of glycogen) and gluconeogenesis (production of glucose). In skeletal muscle insulin stimulates glucose transport into the cells and glycogen synthesis. In adipose tissue insulin promotes glucose uptake by the adipocytes, glycolysis, production of glycerol-3-phosphate and subsequent synthesis of triglycerides. Insulin exerts its regulatory role by coordinating the expression and activity of the enzymes involved in these anabolic processes (reviewed in Koeppen and Stanton, 2008).

Taken together, insulin stimulates glucose utilization and storage in the form of glycogen and suppresses the use of intracellular glycogen stores.

During prolonged fasting blood glucose levels decline, which stimulates the release of glucagon by the α-cells of the pancreas. Glucagon actions are opposite to those of insulin. Glucagon decreases the rate of glycolysis and stimulates glycogenolysis and gluconeogenesis.
in the liver, the principal target organ of the hormone. This regulation results in the release of glucose from the liver into the bloodstream, which prevents hypoglycemia.

2. The endocrine pancreas

The pancreas is composed of exocrine and endocrine tissue. Within the pancreas endocrine cells form clusters called islets of Langerhans. These islets comprise 1 to 2% of pancreas mass and are dispersed in the exocrine tissue. Each pancreatic islet is composed of four major endocrine cell types. β-cells (~70% of islet cells) produce and secrete insulin. They are located in the central core of the islet (Gannon et al., 2000). α-cells (~20% of islet cells) synthesize and release glucagon and are localized at the islet periphery. Somatostatin-producing δ-cells and pancreatic polypeptide-producing cells comprise about 10% and 2% of islet cells, respectively. The aforementioned applies to rodents, while human islets have a less clear peripheral localization of α-cells and have proportionally less β-cells. Recent studies show that in human islets α- and β-cells are dispersed throughout the islet (Brissova et al., 2005; Cabrera et al., 2006).

3. Type 2 diabetes

The failure to maintain normal blood glucose levels results in the development of diabetes mellitus, the most common metabolic disease. According to the World Health Organization, today about 220 million people are affected worldwide (1). It is estimated that by 2030 about 360 million subjects will have diabetes (Wild et al., 2004). There are two main forms of diabetes. Type 1 diabetes is a consequence of the absence of insulin due to autoimmune-mediated destruction of β-cells. It comprises 5-10% of all diabetic cases (Daneman, 2006). Type 1 diabetes usually starts in children and young adults. The patients require insulin injections to compensate for the life-threatening lack of insulin. In contrast, type 2 diabetes arises as a combination of β-cell deficiency of non-autoimmune etiology and impaired insulin action, known as insulin resistance. 90% of all diabetic cases are classified as type 2 diabetes. Genetic predisposition and lifestyle factors such as reduced physical activity and overnutrition cause type 2 diabetes. The disease usually starts in adult age, however, in recent years it is increasingly diagnosed in children. Type 2 diabetes is frequently associated with overweight. Obesity and the resulting lipid accumulation in tissues lead to insulin resistance (reviewed in Muoio and Newgard, 2008). Insulin resistance is a key predisposing factor for the
development of type 2 diabetes. In this condition, insulin fails to act on its target tissues. The organism adapts to this by enhancing β-cell mass and function resulting in increased circulating insulin levels. Elevated insulin (hyperinsulinemia) partially compensates for insulin resistance. This state can be maintained for years or even lifelong (reviewed in DeFronzo et al., 1992; DeFronzo, 2010). Frequently, however, β-cell failure occurs leading to the onset of diabetes. β-cell failure is a key event in the development of type 2 diabetes.

4. Glucose-stimulated insulin secretion

4.1. Consensus model of glucose-stimulated insulin secretion (GSIS)

Glucose is the most important stimulus triggering insulin secretion. Rather than activating a plasma membrane receptor, glucose sensing occurs as β-cells produce glucose-derived metabolites that directly or indirectly promote insulin secretion. Several decades of research lead to a model describing how glucose provokes insulin secretion known as the consensus model of glucose-stimulated insulin secretion. Glucose enters the β-cell via a low affinity high capacity transporter, Glut2 (Thorens, 1992; Newgard and McGarry 1995; Guillam et al., 1997). This allows glucose to equilibrate across the plasma membrane such that the glucose concentration in the cytosol is roughly the same as that in the bloodstream. In the cytoplasm glucose is metabolized by glycolysis. The first step is mediated by glucokinase, which phosphorylates glucose. Glucokinase has low affinity for glucose of about 5 mM and thereby determines the threshold at which glucose starts to be stimulatory for the β-cell (Matschinsky, 1990).

In the β-cell the final product of glycolysis is pyruvate. Lactate is not formed, because the mature β-cell only expresses lactate dehydrogenase at very low levels (Sekine et al., 1994; Thorrez et al., 2011). Pyruvate is transported into the mitochondrial matrix, where it enters the tricarboxylic acid (TCA) cycle via the pyruvate dehydrogenase complex or pyruvate carboxylase. Substrate oxidation in the TCA cycle produces NADH. Electrons from NADH are used by the electron transport chain to create an electrochemical gradient across the inner mitochondrial membrane. The energy of the gradient is used as driving force for the ATP synthase to produce ATP. Mitochondrial ATP synthesis increases the ATP/ADP ratio in the cytoplasm.
4.2. Triggering and amplifying pathways of insulin secretion

The increase of the ATP/ADP ratio leads to closure of ATP-sensitive potassium channels (K$_{ATP}$ channels) and thereby plasma membrane depolarization. This results in the opening of voltage-dependent calcium channels and influx of calcium into the cell. Calcium triggers insulin granule exocytosis. The events described above occur rapidly after β-cell glucose stimulation and lead to a rapid increase of insulin secretion. This pathway is known as the triggering pathway. It is also called K$_{ATP}$-channel-dependent pathway because the closure of K$_{ATP}$ channels and subsequent plasma membrane depolarization are the key events for rapid increase in insulin secretion (Henquin, 2009). Experimentally, insulin exocytosis can be triggered even without glucose by using potassium at high concentrations (usually 30 mM KCl) to depolarize the plasma membrane, causing calcium influx (Wollheim and Sharp, 1981; Gembal et al, 1992).

A sharp peak of insulin release (called first phase of glucose-stimulated insulin secretion) is followed by a rapid decline. In the continuous presence of glucose, a slow increase follows the first phase. This steady increase (second phase) lasts 30-40 minutes before reaching a plateau (Straub and Sharp, 2004). Second phase insulin secretion cannot be mimicked by KCl demonstrating that plasma membrane depolarization is not sufficient for sustained insulin secretion. Under condition where K$_{ATP}$ channels are kept open using diazoxide and the plasma membrane is depolarized by KCl, glucose is able to increase insulin secretion in a concentration-dependent manner (Gembal et al., 1992). This demonstrates that in addition to the regulation of the K$_{ATP}$ channels, glucose acts by producing some other factors which are necessary for second phase insulin secretion. The underlying molecular mechanism remains poorly understood and is known as K$_{ATP}$ – independent or amplifying pathway (Henquin, 2000).

5. Role of mitochondria in GSIS

5.1. Evidence for the central role of the mitochondria in GSIS

Given the regulatory role of ATP described above, it can be appreciated that mitochondrial function is central to GSIS. A number of experiments in primary β-cells and insulin-secreting cell lines have confirmed the essential role of this organelle in metabolism-secretion coupling. A convincing set of results demonstrating the importance of mitochondria has been obtained using insulin-secreting cells lacking mitochondrial DNA (mtDNA) (rho0 cells). These cells can be obtained following treatment with ethidium bromide. The mitochondrial genome
encodes ribosomal RNAs, tRNAs and 13 proteins which are subunits of the respiratory complexes I, III, IV and V (the remaining subunits of these complexes and all subunits of complex II are encoded by the nuclear genome). In rho0 cells the absence of mitochondrially-encoded enzymes results in defective oxidative phosphorylation and impaired ATP production. As a result, these cells completely lose the ability to secrete insulin in response to glucose, but retain responsiveness to membrane depolarizing agents (Soejima et al., 1996; Kennedy et al., 1998; Hayakawa et al., 1998; Tsuruzoe et al., 1998; Noda et al., 2002). In humans, several mtDNA mutations cause mitochondrial diabetes (reviewed in Maassen et al., 2005). The majority of these mutations are located in tRNA genes. The most frequent is the A3243G mutation in Leu tRNA causing maternally inherited diabetes and deafness (MIDD) (van Den Ouweland et al., 1992). In these patients insulin secretion is impaired, however, the cause of β-cell failure in mitochondrial diabetes is not known. It has been proposed that decreased capacity to synthesize insulin, increased oxidative stress and accelerated age-dependent decline in β-cell function and mass contribute to the development of mitochondrial diabetes (reviewed in Maassen et al., 2006). It has been proposed that diminished mitochondrial function in β-cells makes them more susceptible to failure under condition of insulin resistance and thus can predispose to the development of conventional type 2 diabetes (discussed in Maechler et al, 2010). However, a direct demonstration of the link between mitochondrial function in β-cells and development of type 2 diabetes is lacking.

Another evidence for the role of mitochondria in GSIS came from the study on the mitochondrial transcription factor A (TFAM). TFAM is encoded by the nuclear genome and is imported into the mitochondria where it controls mitochondrial DNA transcription and stability (Falkenberg et al., 2007; Scarpulla, 2008). Mice with β-cell specific knockout of TFAM had defective oxidative phosphorylation, impaired GSIS and diabetes (Silva et al., 2000).

The most evident contribution of the mitochondria to the metabolism-secretion coupling is their ability to raise the cytosolic ATP/ADP ratio, as they produce the majority of cellular ATP. In the β-cell, ATP is necessary for the closure of \( K_{\text{ATP}} \) channels and also for insulin synthesis, insulin vesicle maturation and exocytosis. However, evidence is accumulating that ATP production is not the only important role of mitochondria in GSIS.
5.2. Activation of mitochondria in the β-cell during nutrient stimulation

During nutrient stimulation of the β-cell many ATP-hydrolysing processes in the cytosol are activated. In the β-cell, increased utilization of ATP is counteracted by increased mitochondrial ATP synthesis, such that there is no decrease of ATP/ADP ratio in the cytosol. In fact, ATP/ADP ratio increases during nutrient stimulation. Augmented ATP production is a result of mitochondrial activation. During nutrient stimulation β-cell mitochondria also increase oxygen consumption and CO₂ production. It has been suggested that there are two levels of regulation of energy metabolism in the mitochondria (Wiederkehr and Wollheim, 2008). Mitochondrial metabolism is regulated by the concentration of available mitochondrial substrates. Exposure of the β-cell to nutrients leads to increased nutrient oxidation in the cytosol, which provides substrates for mitochondria, thus activating the organelle. Increased substrate availability is not the only mechanism leading to mitochondria activation. Substrate oxidation and oxidative phosphorylation are further activated by mitochondrial signals. These activating signals and mechanisms mediating mitochondrial protein activation during nutrient-stimulated insulin secretion remain largely unknown.

5.3. Role of calcium in β-cell mitochondria

Glucose stimulation of β-cells results in the rapid influx of calcium from the extracellular space. This increases the cytosolic calcium concentration from about 100 nM to 400 nM. The increase of the calcium concentration in the cytoplasm is relayed into the mitochondrial matrix. Calcium enters mitochondria via the calcium uniporter. Its molecular identity remained largely elusive until recently. Perocchi et al. have identified a novel mitochondrial inner membrane protein called mitochondrial calcium uptake 1 (MICU1) which is required for mitochondrial calcium entry (Perocchi et al., 2010). A more recent study reported identification of protein MCU responsible for the mitochondrial calcium uptake in HeLa cells. The pores formed by the MCU protein in lipid bilayers demonstrated electrophysiological properties of the calcium uniporter (De Stefani et al., 2011). Under resting conditions mitochondrial calcium concentrations are close to that in the cytosol. During glucose stimulation, β-cell mitochondrial calcium increases to 600-800 nM (Wiederkehr et al., 2009). The peak of mitochondrial calcium concentration is reached within 1-2 minutes of glucose stimulation. Following this rapid increase mitochondrial calcium concentration returns to levels only modestly increased compared to the resting mitochondrial calcium concentration.
Calcium-sodium or calcium-proton exchangers ensure the lowering of mitochondrial calcium (Lee et al., 2003). Many studies suggest that calcium plays an activating role in mitochondrial metabolism (reviewed in Wiederkehr and Wollheim, 2008). In isolated pancreatic islets removal of extracellular calcium or blocking calcium influx by the $K_{ATP}$-channel opener diazoxide, which prevents glucose-stimulated calcium signaling, reduced CO$_2$ production from glucose (Sener et al., 1990; Hellman et al., 1974). One of the key arguments for the role of mitochondrial calcium in the β-cell came from studies on INS-1 cells treated with *Staphylococcus aureus* α-toxin (Maechler et al., 1997; Maechler et al., 1998). This treatment renders cells permeable to ions and small molecules and thus allows manipulating their cytosolic concentrations. Permeabilized cells were treated with succinate, a substrate of complex II of the electron transport chain. At permissive extramitochondrial calcium concentrations, stimulation with succinate induced mitochondrial calcium rises and increased insulin secretion. These effects were abolished by ruthenium red, an inhibitor of calcium transport via the uniporter (Maechler et al., 1997), demonstrating that nutrient-dependent mitochondrial calcium signaling is necessary for GSIS. These effects were observed under conditions of clamped elevated ATP concentrations, demonstrating that the increase in insulin secretion is due to calcium-dependent production of factors other than ATP (see chapter 5.6).

In another study on permeabilized INS-1E cells, cell calcium was clamped at physiological concentrations. Shifting calcium from 100 nM to 500 nM markedly increased succinate oxidation (Maechler et al., 1998). This demonstrates that increasing the calcium concentration within the physiological range stimulates mitochondrial oxidative metabolism. Mitochondrial calcium oscillations have been demonstrated to correlate with oscillations of NAD(P)H (Pralong et al., 1994). In addition, raising cytosolic calcium experimentally increases NAD(P)H levels and stimulates ATP production (Pralong et al., 1994; Tsuboi et al., 2003; Mármol et al., 2009). Consistent with these results, preventing calcium signaling in INS-1E cells blunts the glucose-dependent increase in cytosolic ATP (Wiederkehr et al., 2011).

Despite the relatively well-defined role of calcium as an activating signal in the mitochondria, only a few calcium-regulated mitochondrial proteins have been identified. These include pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase (also called α-ketoglutarate dehydrogenase) (Denton and McCormack, 1990; reviewed in Duchen, 1999). Isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase are directly stimulated by calcium. Pyruvate dehydrogenase is stimulated by dephosphorylation mediated by calcium-activated pyruvate dehydrogenase phosphatase. ATP synthase has been shown to be stimulated by calcium in isolated porcine heart mitochondria (Territo et al., 2000).
5.4. Pyruvate metabolism in β-cell mitochondria

Glucose stimulation of β-cells results in production of pyruvate by glycolysis. Pyruvate is transported into the mitochondrial matrix where it can be oxidized by pyruvate dehydrogenase complex (PDH) or carboxylated by pyruvate carboxylase (PC). A remarkable feature of β-cell mitochondria is high pyruvate carboxylase activity. It has been shown that in this cell type pyruvate is roughly equally utilized by PDH and PC (Khan et al., 1996; Lu et al., 2002). Carboxylation of pyruvate by PC produces oxaloacetate, which condenses with acetyl-CoA to generate citrate, thus providing carbons to the TCA cycle. Carbon input into the TCA cycle is called anaplerosis (from the Greek Ana “up” and Plerotikos “to fill”). In the β-cell, TCA intermediates can leave the TCA cycle and be exported to the cytosol. Such metabolite transport is necessary for optimal GSIS (see chapter 5.6). This export of metabolites necessitates the replenishment of the cycle, which depends on PC. The importance of PC in the metabolism-secretion coupling has been demonstrated using a number of approaches. siRNA-mediated reduction of PC expression leads to impaired GSIS in INS-1 cells and rat islets (Hasan et al., 2008; Xu et al., 2008). Similar results were obtained when PC was inhibited pharmacologically with phenylacetic acid (Fransson et al., 2006). On the other hand, overproduction of PC resulted in increased GSIS (Xu et al., 2008). Moreover, studies on several INS-1-derived insulin-secreting cell lines showed that cell lines with increased flux through PC had increased insulin-secretory capacity (Lu et al., 2002; Cline et al., 2004).

5.5. Pyruvate cycling

During nutrient stimulation of the β-cell, the concentration of pyruvate in the mitochondria increases. Subsequently, metabolites produced from pyruvate in the TCA cycle also increase. Several TCA cycle products such as citrate, isocitrate, α-ketoglutarate and malate can be transported out of the mitochondrial matrix. Transport of metabolites between mitochondria and cytoplasm is mediated by specific carriers located in the inner mitochondrial membrane. Exported TCA cycle products can be converted to pyruvate via several cytosolic enzymatic reactions. Pyruvate can then reenter mitochondrial matrix. These metabolic reactions and metabolite transport steps have been termed “pyruvate cycling”. There are three pyruvate cycling pathways in the β-cell: pyruvate-malate, pyruvate-citrate and pyruvate-isocitrate cycles (reviewed in MacDonald et al., 2005; Jensen et al., 2008; Jitrapakdee et al., 2010).
In the pyruvate-malate pathway, malate is transported to the cytoplasm via the dicarboxylate carrier (DIC) also called malate carrier. It is then converted to pyruvate by cytosolic malic enzyme. In this reaction NADP$^+$ is reduced to NADPH. Alternatively, malate can be converted to pyruvate in the mitochondrial matrix by the mitochondrial form of malic enzyme.

In the pyruvate-citrate pathway, citrate and isocitrate are transported to the cytoplasm via the citrate-isocitrate carrier (CIC). In the cytosol citrate is converted to oxaloacetate and acetyl-CoA by ATP-citrate lyase. Malate dehydrogenase converts oxaloacetate to malate which then serves as a substrate for cytosolic malic enzyme to produce pyruvate.

The pyruvate-isocitrate pathway (also known as isocitrate-α-ketoglutarate pathway) starts like the pyruvate-citrate pathway. In the cytosol, citrate is converted to isocitrate by cytosolic aconitase. Isocitrate is converted to α-ketoglutarate by cytosolic isocitrate dehydrogenase. This reaction is NADP$^+$-dependent and generates NADPH. α-ketoglutarate can be transported back to mitochondria via the α-ketoglutarate transporter.

5.6. Mitochondria-derived coupling factors

Metabolites derived from the mitochondria or factors linked to their metabolism may act as signals in the amplifying pathway of insulin secretion. The metabolites involved and the molecular mechanism by which they potentiate insulin secretion remain poorly understood. Proposed coupling factors and the evidence suggesting their participation as signals in this biological process are discussed below.

5.6.1. Glutamate

Glutamate levels increase during glucose stimulation of INS-1 cells, islets and purified β-cells (Maechler and Wollheim, 1999; Feldmann et al., 2011). There are two major pathways by which glutamate can be produced in the β-cell during glucose stimulation. Glutamate can be produced from α-ketoglutarate by the mitochondrial enzyme glutamate dehydrogenase (GDH). Alternatively, glutamate can be formed from the amino acid glutamine by glutaminase. Several studies demonstrated that glutamate plays role as an intracellular messenger stimulating GSIS. For instance, in a study on INS-1 cells membrane-permeant forms of glutamate potentiated GSIS (Maechler and Wollheim, 1999). This action of
glutamate was independent of mitochondrial function but appeared to depend on the uptake of glutamate by the vesicles, suggesting that glutamate directly stimulates insulin granule exocytosis. Glutamate has been shown to stimulate insulin secretion in α-toxin permeabilized primary β-cells (Høy et al., 2002). Overexpression of glutamate decarboxylase (GAD65) in INS-1E cells prevented the glucose-dependent increase in cytosolic glutamate concentration and reduced GSIS (Rubi et al., 2001). INS1-E cells and rat pancreatic islets express glutamate carrier 1 (GC1), a mitochondrial protein mediating the transport of glutamate across the inner mitochondrial membrane. In INS-1E cells, GC1 was found to be expressed in the inner mitochondria membrane. Suppressing GC1 expression by shRNA decreased glutamate transport across the mitochondrial membrane leading to reduced GSIS in INS-1E cells and rat islets. Interestingly, both first and second phases of insulin secretion were reduced (Casimir et al., 2009).

However, the role of glutamate as a second messenger is debated. Several studies did not find increase in glutamate levels upon glucose stimulation of rat islets (MacDonald and Fahien, 2000; MacDonald 2002; MacDonald 2003) or did not establish causal link between increased glutamate levels and insulin secretion (Bertrand et al., 2002). Furthermore, glutamine which is metabolized to glutamate in the mitochondria thus increasing glutamate levels did not stimulate GSIS (MacDonald and Fahien, 2000). In addition it is not clear whether during nutrient stimulation GDH preferably catalyzes glutamate production or the reverse reaction producing α-ketoglutarate. The later reaction provides substrate for the TCA cycle and thus would be expected to be important for GSIS.

5.6.2. Malonyl-CoA

Malonyl-CoA is produced in the cytoplasm from acetyl-CoA by the enzyme acetyl-CoA carboxylase and was proposed to act as a potentiating factor in GSIS (Corkey et al., 1989; Chen et al., 1994). The malonyl-CoA/long-chain acyl-CoA hypothesis postulated that malonyl-CoA, which is increased during glucose stimulation of β-cells, can be utilized as substrate for long-chain acyl-CoA synthesis. Long-chain acyl-CoA’s were proposed to directly stimulate insulin exocytosis. In addition, malonyl-CoA inhibits carnitine-palmitoyl-CoA transferase-1 (CPT-1), an enzyme mediating the transport of long-chain acyl-CoA’s into mitochondria. This contributes to the accumulation of long-chain acyl-CoA’s in the cytoplasm. In support of this hypothesis, addition of saturated long-chain fatty acids increased long-chain acyl-CoA levels and potentiated GSIS in the clonal β-cell line HIT (Prentki et al., 1992). In rat pancreas, pharmacological inhibition of ATP-citrate lyase, necessary for
malonyl-CoA synthesis via citrate, inhibited GSIS. Moreover, inhibition of CPT1 facilitated accumulation of long-chain acyl-CoA’s in the cytoplasm and potentiated GSIS (Chen et al., 1994). However, more recent studies provide evidence against this hypothesis. Prevention of the malonyl-CoA increase by overexpression of malonyl-CoA decarboxylase, does not affect GSIS in INS-1 cells (Antinozzi et al., 1998). Furthermore, pharmacological inhibition of long-chain acyl-CoA synthetase prevented long-chain acyl-CoA accumulation but had no effect on GSIS in INS-1 cells and rat islets (Antinozzi et al., 1998; Mulder et al., 2001; Roduit et al., 2004).

5.6.3. α-ketoglutarate

All fuel secretagogues are capable of producing α-ketoglutarate (reviewed in MacDonald et al., 2005). Recent studies suggest an important role of cytosolic α-ketoglutarate in GSIS. siRNA-mediated silencing of cytosolic isocitrate dehydrogenase, an enzyme producing α-ketoglutarate, has been demonstrated to decrease GSIS in INS-1-derived cell lines and rat pancreatic islets (Ronnebaum et al., 2006). In a recent study, the mitochondrial α-ketoglutarate transporter (also called 2-oxoglutarate carrier; OGC) was silenced with siRNA in INS-1 832/13 cells. This resulted in impaired insulin secretion in response to glucose or glucose + KCl, demonstrating that α-ketoglutarate transport is necessary for the $K_{\text{ATP}}$ channel-independent pathway (Odegaard et al., 2010). Interestingly, suppression of OGC inhibited insulin secretion also when α-ketoglutarate levels were selectively increased either in the mitochondria (by using glutamine + glutamate dehydrogenase activator BCH) or in the cytosol (by using membrane-permeable dimethyl-α-ketoglutarate) (Odegaard et al., 2010). These results suggest that the key factor for GSIS is not absolute levels of α-ketoglutarate in the mitochondria or cytosol, but operative transport of α-ketoglutarate between cytosol and mitochondria. The mechanisms of action of α-ketoglutarate in the cytoplasm are not known. α-ketoglutarate is a substrate for α-ketoglutarate dependent hydroxylases. It has been proposed that α-ketoglutarate-dependent hydroxylases can mediate the positive effect of α-ketoglutarate on insulin secretion. In support of this hypothesis, INS-1 832/13 cells as well as human and rat pancreatic islets have been demonstrated to express several proline hydroxylases and their inhibition reduced GSIS (Fallon and MacDonald, 2008).

5.6.4. GTP

Mitochondrial GTP is produced by the TCA cycle enzyme succinyl-CoA synthetase (SCS). There are two nucleotide-specific isoforms of SCS: the ADP-dependent isoform produces
ATP while the GDP-dependent isoform makes GTP. siRNA-mediated silencing of the ADP-dependent SCS in INS-1 832/13 cells resulted in increased activity of the GTP-producing SCS isoform and increased GSIS. In this condition calcium was augmented suggesting that matrix GTP potentiates the glucose-stimulated calcium rise (Kibbey et al., 2007). GTP formed in the matrix cannot be exported from mitochondria, suggesting that its action on GSIS is mediated by a GTP-sensitive pathway in the mitochondria. It was recently discovered that β-cells express the GTP-dependent mitochondrial enzyme phosphoenolpyruvate carboxykinase (PEPCK-M) (Stark et al., 2009). This enzyme uses oxaloacetate and GTP to produce phosphoenolpyruvate (PEP) which is transported to the cytosol. This PEP transport step is active in INS-1 832/13 cells. Mitochondrially-derived PEP constitutes up to 30% of cytosolic PEP (Stark et al., 2009). These results suggest that PEP may be a coupling factor linking mitochondrial GTP production and insulin secretion. GTP-dependent mitochondrial PEP production may be important for the β-cell to monitor the rate of mitochondrial metabolism. This could serve to coordinate mitochondrial metabolism and insulin secretion.

5.6.5. NADPH

NADPH is produced in the cytosol during pyruvate cycling (see chapter 5.5). This explains the observed increase of the NADPH/NADP⁺ ratio during glucose stimulation of β-cells (Ivarsson et al., 2005). NADPH levels are among others sensed in the cytosol by NADPH-dependent glutathione reductase. This enzyme is abundant in the β-cell and converts oxidized glutathione to its reduced form (Laclau et al., 2001). Reduced glutathione is required for processes such as protein folding and disulfide bridges formation and thus can affect activities of many enzymes including proteins involved in GSIS (discussed in MacDonald et al., 2005). Consistent with this idea, recent work shows that glutaredoxin-1 (GRX-1), a protein sensitive to reduced glutathione, is important for GSIS. Silencing of GRX-1 in INS-1 832/13 cells reduced GSIS, while overproduction of GRX-1 increased insulin exocytosis (Reinbothe et al., 2009).

β-cells express voltage-dependent K-channel (Kv) (MacDonald and Wheeler 2003). This channel allows K⁺ efflux from the cell thus repolarizing the plasma membrane. NADPH inhibits Kv activity thus slowing K⁺ efflux. As a result, the plasma membrane remains depolarized longer following the closure of Kᵦₐₜₚ-channels (MacDonald and Wheeler 2003; Tipparaju et al., 2007). This mechanism should result in NADPH-dependent potentiation of GSIS.
6. Protein phosphorylation in the mitochondria

Reversible phosphorylation is one of the most common posttranslational modifications (Hunter, 2000; Cohen, 2002). It has been estimated that about 30% of all proteins can undergo cycles of phosphorylation-dephosphorylation (Hunter, 1995). Phosphorylation of a protein can change its enzymatic activity, subcellular localization, protein stability and interaction with other proteins. Protein phosphorylation is one of the principle mechanisms for the control of signal transduction pathways. Protein phosphorylation is carried out by protein kinases, while dephosphorylation is performed by protein phosphatases. The majority of known kinases and phosphatases are localized in the cytosol. Mitochondrial proteins PDH and branched-chain α-ketoacid dehydrogenase (BCKDH) are known to be regulated by phosphorylation.

6.1. Protein kinases and phosphatases in the mitochondria

Protein kinase activity in the mitochondria was first mentioned in the 1950s after phosphorylation of casein was observed upon incubation with mitochondrial extract (Burnett and Kennedy, 1954). Recent studies demonstrate that mitochondria contain protein kinases and phosphatases. However, most of the kinases and phosphatases found in the mitochondria are known to have a primary role in the cytosol. These include protein kinase A (PKA), protein kinase B (PKB), protein kinase C δ (PKC δ), Src family kinases Src, Lyn, Fyn, Csk, Fgr, c-Jun amino-terminal kinases (cJNK), GSK3β and ERK. Protein phosphatases PP2C-γ, PP2A, PTPD1 and MAPK phosphatase 1 have been detected in the mitochondria (reviewed in Thomson, 2002; Horbinski and Chu, 2005; Salvi et al., 2005; Pagliarini and Dixon, 2006).

Several kinases are associated with the outer mitochondrial membrane. PKA, for instance, is bound to A-kinase anchoring proteins (AKAP) which tethers it to the outer mitochondrial membrane (Chen et al., 1997; Newhall et al., 2006). The PKA/AKAP complex phosphorylates and inactivates BAD, a proapoptotic protein of the BCL-2 family, localized at the mitochondrial surface (Harada et al., 1999). In addition to PKA, AKAP anchors protein tyrosine phosphatase D1 (PTPD1) and Src kinase to the surface of the mitochondrial outer membrane (Cardone et al., 2004; Livigni et al., 2006). The submitochondrial location of many other kinases either to the outer, inner mitochondrial membrane, intermembrane space or matrix remains unclear. Interestingly, there is convincing evidence for the mitochondrial
matrix location of several cytosolic kinases. It is not clear how cytosolic kinases are translocated to the mitochondrial matrix. For PKA, a mechanism of translocation to the mitochondria has been proposed (reviewed in Hüttemann et al., 2007). The PKA/AKAP complex located on the outer side of outer mitochondrial membrane is thought to be internalized by the mitochondria and be transported to the matrix space (Hüttemann et al., 2007). Accordingly, detection of the PKA protein and activity in mitochondria has been reported in a number of studies (Dimino et al., 1981; Schwoch et al., 1990; Technikova-Dobrova et al., 1993; Technikova-Dobrova et al., 2001). Src kinase was found in the intermembrane space of mitochondria from rat brains (Salvi et al., 2002). Using biochemical methods and immunoelectron microscopy Miyazaki and coworkers detected Src kinase in the inner mitochondrial membrane of HEK293 cells and osteoclasts (Miyazaki et al., 2003).

Importantly, there are a number of protein kinases and phosphatases specifically localized in the mitochondrial matrix. Early studies showed mitochondrial matrix localization of PDKs, PDPs, BCKDH kinase and BCKDH phosphatase. More recent studies identified matrix localization of kinase PINK1 (Valente et al., 2004) and phosphatases PTPMT1 (Pagliarini et al., 2005), PP2Cm (Lu et al., 2007) and Tim50 (Guo et al., 2004). These kinase and phosphatases contain the mitochondrial localization signal within their sequences and function as resident mitochondrial molecules.

PINK1 (PTEN-induced kinase 1) is a mitochondrial serine/threonine kinase. Inactivating mutations or absence of PINK1 lead to impaired mitochondrial respiration, reduced ATP production, increased ROS production and apoptosis and have been associated with Parkinson disease (reviewed in Deas et al., 2009). PINK1 is required for the regulation of respiratory complex activity, regulation of mitochondrial chaperones TRAP1/Hsp75, mitochondrial fusion and fission and regulation of calcium efflux from mitochondria through the Na⁺/Ca⁺ exchanger (reviewed in Deas et al., 2009).

The novel tyrosine phosphatase PTPMT1 (Protein Tyrosine Phosphatase localized to the Mitochondrion 1) has been identified in the mitochondria of insulin-secreting INS-1 cells (Pagliarini et al., 2005). Knockdown of PTPMT1 expression in INS-1 832/13 cells increased ATP levels by about 50% which was accompanied by significantly increased GSIS. Although the substrate of this enzyme has not been identified, these results illustrate the importance of reversible phosphorylation in mitochondria for β-cell function.
PP2Cm is a serine/threonine phosphatase found in human, mouse and zebrafish. In mouse liver, COS-1 and HEK293 cells this phosphatase is expressed in the mitochondrial matrix. Mitochondria from mice treated with adenovirus-delivered shRNA against PP2Cm showed elevated susceptibility to calcium-induced mitochondrial membrane permeability transition pore (MPTP) opening. In zebrafish, suppression of PP2Cm induced apoptosis, defective development of the liver, central nervous system and heart (Lu et al., 2007). Recently it has been reported that PP2Cm binds to branched-chain α-ketoacid dehydrogenase (BCKDH) and dephosphorylates the enzyme leading to its activation. The physiological significance of PP2Cm has been demonstrated in PP2Cm knockout mice, which have decreased BCKDH activity and increased branched-chain amino acids levels in plasma compared with wild-type littermates (Lu et al., 2009).

The phosphatase Tim50 is part of the mitochondrial translocase complex mediating protein transport from the cytosol to mitochondria. Tim50 is expressed in the inner mitochondrial membrane and interacts with another component of the translocase complex, Tim23. Tim50 has been shown to have phosphatase activity towards phosphotyrosine and phosphothreonine residues. Decreasing Tim50 expression by siRNA in 293T cells resulted in increased cytochrome c release from mitochondria and apoptosis, compared to non-treated cells. Furthermore, loss of Tim50 function resulted in increased apoptosis in zebrafish (Guo et al., 2004). However, it has not been shown whether these phenotypes are due to loss of phosphatase activity.

### 6.2. Mitochondrial proteins regulated by phosphorylation

In addition to identification of mitochondrial kinases and phosphatases many studies revealed their specific substrates in the mitochondria. For more than 60 mitochondrial proteins phosphorylation has been confirmed by several studies (reviewed in Pagliarini and Dixon, 2006). Importantly, for a number of mitochondrial proteins phosphorylation has been shown to regulate protein activity and function. Well-known examples are PDH and BCKDH, which are activated by dephosphorylation (Linn et al., 1969 a, b). Several subunits of the respiratory complex I have been shown to be phosphorylated by PKA in a cAMP-dependent manner (Papa et al., 1996; Raha et al., 2002; Chen et al., 2004; Schilling et al., 2005). In mouse fibroblasts, cAMP-dependent phosphorylation of the 18 kDa subunit of complex I has been reported to increase complex I activity (Seacco et al., 2000), demonstrating the importance of
protein phosphorylation for mitochondrial function. Miyazaki et al. showed that phosphorylation of subunit II of the respiratory complex IV (cytochrome c oxidase; COX) by Src kinase positively regulates its activity. Deletion of the c-src gene prevented tyrosine phosphorylation and reduced complex IV activity (Miyazaki et al., 2003). In an in vivo study, Lee and coworkers showed that phosphorylation of the Tyr304 of subunit I of COX is inhibitory (Lee et al., 2005). Tyrosine phosphorylation was also identified in subunit IV of COX (Lee et al., 2005). The β subunit of ATP synthase has also been demonstrated to be phosphorylated (Højlund et al., 2003). Taken together, these results demonstrate that respiratory chain complexes are regulated by reversible phosphorylation.

7. Pyruvate dehydrogenase

7.1. Reactions catalyzed by the PDH complex

Pyruvate dehydrogenase (PDH) is part of the multi-subunit PDH complex (PDC) located in the mitochondrial matrix. PDC converts pyruvate, produced in glycolysis, to acetyl-CoA, thus linking glycolysis and downstream mitochondrial energy metabolism. The overall reaction catalyzed by the PDC is the following:

\[
\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+
\]

Mammalian PDC is composed of the catalytic components pyruvate dehydrogenase (E1), dihydrolipoyl acetyltransferase (E2) and dihydrolipoyl dehydrogenase (E3), regulatory components pyruvate dehydrogenase kinases (PDK), pyruvate dehydrogenase phosphatases (PDP) and a binding protein E3-binding protein (E3BP) (see Table 1, reviewed in Harris et al., 2002; Roche and Hiromasa, 2007).

The PDC reaction is performed in several consecutive steps by the PDC subunits E1, E2 and E3 (reviewed in Roche and Hiromasa, 2007). The first step is performed by E1 and requires a cofactor thiamin pyrophosphate (TPP). E1 cleaves off carbon 1 of pyruvate (pyruvate decarboxylation). The resulting CO₂ is released and an acetyl group is covalently attached to TPP. In the second step, E1 transfers the acetyl group from TPP to the (-S-S-) group of the lipoyl domain of E2 (acetylation of lipoyl domain). Then E2 transfers the acetyl group from the lipoyl domain to the (-SH) thiol group of coenzyme A. The result is production of acetyl-CoA and regeneration of TPP for the next round of E1-catalyzed reaction. In the next step, the
(-SH) groups of E2 lipoyl domain are oxidized in the active site of E3 to regenerate (-S-S-). This is coupled to reduction of FAD to FADH$_2$. The latter is used by NAD$^+$, yielding NADH as a final product.

### 7.2. Structure of the PDH complex

Components of the PDC are presented in multiple copies. E2 and E3BP form structural core of the complex. This core is a 60mer structure formed by 48 E2 subunits and 12 E3BP subunits (Hiromasa et al., 2004). The core is also called E2$_{48}$·E3BP$_{12}$ structure. Other subunits of the complex are organized around the central core. Each E2 subunit has an inner core-forming region and an outer region that extends from the core. The outer region has binding sites for the non-covalent interaction which E1, PDKs and PDPs. Each E3BP subunit has an E3-binding site to which one E3 subunit binds. E1 subunits form a tetramer composed of 2 α and 2 β subunits. The mammalian PDC contains 20-30 E1 tetramers. The molecular weight of the PDC is about 9 MDa.

### Table 1. Components of the human pyruvate dehydrogenase complex

<table>
<thead>
<tr>
<th>Name</th>
<th>Length, amino acids</th>
<th>Molecular weight, kDa</th>
<th>UniProt accession number</th>
<th>EC accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate dehydrogenase E1α</td>
<td>390</td>
<td>43.29</td>
<td>P08559</td>
<td>EC=1.2.4.1</td>
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<tr>
<td>Pyruvate dehydrogenase E1β</td>
<td>359</td>
<td>39.23</td>
<td>P11177</td>
<td>EC=1.2.4.1</td>
</tr>
<tr>
<td>Dihydrolipoyl acetyltransferase (E2)</td>
<td>647</td>
<td>68.99</td>
<td>P10515</td>
<td>EC=2.3.1.12</td>
</tr>
<tr>
<td>Dihydrolipoyl dehydrogenase (E3)</td>
<td>509</td>
<td>54.18</td>
<td>P09622</td>
<td>EC=1.8.1.4</td>
</tr>
<tr>
<td>E3BP (E3 binding protein)</td>
<td>501</td>
<td>54.12</td>
<td>O00330</td>
<td></td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase 1 (PDK1)</td>
<td>436</td>
<td>49.24</td>
<td>Q15118</td>
<td>EC=2.7.11.2</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase 2 (PDK2)</td>
<td>407</td>
<td>46.15</td>
<td>Q15119</td>
<td>EC=2.7.11.2</td>
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<tr>
<td>Pyruvate dehydrogenase kinase 3 (PDK3)</td>
<td>406</td>
<td>46.94</td>
<td>Q15120</td>
<td>EC=2.7.11.2</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase 4 (PDK4)</td>
<td>411</td>
<td>46.47</td>
<td>Q16654</td>
<td>EC=2.7.11.2</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase phosphatase 1 (PDP1)</td>
<td>537</td>
<td>61.05</td>
<td>Q9P0J1</td>
<td>EC=3.1.3.43</td>
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<td>59.98</td>
<td>Q9P2J9</td>
<td>EC=3.1.3.43</td>
</tr>
</tbody>
</table>
7.3. Regulation of PDH activity

PDH is one of the key enzymes in energy metabolism. Consistent with its important role, PDH is tightly controlled by several mechanisms. These include posttranslational modification (reversible phosphorylation of E1α subunit), regulation of regulatory components PDKs and PDPs by metabolites and transcriptional control of PDKs and PDPs (reviewed in Harris et al., 2002; Holness and Sugden, 2003; Patel and Korotchkina, 2006).

The regulation by reversible phosphorylation is the most important mechanism regulating PDH activity. Phosphorylation is performed by specific PDH kinases (PDK) and results in inactivation of the enzyme. Dephosphorylation by specific PDH phosphatases (PDP) activates the enzyme. PDH activity is determined by the ratio of unphosphorylated to the phosphorylated form. Phosphorylation-dephosphorylation occurs on three serine residues on the E1α subunit: Ser 293 (site 1), Ser 300 (site 2) and Ser 232 (site 3). The amino acids are numbered with respect to the PDH E1α precursor protein. In some studies phosphorylation sites are numbered according to the mature PDH E1α protein as Ser 264 (site 1), Ser 271 (site 2) and Ser 203 (site 3). Phosphorylation of any one of three phosphorylation sites is sufficient to render PDH inactive (Korotchkina and Patel, 1995).

In mammals four PDK isoforms (PDK1, 2, 3 and 4) and two PDP isoforms (PDP 1 and 2) are expressed (Gudi et al., 1995; Huang et al., 1998). PDH activity is, therefore, determined by the relative activities of PDKs and PDPs. PDKs have been shown to have different activities towards individual phosphorylation sites. All PDK isoforms can phosphorylate sites 1 and 2, but site 3 can only be phosphorylated by PDK1 (Korotchkina and Patel, 2001; Kolobova et al., 2001). In addition, PDKs differ in activity towards a given site. For instance, among the four PDK isoforms that phosphorylate site 1, PDK2 has the highest activity and PDK3 has the lowest activity to this site (Korotchkina and Patel, 2001). The different PDH phosphorylation sites are modified at different rate, with phosphorylation of site 1 being the most rapid and phosphorylation of site 3 the least rapid (Sale and Randle, 1981; Korotchkina and Patel, 1995). Differential activity of PDK towards phosphorylation sites and differences in rates of their phosphorylation are expected to allow different rates of PDH inactivation which is likely to be important under variable physiological conditions.

PDK2 is ubiquitously expressed, whereas other PDKs are expressed in a tissue-specific manner (Bowker-Kinley et al., 1998). Tissue-specific expression is also observed for PDPs. PDP1 is expressed in heart and skeletal muscle, while PDP2 is detected in liver, adipose tissue, kidney and heart (reviewed in Holness and Sugden, 2003). Given the differences in
PDKs described above, their tissue-specific expression should result in differences in the regulation of PDH in different tissues.

PDH activity is affected by the levels of a number of intramitochondrial metabolites. Recent studies demonstrate that metabolite regulation occurs primarily by affecting phosphorylation-dephosphorylation, rather than PDH activity directly. Regulation by metabolites is a means of rapid acute regulation of PDH activity. The main parameters affecting PDH activity are the ratio of acetyl-CoA/CoA, NADH/NAD⁺, ATP/ADP and the levels of pyruvate. High levels of ATP and products of the PDC reaction acetyl-CoA and NADH stimulate PDK activity and hence inhibit PDH. Increased NADH/NAD⁺ and acetyl-CoA/CoA synergistically stimulate PDK activity. These metabolites regulate PDKs via different mechanisms. NADH promotes reduction of -S-S- to -SH-SH- in the lipoyl domain of E2, while increased acetyl-CoA facilitates subsequent acetylation of -SH-SH- groups. This increases activity of PDKs bound to the E2 subunit (Yang et al., 1998; Roche et al., 2003). PDK isoforms have different sensitivities to regulation by NADH and acetyl-CoA. PDK2 and PDK4 are the most sensitive to stimulation by NADH and acetyl-CoA (reviewed in Patel and Korotchkina, 2006).

ADP and substrates of the PDH reaction pyruvate, CoA and NAD⁺ inhibit PDK activity, therefore keeping PDH active. The inhibitory effect of ADP is due to its competition with ATP for binding to PDKs. Pyruvate also binds to PDKs and reduces their activity. Combination of ADP and pyruvate results in more pronounced PDK inhibition than for each substance alone (Pratt and Roche, 1979; Bao et al., 2004). Inhibition of PDK by ADP and pyruvate and subsequent activation of PDH constitutes a positive feedback loop, mediating PDH activation under conditions of low energy (increased ADP levels) and increased substrate availability (increased pyruvate levels).

Long-term regulation of PDH activity is accomplished by changes in the expression of PDKs and PDPs. This mode of regulation is relevant for such conditions as physical endurance training or extended starvation. During prolonged starvation expression and activity of PDKs increases. In skeletal muscle, heart, kidney and liver PDK4 is the main PDK isoform activated by starvation. In addition, PDK2 expression is also increased in liver and kidney (Wu et al., 2000; reviewed in Holness and Sugden, 2003). Inhibition of PDH by PDK expression is an essential mechanism limiting glucose oxidation, while promoting glucose conservation and re-direction of three carbon compounds for gluconeogenesis (reviewed in Holness and Sugden, 2003). Reduction of PDH activity also facilitates the switch to the utilization of fatty acids and ketone bodies as the main energy sources. There are several mechanisms mediating
the increase of PDK expression during starvation. Decline of insulin levels during starvation leads to increase of white adipose tissue lipolysis, which results in increased circulating non-esterified fatty acid concentrations. Fatty acids increase PDK4 expression, in part, via activation of the transcription factor PPARα (Wu et al., 2001; Sugden et al., 2002). Furthermore, reduced insulin levels result in decreased phosphorylation and increased activity of the FoxO transcription factors, regulating expression of PDK4 (Kwon et al., 2004; Kwon and Harris, 2004). Moreover, glucocorticoid levels are increased during starvation. Glucocorticoid signaling promotes histone acetyltransferase p300/CREB recruitment to the PDK4 promoter and an increase of PDK4 expression (Kwon et al., 2006).

In addition to an increase in PDK activity, starvation-dependent decrease in PDH activity is mediated by a decrease of PDP activity. During starvation in rats, PDP2 expression is decreased and a result total PDP activity reduced (Huang et al., 2003), which prevents PDH activation.

7.4. Unclear role of regulation of PDH in the β-cell

The importance of PDH for glucose metabolism suggests that regulation of the PDH reaction can be one of the key regulatory steps for β-cell function. In contrast to the relatively well-defined role of PC, the role of PDH regulation in nutrient-stimulated insulin secretion is much less clear. The relationship between activity of PDH and GSIS is not well elucidated. Published data on activity of PDH in basal and nutrient-stimulated state in the β-cell are controversial. In a study on rat pancreatic islets, Paxton et al. reported high proportion of active PDH (94%) in the basal state (Paxton et al., 1988). However, two other studies report the opposite result. In a study by McCormack et al. in isolated rat islets only about 16% of PDH was active at 3 mM glucose. PDH activity was raised to about 50% within 5 minutes after glucose stimulation (12-20 mM). Such activation correlated with increase of insulin secretion (McCormack et al., 1990). Nicholls et al. reported that glucose stimulates PDH activity in rat islets and the mouse insulin-secreting cell line MIN6 (Nicholls et al., 2002).

It is not clear whether PDH regulation is important for β-cell function. Recently glucose-dependent insulin secretion has been studied in mice with β-cell specific PDH knockout. Loss of PDH in β-cells decreased GSIS. In this study high glucose increased insulin secretion ~2.4-fold in islets from mutant mice, compared to ~4-fold in islets from wild-type littermates (Srinivasan et al., 2010, Figure 5), demonstrating that PDH is relevant for GSIS. However, hyperglycemic clamp analysis demonstrated that insulin secretion was almost similar between
the two groups of animals during the first 30 minutes of glucose stimulation (Srinivasan et al., 2010, Figure 7B, C). This finding and the fact that GSIS was not completely blunted by PDH knockout in β-cells do not allow making an unequivocal conclusion about the role of PDH in GSIS. In another study role of regulation of PDH activity on GSIS was studied in rat islets. Expression of exogenous PDP and PDK3 in rat islets was used to increase and respectively decrease PDH activity. This had no effect on GSIS (Nicholls et al., 2002). A recent study on INS-1 832/13 clonal β-cells shows that upon siRNA-mediated decrease of PDK1 expression, PDH activity increases. This is accompanied by enhanced GSIS (Krus et al., 2010), suggesting that regulation of PDH activity is essential for GSIS.

Thus, the role of PDH in GSIS is not well defined and further studies are necessary to delineate the mechanisms regulating PDH activity during nutrient-stimulated insulin secretion and relevance of such regulation for β-cell function.

8. Mitochondrial matrix pH in the β-cell

Mitochondrial metabolism is central to insulin secretion. During nutrient stimulation, β-cell mitochondria are activated. Mitochondrial activation is accompanied by establishment of proton electrochemical gradient across the inner mitochondrial membrane. This gradient is composed of electrical potential across the inner mitochondrial membrane and proton chemical gradient. Both components are essential for increasing driving force on the ATP synthase (Das, 2003). Proton electrochemical gradient is also important for transport of metabolites across inner mitochondrial membrane. Proton chemical gradient is proportional to the ΔpH across the inner mitochondrial membrane. In most cell types the mitochondrial matrix is more alkaline than cytosol. Mitochondrial matrix pH has been reported to be between 7.6 and 8.3 in different cell types (Llopis et al., 1998; Abad et al., 2004), which is about 1 pH unit more alkaline than cytosol.

In a recent study from our group it has been shown that pH in the β-cell mitochondrial matrix is about 7.25, which is lower than in other cell types (Wiederkehr et al., 2009). In the same study it has been demonstrated that matrix pH increases upon glucose and leucine stimulation in primary β-cells and INS-1E cells. Cytosolic pH did not change during nutrient stimulation. Matrix alkalinization has been shown to be important for ATP production. When alkalinization was prevented by using K⁺/H⁺ ionophore nigericin, ATP production from exogenously added succinate was blocked (Wiederkehr et al., 2009).
Project I.

Mitochondrial protein phosphorylation in INS-1E cells

9.1. Context

In the β-cell, mitochondria are activated during nutrient stimulation. Today we know little on how mitochondrial proteins are regulated in the β-cell. Reversible phosphorylation has emerged as a means of regulating protein activity in the mitochondria. We hypothesize that in the β-cell calcium and other yet unidentified mitochondrial signals could exert their regulatory role by altering protein phosphorylation-dephosphorylation. Such regulation may affect a number of mitochondrial functions including nutrient oxidation, electron transport and ATP production. This mechanism would likely be important for nutrient-stimulated insulin secretion.

9.2. Aims

In this project, we aimed to identify novel mitochondrial phosphoproteins in the pancreatic β-cell. More specifically, we wanted to identify mitochondrial proteins that undergo nutrient-dependent phosphorylation. Of particular interest would be phosphorylation events that increase mitochondrial energy metabolism, thereby augmenting nutrient-stimulated insulin secretion.
9.3. Results

9.3.1. Detection of mitochondrial phosphoproteins

Mitochondria from INS-1E cells were isolated and mitochondrial proteins were separated on SDS-polyacrylamide gels. To detect phosphorylated proteins, the gels were stained with Pro-Q Diamond (see Materials and Methods). This fluorescent dye detects phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins directly in SDS-polyacrylamide gels. This strategy has previously been applied successfully to identify protein phosphorylation (Schulenberg et al., 2003; Schulenberg et al., 2004). We detected a large number of Pro-Q Diamond-positive bands suggesting that many mitochondrial proteins are phosphorylated (Figure 1). The staining pattern obtained with Pro-Q Diamond was different from that obtained with the general protein staining dye Sypro Ruby. To confirm that Pro-Q Diamond specifically bound to phosphoproteins, we compared alkaline phosphatase-treated and control protein samples. Removal of phosphate groups by alkaline phosphatase resulted in a significant decrease of the intensity of Pro-Q Diamond-positive bands (Figure 1; compare lanes 2 and 3; lanes 4 and 5), demonstrating that Pro-Q Diamond is specific for phosphoproteins.

To test if resident mitochondrial proteins are phosphorylated, we treated intact mitochondria with alkaline phosphatase. Matrix and intermembrane space phosphoproteins are protected from alkaline phosphatase by mitochondrial membranes and, therefore, are expected to remain phosphorylated after alkaline phosphatase treatment. Indeed, several bands were protected from alkaline phosphatase (e.g. the band labeled with a blue arrow in Figure 1), suggesting that these are resident mitochondrial phosphoproteins. Phosphoproteins located outside mitochondria such as those attached to the mitochondrial surface and unrelated contaminating phosphoproteins are accessible to alkaline phosphatase and should be dephosphorylated by the enzyme. The intensity of many bands decreased upon alkaline phosphatase-treatment (Figure 1; compare lanes 2 and 3; e.g. the band labeled with a red arrow), suggesting that these proteins are not located inside mitochondria. In mitochondrial lysates all phosphoproteins are accessible to alkaline phosphatase. This explains why following alkaline phosphatase treatment, the Pro-Q Diamond signal was markedly reduced in mitochondrial lysates compared to intact mitochondria (Figure 1; compare lanes 3 and 5), while the intensity of the protein bands stained with Sypro Ruby was similar. Taken together,
these results strongly suggest that many matrix or intermembrane space mitochondrial proteins are phosphorylated in INS-1E cells.

Figure 1. Detection of mitochondrial protein phosphorylation in INS-1E cells

Intact INS-1E cell mitochondria and mitochondrial lysate were incubated with or without calf intestinal alkaline phosphatase (1 unit per μg protein) as described in Materials and Methods. 7 μg of protein was loaded on each lane of a 12% SDS-polyacrylamide minigel. The gel was stained with Pro-Q Diamond and then with Sypro Ruby. Lane 1: molecular weight marker, the 45 kDa protein is phosphorylated ovalbumin which served as a positive control for phosphoprotein staining. Other proteins of the weight marker are non-phosphorylated and were detected only with Sypro Ruby. Note that alkaline phosphatase (69 kDa protein) is detected in lanes 3 and 5 after Sypro Ruby staining as a pronounced band around 66 kDa. See Materials and Methods for details.

9.3.2. Detection of nutrient-induced mitochondrial protein phosphorylation

To probe for nutrient-dependent phosphorylation of mitochondrial proteins, we considered two possible approaches. The first one is to expose cells to nutrients and then isolate mitochondria, resolve mitochondrial proteins by SDS-PAGE and stain the gel with Pro-Q Diamond. The major drawback of this approach is that the phosphorylation of proteins may be altered during the isolation of mitochondria, for instance due to the action of protein phosphatases. An alternative approach is to isolate mitochondria and incubate them with substrates such as pyruvate or succinate. Following the exposure, mitochondria can be rapidly
lysed which minimizes the risk of protein degradation or modification by protein phosphatases. A possible disadvantage is that stimulation with nutrients in vitro may not necessarily reflect the glucose responses in intact cells. The second approach was chosen for the following experiments. Mitochondria were isolated by differential centrifugation from INS-1E cell homogenates. The functional state of the mitochondria was assessed by measuring oxygen consumption of mitochondrial suspensions. These measurements were performed using a Clark-type electrode as described in Materials and Methods. In the absence of exogenous nutrients, mitochondria consumed little oxygen (baseline in Figure 2A). Addition of 5 mM succinate immediately increased oxygen consumption (Figure 2A). Mitochondria were collected before addition of succinate (time point 1 in the graph) or after (time point 2). Mitochondrial proteins were separated by SDS-PAGE. Phosphorylated proteins were detected by Pro-Q Diamond staining (Figure 2B). The overall pattern of phosphoprotein staining was similar for the mitochondria collected before and after succinate stimulation. However, one protein band of about 60 kDa was more intensively stained with Pro-Q Diamond in the sample collected before succinate stimulation (shown by a red arrow in Figure 2B). This result suggests that this protein undergoes dephosphorylation upon stimulation of mitochondria with substrate. The neighboring band of lower molecular weight was stained more intensively following substrate stimulation, suggesting that this protein undergoes nutrient-dependent phosphorylation (shown by a green arrow in Figure 2B).
Figure 2. Detection of nutrient-dependent mitochondrial protein phosphorylation

(A) Oxygen consumption of mitochondrial suspension was measured using a Clark-type electrode. Addition of mitochondria, succinate and ADP is indicated by arrows in the graph. Mitochondria were collected before addition of substrates (time point 1) or after addition of substrates (time point 2).

(B) Mitochondria collected in (A) were quickly pelleted by centrifugation and snap-frozen in liquid nitrogen. Mitochondria were lysed and proteins were resolved by SDS-PAGE. Lane 1: molecular weight marker, the 45 kDa protein is phosphorylated ovalbumin which served as a positive control for phosphoprotein staining. Lane 2: mitochondria collected at time point 1. Lane 3: mitochondria collected at time point 2. The gel was stained with Pro-Q Diamond and then re-stained with Sypro Ruby. See Materials and Methods for details.
9.3.3. Mitochondrial purification

Having detected mitochondrial protein phosphorylation in INS-1E cells, we aimed at identifying individual mitochondrial phosphoproteins. To this end, mitochondrial proteins from INS-1E cells were separated by two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Pro-Q Diamond-positive candidate phosphoproteins were identified by mass-spectrometry.

For such experiments, it was critical to obtain highly purified mitochondria virtually free of contaminating cytosol, plasma membrane or endoplasmic reticulum. To reduce the amount of non-mitochondrial proteins, we decided to purify mitochondria by discontinuous Percoll gradient centrifugation (Sims and Anderson, 2008). We adapted this method for the isolation of INS-1E cell mitochondria (described in Materials and Methods). The crude mitochondrial fraction was layered on top of a discontinuous Percoll gradient containing a lower layer of 40% Percoll and an upper layer of 23% Percoll. Following centrifugation at 31000 g for 20 min, several bands were observed. The mitochondria were concentrated at the interphase between the two Percoll layers. To follow the purity of the mitochondria, we employed Western blotting using antibodies to markers of different subcellular compartments: subunit IV of cytochrome c oxidase (COX) for mitochondria, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for cytosol, and calnexin for endoplasmic reticulum (ER). The respective marker proteins were detected in total INS-1E cell lysate (Figure 3, lanes 5-7). The crude mitochondrial fraction obtained after 9000 g centrifugation of the cell homogenate (9000 g pellet, see Materials and Methods) was enriched in COX signal, and had no GAPDH (Figure 3, compare lanes 3 and 4). However, it was also enriched in calnexin. This result suggests that the crude mitochondrial fraction had no or little contamination by soluble cytosolic proteins but is highly contaminated by ER proteins. Percoll gradient-purified mitochondria (Figure 3, lane 2) had high COX signal while the calnexin signal was about 8-fold decreased compared with the crude mitochondrial fraction (Figure 3, lane 3). Such highly purified mitochondrial fractions were used for the identification of phosphoproteins.
Figure 3. Western blot analysis of subcellular fractions

Percoll fractions #1 and #2 were collected after 31000 g centrifugation from the top Percoll layer (fraction #1) and the interphase between the two Percoll layers (fraction #2). Crude mitochondrial fraction (pellet, P) and cytosolic fraction (supernatant, S) were obtained after 9000 g centrifugation of the INS-1E cell homogenate. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to an ECL-Hybond membrane. The membrane was cut into three fragments. The top fragment was probed with an antibody to calnexin, the middle fragment with an antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the bottom fragment with an antibody to subunit IV of cytochrome c oxidase (see Materials and Methods for details).

9.3.4. Detection of mitochondrial phosphoproteins on 2D gels

To identify mitochondrial proteins phosphorylated in a nutrient-dependent manner, we decided to stimulate INS-1E cells with 16.7 mM glucose, isolate mitochondria and subject their proteins to 2D PAGE, followed by Pro-Q Diamond staining and phosphoprotein identification by mass spectrometry. Unlike analyzing mitochondrial protein phosphorylation following treatment of isolated mitochondria with substrates (see chapter 9.3.2), stimulation of intact INS-1E cells with glucose may reveal physiologically-relevant phosphorylation events.
In two-dimensional gel electrophoresis, proteins are first separated according to their isoelectric point. In the second dimension, they are separated according to their molecular weight. As 2D gels separate proteins according to two properties of proteins, they result in better separation than 1D gels. Prior to separation on 2D gels, mitochondria from INS-1E cells were purified by centrifugation on Percoll gradient and proteins were precipitated as described in Materials and Methods. Proteins were separated on 2D polyacrylamide gels. Staining with Pro-Q Diamond revealed many phosphorylated proteins detected as individual spots in the gel (Figure 4). For the detection of total protein the gels were re-stained with Sypro Ruby. By comparing images corresponding to phosphoprotein and total protein staining, we detected phosphorylated proteins (seen on both images) and non-phosphorylated proteins (detected only with total protein stain) (Figure 4). The attribution of a given protein to either phosphorylated or non-phosphorylated should be taken cautiously as it depends on the sensitivity of Pro-Q Diamond. Proteins categorized as non-phosphorylated could in fact be phosphorylated but such weak phosphorylation could be below the detection limit. Some of the abundant proteins may bind small amounts of Pro-Q Diamond without being actual phosphoproteins. Among the phosphorylated proteins we detected proteins with strong and weak Pro-Q Diamond signals, suggesting different levels of phosphorylation. For further analysis we concentrated on proteins with the strongest Pro-Q Diamond signal. Among those there were abundant proteins (intensively stained with Sypro Ruby, ex. spots 3, 4, 8, 9, 18, 20, Figure 4) and non-abundant proteins (faintly stained with Sypro Ruby, ex. spots 29, 34, 35, 39, 40).
Mitochondria were purified by centrifugation on Percoll gradient. 250 μg of mitochondrial protein was resolved on a 2D polyacrylamide gel (16 x 16 cm). The gel was stained with Pro-Q Diamond (A) and then re-stained with Sypro Ruby (B) as described in Materials and Methods. Protein spots corresponding to phosphorylated proteins were numbered for further analysis.

We next assessed whether Pro-Q Diamond-positive spots were indeed phosphoproteins. To this end, Percoll gradient-purified mitochondria were lysed and a portion of the sample was treated with alkaline phosphatase, while another portion was treated similarly, omitting alkaline phosphatase (described in Materials and Methods). Alkaline phosphatase-treated and control protein samples were resolved on two separate 2D gels, the gels were stained with Pro-Q Diamond and then with Sypro Ruby. Less phosphorylated proteins were detected in
alkaline phosphatase-treated sample (Figure 5). However, the most abundant proteins intensively stained with total protein stain, were also detected by Pro-Q Diamond. This suggests that non-phosphorylated proteins can be stained by Pro-Q Diamond if present in large quantities. For instance, alkaline phosphatase was stained by Pro-Q Diamond in the alkaline-phosphatase treated sample (Figure 5C).

Figure 5. Pro-Q Diamond specificity for phosphoproteins

Mitochondria were purified by Percoll gradient centrifugation. 60 μg of mitochondrial proteins was separated on the 2D gel (8 x 8 cm), stained with Pro-Q Diamond (A) and re-stained with Sypro-Ruby (B). 60 μg of mitochondrial proteins was treated with alkaline phosphatase, separated on the 2D gel (8 x 8 cm), stained with Pro-Q Diamond (C) and re-stained with Sypro-Ruby (D). Alkaline phosphatase was detected as an abundant protein with a molecular weight of 70 kDa (labeled with a yellow arrow).
9.3.5. Identification of mitochondrial phosphoproteins

We aimed at identifying proteins with the highest intensity of Pro-Q Diamond signal. Only proteins sufficiently abundant to be detected by Sypro Ruby could be identified by mass spectrometry. This limited us to the identification of abundant Pro-Q Diamond-positive proteins. Several protein spots with a strong Pro-Q Diamond signal were cut from the gel and used for protein identification by mass spectrometry. The gel from one of these experiments is shown in Figure 4. The following protein spots were cut from the gel and analyzed by mass spectrometry: 18, 20, 24, 37, 38 and 41 (Figure 4). Only the first three of them were identified (Table 2). Proteins corresponding to spots 37, 38 and 41 could not be identified by mass spectrometry due to low abundance. Two of the identified proteins are ER proteins, which suggests that mitochondria preparation contained ER proteins even after purification on Percoll gradient.

Table 2. Phosphorylated proteins analyzed by mass spectrometry

<table>
<thead>
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<th>Spot number</th>
<th>Protein name</th>
<th>Synonyms</th>
<th>Molecular weight</th>
<th>UniProt accession number</th>
<th>EC accession number</th>
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</thead>
<tbody>
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<td>Spot 18</td>
<td>Protein disulfide-isomerase A3</td>
<td>58 kDa glucose-regulated protein (GRP58), 58 kDa microsomal protein, HIP-70</td>
<td>57 kDa</td>
<td>P11598</td>
<td>EC=5.3.4.1</td>
</tr>
<tr>
<td>Spot 20</td>
<td>Protein disulfide-isomerase</td>
<td>Cellular thyroid hormone-binding protein, prolyl 4-hydroxylase subunit β</td>
<td>57 kDa</td>
<td>P04785</td>
<td>EC=5.3.4.1</td>
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<tr>
<td>Spot 24</td>
<td>ATP synthase subunit α</td>
<td></td>
<td>60 kDa</td>
<td>P15999</td>
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</tr>
<tr>
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<td></td>
<td>25-30 kDa</td>
<td></td>
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</tr>
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</tr>
<tr>
<td>Spot 41</td>
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</table>
Project II.

Regulation of pyruvate dehydrogenase by phosphorylation in the pancreatic β-cell

10.1. Context

The controversial data on the relevance of PDH for β-cell function are described in chapter 7.4. One of the aspects which have not been studied is whether and how PDH is regulated in the β-cell during acute nutrient stimulation. Given the key role of PDH in mitochondrial metabolism, it can be hypothesized that acute regulation of PDH activity by nutrients or nutrient-derived signals can be an important regulatory step in the β-cell.

10.2. Aims

We investigated whether PDH is regulated by phosphorylation during nutrient stimulation, what signals regulate PDH phosphorylation and whether PDH phosphorylation is important for metabolism-secretion coupling.
10.3. Results

10.3.1. High glucose stimulates PDH E1α phosphorylation in INS-1E cells and rat pancreatic islets

We first tested whether the phosphorylation state of PDH E1α is affected during glucose stimulation of INS-1E cells. To this end, we exposed INS-1E cells to 2.5 or 16.7 mM glucose and analyzed phosphorylation of Ser^{293} (phosphorylation site 1) of PDH E1α subunit by Western blotting. Stimulation with 16.7 mM glucose resulted in a remarkable increase in PDH E1α phosphorylation (Figure 6A). This effect was time-dependent. An increase in phosphorylation could be seen already after 2 minutes of glucose stimulation. Using varying glucose concentrations revealed concentration-dependence of PDH E1α phosphorylation. The minimal glucose concentration required for PDH E1α phosphorylation was 7.5 mM (Figure 6B), which corresponds to threshold glucose concentration necessary for stimulation of GSIS. A similar concentration-dependence was observed for phosphorylation of Ser^{300} (phosphorylation site 2) of PDH E1α (Figure 6C). The relevance of glucose-induced PDH E1α phosphorylation was also tested in primary cells. Indeed, glucose stimulated PDH E1α phosphorylation in rat pancreatic islets to a similar extent as observed in INS-1E cells (Figure 6D). We next tested if other secretagogues could affect PDH E1α phosphorylation. Leucine and monomethyl succinate are two insulin secretagogues which stimulate mitochondria independently of PDH. Both secretagogues increase ATP production (Akhmedov et al., 2010), but nevertheless had no effect on the PDH E1α phosphorylation state (Figure 6E, F). These results suggest that PDH E1α phosphorylation is caused specifically by glucose and not during any nutrient stimulation. In order to test if increased NADH levels can stimulate PDH E1α phosphorylation, INS-1E cells were incubated with rotenone, an inhibitor of complex I of the respiratory chain. Rotenone was included for 2 to 10 minutes during incubation of cells at 2.5 and 7 mM glucose. This maneuver did not increase PDH E1α phosphorylation compared with that observed at 2.5 mM glucose (Figure 6G). Thus, glucose-stimulated PDH phosphorylation is not mediated solely by increase of NADH level.
Figure 6. Glucose stimulates PDH E1α phosphorylation in INS-1E cells and rat islets

(A) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated with KRBH containing 16.7 mM glucose for the indicated amount of time. Cell lysates (50 µg) were analyzed by Western blotting using an antibody specifically recognizing site 1 phosphorylated PDH E1α (p-Ser^293) and re-probed with an antibody recognizing PDH E1α independently of phosphorylation (total) as described in Materials and Methods.

(B, C) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated for 15 min with KRBH containing indicated glucose concentrations. Cell lysates (50 µg) were analyzed as in (A). In (C) an antibody recognizing site 2 phosphorylated PDH E1α (p-Ser^300) was used.

(D) Rat islets were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated for 15 min with KRBH containing 2.5 or 16.7 mM glucose. Whole islet lysates (50 µg) were analyzed as described in (A) using the antibodies indicated in the figure.
(E, F) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated for the indicated amount of time with KRBH containing different secretagogues as shown in the figure. Cell lysates (50 µg) were analyzed as in (A).

(G) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated for 15 min with KRBH containing the indicated glucose concentrations. Then 5 µM rotenone was added for the indicated amount of time. Cell lysates (50 µg) were analyzed as in (A).

10.3.2. Calcium stimulates PDH E1α dephosphorylation in INS-1E cells

Glucose-dependent increase in PDH E1α phosphorylation is a surprising result. Glucose stimulation is known to raise mitochondrial calcium and thus is expected to increase activity of PDP1 resulting in dephosphorylation of PDH E1α, a result opposite to our observation. Interestingly, the glucose-evoked increase in PDH E1α phosphorylation coincided with the glucose-stimulated mitochondrial calcium rise. Thus it was of interest to determine the role of calcium in the observed PDH E1α phosphorylation. To do this, we prevented glucose-stimulated calcium signals by applying EGTA at 16.7 mM glucose. This resulted in increased PDH E1α phosphorylation compared with 16.7 mM glucose alone (Figure 7A). On the other hand, in the condition where glucose-evoked calcium signals were further increased using 30 mM KCl, which depolarizes plasma membrane leading to calcium influx through voltage-dependent calcium channels, glucose was no longer able to stimulate PDH E1α phosphorylation (Figure 7A). This is likely due to increased activity of calcium-dependent PDP1. These results show that in INS-1E cells calcium-dependent regulation of PDH E1α phosphorylation is similar to other cell types. Under resting glucose (2.5 mM) augmenting or depleting calcium did not affect PDH E1α phosphorylation. These results show that nutrient stimulation and not calcium per se is the principal signal for PDH E1α phosphorylation. Dichloroacetate (DCA), a general inhibitor of PDKs, prevented glucose-stimulated increase in PDH E1α phosphorylation (Figure 7B), demonstrating that the observed PDH E1α phosphorylation is caused by PDKs.
Figure 7. Calcium and PDKs regulate PDH E1α phosphorylation

(A) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated for the indicated amount of time in KRBH containing 2.5 or 16.7 mM glucose and either EGTA (1 mM) or KCl (30 mM) as shown in the figure. Cell lysates (50 µg) were analyzed by Western blotting using the indicated antibodies.

(B) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose or in KRBH containing 2.5 mM glucose + 5 mM DCA. The cells were then incubated for 15 min in KRBH with 2.5 or 16.7 mM glucose and 5 mM DCA as indicated. Cell lysates (50 µg) were analyzed by Western blotting using the indicated antibodies.

10.3.3. Glucose-stimulated PDH E1α phosphorylation causes decrease in PDH activity

We proceeded to determine the effect of PDH E1α phosphorylation on PDH activity. We first measured PDH activity in INS-1E cell lysates prepared after cell exposure to 2.5 or 16.7 mM glucose. Samples were treated with PDP1 to convert PDH to fully active dephosphorylated form and determine maximal total PDH activity. Percentage of active PDH at 2.5 mM glucose was 91%. Following 15 minutes incubation at 16.7 mM glucose PDH activity decreased to 78% of maximal activity (Figure 8A). To assess PDH activity in living cells, we measured [1-14C] pyruvate oxidation. Carbon 1 of pyruvate is cleaved off by PDH and thus measurement of pyruvate oxidation can be used as readout of PDH activity. Pyruvate oxidation was decreased by ~30% in cells incubated at 16.7 mM glucose compared with cells incubated at
2.5 mM glucose (Figure 8B). These results demonstrate that PDH activity in INS-1E cells is close to maximal in basal non-stimulated state. Glucose-dependent PDH E1α phosphorylation lowers PDH activity, which is in agreement with the known inhibitory role of PDH E1α phosphorylation in PDH activity.

![Figure 8](image)

**Figure 8. Glucose stimulation decreases PDH activity in INS-1E cells**

(A) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then incubated for 15 min in KRBH containing 2.5 or 16.7 mM glucose. Cells were collected, lysed and PDH activity was measured as described in Materials and Methods. Parallel samples were treated with PDP1 in order to determine total PDH activity. Results are expressed as percentage of total PDH activity. Values represent the mean of four independent experiments +/- SEM.

(B) INS-1E cells were preincubated for 30 min in KRBH containing 2.5 mM glucose, then washed in KRBH without glucose and incubated with KRBH containing 2.5 or 16.7 mM glucose plus 0.1 mM cold pyruvate and [1-14C] pyruvate as described in Materials and Methods. Data are expressed as percentage of pyruvate oxidation at 2.5 mM glucose. Values represent the mean of three independent experiments +/- SEM. Statistical significance was determined by Student’s t-test for unpaired data; * p<0.05, ** p<0.01.

**10.3.4. Expression of PDK isoforms in INS-1E cells and rat tissues**

To determine which PDK isoforms are responsible for the observed phosphorylation we first assessed the expression of PDK isoforms in INS-1E cells. We compared expression of PDK1, PDK2, PDK3 and PDK4 in INS-1E cells, FACS-sorted rat α- and β-cells, pancreatic islets and a number of rat tissues by quantitative RT-PCR. In INS-1E cells PDK1 mRNA level was similar to that of β-cells, islets and comparable to most of the tissues tested (Figure 9A). Expression of PDK2 in INS-1E cells was similar to β-cells, islets and lower than in most
tissues (Figure 9B). PDK3 was well expressed in INS-1E cells and both α- and β-cells unlike the very low expression in liver and heart (Figure 9C). PDK4 was expressed in α-cells, islets and all tissues tested, but it was not detected in INS-1E cells (Figure 9D). The observed modest expression of PDK4 in β-cells can be explained by the presence of contaminating α-cells in the β-cell preparation. PDK4 is unlikely to be of importance for PDH regulation in INS-1E cells and β-cells.
Figure 9. Tissue- and cell-specific expression of PDK isoforms

PDK1 (A), PDK2 (B), PDK3 (C) and PDK4 (D) transcript levels were examined in INS-1E cells, α- and β-cells, and rat tissues by quantitative RT-PCR and normalized to cyclophilin or RPS-29 (muscle, heart). Expression levels are shown relative to INS-1E cells (A, B, C) or heart (D). The RNA was isolated from tissues obtained from two animals. The RNA from α- and β-cells was obtained after FACS sorting of islets (3 independent isolations). Values represent the mean of four experiments +/- SEM.
10.3.5. Role of PDK isoforms in PDH E1α phosphorylation

To test the role of PDK1, PDK2 and PDK3 in PDH E1α phosphorylation, we used siRNA to specifically decrease expression of these isoforms in INS-1E cells. Each PDK isoform was targeted by siRNA to allow selective knockdown. Transfection with siRNAs resulted in 58.8%, 64.6% and 73.6% decrease in expression of PDK1, PDK2 and PDK3 respectively (Figure 10A). Each siRNA was able to inhibit expression in an isoform-specific fashion. Knockdown of individual PDK isoforms caused neither a decrease nor a compensatory increase in expression of the other two isoforms (data not shown). PDK expression was not affected by treatment with transfection reagent alone or transfection with control siRNA. Following knockdown of the different PDK isoforms, INS-1E cells were assessed for glucose-dependent PDH E1α phosphorylation. Mock treatment or control siRNA did not alter glucose-induced PDH E1α phosphorylation. Glucose-dependent PDH E1α phosphorylation was remarkably reduced in cells transfected with siRNA against either PDK1 or PDK3. Knocking down PDK2 only modestly decreased glucose-stimulated PDH E1α phosphorylation. Using different combinations of siRNAs showed that PDH E1α phosphorylation is markedly decreased in all conditions where PDK1 or PDK3 are knocked down (Figure 10B). Decrease of PDH E1α phosphorylation upon knockdown of PDK1 or PDK3 could not be compensated by the other two PDK isoforms. Taken together, these results suggest that all three PDK isoforms contribute to PDH E1α phosphorylation with PDK1 and PDK3 being the most important isoforms in INS-1E cells.
Figure 10. Knocking-down PDKs expression decreases glucose-stimulated PDH E1α phosphorylation

(A) INS-1E cells were transfected with siRNAs as indicated in the figure. Mock represents cells treated with transfection reagent alone. The control siRNA was Negative Control siRNA #2 (Ambion). Expression of PDK1, PDK2 and PDK3 was measured by quantitative RT-PCR 96 h after transfection and is shown relative to mock-treated INS-1E cells. Values represent mean of three independent experiments +/- SEM. Statistical significance was determined by Student’s t-test for unpaired data; * p<0.05, ** p<0.01, # p>0.05 (not statistically significant).

(B) INS-1E cells were transfected with control siRNA, individual siRNA against PDK1, PDK2 or PDK3 or combinations of siRNAs as indicated in the figure. 96 h after transfection cells were shifted for 30 min in KRBH containing 2.5 mM glucose, then incubated for 15 min in KRBH containing 2.5 or 16.7 mM glucose. Cell lysates (50 µg) were analyzed by Western blotting using the indicated antibodies. A representative Western blot is shown.

10.3.6. Glucose-stimulated PDH E1α phosphorylation is not essential for metabolism-secretion coupling

We hypothesized that decrease of PDH activity upon PDH E1α phosphorylation should divert pyruvate towards PC, favoring anaplerosis. As this pathway is essential for the export of mitochondrial metabolites and formation of mitochondria-derived coupling factors, GSIS may
be potentiated. We first analyzed whether PDH E1α phosphorylation alters the glucose-evoked mitochondrial calcium rise, a key regulator of GSIS (Wiederkehr et al., 2011). We measured mitochondrial calcium in control INS-1E cells and under conditions where PDH E1α phosphorylation is prevented by siRNAs. For these experiments INS-1E cells were transfected with control or PDK-specific siRNAs and then infected with adenovirus expressing mitochondrially targeted aequorin. Stimulation of control INS-1E cells with 16.7 mM glucose resulted in a sharp increase in mitochondrial calcium concentrations. The same result was obtained in cells transfected with a mixture of siRNAs against PDK1, PDK2 and PDK3 (Figure 11A). Mitochondrial calcium rise in response to plasma membrane depolarization by 30 mM KCl was also not affected by PDKs knockdown. K<sub>ATP</sub> channel-dependent calcium influx stimulated by glucose and mitochondrial calcium handling are normal in INS-1E cells that fail to phosphorylate PDH E1α.

We next examined insulin secretion in INS-1E cells after PDK knockdown. In control conditions shifting cells from 2.5 to 16.7 mM glucose resulted in ~4-fold increase in insulin secretion. The same glucose-stimulated increase was observed in PDK siRNA-treated cells including the condition where all three PDK isoforms were knocked down (Figure 11B). Similarly, basal insulin secretion was normal following knockdown of one or a combination of PDK isoforms.

![Graph showing mitochondrial calcium rise](image)
Figure 11. Metabolism-secretion coupling is not affected by PDH E1α phosphorylation

(A) Mitochondrial calcium was measured in INS-1E cells transfected with control siRNA (black trace) or a combination of three siRNAs targeting PDK1, PDK2 and PDK3 (grey trace) and infected with adenovirus expressing mitochondrially-targeted aequorin. Cells were perifused with KRBH containing the indicated glucose concentrations. At the end of the experiment calcium influx was triggered using 30 mM KCl. Values represent the mean of five independent experiments. SEM are shown every 60 sec.

(B) INS-1E cells were incubated for 30 min in KRBH containing 2.5 or 16.7 mM glucose and insulin secretion and content were measured. Secreted insulin is shown as percentage of insulin content. Values represent the mean of four independent experiments +/-SEM.

10.3.7. PDH E1α phosphorylation is not important for INS-1E cell viability during glucotoxicity

Prolonged exposure of β-cells to elevated glucose has toxic effects known as glucotoxicity. The underlying mechanism, although not completely understood, involves activation of pro-apoptotic and lipogenic genes, resulting in increased apoptosis, lipid accumulation and reduced GSIS (Wang et al., 2005). We hypothesized that increase of PDH E1α phosphorylation leading to decreased PDH activity will result in lowered acetyl-CoA synthesis in the mitochondria, and subsequently decreased formation of the TCA cycle intermediates such as citrate. Decreased citrate production in the mitochondria is expected to decrease citrate levels in the cytosol, because citrate is exported to the cytosol via citrate-
isocitrate carrier (CIC). This should result in the decrease of citrate-derived acetyl-CoA in the cytosol and subsequently lowered fatty acid synthesis \textit{de novo}. As lipid accumulation is one of the central factors in glucotoxicity, reduced PDH activity and subsequent decreased triacylglycerol production in the cytosol should have protective effect under conditions of prolonged exposure to high glucose. To test this hypothesis, we first assessed PDH E1α phosphorylation after 48 h of culturing in regular INS medium containing 11 mM glucose and INS medium containing 30 mM glucose. PDH E1α phosphorylation was analyzed by Western blotting. In lysate prepared immediately after removal of regular INS medium PDH E1α phosphorylation was increased (Figure 12A, lane 3). Remarkably, PDH E1α phosphorylation was massively increased following incubation with 30 mM glucose (Figure 12A, lane 1). Interestingly, this hyperphosphorylation of PDH E1α was lost within 2 h in INS medium containing 4 mM glucose (Figure 12A, lanes 2 and 4). We wondered whether the glucotoxicity caused by 30 mM glucose would impair acute glucose-stimulated PDH E1α phosphorylation. To test this, INS-1E cells cultured for 48 h either in 11 or 30 mM glucose, were shifted for 30 minutes to KRBH containing 2.5 mM glucose and then to 16.7 mM glucose. Glucose-stimulated PDH E1α phosphorylation was much more pronounced in cells cultured at 30 mM glucose (Figure 12B). This can be explained by increased expression of PDKs during prolonged glucose exposure, which leads to an increase in total PDK activity. Increased expression of PDK1 and PDK2 resulting in augmented total PDK activity has been shown in mouse islets cultured for 72 h in 30 mM glucose (Xu et al., 2006).

We next analyzed cell viability after 48 h culturing in 11 and 30 mM glucose INS medium. Cell death was increased about 4-fold in cells cultured in 30 mM glucose compared to cells cultured in 11 mM glucose (Figure 12C). To test if PDH E1α phosphorylation affects cell viability, we compared cell death in cells treated with combination of siRNAs against PDK1, PDK2 and PDK3 and in control siRNA-treated cells. Down-regulation of PDK isoforms 1, 2 and 3 was neither protective nor did it enhance glucose-induced cell death (Figure 12C). This result suggests that despite the enhanced phosphorylation of PDH E1α at glucose levels inducing glucotoxicity, this regulatory mechanism does not affect β-cell viability.
Figure 12. PDH E1α phosphorylation does not affect cell viability at 11 and 30 mM glucose

(A) INS-1E cells were incubated for 48 h in INS medium containing 11 or 30 mM glucose as indicated in the figure. Cell lysates were prepared immediately after medium removal (lanes 1 and 3) or following 2 h incubation in INS medium containing 4 mM glucose (lanes 2 and 4). Cell lysates (50 µg) were analyzed by Western blotting using the indicated antibodies.
(B) INS-1E cells were incubated for 48 h in INS medium containing 11 or 30 mM glucose. The cells were then switched for 30 min in KRBH containing 2.5 mM glucose followed by 15 min incubation in KRBH with the indicated glucose concentrations. Cell lysates (50 µg) were analyzed by Western blotting using the indicated antibodies.

(C) INS-1E cells were transfected with siRNAs as indicated. Mock represents cells treated with transfection reagent alone. 48 h post-transfection the cells were changed to medium containing either 11 or 30 mM glucose as shown in the figure. Cell death was assessed 48 h later (see Materials and Methods). Values represent the mean of three independent experiments +/- SEM.
Project III.

Role of the calcium-binding protein S100A1 in the regulation of ATP production in INS-1E cells

11.1. Context

Glucose stimulation of the β-cell causes a mitochondrial calcium transient. One proposed function of this calcium rise is to stimulate the activities of several mitochondrial matrix dehydrogenases. It is likely that other proteins of the mitochondrial matrix are also regulated by calcium. For instance, calcium-binding proteins may serve as mediators of calcium signals by binding to mitochondrial matrix proteins. In other cellular compartments, calcium-binding proteins of the S100 family act as calcium sensor proteins and transmit the signal by calcium-dependent binding to target proteins thereby regulating their activity. S100 proteins are involved in the control of a large variety of calcium-dependent processes, including cell growth, differentiation, cell cycle progression and transcription (reviewed in Heizmann, 2002; Heizmann et al., 2002; Marenholz et al., 2004). All members of the S100 family (20 proteins) are of low molecular weight (10-12 kDa) and contain two EF-hand calcium-binding domains. The C-terminal EF-hand is common to all EF-hand calcium-binding proteins, while the N-terminal EF-hand is specific for the S100 proteins.

S100A1 is a member of this family of calcium binding proteins. S100A1 (also called S100α) is a 94 aminoacid protein (10 kDa). In the cell, S100A1 forms homodimers or heterodimers with the S100B protein (also called S100β). Among other tissues S100A1 is expressed in rat cardiomyocytes and, interestingly, has been shown to localize to mitochondria. Immunoprecipitation experiments demonstrated that S100A1 interacts with several mitochondrial proteins, including the α- and β-subunits of the ATP synthase. Moreover, overexpression of S100A1 resulted in increased ATP production, whereas its knockdown decreased ATP levels. Mice lacking expression of S100A1 had decreased activity of ATP synthase, comparing to wild-type controls (Boerries et al., 2007). Taken together, these results suggest a physiological significance of S100A1 for energy homeostasis in heart.
11.2. Aims

In this study, we wanted to test if the calcium-binding protein S100A1 functions as calcium sensor in β-cell mitochondria. More specifically, it was of interest to test if S100A1 can modulate ATP synthesis and metabolism-secretion coupling in the β-cell.
11.3. Results

11.3.1. Expression and localization of endogenous S100A1 protein in INS-1E cells

Given the central role of ATP production for GSIS, it was of interest to test if in the β-cell S100A1 has a similar function as in cardiomyocytes. First we tested whether S100A1 is expressed in insulin-secreting cells. S100A1 transcript levels were measured by quantitative RT-PCR in INS-1E cells, kidney, liver, brain, spleen and heart. S100A1 mRNA was detected in INS-1E cells and all tissues tested, with the highest levels measured in kidney, brain and heart (Figure 13A). S100A1 expression in INS-1E cells was about 3.5-fold lower than in heart, which is known to have especially high levels of S100A1. To analyze S100A1 protein levels, tissue lysates were analyzed by Western blotting using an S100A1 antibody (Figure 13B). The highest S100A1 protein levels were observed in kidney, brain and heart consistent with the S100A1 transcript levels measured by quantitative RT-PCR.
Figure 13. S100A1 expression in INS-1E cells and rat tissues

(A) Results of quantitative RT-PCR. Expression was normalized to the housekeeping gene cyclophilin and is shown relative to INS-1E cells. Values represent the mean of three independent experiments +/-SEM.

(B) Western blot detection of S100A1 protein. 160 μg of total tissue lysate was loaded in each lane of 15% polyacrylamide gel. The membrane was probed with S100A1 antibody and then stripped and re-probed with an antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Next, it was of interest to analyze the subcellular distribution of S100A1, in particular to test whether the protein is localized to mitochondria. To this end, mitochondrial and cytosolic fractions obtained by differential centrifugation of INS-1E cell homogenate were analyzed by Western blotting (Figure 14). Antibodies to subunit IV of cytochrome c oxidase (COX) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as mitochondrial and soluble cytosolic fraction markers, respectively. The mitochondrial protein was detected in the 9000 g pellet fraction (Figure 14, lane 1), while GAPDH was observed in the supernatant (Figure 14, lane 2). Likewise, S100A1 was detected exclusively in the supernatant (Figure 14, lane 2). This result suggests that S100A1 is a soluble cytosolic protein in INS-1E cells.
Figure 14. Western blot analysis of S100A1 subcellular distribution in INS-1E cells

Total INS-1E cell lysate, crude mitochondrial fraction (9000 g pellet, P) and cytosolic fraction (9000 g supernatant, S) were separated on 10% polyacrylamide gel. The membrane was probed with S100A1 antibody, then stripped and re-probed with antibodies to subunit IV of cytochrome c oxidase (COX) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As a positive control for S100A1 detection, INS-1E cells expressing adenovirus-encoded S100A1 were used (lane 7).

11.3.2. Cloning of the S100A1 gene

Although endogenous S100A1 displays cytosolic localization in INS-1E cells, it was of interest to test whether targeting S100A1 to the mitochondrial matrix compartment would affect ATP production as it does in cardiomyocytes. To this end, we constructed an adenovirus encoding the S100A1 fused to a mitochondrial targeting sequence. An adenovirus encoding S100A1 without the mitochondrial targeting sequence was constructed in parallel and used as a control.

The S100A1 cDNA (coding sequence) was cloned using reverse transcriptase and mRNA derived from INS-1E cells as a template. The main steps are illustrated in Figure 15.
INS-1E cell RNA was used to synthesize single-stranded DNA (ssDNA) by the reverse transcriptase reaction as described in Materials in Methods. For the subsequent cloning procedures it was necessary to introduce an NcoI restriction site (CCATGG) upstream of the S100A1 and an XbaI site (TCTAGA) downstream of the S100A1 coding sequence. To this end, PCR was used with specially designed primers (see Materials and Methods). The results of the reaction are shown in Figure 16A. To prevent digestion of S100A1 during NcoI and XbaI restriction, these sites should not be present in the S100A1 coding sequence. S100A1 has no XbaI sites however one NcoI site is present: … GAG ACC GCC ATG GAG ACC … To remove the NcoI site we decided to make a C to T substitution changing the NcoI consensus sequence CCATGG to CTATGG. This substitution changed Ala codon GCC to synonymic GCT, thus keeping the protein sequence unchanged. To introduce the substitution, we used PCR with specific primers one of which bearing a single nucleotide substitution (see Materials and Methods).

S100A1 cDNA was then ligated into pGEM®-T Easy vector as described in Materials and Methods. To confirm the insertion of S100A1, several clones of pGEM-S100A1 plasmid were analyzed by double digestion with NcoI and XbaI restriction enzymes (Figure 16B). Clones pDA011, pDA012 and pDA013 were sequenced by the University DNA Sequencing Facility. pDA012 was selected for further work.
(A) Results of PCR amplification of S100A1 using ssDNA as template and S100A1-specific primers. The amount of RNA used to synthesize specific ssDNA is indicated in the figure. After the PCR, the reaction mix was subjected to electrophoresis on 2.5% agarose gel. Lane 1: 100 bp DNA Ladder. Lanes 4 and 9; no reverse transcriptase was used in the ssDNA synthesis. Lane 10 is a negative control; no template was included in the PCR mix. Prior to ssDNA synthesis, RNA was treated with DNase to remove contaminating genomic DNA. In lanes 2-4, RNA was treated with DNase provided with the RNeasy Mini kit. In lanes 5-9, RNA was treated with DNase I (Ambion). The 300 bp band corresponding to S100A1 was excised from the gel and extracted. See Materials and Methods for details.

(B) Three clones of pGEM-S100A1 plasmid, called pDA011, pDA012 and pDA013 were analyzed by restriction with NcoI and XbaI. The reaction mixture was analyzed on agarose
gel as in (A). Lane 7 and 8 show pGEM-T Easy vector and S100A1 cDNA used in the ligation reaction. The S100A1 band is visible at 300 bp. See Materials and Methods for details.

11.3.3. Construction of the S100A1 adenoviral vector (Ad-S100A1)

The method used to create recombinant adenovirus encoding S100A1 is based on cloning of the gene of interest into the Adeno-X plasmid (32.7 kb) containing the Ad5 adenoviral genome (Clontech Laboratories, for details see “Adeno-X™ Tet-Off® & Tet-On® Expression System 1 User Manual” PT3496-1, http://www.clontech.com ). The resulting vector is linearized and used to transfect HEK293 cells. Following transfection, virus particles are produced which are then purified to prepare virus stocks. In the Adeno-X adenoviral genome the E1 and E3 genes necessary for viral DNA replication, transcription and assembly of viral particles are deleted. The virus is not able to replicate and propagate in any cell type except HEK293 cells which express the adenoviral genes E1 and E3. In the recombinant adenovirus, the transcription of the gene of interest is under control of the tetracycline-responsive element (TRE). To express a Tet-responsive gene in a target cell line, the recombinant virus should be co-infected with Adeno-X Tet-On® Virus, encoding reverse tetracycline-controlled transactivator (rtTA). rtTA binds the TRE element and activates transcription in the presence of tetracycline or its analog doxycycline. The transcription of the target gene can be turned on by addition of doxycycline to the culture medium. Moreover, expression can be modulated by varying the doxycycline concentration. The construction was done as described in the Adeno-X Tet-Off & Tet-On Expression Systems User Manual (PT3496-1). The main steps are shown in Figure 17.

```
Cloning S100A1 cDNA into pTRE-Shuttle2
  ↓
Cloning Tet-responsive expression cassette into Adeno-X vector
  ↓
Amplification of adenovirus in low-passage HEK293 cells
  ↓
Purification of adenovirus
```

Figure 17. Construction of recombinant adenovirus
11.3.3.1. Cloning S100A1 cDNA into the pTRE-Shuttle2 vector

The first step was to re-clone S100A1 from the pDA012 plasmid to the pTRE-Shuttle2 vector (Clontech Laboratories). The pTRE-Shuttle2 contains a TRE element upstream of the multiple cloning site. Thus, an insert cloned into the multiple cloning site is located downstream of the TRE element, so that in the resulting plasmid the inserted gene is expressed in a tetracycline-dependent manner. pDA012 was digested with NcoI and XbaI, the resulting DNA fragments were separated on the agarose gel and S100A1 was excised from the gel and purified. S100A1 was ligated into NcoI/XbaI-digested pTRE-Shuttle2. The reaction mixture was used to transform DH5α competent bacteria. To confirm the insertion of S100A1, several kanamycin-resistant clones were analyzed by double digestion with NcoI and XbaI restriction enzymes. Clones pDA031, pDA032 were sequenced by the University DNA Sequencing Facility and were found to carry the transgene without any unwanted mutations. pDA031 was selected for the next step.

11.3.3.2. Cloning of the S100A1 expression cassette into the Adeno-X vector

Next, the expression cassette containing S100A1 was re-cloned into the pAdeno-X plasmid. The expression cassette (1891 bp) was removed from the pDA031 by digestion with PI-SceI and I-CeuI restriction enzymes (Figure 18A). The expression cassette was ligated into pAdeno-X and transformed into E. coli DH10B competent bacteria. Ampicillin-resistant colonies were selected. Recombinants containing the expression cassette were identified by PCR using primers amplifying a 952 bp region within the expression cassette:

p206 (forward): 5’ GGC TTG ACC GAC AAT TGC 3’
p207 (reverse): 5’ GGT GTG GGA GGT TTT TTA AAG C 3’

Bacteria from ampicillin-resistant colonies served as a template for the PCR. In parallel bacteria from the same clones were grown in small liquid cultures. PCR products were analyzed on agarose gel electrophoresis. Two recombinant clones were identified by the presence of a 952 bp amplified fragment. They were grown overnight in ampicillin-containing LB medium and used to purify the recombinant pAdeno-S100A1 DNA. The presence of the expression cassette was confirmed by PCR as shown in Figure 18B. pAdeno-S100A1 DNA was used to transfect HEK293 cells. The adenovirus was amplified and purified as described in Materials and Methods.
Figure 18. Construction of the S100A1 adenoviral vector

(A) A clone of pTRE-S100A1 called pDA031 (2.5 μg) was digested with restriction enzymes PI-SceI and I-CeuI. The DNA from the reaction mixture was extracted by the phenol:chloroform:isoamyl method. The aqueous fraction was collected and applied on 0.9% agarose gel. 1 kb DNA ladder is loaded on the first lane. DNA fragments of the expected sizes are visible: the expression cassette containing S100A1 (1891 bp) and the remaining part of the vector (2816 bp). The upper band is undigested pDA031 vector.

(B) Results of PCR. 30 ng of the indicated constructs was used in each PCR. Reaction mixtures were analyzed on 0.8% agarose gel. Lane 1: 1 kb DNA Ladder. Lane 6: negative control: no template was included in the reaction mixture. DNA fragments of the expected size (952 bp) were detected. pAW322 (lane 7, amplified fragment size 1452 bp) and pDA031 (lane 8, amplified fragment size 952 bp) were used as positive controls.
11.3.4. Testing S100A1 adenovirus in INS-1E cells

To use the constructed adenovirus to overexpress S100A1 in target cells it was first necessary to optimize the amount of S100A1 adenovirus and Tet-ON adenovirus needed to achieve maximal percentage of infected cells and maximal S100A1 induction. The amount of virus used is expressed as infection units per cell (ifu/cell) and is called multiplicity of infection (M.O.I.). In addition to the amounts of viruses used, the ratio of Ad-S100A1/Tet-ON (coinfection ratio) can affect the efficiency of expression of the adenovirus-encoded gene. INS-1E cells were co-infected with the two adenoviruses: Ad-S100A1 and Tet-ON. M.O.I. ranged between 10-80 ifu/cell for Ad-S100A1 and 10-30 ifu/cell for Tet-ON adenovirus. Ad-S100A1/Tet-ON coinfection ratios were varied between 1:1 and 8:1. At 48 h post-infection cells were fixed and prepared for immunofluorescence to detect S100A1 expression (Figure 19A). The proportion of infected cells was calculated as a percentage of S100A1-positive cells out of total number of cells revealed by staining of cell nuclei with DAPI (Figure 19B). Maximal percentage of infected cells (>90% cells expressing the transgene) was observed when the combination of 60 ifu/cell of Ad-S100A1 and 30 ifu/cell of Tet-ON virus was used (Figure 19B).

In infected cells, massively overexpressing the transgene, S100A1 showed cytosolic localization. Endogenous S100A1 could also be detected but the signal was quite faint (Figure 19A). Endogenous S100A1 did not show the typical cytosolic distribution, but displayed a grain-like pattern. This pattern was distinct from that observed for mitochondrial proteins. This result demonstrates that in INS-1E cells endogenous S100A1 has neither a typical cytosolic nor mitochondrial location.

To determine the effective concentration of doxycycline needed to induce S100A1 expression, INS-1E cells were co-infected with 60 ifu/cell of Ad-S100A1 and 30 ifu/cell of Tet-ON and incubated for 48 h with varying concentrations of doxycycline. S100A1 protein levels were analyzed by Western blotting. S100A1 protein was detectable starting at 8 ng/ml of doxycycline, with maximal expression observed at 1000 and 5000 ng/ml (Figure 19C). To reveal the time-course of S100A1 expression, INS-1E cells were infected as above and incubated with 1 µg/ml doxycycline for different amount of time. S100A1 protein was detected after 18 h, maximal expression was observed after 36 to 48 h of doxycycline treatment (Figure 19D).
11.3.5. Effects of S100A1 overexpression on ATP production in INS-1E cells

We next tested if S100A1 overexpression altered glucose-induced ATP changes in INS-1E cells. ATP production was measured kinetically in INS-1E cells infected with adenovirus expressing luciferase in the cytosol. Cells were perifused with 2.5 mM glucose followed by 16.7 mM glucose (described in Materials and Methods). In control cells glucose stimulation resulted in a steady increase of luminescence, reflecting cytosolic ATP levels. After 40 min of glucose stimulation, ATP levels had increased by about 45% (Figure 19E; green trace). In cells infected with Ad-S100A1 and Tet-ON virus, glucose increased ATP levels by about 35% after 40 min of stimulation whether or not the transgene had been induced with doxycycline (Figure 19E; red and blue traces). This result demonstrates that S100A1 overexpression in the cytosol does not affect the glucose-induced rise in ATP in INS-1E cells.

To measure metabolic activity in control cells and cells infected with Ad-S100A1, Tet-ON and luciferase virus, we performed the MTT assay (Janjic and Wollheim, 1992; see Materials and Methods). In cells infected with the three adenoviruses, metabolic activity was reduced by about 10% compared to cells infected with luciferase adenovirus alone (Figure 19F). This effect was independent of induction of S100A1 with doxycycline. Reduced metabolic activity is a consequence of the presence of additional adenoviruses. Decrease of metabolic activity explains decreased rate of ATP production in cells infected with the three adenoviruses.
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B

![Bar graph showing the percentage of S100A1-positive cells for different coinfection ratios. The coinfection ratio is indicated at the bottom of the graph. The bars represent the percentage of S100A1-positive cells for each coinfection ratio: Ad-S100A1/TetON 10/10, 20/20, 20/10, 40/20, 60/30, 40/10, and 80/10. The x-axis represents the coinfection ratios, and the y-axis represents the percentage of S100A1-positive cells.]

C

![Western blot analysis showing the expression of S100A1 and GAPDH proteins with varying concentrations of dox (ng/ml). The kDa markers are labeled: 6.0, 37.1, and 25.9.]

D

![Western blot analysis showing the expression of S100A1 and GAPDH proteins over time with dox 1 μg/ml. The kDa markers are labeled: 6.0, 37.1, and 25.9. The x-axis represents the hours with dox 1 μg/ml, ranging from 0 to 48 hours.]
Figure 19. Testing the Ad-S100A1 in INS-1E cells

(A) Immunofluorescence analysis of S100A1 expression in INS-1E cells. Cells were left untreated (non-infected) or co-infected with Ad-mitoS100A1 and Tet-ON virus. Infected cells were incubated for 48 h without doxycycline (- dox) or with 1 μg/ml of doxycycline (+ dox) and prepared for immunofluorescence as described in Materials and Methods. S100A1 antibody was used to detect S100A1 protein. DAPI was used to stain cell nuclei. To test the specificity of S100A1 detection, the same protocol was performed without using primary antibody (no antibody control).

(B) INS-1E cells were infected at different M.O.I. (ifu/cell is indicated in the figure) and variable coinfection ratios and analyzed by immunofluorescence. Percentage of S100A1-expressing cells out of total number of cells (revealed by DAPI staining) is shown. N=1.

(C) Western blot analysis of S100A1 expression. INS-1E cells were infected with 60 ifu/cell of Ad-S100A1 and 30 ifu/cell of Tet-ON virus and incubated with the doxycycline concentrations (ng/ml) indicated in the figure. 48 h post-infection cells were lysed and lysates
(10 μg) were subjected to SDS-PAGE. The membrane was probed with S100A1 antibody, then stripped and re-probed with glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH).

(D) Western blot analysis of S100A1 expression. INS-1E cells were infected as in (C) and incubated with 1 μg/ml of doxycycline for the times indicated in the figure. Western blotting was performed as in (C).

(E) Effects of S100A1 overexpression on cytosolic ATP levels. INS-1E cells infected with luciferase-expressing adenovirus (green trace), or with luciferase adenovirus + Ad-S100A1 + Tet-ON without doxycycline (blue trace) or with doxycycline (red trace). Cytosolic ATP levels were monitored over time in the presence of 2.5 mM glucose (dashed line) and 16.7 mM glucose (solid line) by ATP-dependent luciferase luminescence. A representative result is shown from one of the three independent experiments.

(F) Metabolic activity of INS-1E cells infected with different adenoviruses measured using the MTT assay (see Materials and Methods). Amounts of viruses and doxycycline concentration are indicated in the figure. N=1.

11.3.6. Construction of adenoviral vector for expression of S100A1 in mitochondria (Ad-mitoS100A1)

Adenovirus encoding mitochondrially-targeted S100A1 was constructed using methodology similar to that described for the Ad-S100A1 virus. To target S100A1 to the mitochondrial matrix, it was necessary to clone the S100A1 cDNA downstream of a mitochondrial targeting sequence. For this purpose, the S100A1 cDNA was recloned from the pDA012 plasmid (pGEM-T Easy-S100A1) into the pTRE-Shuttle2 containing a mitochondrial localization sequence from the cytochrome c oxidase gene (pAW324 plasmid). The sequence of the resulting plasmid (pDA054) was confirmed by the University DNA Sequencing Facility.

The expression cassette containing the TRE element and the S100A1 fused to the mitochondrial localization sequence was cloned into the pAdenoX genome. The Ad-mitoS100A1 adenovirus was produced as described for the Ad-S100A1 virus. To test expression of mitoS100A1, INS-1E cells were co-infected with Ad-mitoS100A1 and Tet-ON virus. S100A1 protein was detected by immunofluorescence. MitoS100A1 localized to a filamentous network typical for mitochondrial proteins (Figure 20).
Figure 20. Immunofluorescence analysis of mitoS100A1 expression in INS-1E cells

Cells were left untreated (non-infected) or co-infected with 80 ifu/cell of Ad-mitoS100A1 and 40 ifu/cell Tet-ON virus. Infected cells were incubated for 48 h without doxycycline (- dox) or with 1 μg/ml of doxycycline (+ dox) and prepared for immunofluorescence as described in Materials and Methods. S100A1 antibody was used to detect S100A1 protein. DAPI was used
to stain nuclei. To test the specificity of S100A1 detection, the same protocol was performed without primary antibody (no antibody control).

11.3.7. Effects of mitochondrial S100A1 expression on metabolism-secretion coupling in INS-1E cells

To test the effect of S100A1 overexpression in the mitochondrial matrix, INS-1E cells were infected with Ad-mitoS100A1 and Tet-ON adenovirus. Infected cells were used to measure GSIS. Control cells infected with the viruses but not treated with doxycycline demonstrated a 3.5-fold increase in insulin secretion when shifted from 2.5 to 16.7 mM glucose. Induction of mitoS100A1 did not affect GSIS (Wiederkehr et al., 2011, Figure 3D). To measure mitochondrial calcium INS-1E cells were infected with adenovirus encoding mitochondrially targeted aequorin. Cells were perifused with 2.5 mM glucose and subsequently with 16.7 mM glucose (see Materials and Methods). In control cells, mitochondrial calcium rapidly increased from 200 nM to 1000 nM upon stimulation with 16.7 mM glucose. The same mitochondrial calcium response was observed in cells overexpressing S100A1 in the mitochondria (Wiederkehr et al., 2011, Figure 3C). This result argues against the participation of S100A1 in calcium signaling in the mitochondria. MitoS100A1 does not buffer the mitochondrial calcium signal. In addition, as it fails to augment insulin secretion, the protein does not augment mitochondrial function, which is inconsistent with the potentiating effect of S100A1 on mitochondrial function in cardiomyocytes.
Project IV.

Role of mitochondrial matrix pH in the β-cell

12.1. Context

Mitochondrial matrix alkalinization has been shown to occur in the β-cell and INS-1E cells during nutrient stimulation. Mitochondrial matrix alkalinization has been demonstrated to be essential for ATP production (see chapter 8). As ATP is a key factor for metabolism-secretion coupling, this suggests that matrix alkalinization can be an important biological process contributing to the control of nutrient-dependent insulin secretion.

12.2. Aims

The principal aim of this study was to test if the increase of mitochondrial matrix pH, observed in the β-cell during glucose stimulation, is of physiological importance. We investigated whether the matrix pH controls oxidative phosphorylation in intact INS-1E cells and acts as an intermediate signal in GSIS.

12.3. Results (article published in the FASEB Journal)
Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal β cells

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ABSTRACT Glucose-evoked mitochondrial signals augment ATP synthesis in the pancreatic β cell. This activation of energy metabolism increases the cytosolic ATP/ADP ratio, which stimulates plasma membrane electrical activity and insulin granule exocytosis. We have recently demonstrated that matrix pH increases during nutrient stimulation of the pancreatic β cell. Here, we have tested whether mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in the rat β-cell line INS-1E. Acidification of the mitochondrial matrix pH by nigericin blunted nutrient-dependent respiratory and ATP responses (continuously monitored in intact cells). Using electrophysiology and single cell imaging, we find that the associated defects in energy metabolism suppress glucose-stimulated plasma membrane electrical activity and cytosolic calcium transients. The same parameters were unaffected after direct stimulation of electrical activity with tolbutamide, which bypasses mitochondrial function. Furthermore, lowered matrix pH strongly inhibited sustained, but not first-phase, insulin secretion. Our results demonstrate that the matrix pH exerts a control function on oxidative phosphorylation in intact cells and that this mode of regulation is of physiological relevance for the generation of downstream signals leading to insulin granule exocytosis. We propose that matrix pH serves a novel signaling role in sustained cell activation.—Akhmedov, D., Braun, M., Mataki, C., Park, K.-S., Pozzan, T., Schoonjans, K., Rorsman, P., Wollheim, C. B., Wiederkehr, A. Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal β cells. FASEB J. 24, 4613–4626 (2010). www.fasebj.org

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The pancreatic β cell is able to sense a large number of secretagogues to adapt insulin release to blood nutrient concentrations. Nutrients such as glucose or amino acids are sensed via their uptake and metabolism, which results in downstream signals that lead to insulin granule exocytosis. Mitochondria play a central role in this metabolism-secretion coupling. They are required for the oxidative metabolism of nutrients and oxidative phosphorylation. The resulting increase of the ATP/ADP ratio causes closure of KATP channels, thereby initiating β-cell electrical activity (triggering pathway). Other mitochondria-derived metabolites may potentiate exocytosis by mechanisms that act in parallel with the regulation of the KATP channel (amplifying pathway) (1–3). Therefore, inhibition of mitochondrial function, in particular, respiratory chain activity, impairs insulin secretion (4–6).

The respiratory chain oxidizes NADH and FADH2 derived from oxidative metabolism to reduce molecular oxygen, which is coupled to the establishment of a proton electrochemical gradient across the inner mitochondrial membrane that drives the mitochondrial ATP synthase. The electrochemical potential is the sum of the electrical potential across the inner mitochondrial membrane and a chemical component proportional to the pH difference between the mitochondrial matrix and the cytosol (7).

Glucose stimulation of the β cell results in the activation of a large number of energy-demanding processes, including plasma membrane electrical activity, associated ion handling, Ca2+ cycling, gene transcription/translation, and insulin granule transport, as well as exocytosis. β-Cell mitochondria are able to cope with this increased energy demand by adjusting oxygen consumption and ATP synthesis rates (4, 8–10). Rapid adjustment of ATP synthesis to energy demand has been proposed to depend on a feedback control mech-
anism, whereby an increase in ADP and inorganic phosphate (P_i) due to ATP hydrolysis accelerates mitochondrial respiration to maintain a normal ATP/ADP*P_i ratio (phosphorylation potential). This mode of regulation may not necessarily apply to all tissues. For instance, in the heart, the energy demand increases severalfold during enhanced workload. Nevertheless, no changes of ATP or ADP levels during physiological stimulation were observed (reviewed in ref. 11).

In the pancreatic β cell, the phosphorylation potential increases during nutrient stimulation despite elevated energy consumption (9, 12, 13). This is achieved through a rise in oxygen consumption and ATP synthesis rates (8, 10). As ADP levels following nutrient activation are reduced (12, 14, 15), a feedback control mechanism for the acceleration of mitochondrial oxidative phosphorylation is unlikely. Therefore, other signals are needed to coordinate cytosolic energy demand and mitochondrial ATP synthesis. For instance, Ca^{2+} signals exert such a function, as they are relayed into mitochondria, where they activate matrix dehydrogenases, as well as the ATP synthase (16–18).

We have recently identified matrix pH as a potential signal that, like Ca^{2+}, could link nutrient stimulation to β-cell energy metabolism (10). Matrix pH has been studied in a number of cell types in situ and ranges from 7.7 to 8.2 (19–21). Matrix pH is therefore ~1 pH unit more alkaline than the cytosolic pH, which is close to 7. This proton concentration gradient adds about ~60 mV to the electrochemical potential (about ~150 mV) across the inner mitochondrial membrane (22), thus significantly contributing to the driving force on the ATP synthase. The proton chemical gradient is also linked to a number of transport processes exchanging metabolites or ions between mitochondria and the cytosol (23, 24).

We have previously shown that in INS-1E cells (clonal β cells) and primary rat β cells, the matrix pH is unusually low but alkalinizes following nutrient stimulation, paralleling the net cytosolic ATP increase (10). Furthermore, we demonstrated that preventing matrix alkalization in permeabilized INS-1E cells using the ionophore nigericin abolished substrate-driven ATP synthesis from exogenously added ADP (10). On the basis of these findings, we have tested here whether the mitochondrial pH is a modulator of oxidative phosphorylation and metabolism-secretion coupling in intact INS-1E cells.

**MATERIALS AND METHODS**

Most chemicals and reagents used for the experiments were from Sigma (Buchs, Switzerland) and Fluka Chemie (Buchs, Switzerland). Coelenterazine was purchased from Carbocell (La Jolla, CA, USA). Beetle luciferin was obtained from Promega (Basel, Switzerland). BCECF and JC-1 were from Molecular Probes (Invitrogen, Basel, Switzerland). BSA fraction V was from AppliChem (Darmstadt, Germany).

**Cell culture conditions**

INS-1E cells were cultured in RPMI 1640 medium containing 11 mM glucose (Invitrogen) supplemented with 10 mM HEPES (pH 7.3), 10% (vol/vol) heat-inactivated FCS (Brunschwig AG, Basel, Switzerland), 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 50 μg/ml penicillin, and 100 μg/ml streptomycin (INS medium).

**Recombinant adenoviruses**

The adenoviruses expressing mitochondrially targeted aequorin (Ad-RIP-mitoAequorin), cytoplasmic luciferase (Ad-RIP-Luciferase), and the Ad-RIP-mtAlpHi were constructed as described previously (10, 25). For cytosolic Ca^{2+} measurements, the Ca^{2+}-responsive protein construct YC3.6 (26) was cloned behind the rat insulin promoter. This construct was recloned into an adenovirus vector backbone (Clontech Laboratories, Mountain View, CA, USA). Adenoviruses were amplified in HEK293 cells.

**Luminescence measurements**

One day after plating, cultured cells were infected for 90 min at 37°C with either Ad-RIP-luciferase or Ad-RIP-mitoAequorin and analyzed 1 or 2 d later. All measurements described in this study were performed in Krebs-Ringer bicarbonate HEPES buffer (KRBH): 140 mM NaCl, 3.6 mM KCl, 0.5 mM MgSO_4, 0.5 mM NaH_2PO_4, 0.5 mM MgSO_4, 1.5 mM CaCl_2, 10 mM HEPES, 5 mM NaHCO_3, and 2.5 mM glucose, pH 7.4. The cells were perfused at a rate of 1 ml/min.

**Single-cell imaging**

For cytosolic and mitochondrial pH measurements, 10^6 INS-1E cells were plated onto polyornithine-coated 25-mm glass coverslips (Menzel, Bielefeld, Germany) in a 6-well plate. One day after plating, the INS-1E cells were infected with Ad-RIP-mtAlpHi (10) or Ad-RIP-YC3.6. Titration of the mitochondrial pH was performed as described previously (20). Image acquisition was performed on an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss AG, Zurich, Switzerland) with an array laser confocal spinning disk (QLC100; VisiTech, Sunderland, UK). Cells were imaged using a ×65 (numerical aperture 1.4) oil-immersion objective (Carl Zeiss AG). For mitochondrial pH measurements, mtAlpHi was excited using 488-nm laser light. The emission wavelength was 535 nm. Images were acquired every 10 s and analyzed using Metafluor 6.3 software (Universal Imaging: Molecular Devices, Sunnyvale, CA, USA). For cytosolic Ca^{2+} measurements, YC3.6 was excited with 440-nm light, and emission was followed at 480 and 535 nm. Images were acquired every 2 s. For cytosolic pH measurements, INS-1E cells were washed once in KRBH and loaded with the pH indicator BCECF (1 μM) for 8 min at 37°C. The cytosolic pH was measured ratiometrically, exciting with laser light of 440/488 nm and measuring the emission at 535 nm in KRBH containing sulfinpyrazone (100 μM).

**Mitochondrial membrane potential measurements**

INS-1E cells were plated into black-walled polyornithine-coated 96-well plates (75,000 cells/well; Greiner Bio-One,
Frickenhausen, Germany). Cells were washed with KRBH and 0.1% BSA and incubated for 30 min at 37°C in the presence of 350 nM JC-1 (Invitrogen). The cells were washed twice with KRBH and 0.1% BSA and incubated for 20 min. JC-1 fluorescence was measured ratiometrically at 37°C. The wavelengths used were 490 nm excitation/540 nm emission (green; monomer) and 540 nm excitation/590 nm emission (red; J aggregates) in a multiwell fluorescence reader (FlexStation; Molecular Devices) as described previously (27).

**Oxygen consumption**

INS-1E cells were seeded onto polyornithine-coated plates (Seahorse Biosciences, North Billerica, MA, USA). The cells were grown for 2 or 3 d in INS medium, reaching ~80% confluence (10^5 cells/well). The cells were washed 2 times with KRBH and 2.5 mM glucose. The plates were placed into the Seahorse XF24 instrument, and oxygen consumption rates were determined every 9 min. Stock solutions of glucose or nigericin were added during the run and immediately mixed to reach final concentrations as indicated in the figures.

**Electrophysiology**

Electrophysiological measurements were performed using the perforated-patch whole-cell configuration of the patch-clamp technique. Patch pipettes were pulled from borosilicate glass and fire-polished (tip resistance 4–8 MΩ). Recordings were performed using an EPC-10 amplifier and Pulse software (HEKA Electronics, Malone Bay, NS, Canada). The temperature in the recording chamber was kept at 32–33°C by continuous superfusion with heated KRBH. The pipette solution consisted of (mM) 76 K_2SO_4, 10 KCl, 10 NaCl, 1 MgCl_2, and 5 HEPES (pH adjusted to 7.35 with KOH). Electrical contact was established by the addition of the pore-forming antibiotic amphotericin B (0.24 mg/ml) to the pipette solution.

**Cell loss and apoptosis measurements**

INS-1E cells were incubated in KRBH and 16.7 mM glucose with or without 500 nM nigericin. After incubation, the cells were washed with phosphate-buffered saline and detached by trypsinization. Cell numbers were determined using a counting chamber (Neubauer, Marienfeld, Germany). Apoptosis was measured using a cell death detection ELISA Plus kit (Hoffman-La Roche, Basel, Switzerland).

**Insulin secretion**

INS-1E cells were plated into polyornithine-coated 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA; 4×10^5 cells/well) and grown for 48 h. Prior to the experiments, the cells were washed 3 times with KRBH, 2.5 mM glucose, and 0.1% BSA. The cells were preincubated for 30 min at 37°C in the same buffer. Before incubation, the wells were washed once with KRBH, 2.5 mM glucose, and 0.1% BSA and incubated for 30 min in the presence of different stimuli. Supernatants were saved for insulin measurements. Cells were extracted in acid ethanol overnight at 4°C to determine the insulin content. Insulin was measured using an enzyme immunoassay kit (SPI bio, Massy, France). For kinetic secretion measurements, 0.5-ml insulin samples were collected every 30 s in polystyrol tubes (PSN-55; Millian, Basel, Switzerland) containing BSA (final concentration 0.05%) during perfusion experiments also continuously recording the mitochondrial Ca^{2+} signal.

**Static ATP and ADP measurements**

INS-1E cells were prepared as for insulin secretion measurements. ATP was determined using a bioluminescence kit (HS II; Roche Diagnostics, Rotkreuz, Switzerland). ADP was measured after enzymatic removal of ATP, as described previously (28).

**Results**

During glucose stimulation, mitochondrial matrix pH in the insulin-secreting INS-1E cells increased from pH 7.25 ± 0.04 to 7.78 ± 0.02 (n=5) (Fig. 1A), consistent with our earlier findings (10). Mitochondrial pH was determined using the pH-sensitive probe mtAlpHi, as described previously (10, 20). The resting matrix pH was only 0.19 pH units higher than the cytosolic pH determined under identical conditions using the fluorescent indicator BCECF (Fig. 1C). Fifteen minutes after initiation of the glucose response, the ΔpH increased 3.5-fold to 0.66 (Fig. 1C). To assess the role of mitochondrial matrix pH and the alkalinization associated with glucose stimulation, we have used nigericin. This ionophore mediates the electroneutral exchange of potassium and protons across biological membranes. Matrix pH was lowered rapidly by nigericin (500 nM) and partially recovered over the following 15 min (Fig. 1B, D). In the presence of nigericin, matrix pH remained 0.31–0.49 pH units lower than the control along the entire time course of the glucose response.

**Matrix acidification lowers ΔpH and causes hyperpolarization of the mitochondrial electrical potential**

Nigericin is not specific for the inner mitochondrial membrane but also acts on other cellular membranes (22). For instance, at the plasma membrane, nigericin may mediate the export of potassium down its concentration gradient in exchange for the uptake of protons, and as a consequence cause acidification of the cytosol. However, 500 nM nigericin did not alter the cytosolic pH under conditions that caused a pronounced acidification of the mitochondrial matrix (Fig. 1C, D). Thus, the ΔpH across the inner mitochondrial membrane collapsed shortly after nigericin addition and partially recovered thereafter (Fig. 1D). At the concentration applied here, nigericin lowers the ΔpH across the inner mitochondrial membrane by ~0.4 pH units throughout.

Mitochondria attempt to maintain their electrochemical gradient constant. A decrease of the mitochondrial matrix pH of 0.4 pH units (equivalent to ~24
mV of proton motive force) as observed here should therefore be compensated for by an increase of the electrical potential. To study the electrical potential, we used the ratiometric probe JC-1 (27). Consistent with earlier studies, glucose stimulation of INS-1E cells causes hyperpolarization of the inner mitochondrial membrane (Fig. 2A) (27, 29). Nigericin further hyperpolarized the mitochondrial electrical gradient in a concentration-dependent manner (Fig. 2A). Hyperpolarization by the ionophore was also observed when cells were maintained continuously in the presence of 2.5 mM glucose (Fig. 2B). Such compensatory hyperpolarization of the electrical gradient across the inner mitochondrial membrane in response to nigericin has been described previously in cerebellar granule neurons (22). Unlike the protonophore FCCP, nigericin does not cause uncoupling of the inner mitochondrial membrane (Fig. 2A, B).

Matrix acidification inhibits the normal ATP response to glucose

The compensatory hyperpolarization should maintain the driving force on the ATP-synthase, thereby preserving ATP homeostasis. Therefore, we tested whether matrix acidification affects ATP synthesis in response to glucose. Relative changes of ATP were measured kinetically by expressing luciferase in the cytosol of INS-1E cells (25) (Fig. 2C). Following glucose stimulation, the cytosolic ATP signal increased. This glucose-stimulated response was strongly reduced by nigericin at both 100 and 500 nM (Fig. 2D). During the first 5 min of the glucose response, a reduction of the matrix pH had little or no effect. Thereafter, ATP levels steadily increased in the control but were strongly attenuated when the matrix pH was lowered by nigericin (Fig. 2D). Under basal glucose conditions, manipulation of the matrix pH with 500 nM nigericin caused a rapid reduction of the cytosolic ATP levels during the first 3 min (Fig. 2E). After 20 min, 500 nM had lowered the cytosolic ATP levels by 17.3 ± 1% (Fig. 2E; n=3). Thus, the matrix pH or ΔpH is relevant for maintenance of ATP homeostasis, as well as glucose-stimulated net ATP changes in INS-1E cells.

The effect of nigericin was further studied by measuring total cellular ATP and ADP after extraction of INS-1E cells 5 and 20 min following stimulation with 16.7 mM glucose (Fig. 2F). After 5 min, 16.7 mM glucose significantly elevated the ATP/ADP ratio com-
Matrix acidification causes hyperpolarization of the inner mitochondrial membrane and reduces glucose-dependent cytosolic ATP rises. A, B) INS-1E cells were loaded for 30 min with 350 nM of JC-1. Hyperpolarization was expressed as an increase of the JC-1 fluorescence ratio over basal (100%). Ratio after depolarization with FCCP (10 \mu M) was set to 0. INS-1E cells were stimulated with 16.7 mM glucose (A) or maintained at 2.5 mM glucose (B). Nigericin was added at increasing concentrations (20 nM, 100 nM, 500 nM), indicated by different grayscale levels. Control responses are shown in black. Dashed line indicates the time point of nigericin addition (n=3; each experiment performed in duplicate). Error bars = se; displayed every 120 s.

C) Relative cytosolic ATP changes were monitored in INS-1E cells 2 d after infection with Ad-RIP-luciferase. Net ATP changes were monitored during glucose stimulation in the presence (gray trace) or absence (black control trace) of 500 nM nigericin (n = 5). Error bars = se; displayed every 120 s. D) ATP responses were quantified after 20 min in the presence of 16.7 mM glucose and increasing concentrations of nigericin. Data are expressed as mean ± se percentage of control response to glucose performed on the same day (n=4).

E) ATP changes were measured as shown in C. INS-1E cells were perifused with KRBH containing 2.5 mM glucose; 500 nmol was added, as indicated by the dashed line (n=3). Error bars = se; displayed every 120 s. F) For static measurement of the ATP/ADP ratio, INS-1E cells were incubated for 5 and 20 min at the indicated glucose concentrations in the presence or absence of nigericin. ATP and ADP concentrations in lysates were determined and expressed as the ATP/ADP ratio (n=4). Values are means ± se. *P < 0.05.
pared to the cells maintained at 2.5 mM glucose. At this early time point, the ATP/ADP response had a tendency to be lower in the presence of nigericin, but the difference was not significant. At 20 min, nigericin had lowered the glucose-stimulated increase of the ATP/ADP ratio by >50% (Fig. 2F).

**Matrix acidification inhibits accelerated oxygen consumption in response to glucose**

The presented results are in agreement with the interpretation that matrix acidification slows mitochondrial ATP synthesis despite hyperpolarization of the electrical potential across the inner mitochondrial membrane. Through its effect on other biological membrane proteins, such as the Na+/K+ ATPase or the proton pumping vacuolar ATPase. To test whether matrix pH indeed affects mitochondrial energy metabolism, we studied respiration after manipulation of the mitochondrial pH. In control INS-1E cells, glucose caused a rapid increase in oxygen consumption. At 9 min after exposure to 16.7 mM, glucose respiration was already significantly increased, from a basal rate of 228 ± 4.4 pmol/min/10⁵ cells (basal respiration) to 531 ± 26.2 pmol/min/10⁵ cells. This initial rapid increase was followed by a slower phase reaching 837 ± 71 pmol/min/10⁵ cells 45 min after initiation of the glucose stimulus (Fig. 3A). The kinetics of the respiratory responses resembles the net changes of ATP as described in Fig. 2C.

The initial rapid glucose-dependent acceleration of respiration was unaffected by nigericin. In contrast, the slower second phase of the respiratory response to glucose was strongly reduced or even abolished when 100 or 500 nM nigericin was added at the time of glucose addition (Fig. 3A). To ensure that the initial increase in respiratory rate is unaffected by the matrix pH, we performed similar glucose-dependent oxygen consumption experiments after preincubation with nigericin. The result confirms that nigericin is unable to suppress the early effect of glucose on respiration (Fig. 3B). The data can therefore not be explained by a putative slow action of nigericin on mitochondrial function. Under basal conditions (2.5 mM glucose), nigericin caused little or no decrease of the oxygen consumption rate (Fig. 3B) and data not shown).

We conclude that matrix pH is a key regulator of mitochondrial oxidative phosphorylation. As alkalinization of the matrix pH occurs during glucose stimulation, this may be of particular importance for the second phase of the net ATP (Fig. 2C) and respiratory response (Fig. 3A, B) to glucose.

Using alternative substrates to stimulate mitochondrial respiration, we narrowed the possible control sites modulated by matrix pH. Leucine is a nutrient secretagogue that is metabolized to form acetyl-CoA without a direct involvement of glycolysis or pyruvate dehydrogenase. Leucine increases the net ATP levels (Fig. 3C) and causes matrix alkalinization similar to glucose (10). Leucine-dependent induction of oxygen consumption was rapid, reaching a maximal value at 9 min, slowly decreasing thereafter (Fig. 3D). Nigericin lowered the amplitude of the respiratory response by 41.4 ± 9.0% and abolished the leucine-dependent net ATP increase.

Methyl succinate is membrane permeable and can be used in intact cells to stimulate respiration. Oxidation of this substrate to fumarate by complex II provides reducing equivalents in the form of FADH2 to the respiratory chain. Direct stimulation of INS-1E cell by methyl succinate caused a sharp rise of respiration and cytosolic ATP levels. Both responses were blunted by 500 nM nigericin (Fig. 3E, F). The result with methyl succinate argues that matrix pH regulates oxidative phosphorylation downstream of complex II.

**Effect of nigericin on mitochondrial functions in HeLa and HepG2 cells**

To assess the general importance of this regulatory mechanism in the control of oxidative phosphorylation, we also tested the effect of nigericin on mitochondrial functions on two transformed widely used cell lines, HeLa and HepG2 (Fig. 4). To augment the contribution of mitochondria to total ATP synthesis in HeLa cells, glucose was omitted from the KRBH and replaced by 1 mM lactate and 0.1 mM pyruvate. Nigericin hyperpolarized the mitochondrial electrical potential in HeLa cells (Fig. 4A), similar to the results obtained with INS-1E cells (Fig. 2A, B). The effect of nigericin on cytosolic ATP levels was, however, weak (reduced by 5.9±3.0% after 30-min incubation in the presence of 500 nM nigericin; Fig. 4C). Nigericin (500 nM) also inhibited respiration of HeLa cells, but this was only apparent during prolonged incubation with the ionophore (Fig. 4B). In HepG2 cells, respiration was not affected by nigericin (Fig. 4D). We conclude that mitochondrial functions of INS-1E cells are particularly sensitive to the manipulation of the matrix pH by nigericin.

**Nigericin does not result in cell death or mitochondrial fragmentation**

The observed strong effects on respiration and ATP changes in INS-1E cells could be due to cell loss or death. To distinguish this possibility from a true effect of the matrix pH changes on mitochondrial respiration, cell numbers were determined at the end of a static incubation. One- or 2-h incubations in the presence of nigericin (500 nM) did not lower the number of cells when compared to control (KRBH and 16.7 mM glucose; Fig. 5A). A majority of cells was lost when
hydrogen peroxide (0.1 or 1 mM) was added as a positive control.

As a readout for apoptosis, we quantified cytosolic histone-bound DNA fragments using a cell-death detection kit (see Materials and Methods). Under control conditions, the number of apoptotic cells is low. Longer incubations in KRBH and 16.7 mM glucose resulted in doubling of cells undergoing apoptotic cell death, but at no time point did nigericin significantly enhance apoptosis (Fig. 5B). Furthermore, no histone-bound DNA fragments were detected in the cell supernatant, arguing against nigericin-induced necrotic cell death (data not shown).

Furthermore, mitochondria remained filamentous and retained JC-1 when incubated in KRBH and 16.7 mM glucose containing 500 nM nigericin (Fig. 5C). In

Figure 3. Nigericin inhibits substrate-dependent acceleration of mitochondrial respiration in intact INS-1E cells. INS-1E cells were stimulated with 16.7 mM glucose (A, B), 10 mM leucine (C, D), or 5 mM methyl succinate (E, F). Oxygen consumption rates (A, B, D, F) were measured every 9 min before and after substrate stimulation. ATP changes (C, E) were monitored as described in Fig. 2D. Nigericin was added at the same time as the indicated nutrient except in B, where nigericin (500 nM) was added 9 min prior to the initiation of the glucose response (open triangles). At the same time point, nigericin was also added to cells maintained at 2.5 mM glucose (B, open squares). For respiration experiments, se is shown for each condition and time point. A, B) Measurements were performed in quadruplicate in 3 independent experiments (n=5; n=12). C, E) Error bars = se; shown every 60 s (n = 3). D, F) Measurements were performed in quadruplicate; values are means ± se (n=4). Qualitatively similar results were obtained in 3 independent experiments. Solid diamonds, control; open triangles, 500 nM nigericin; open circles, 100 nM nigericin.
contrast, hydrogen peroxide induced mitochondrial fragmentation and partial loss of the dye (Fig. 5C and data not shown).

Most important, hyperpolarization of the inner mitochondrial membrane was more pronounced in the presence of nigericin even when analyzed for extended incubation times (Fig. 5D, 90 min). Mitochondrial integrity and cell viability were not compromised by nigericin over the time course studied here.

**Nigericin suppresses plasma membrane electrical activity and thereby cytosolic Ca2+ signals**

A main goal of this study was to assess whether matrix pH changes and the associated regulation of mitochondrial function is of physiological importance for metabolism-secretion coupling. We therefore tested whether the inhibitory effect of nigericin on cytosolic ATP levels is sufficient to blunt electrical activity resulting from closure of the KATP channels. To this end, we monitored the electrical activity of INS-1E cells using perforated-patch membrane potential recordings (30). In this recording configuration, electrical activity is controlled by endogenously synthesized ATP. Stimulation of resting INS-1E cells with 16.7 mM glucose caused plasma membrane depolarization and initiated the firing of action potentials (Fig. 6A, B). Nigericin dramatically decreased the frequency of glucose-induced action potentials (Fig. 6A, C) and partially repolarized the cell (Fig. 6B). Nigericin does not appear to have an acute effect on the membrane potential, as action potential frequency started to decrease only after a delay of 1–3 min, which was also associated with a partial membrane hyperpolarization (Fig. 6A, B). Lowering matrix pH increased plasma membrane KATP conductance (Fig. 6D, E). This is likely the consequence of lowered cytosolic ATP levels after nigericin treatment (Fig. 2C, D, F). Therefore, the link between mitochondrial activation and the plasma membrane electrical activity is perturbed by nigericin.

As an additional kinetic readout for a defect in metabolism secretion coupling, we monitored cytosolic Ca2+ transients, using the ratiometric probe YC3.6 (26). The YC3.6 coding sequence was cloned into an adenovirus vector backbone under the control of the rat insulin promoter. Following infection with the Ad-RIP-YC3.6, groups of INS-1E cells (6–12 cells) expressing the Ca2+-sensitive protein were assessed for their ability to respond to glucose (Fig. 7A). Stimulation with 16.7 mM glucose induced Ca2+ transients in 81% of the cells. Glucose increased the frequency of Ca2+ transients 5.1-fold over the frequency observed at resting glucose concentrations, whereas the average amplitude of the Ca2+ transients was only slightly increased (Fig. 7B, C). After the addition of nigericin to glucose-activated INS-1E cells, the frequency of cytosolic Ca2+ transients decreased almost back to basal (Fig. 7A, B). This attenuation was reversible (Fig. 7A).

Tolbutamide, a blocker of the KATP channel, was able to initiate action potentials and thereby cytosolic Ca2+ transients, even in the presence of nigericin (Figs. 6A, C and 7D). These results argue that nigericin does not suppress action potentials or Ca2+ influx by a direct effect but indeed acts by altering mitochondrial ATP synthesis and regulation of the KATP channel conductance. Taken together, the electrophysiological recordings and cytosolic Ca2+ measurements demonstrate a very strong inhibition of the KATP-channel-dependent
pathway of glucose-stimulated insulin secretion when the mitochondrial matrix pH was manipulated with nigericin.

Nigericin strongly reduces insulin secretion from INS-1E cells

This marked effect on metabolism-secretion coupling was also revealed in static insulin secretion experiments. In control INS-1E cells, 16.7 mM glucose raised insulin secretion 5.6-fold. Basal insulin secretion was not reduced by nigericin, but glucose-stimulated insulin secretion was only 1.6-fold over basal when the ionophore was present (Fig. 8A).

Given the pronounced suppression of insulin secretion by nigericin, we further tested whether the ionophore interferes directly with the exocytotic machinery, in addition to its upstream attenuation of mitochondrial function. To address this, we used
phorbol 12-myristate 13-acetate to stimulate PKC and thereby proteins of the exocytotic machinery (31). The addition of the phorbol ester neither increased mitochondrial pH nor affected cytosolic ATP levels (data not shown). We therefore conclude that it stimulates insulin secretion independently of mitochondrial activation. Nigericin did not affect phorbol 12-myristate 13-acetate-stimulated insulin secretion (Fig. 8B). This experiment demonstrates that nigericin does not interfere with the function of the exocytotic machinery per se.

The measurements of net cytosolic ATP levels (Fig. 2C, F) and mitochondrial respiration (Fig. 3) indicate that the initial phase of glucose-dependent mitochondrial activation is unaffected when the mitochondrial matrix is acidified. We, therefore, tested whether this early activation of mitochondria is sufficient to initiate metabolism-secretion coupling. For this, we followed the mitochondrial Ca$^{2+}$/H$^{+}$ signal in a population of INS-1E cells after infection with an adenovirus expressing mitochondrially targeted aequorin. Mitochondrial Ca$^{2+}$ rises are closely correlated with the cytosolic Ca$^{2+}$ signals, and therefore faithfully report on the kinetics of β-cell activation. During these experiments, samples were also taken from the efflux of the perifusion to determine the effect of nigericin on the kinetics of insulin secretion. Following glucose stimulation, Ca$^{2+}$ rose from 170 nM to a maximal concentration of 950 nM in the mitochondrial matrix (Fig. 8C). Stimulation with glucose in the presence of 500 nM nigericin resulted in an initial mitochondrial Ca$^{2+}$ rise, similar to the control (Fig. 8C and data not shown). However, when nigericin was present, the mitochondrial Ca$^{2+}$ signal was of much shorter duration than the control response, as the signal rapidly returned to basal (Fig. 8C). Perfusion with nigericin prior to the glucose stimulus did not prevent this transient mitochondrial Ca$^{2+}$ rise, demonstrating that nigericin was not simply acting too slowly to prevent the early phase of the matrix Ca$^{2+}$ signaling (data not shown). We conclude that initiation of metabolism-secretion coupling, and therefore, the initial mitochondrial Ca$^{2+}$ rise to glucose is resistant to the acidification of the mitochondrial matrix pH. During these perifusion experiments, control INS-1E cells augmented insulin secretion 5-fold when stimulated with 16.7 mM glucose, and insulin secretion remained elevated thereafter (Fig. 8D). In contrast, after acidification of the mitochondrial pH by nigericin, glucose-dependent insulin secretion was only transiently stimulated, peaked 2.5 min after initiation of the glucose stimulus, and then rapidly returned to basal rates (Fig. 8D). We conclude that the ΔpH across the inner mitochondrial membrane or the matrix pH has a very strong effect on metabolism-secretion coupling and becomes increasingly important during sustained-phase insulin secretion.
In the pancreatic β cell, the ATP/ADP ratio acts as an essential upstream signal for insulin granule exocytosis (3). This signaling role of the ATP/ADP ratio may explain why β-cell energy metabolism must prevent a rise in ADP even during times of enhanced ATP hydrolysis (9, 12, 13). In this cell type, feedback activation of oxidative phosphorylation by ADP is therefore not a likely mechanism to explain nutrient stimulation of the mitochondrial ATP synthesis rate. Recently, we proposed that the mitochondrial matrix pH contributes to the control of mitochondrial ATP synthesis in β cells. This concept is based on our findings that matrix pH is low in the resting β cell (pH 7.25) and increases to pH 7.7 following stimulation with nutrient secretagogues (10). By contrast, in a large number of other cell types, matrix pH in situ was shown to be alkaline (close to pH 8) (10, 19–21). Furthermore, our earlier results demonstrate that preventing matrix alkalinization in permeabilized INS-1E cells almost completely abolished ATP synthesis from exogenous ADP (10).

In the present study, we have manipulated the mitochondrial matrix pH in intact insulin-secreting cells, in order to test whether mitochondrial pH regulates oxygen consumption and ATP synthesis and to assess whether the matrix pH has an effect on metabolism-secretion coupling and hormone release. For this purpose, we have used nigericin, which mediates the electroneutral exchange of protons against potassium across biological membranes. Nigericin caused a rapid acidification of the mitochondrial matrix pH driven by the pH gradient across the inner mitochondrial membrane. We point out that weak acids failed to cause matrix acidification, probably because of the high buffering capacity and pH-controlling mechanisms in the cytosol (data not shown). Nigericin (500 nM) caused mitochondrial acidification starting within the first 10 s after addition but, remarkably, had no acute effects on the cytosolic pH or the plasma membrane potential. Partial repolarization of the plasma membrane, cessation of action potentials, and cytosolic Ca\(^{2+}\) transients were only observed after 1–3 min following the addition of the compound. We conclude that these actions of nigericin are mainly a secondary consequence of the inhibition of mitochondrial function. By lowering the mitochondrial ATP response to glucose, nigericin increases the \(K_{\text{ATP}}\) channel conductance, which prevents plasma membrane depolarization, Ca\(^{2+}\) influx, and insulin granule exocytosis.

On average, nigericin lowered the \(\Delta p\text{H}\) across the inner mitochondrial membrane by 0.4 pH units, thereby decreasing the electrochemical gradient, the driving force of the ATP synthase. This reduction was at least partially compensated by a hyperpolarization of the electrical gradient across the mitochondrial membrane. Despite this hyperpolarization, we observed a pronounced reduction of the cytosolic ATP,
as well as ATP/ADP ratio, in response to glucose. This blunting is likely due to inhibition of mitochondrial function rather than accelerated ATP hydrolysis in the cytosol, a conclusion that is supported by the inhibition of mitochondrial oxygen consumption by nigericin. Our data demonstrate that matrix pH or the linked pH effectively controls oxidative phosphorylation in insulin-secreting cells.

Glucose caused a very pronounced time-dependent increase of the respiration rate in INS-1E cells, which appeared to be biphasic. A rapid initial acceleration of oxygen consumption was followed by a slower gradual rise. Interestingly, the initial increase of the oxygen consumption rate and ATP response to glucose were unchanged and slightly reduced, respectively, when the matrix pH was lowered. In contrast, the slower second phase leading to a gradual acceleration of mitochondrial energy metabolism depends on matrix alkalinization and possibly other mitochondrial signals. Similarly, nigericin strongly reduced leucine- and methyl succinate-stimulated mitochondrial energy metabolism. Both of these substrates mimic the effects of glucose on insulin secretion. These substrates were even more sensitive to matrix acidification than glucose, as illustrated by the more rapid onset of inhibition. The pronounced attenuation of oxidative phosphorylation after stimulation with methyl succinate suggests that the main control function by matrix pH is exerted downstream of complex II.

ΔpH is also important as a driving force for a number of metabolite transport steps across the inner mitochondrial membrane (24). For instance, the uptake of the energy substrates pyruvate and glutamate or the uptake of inorganic phosphate for ATP synthesis is coupled to the net import of protons, and therefore depends on ΔpH. Alkalinization of the mitochondrial matrix in the β cell is therefore a plausible mechanism to ensure continued provision of these substrates during sustained insulin secretion.

Figure 8. Mitochondrial matrix acidification blunts insulin secretion in INS-1E cells. A, B) INS-1E cells were incubated for 30 min in KRBH buffer containing glucose (2.5 or 16.7 mM; A), nigericin (500 nM), or PMA (100 nM; B) as indicated. Insulin was measured in the supernatant and the cell content after 30 min of incubation and expressed as percentage of content. C) INS-1E cells were infected with an adenovirus for the expression of mitochondrial aequorin 2 d prior to the experiment. After loading with coelenterazine, cells were perifused in KRBH and 2.5 mM glucose and stimulated with 16.7 mM glucose (black traces) or 16.7 mM glucose and 500 nM nigericin (gray traces). Mitochondrial aequorin-derived luminescence was monitored and recalculated to Ca²⁺ concentrations. D) Samples in the efflux of the mitochondrial Ca²⁺ measurements were taken every 30 s, and insulin concentration was determined. Flux rate was 1 ml/min. Error bars = SE (n=3).
On the basis of our findings, we propose that matrix pH acts together with matrix Ca\(^{2+}\) to stimulate mitochondrial energy metabolism. Ca\(^{2+}\) has been shown to accelerate oxidative metabolism by activating matrix dehydrogenases (16). Matrix pH, on the other hand, may control respiration and ATP synthesis rates. Matrix Ca\(^{2+}\) and pH may not only complement each other but also directly depend on each other. However, our results in β cells argue against this possibility. First, the time courses of glucose-stimulated increases in mitochondrial Ca\(^{2+}\) and pH are strikingly different. Second, raising cytosolic Ca\(^{2+}\) without nutrient stimulation failed to induce matrix alkalization (10). Third, matrix acidification by nigericin neither caused mitochondrial Ca\(^{2+}\) uptake by itself nor potentiated the initial glucose-dependent mitochondrial Ca\(^{2+}\) rise. This is also consistent with our recent findings that stress the importance of the Na\(^+\)/Ca\(^{2+}\) exchanger as opposed to the Ca\(^{2+}\)/H\(^+\) transporter in the β cell. Inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger rescues mitochondrial dysfunction and glucose-stimulated insulin secretion in rat islets overexpressing dominant-negative PDx1 by increasing the glucose stimulated matrix Ca\(^{2+}\) signal (32).

The principal effect of matrix pH is on sustained nutrient-dependent stimulation of energy metabolism. Glucose-stimulated insulin secretion increased initially but was almost completely blocked thereafter. On the basis of its effect on metabolism-secretion coupling, matrix acidification progressively lowers mitochondrial Ca\(^{2+}\) signals as well. The described blunting of second-phase insulin secretion is therefore due to the absence of both mitochondrial activating signals. Initial metabolism-secretion coupling is much less affected, as mitochondrial Ca\(^{2+}\) reached similar maximal concentrations in the presence and absence of nigericin.

We propose that the matrix pH and mitochondrial calcium transients are complementary mechanisms regulating mitochondrial oxidative phosphorylation. Calcium signals are rapid and transient, which allows the adjustment of mitochondrial energy metabolism on a second-to-second basis. On the other hand, the matrix pH changes are slow and may modulate oxidative phosphorylation in response to long-lasting signals, as such changes in nutrient conditions.

This study demonstrates that the mitochondrial matrix pH controls the rate of oxidative phosphorylation. We propose that the low mitochondrial matrix pH observed in resting β cells is a physiological regulator that slows ATP synthesis to maintain the ATP/ADP ratio below threshold in order to prevent initiation of electrical activity. Once the matrix pH rises in response to nutrients, this inhibition is gradually lost with potentiating effects on both triggering and amplifying pathways of insulin secretion.

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DISCUSSION

Project I.

In this work we studied mitochondrial protein phosphorylation in INS-1E cells using PAGE-based phosphoprotein detection technique employing phosphoprotein-sensitive dye Pro-Q Diamond. Mitochondrial protein phosphorylation was detected in INS-1E cells using one-dimensional and two-dimensional PAGE. Together these results suggest that many mitochondrial proteins in this cell type are phosphorylated. Alkaline phosphatase treatment of intact mitochondria and mitochondrial lysates was used to discriminate between resident mitochondrial phosphoproteins and phosphoproteins associated with the outer surface of the organelle. In mitochondrial lysates all proteins are accessible to alkaline phosphatase and are efficiently dephosphorylated. In intact mitochondria a set of mitochondrial proteins are protected from alkaline phosphatase. A significant number of protein bands remained Pro-Q Diamond-positive even after alkaline phosphatase treatment of the intact mitochondria, which is a strong indication that mitochondrial resident proteins are phosphorylated.

We hypothesized that nutrients and nutrient-derived signals can regulate mitochondrial protein phosphorylation in the β-cell. Nutrient-dependency of mitochondrial protein phosphorylation was assessed in isolated mitochondria treated with the substrate succinate. Succinate stimulates mitochondrial metabolism and has been shown to stimulate insulin secretion in permeabilized cells (see chapter 5.3). Two bands changed their phosphorylation state in response to succinate stimulation: one demonstrating succinate-dependent phosphorylation and another – succinate-dependent dephosphorylation. These results suggest that mitochondrial proteins can be phosphorylated in a nutrient-dependent manner in INS-1E cells. Such regulation may serve a nutrient-sensing role in the β-cell. We speculate that phosphorylation of mitochondrial proteins such as subunits of respiratory chain complexes involved in energy metabolism, is important in the β-cell to adapt metabolism to the amount of available nutrients. Such regulation would be important in the control of insulin secretion. Nutrient-dependent change in phosphorylation status was detected using crude mitochondrial fraction. As shown in Figure 3, crude mitochondrial fraction is heavily contaminated with ER proteins. It is thus possible that proteins that changed their phosphorylation state upon succinate treatment are not mitochondrial proteins. Further work is necessary to make a link between nutrient stimulation, phosphorylation of mitochondrial proteins and the regulation of mitochondrial function.
To identify mitochondrial proteins regulated by nutrient-dependent phosphorylation, a second alternative approach was used; intact INS-1E cells were stimulated with 16.7 mM glucose followed by the isolation of mitochondria of the stimulated cells and the analysis of mitochondrial proteins by 2D gel electrophoresis. Several highly abundant phosphorylated proteins were identified by mass spectrometry (see Table 2). However, only one of these proteins, subunit α of ATP synthase, is a mitochondrial protein. Phosphorylation of subunit α of ATP synthase has been previously demonstrated in different cell types. In the reports by Hopper et al. and Aponte et al. isolated porcine heart mitochondria were energized by incubation with glutamate, malate, inorganic phosphate and ATP to mimic the situation of nutrient availability. Mitochondrial proteins were analyzed by 2D PAGE. Following staining of the gels with Pro-Q Diamond, Hopper et al. identified subunit α of ATP synthase as a phosphoprotein (Hopper et al., 2006). Aponte et al. detected phosphorylation of subunit α of ATP synthase in porcine heart and liver mitochondria using Phos-Tag 540 phosphoprotein stain and ³²P labeling (Aponte et al., 2009). In the follow-up study, de-energizing mitochondria by incubation in the absence of glutamate, malate, inorganic phosphate and ATP resulted in dephosphorylation of Ser65 of subunit α of ATP synthase (Boja et al., 2009). Taken together, these findings suggest that subunit α of ATP synthase is phosphorylated in conditions of nutrient availability and dephosphorylated in conditions of nutrient shortage. Our result show that this regulation could be important for regulating ATP production in response to varying nutrient concentrations in the β-cell and, as a consequence, may be essential for nutrient-dependent insulin-secretion. The importance of subunit α of ATP synthase for GSIS has been demonstrated in several studies. Rats bearing inactivating point mutation in the gene encoding subunit α of ATP synthase (BHE/cdb rats) develop diabetes with age (Berdanier, 1991; Mathews et al, 1995; Kim and Berdanier, 1999). Pancreatic islets from these rats have reduced glucose-dependent ATP production and decreased GSIS compared to wild-type rats (Liang et al., 1994; Saleh et al., 2008).

Phosphorylation of subunit β of ATP synthase has also been observed in several studies. In rabbit cardiomyocytes proteomics analysis revealed that upon treatment with adenosine and diazoxide, subunit β of ATP synthase is phosphorylated on five serine and threonine sites (Arrell et al., 2006). The functional consequences of four phosphorylation events were investigated in yeast Saccharomyces cerevisiae. For each of the four residues a mutant strain was generated in which phosphomimetic (substitution of serine/threonine to aspartic or glutamic acid) or nonphosphorylatable mutation (substitution of serine/threonine to alanine)
was introduced into these phosphorylation sites. Assembly of the F1Fo ATP synthase complex and ATPase activity were assessed. Phosphomimetic mutations of three sites (Thr58, Thr262 and Thr 318, corresponding to rabbit Thr107, Thr312 and Thr368) reduced ATPase activity compared to the wild-type strain. For the Thr58 and Thr262 mutants, this effect was accompanied by altered F1Fo complex formation (Kane et al., 2010), suggesting that phosphorylation decreases ATP synthase activity due to impaired F1Fo complex formation. Phosphorylation of subunit β of ATP synthase has also been demonstrated in human skeletal muscle. In muscle from type 2 diabetic patients, ATP synthase subunit β protein levels and phosphorylation were altered compared to that in healthy individuals (Højlund et al., 2003; Højlund et al., 2010). Taken together, these results suggest that phosphorylation of the β subunit of ATP synthase may be of physiological importance, however a direct demonstration of biological relevance of such regulation in mammalian cells is lacking. Regulation of subunit β of ATP synthase has been demonstrated to be relevant for GSIS. In rat islets several day exposure to leucine increased expression of subunit β of ATP synthase, resulting in increased ATP levels and enhanced GSIS (Yang et al., 2004; Yang et al., 2006). In INS1 cells siRNA-mediated decrease in expression of subunit β of ATP synthase resulted in decreased ATP production and diminished GSIS (Yang et al., 2004).

Although Pro-Q Diamond has been validated as a phosphoprotein dye in several publications (Schulenberg et al., 2004; Hopper et al., 2006; Kinoshita et al., 2006), identification of a protein based on Pro-Q Diamond staining is not sufficient to conclude that this protein is phosphorylated. Additional methods should be used to confirm protein phosphorylation, such as $^{32}$P labeling and identification of the phosphorylation site by mass spectrometry. In addition, Pro-Q Diamond has been reported to be less sensitive than $^{32}$P labeling. However, unlike $^{32}$P incorporation, phosphoprotein staining with Pro-Q Diamond is not dependent on phosphate turnover and thus better suited for identification of steady state protein phosphorylation (Hopper et al., 2006). An interesting alternative method for the identification of phosphoproteins has been reported. Prior to separation on 2D gels, the protein samples were divided into two aliquots, one of which was treated with λ-protein phosphatase and the other left untreated. The aliquots were resolved on two separate 2D gels. In phosphatase-treated samples removal of the phosphate group resulted in a shift of the protein spot toward a more alkaline area in the gel when compared to untreated sample (Yamagata et al., 2002). As mentioned earlier, only proteins sufficiently abundant to be detected with Sypro Ruby could be identified by mass spectrometry. This is a significant limitation of the method used here. It allowed identification of only the most abundant phosphorylated proteins. This
technique requires comparison of two images: phosphoprotein and total protein stains. A protein spot of interest should be found on both images. Often, the protein spot of interest is surrounded by many protein spots and the wrong spot can be selected for mass spectrometry identification by mistake. In the gel shown on Figure 4, for instance, protein spots 14 and 15 are not visible when looking for total protein staining. However, two neighboring spots with higher molecular weight are stained intensively with Sypro Ruby. Moreover, during washes and re-staining for total protein the gel size slightly changes making it difficult to identify the originally Pro-Q Diamond-positive protein spot beyond doubt.

Another difficulty of identification of phosphorylated mitochondrial proteins comes from the necessity of organelle purification and inevitable contamination with non-mitochondrial proteins. Although the mitochondria used in this study were purified by centrifugation on Percoll gradient and show remarkable decrease of contamination with ER proteins comparing to the crude mitochondrial fraction (Figure 3), two of the three identified phosphoproteins were ER resident proteins (Table 2).

In sum, mitochondrial protein phosphorylation in the β-cell is likely one of the mechanisms mediating mitochondria activation during nutrient stimulation. Protein phosphorylation in the mitochondria may be required to translate nutrient-derived signals into changes in mitochondrial protein activity. In the β-cell such regulation would contribute to nutrient-stimulated insulin secretion.

**Project II.**

A key finding of this study is that high glucose stimulates PDH E1α phosphorylation in INS-1E cells and rat islets. As balance between phosphorylated and dephosphorylated form of PDH E1α is determined by relative activities of PDK and PDP, the increase in PDH E1α phosphorylation can be a result of increased PDK activity, decreased PDP activity or combination of both. Simultaneous decrease of PDK activity and increase of PDP activity has been shown to activate PDH in an *in vitro* study (McLean et al., 2008). Reciprocal regulation of PDK and PDP activities has also been reported in rat heart and kidney (Huang et al., 2003).

PDK activity is stimulated by increased NADH/NAD⁺, acetyl-CoA/CoA and ATP/ADP ratios (Yang et al., 1998; Roche et al., 2003). The increased NADH/NAD⁺ ratio has been shown to inhibit PDPs which would also favor PDH E1α phosphorylation (Pettit et al., 1975). In the β-cell NADH/NAD⁺, acetyl-CoA/CoA and ATP/ADP ratios are rapidly increased following glucose stimulation (Patterson et al., 2000; Nilsson et al., 1996) and would be expected to
activate PDK, resulting in PDH E1α phosphorylation. If this increase in NADH/NAD\(^+\), acetyl-CoA/CoA and ATP/ADP ratios is a key factor stimulating PDK activity and PDH E1α phosphorylation during glucose stimulation, then any nutrient that increases production of NADH, acetyl-CoA and ATP should stimulate PDH E1α phosphorylation. However exposure of INS-1E cells to monomethyl succinate and leucine, both of which produce NADH and acetyl-CoA, did not stimulate PDH E1α phosphorylation. Monomethyl succinate is a membrane-permeant compound, which is converted to succinate in the cytosol. Succinate enters mitochondria and is converted to fumarate and then to malate. Malate is converted to oxaloacetate generating NADH. Alternatively, malate can be converted to pyruvate by mitochondrial or cytosolic malic enzyme. Pyruvate is converted to acetyl-CoA by PDH. Thus, stimulation with monomethyl succinate is expected to raise NADH and acetyl-CoA in the mitochondria. Leucine is transported to the mitochondria where it activates glutamate dehydrogenase stimulating conversion of glutamate to α-ketoglutarate, which feeds into the TCA cycle. TCA cycle activity raises mitochondrial NADH levels. Leucine can also be converted to ketoisocaproate (KIC) and subsequently to acetyl-CoA.

Why do monomethyl succinate and leucine not have the same effect on PDH E1α phosphorylation as glucose? Increased NADH/NAD\(^+\) and acetyl-CoA/CoA synergistically stimulate PDK activity. For instance PDK1 and PDK2 are only modestly stimulated by increased NADH/NAD\(^+\) ratio. Combination of increased NADH and acetyl-CoA was about ten times more efficient in stimulation of PDK1 and PDK2 activity compared to increased NADH alone (Bowker-Kinley et al., 1998). It is possible that glucose is more efficient in simultaneous production of NADH and acetyl-CoA than monomethyl succinate or leucine. In addition, monomethyl succinate and leucine differ from glucose in that they stimulate mitochondria directly bypassing glycolysis (reviewed in MacDonald et al., 2005). It is therefore possible that glucose-dependent PDH E1α phosphorylation is caused by a factor produced during glucose metabolism such as a glycolysis-derived metabolite. Another possible explanation comes from the fact that PDKs are physically associated with the PDH complex (Harris et al., 2002; Roche and Hiromasa, 2007). One can hypothesize that PDK activity is modulated by means of conformational changes depending on the flux through PDH in such a way that the presence of the PDH substrate pyruvate is required to activate PDKs. This condition is met in case of glucose but not monomethyl succinate or leucine which stimulate mitochondrial metabolism independently of PDH.
Glucose stimulation increases mitochondrial calcium and thus would be expected to stimulate PDP1 resulting in dephosphorylation of PDH E1α. Surprisingly, in INS-1E cells glucose raised mitochondrial calcium and stimulated PDH E1α phosphorylation at the same time. This suggests that during glucose stimulation total PDK activity is proportionally more activated than the PDP activity leading to enhanced PDH E1α phosphorylation despite the glucose-stimulated mitochondrial calcium rise. Preventing calcium signals during glucose stimulation further increased PDH E1α phosphorylation while raising calcium by KCl decreased phosphorylation. These findings are consistent with the known regulation of PDP1 by calcium. Manipulating calcium concentration had an effect only when glucose was augmented. Thus, high glucose is a regulator of PDH E1α phosphorylation status in INS-1E cells.

Measurement of PDH activity in INS-1E cells revealed that in basal condition (2.5 mM glucose) almost all of the PDH (~90%) was in its active form. This result is in agreement with Western blots that show little or no PDH E1α phosphorylation at 2.5 mM glucose. Glucose-induced increase in PDH E1α phosphorylation resulted in a ~ 13 and 30% decrease in PDH activity measured in vitro and in vivo, respectively. In agreement with our results, a high proportion of active PDH (94%) was reported in rat islets at resting glucose concentrations (Paxton et al., 1988). However, two publications report the opposite result. In a study by McCormack et al. in isolated rat islets only about 16% of PDH was active at 3 mM glucose. PDH activity rose to about 50% of total activity within 5 minutes after glucose stimulation (12-20 mM). This correlated with increased insulin secretion (McCormack et al., 1990). Nicholls et al. demonstrated glucose-stimulated increase in PDH activity in rat islets and a mouse insulin-secreting cell line MIN6 (Nicholls et al, 2002). Our results however strongly argue that both in INS-1E cells and rat islets PDH is phosphorylated and therefore its activity is inhibited following glucose stimulation.

In the present study we show that three PDK isoforms: PDK1, PDK2 and PDK3 are expressed in INS-1E cells. Although PDK4 expression was earlier reported in rat islets (Sugden et al., 2001), recent publications show no expression of PDK4 in INS-1 cells (Arunugam et al., 2010) and rat β-cells obtained by laser-capture microdissection (Jermendy et al., 2011). Specific knockdown of PDK isoforms by siRNAs revealed that PDH E1α phosphorylation is mainly due to the action of PDK1 and PDK3 while PDK2 is less important. Preventing PDH E1α phosphorylation under experimental conditions where all three PDKs were knocked down simultaneously did neither affect the glucose-dependent mitochondrial calcium rise nor
insulin secretion. This result agrees with the findings that decreasing or increasing PDH activity by overexpression of PDK or PDP, respectively, in rat islets did not affect GSIS (Nicholls et al., 2002). However, a more recent study on INS-1 832/13 cells had demonstrated enhanced GSIS upon siRNA-mediated knockdown of PDK1 (Krus et al., 2010). How can we explain different result obtained in our study? In INS-1E cells, PDH activity is close to maximal under basal conditions. PDH E1α phosphorylation only modestly decreases PDH activity. Preventing PDH E1α phosphorylation by PDK inhibition does not affect GSIS, demonstrating that slightly reduced activity of phosphorylated PDH E1α is sufficient for normal GSIS. We hypothesize that for normal GSIS PDH activity should be above a certain threshold. Any change of PDH activity will not affect GSIS unless the PDH activity drops below that threshold level. In the study by Krus et al. PDH phosphorylation was not examined and PDH activity was not measured as percent of total maximal PDH activity. It is possible that in INS-1 832/13 cells, unlike INS-1E cells, basal PDH activity is much lower than maximal and phosphorylation further decreases PDH activity below the critical threshold level necessary for normal GSIS. Preventing phosphorylation with PDK1 siRNA may enhance PDH activity above the threshold during glucose stimulation and thereby improve GSIS. In addition, in INS-1 832/13 cells, suppression of PDK1 expression increased PDH activity 1.7 fold (Krus et al., 2010). It thus seems that in this cell type phosphorylation has a more pronounced impact on PDH activity than in INS-1E cells.

In the present study we furthermore tested the hypothesis that PDH E1α phosphorylation protects from glucotoxicity. Consistent with published results, incubation with 30 mM glucose increased cell death compared with 11 mM glucose. In our experiments, PDH E1α phosphorylation did not affect INS-1E cell viability at 11 and 30 mM glucose. The likely explanation is that a slight decrease of PDH activity as a result of PDH E1α phosphorylation is not sufficient to significantly cause a protective effect. Decreased PDH activity has been shown to have a protective effect in rat islets under conditions of long-term high glucose exposure (Liu et al., 2004). After 48 h incubation at 16.7 mM glucose, PDH activity was reduced by 65%. Reduced PDH activity was shown to be essential to preserve normal GSIS as preventing this decrease in PDH activity blunted GSIS (Liu et al., 2004). The decrease of PDH activity was also reported in mouse islets after 72 h incubation with 30 mM glucose (Xu et al., 2006).

Although in this study we did not find any biological relevance of glucose-dependent PDH E1α phosphorylation, it is possible that under certain conditions this regulation is of
physiological importance. One of the important aspects of cell physiology is production of reactive oxygen species (ROS). ROS are produced when electrons from complex III of the electron-transport chain escape to directly reduce oxygen (reviewed in Klimova and Chandel, 2008). Interestingly, acute and chronic exposure of INS-1 cells to 20 mM glucose did not increase ROS production (Produit-Zengaffinen et al., 2007). Moreover, increasing glucose concentration up to 20 mM decreased ROS production in isolated rat pancreatic β-cells, despite the increase of mitochondrial respiration (Martens et al., 2005). The mechanism preventing ROS production under conditions of increased metabolic activity is not clear. It has been proposed that the protective effect of glucose is mediated by increased NADPH levels in the cytosol. NADPH is necessary to produce reduced glutathione by NADPH-dependent glutathione reductase, which is abundant in the β-cell (Laclau et al., 2001). In fact, glucose has been shown to stimulate concomitant increase in reduced glutathione and NADPH in rat islets (Ammon et al., 1980). Reduced glutathione oxidizes to reduce ROS. It can be hypothesized that PDH E1α phosphorylation favors pyruvate metabolism via PC and may thereby indirectly influence ROS formation. Oxaloacetate produced from pyruvate can be converted to malate by mitochondrial malate dehydrogenase. Malate can be exported to the cytosol, where it can be converted to pyruvate by malic enzyme, a reaction coupled to NADPH production. It thus possible that reduced PDH activity can favor cytosolic NADPH production, which in turn can be used to maintain high levels of reduced glutathione, necessary to decrease ROS levels.

Project III.

In this study we showed by subcellular fractionation and immunofluorescence that, in INS-1E cells, endogenous S100A1 exhibits cytosolic localization. Immunofluorescence experiments showed that in the cytosol endogenous S100A1 displays a granular-like pattern, not typical for soluble cytosolic proteins. The protein may therefore be loosely associated with cellular organelles, an association not preserved during biochemical fractionation. Previously in rat cardiomyocytes S100A1 has been shown to localize to the mitochondrial matrix (Boerries et al., 2007). Interestingly, in another study on the same cell type, immunofluorescence experiments showed that endogenous S100A1 co-localizes with the sarcoplasmic reticulum protein SERCA2a (Most et al., 2005). Given the granular-like pattern of S100A1 expression in the cytosol, we speculated that in INS-1E cells the protein is associated with the endoplasmic reticulum (ER), similar to its localization to the sarcoplasmic reticulum in cardiomyocytes. In this case S100A1 would be expected to be found in the 9000 g pellet
fraction, similar to the ER protein calnexin. This was not observed in INS-1E cells arguing against strong association between S100A1 and the ER.

It has been shown that in rat cardiomyocytes S100A1 physically interacts with the ATP synthase, stimulating ATP production (Boerries et al., 2007). Overexpression of S100A1 in the cytosol of INS-1E cells had no effect on ATP production. Moreover, overexpression of S100A1 specifically in the mitochondria did neither affect the mitochondrial calcium signals, nor GSIS. If S100A1 would have improved metabolism-secretion coupling, overexpression of the protein in the mitochondria would be expected to enhance the glucose-stimulated calcium rise and insulin secretion. It is also unlikely that S100A1 buffers mitochondrial calcium. If this were the case, the glucose-dependent mitochondrial calcium rise would have been decreased as observed in INS-1E cells overexpressing S100G in the mitochondria (Wiederkehr et al., 2011, Figure 3A).

The lack of effect of S100A1 expression on GSIS suggests that S100A1 does not stimulate ATP synthase in INS-1E cells. There are several explanations for these findings. One possibility is that the interaction between S100A1 and ATP-synthase requires a third protein factor. INS-1E cells may not express this cofactor, preventing activation of ATP synthase by S100A1 in this cell type. Another possibility is that S100A1 requires a specific pH in the mitochondria, which may be different in cardiomyocytes and INS-1E cells. It is also possible that mitochondrial calcium concentrations are different in cardiomyocytes and INS-1E cells.

When S100A1 was overexpressed in the mitochondria, no decrease of mitochondrial calcium signals was observed, which is likely due to the low calcium-binding affinity of S100A1 (Kd=290 μM, Heizmann and Cox, 1998). By contrast, overexpression of another S100-family protein in the mitochondrial matrix, S100G, efficiently attenuated mitochondrial calcium signals in INS-1E cells (Wiederkehr et al., 2011, Figure 3A), which is explained by its calcium binding affinity in the range of the mitochondrial calcium signal (Kd=0.3-0.5 μM, Linse et al., 1991).

**Project IV.**

Consistent with a previous report (Wiederkehr et al., 2009), glucose stimulation resulted in alkalinization of the mitochondrial matrix pH in INS-1E cells. Nigericin prevented this effect without altering the cytosolic pH. Under these conditions, the nutrient-dependent increase in cytosolic ATP levels and oxygen consumption were blunted. This demonstrates that oxidative
phosphorylation is strongly inhibited when matrix alkalinization is reduced. With time nigericin lowered the ATP/ADP ratio until $K_{ATP}$ channels reopened despite the continuous presence of glucose and as a result plasma membrane action potentials were abolished. Consistent with these findings, glucose-dependent cytosolic $Ca^{2+}$ transients and GSIS were inhibited.

Remarkably, the effects of nigericin on ATP levels and insulin secretion were most pronounced during the second phase of GSIS. This demonstrates that matrix pH alkalinization is primarily required for sustained cell activation.

Taken together, these results show that mitochondrial matrix pH is an important signal controlling oxidative phosphorylation and hence metabolism-secretion coupling.
CONCLUSION AND PERSPECTIVES

Project I.

- Mitochondrial phosphoproteins have been detected in INS-1E cells using the phosphospecific gel stain Pro-Q Diamond

- Many of the detected phosphoproteins are located in the mitochondrial matrix

- Two detected phosphoproteins display nutrient-dependent phosphorylation and dephosphorylation

- Among mitochondrial phosphoproteins detected on 2D gels, subunit α of ATP synthase has been identified by mass spectrometry

Future work should be aimed at confirming these results using another method of phosphoprotein detection such as $^{32}$P labeling. Novel mitochondrial phosphoproteins can be identified by mass spectrometry following protein separation on 2D gels. Mass spectrometrical analysis can be done to identify phosphorylation site in the subunit α of ATP synthase. This will allow directly testing the physiological importance of phosphorylation of this protein in INS-1E cells. For instance, the phosphorylated residue within the phosphorylation site can be substituted to alanine, making the phosphorylation site unphosphorylatable. Alternatively, the phosphorylation residue can be mutated to aspartic or glutamic acid, mimicking phosphorylation.
Project II.

- PDH E1α is rapidly phosphorylated following glucose stimulation in INS-1E cells and rat pancreatic islets. Stimulation with leucine or monomethyl succinate does not affect PDH E1α phosphorylation.

- Glucose stimulation decreases PDH activity.

- INS-1E cells express PDK1, PDK2 and PDK3. Glucose-stimulated PDH E1α phosphorylation is mainly mediated by PDK1 and PDK3.

- PDH E1α phosphorylation does not affect mitochondrial calcium signaling and insulin secretion in INS-1E cells.

- Chronic exposure to 30 mM glucose leads to hyperphosphorylation of PDH E1α in INS-1E cells. However this phenotype is not essential for cell survival under conditions of elevated glucose concentrations.

Future studies could be focused on the analysis of metabolic fluxes in the β-cell during glucose stimulation. It is of interest to compare relative fluxes through PDH and PC during stimulation with glucose, which increases PDH E1α phosphorylation, and leucine and monomethyl succinate which do not affect PDH E1α phosphorylation.
Project III.

- S100A1 displays cytosolic localization in INS-1E cells

- Overexpression of S100A1 in the cytosol or in the mitochondrial matrix does not affect ATP production, glucose-induced mitochondrial calcium rise and GSIS

Given the crucial role of mitochondrial calcium for the regulation of GSIS, it is likely that other members of the S100 family may function as calcium sensors in the mitochondrial matrix in INS-1E cells and β-cells. Future work can be aimed at testing the expression of several S100 family members in the β-cell mitochondria. To identify mitochondrial matrix proteins physically interacting with the expressed S100 proteins, immunoprecipitation experiments could be performed. To directly test the physiological roles of the expressed S100 proteins in the β-cell, the effects of siRNA-mediated decrease in their expression can be studied.

Project IV.

- Alkalization of mitochondrial matrix pH in INS-1E cells and rat islets during nutrient stimulation is required for oxidative phosphorylation and GSIS

- Mitochondrial matrix alkalization is particularly important for the second phase of insulin secretion

It is likely that in the β-cell changes of mitochondrial matrix pH are translated into changes of mitochondrial protein activity. It is possible that in the mitochondrial matrix pH affects posttranslational protein modifications such as protein phosphorylation. To detect pH-dependent protein phosphorylation events, a screen for mitochondrial phosphoproteins can be performed in the presence and absence of nigericin, which prevents mitochondrial matrix pH alkalization without affecting pH in the cytosol.
MATERIALS AND METHODS

13. Reagents

Chemicals were from Sigma (Buchs, Switzerland) and Fluka Chemie (Buchs, Switzerland) unless otherwise indicated.

14. Cell culture conditions

INS-1E cells were cultured at 37°C in humidified atmosphere (5% CO₂) in RPMI-1640 medium containing 11 mM glucose (Invitrogen, Cat. No. 21875), supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum (Brunschewig AG, Switzerland), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 μg/ml penicillin, 100 μg/ml streptomycin (INS medium). Cells were cultured in 75cm² tissue culture flasks (BD Biosciences, Bedford, MA, USA, Cat. No. 353135) and passaged weekly. Cells were washed with PBS without Ca²⁺ and Mg²⁺ and then treated with 0.05% trypsin-EDTA (Invitrogen, Cat. No. 25300054). 3-4 x 10⁶ cells were plated in 20 ml INS medium per flask. The growth medium was changed 3-4 days after plating.

Most INS-1E cell experiments were performed in Krebs-Ringer bicarbonate Hepes buffer (KRBH): 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 1.5 mM CaCl₂, 10 mM Hepes, 5 mM NaHCO₃, pH 7.4.

HEK293 cells were cultured in DMEM medium (Invitrogen, Cat.No. 41965-039) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 μg/ml penicillin and 100μg/ml streptomycin.

15. Bacterial strains and transformation

For DNA transformation DH5α and DH10B E.coli strains were used. DH5α bacteria were transformed by heat-shock. DH10B was transformed by electroporation. Transformants were selected on LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, 15 g/L agar, pH 7.2) containing 50 μg/ml ampicillin or 25 μg/ml kanamycin. Transformants were grown in liquid LB medium (5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl, pH 7.2) containing 50
μg/ml ampicillin or 25 μg/ml kanamycin to maintain selective pressure. Plasmid DNA was extracted from bacteria using QIAprep Spin Miniprep Kit (Qiagen, Cat. No. 27104) as described in the user manual.

16. DNA ligation

S100A1 and mitoS100A1 were amplified by PCR using Taq-polymerase which adds a single deoxyadenosine, in a template-independent manner, to the 3’-ends of the amplified fragments. Ligation of S100A1 cDNA into pGEM®-T Easy vector (Promega, Madison, WI, USA) was performed as described in “pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual” (can be found at: http://www.promega.com/). pGEM®-T Easy vector is provided in precut form with 3’-thimidine overhangs at both sides of the insertion site. This facilitates ligation with PCR products. The ligation mix was used to transform DH5α competent bacteria. Transformed bacteria were plated on LB agar plates containing 50 μg/ml ampicillin. Several colonies were picked the following day and grown in liquid LB containing 50 μg/ml ampicillin.

17. Polymerase chain reaction (PCR)

To amplify S100A1 specific ssDNA was used as template. Primers were designed to introduce a NcoI site and XbaI site upstream and downstream of the S100A1 coding sequence, respectively. In each primer only the 3’ part was specific to the target, while 5’ part contained desired restriction site sequence:

pcA1-1 (forward, 30 bp, the underlined part is specific to the S100A1 coding sequence. The NcoI site is shown in bold): 5’– GCTGCCATGGGCTCTGAGCTGGAGACCAGCC – 3’

pcA1-2 (reverse, 26 bp, the underlined part is specific to the S100A1 coding sequence. The XbaI site is shown in bold): 5’ – CTGCTTAGATGCTCAACTGTGTTCTCC – 3’

An undesirable NcoI site within the S100A1 coding sequence was mutated using PCR with the following primers:

pcA1-3 (forward, 40 bp, the underlined part is specific to the S100A1 coding sequence. The C to T single nucleotide substitution is shown in bold): 5’– GCTGCCATGGGCTCTGAGCTGGAGACCACGTATGGAGACC – 3’

pcA1-2 (reverse, 26 bp): 5’ – CTGCTTAGATGCTCAACTGTGTTCTCC – 3’
The reaction mix contained the following components (final concentrations are shown):

3-3.5 ng/µl DNA  
0.8 µM forward primer  
0.8 µM reverse primer  
ThermoPol Buffer 1x (New England Biolabs, provided with Taq Polymerase)  
Taq polymerase (New England Biolabs, Cat. No. M0267S) 0.02 Units/µl

The following thermocycle program was used:

Stage 1 (1 cycle): 95°C, 5 min  
Stage 2 (30 cycles): 95°C, 45 sec  
52°C, 45 sec  
72°C, 1 min 20 sec  
Stage 3 (1 cycle): 72°C, 2 min

18. DNA gel electrophoresis

DNA was resuspended in 6x loading buffer (0.25% bromophenol blue, 30% (v/v) glycerol, 120 mM EDTA) and subjected to electrophoresis in agarose gels. The gels contained 0.8-2.5% agarose (Invitrogen, Cat. No. 15510-027) and 1x SYBR Safe stain (Invitrogen, Cat. No. S33102) in 1x TAE buffer (40 mM Tris, 0.114% (v/v) acetic acid, 1 mM EDTA). The following DNA ladders were used: BenchTop 100 bp DNA Ladder (Cat. No. G8291, Promega, Madison, WI, USA) (Figure 16A) and 1 kb DNA Ladder (New England Biolabs, Cat. No. N3232) (Figure 18A,B). For subsequent cloning, DNA fragments were excised from the gels and extracted using MinElute Gel Extraction kit (Qiagen, Cat. No. 28606).

19. RNA isolation

Total RNA was extracted from INS-1E cells, rat pancreatic islets and tissues using RNeasy Mini Kit (Qiagen, Cat. No. 74104). INS-1E cells and pancreatic islets were suspended in lysis buffer (provided with the kit) and passed through a needle (0.8 x 40 mm 21G 1.5 inch Nr.2, BD Microlance, Ref. 304432) fitted onto a syringe (1 ml, BD Plastipak, Ref. 300013). Rat tissues were surgically removed, rinsed in ice-cold 0.9 mM NaCl. The tissue was cut into small pieces (~5 x 5 x 5 mm) and flash-frozen in liquid nitrogen. Then frozen tissues were grinded to powder, mixed with lysis buffer and processed as described in the RNeasy Mini Kit protocol. Following isolation, RNA samples were treated with 0.5 Units of RNase-free DNase I (Ambion, Austin, TX, USA) / 1 µg of RNA to remove contaminating genomic DNA.
The DNase digest was performed for 30 min at 37°C. DNase I was inactivated by heating for 10 min at 75°C. RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

20. Reverse transcription

ssDNA (single-stranded DNA) was synthesized using RNA as a template. The reaction was catalyzed by reverse transcriptase (SuperScript™ II, Invitrogen). The reaction mixture for ssDNA synthesis for quantitative RT-PCR contained the following components (final concentrations are shown):

75 ng/µl total RNA
15 ng/µl random hexamer primer (Invitrogen, Cat. No. 48190-011)
0.25 mM (each) dNTPs
10 mM DTT
1x First Strand buffer (Invitrogen)
10 Units/µl SuperScript™ II Reverse Transcriptase (Invitrogen, Cat. No. 18064-022)

First, RNA and hexamer primer were heated for 5 min at 95°C, and placed for 2 min on ice. Then the other components were added and the reaction mixture was incubated for 90 min at 37°C.

For specific S100A1 ssDNA synthesis, a gene-specific primer was used instead of the random primer:

pcA1-1: 5’– GCTGCCATGGGCTCTGAGCTGGAGACCGCC – 3’

The reaction mix contained the following components (final concentrations are shown):

50-250 ng/µl total RNA
0.1 µM pcA1-1 primer
0.5 mM (each) dNTPs
10 mM DTT
1x First-Strand buffer (Invitrogen)
10 Units/µl SuperScript™ II Reverse Transcriptase (Invitrogen, Cat. No. 18064-022)

A mixture containing RNA, pcA1-1 primer and dNTPs was incubated for 5 min at 65°C. Next, First-Strand buffer and DTT were added followed by incubation for 2 min at 42°C (primer annealing). Finally, reverse transcriptase was added and reaction was incubated for 50 min at 42°C. The enzyme was inactivated by heating for 15 min at 70°C.
21. Quantitative reverse-transcriptase PCR

ssDNA was used as a template in quantitative PCR using SYBR Green mix (FastStart Universal SYBR Green Master (Rox), Roche Diagnostics GmbH, Mannheim, Germany, Cat. No. 04 913 914 001) and specific primer pairs. The primers were designed using Primer Express 2.0 software. The primers used are listed in Table 3. The reactions were performed in 96-well plate format on ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using ABI PRISM 7000 SDS software. The reaction mix (25 µl) contained the following components (final concentrations are shown):

4-8 ng/µl ssDNA
0.3 µM forward primer
0.3 µM reverse primer
50% (v/v) SYBR Green mix

The following thermocycle program was used:

Stage 1 (1 cycle): 50°C, 2 min
Stage 2 (1 cycle): 95°C, 10 min
Stage 3 (50 cycles): 95°C, 15 sec
60°C, 1 min
Stage 4 (1 cycle): 95°C, 15 sec
60°C, 1 min
95°C, 15 sec

Expression levels were calculated according to Pfaffl method (Pfaffl, 2001). This a relative quantification method, which shows expression levels in number of folds as compared to expression level in the control sample. In each sample expression of gene of interest was normalized to the expression of a reference house-keeping gene cyclophilin or RPS-29. The method takes into account the difference in amplification efficiency between the gene of interest and reference gene. The amplification efficiency of the gene of interest (E gene) and reference gene (E ref gene) was calculated from the plot of Ct number against logarithm cDNA input according to the formula (Pfaffl, 2001):

\[
E = 10^{-\frac{1}{\text{slope}}}
\]

ΔCt for each gene was then calculated by subtracting the Ct number of treated sample from that of control sample. The expression of the gene of interest (normalized to reference gene) in treated sample compared to control sample was calculated according to the formula (Pfaffl, 2001):

\[
E = 10^{-\frac{1}{\text{slope}}}
\]
\[
\text{ratio} = \frac{(E \text{ gene})^{ΔCt \text{ control-treated}}}{(E \text{ ref gene})^{ΔCt \text{ control-treated}}}
\]

Table 3. Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophilin</td>
<td>Forward: CGT GGG CTC CGT TGT CTT</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGA CTT TAG GTC CCT TCT TAT CG</td>
</tr>
<tr>
<td>RPS-29</td>
<td>Forward: GGT CGC TTA GTC CAA CTT AAT GAA G</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCT GAA CAT GTG CCG ACA GT</td>
</tr>
<tr>
<td>S100A1</td>
<td>Forward: GCT GAG CAA GAA GGA GCT GAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAC AGC ATC TGC ATC CTT CTG</td>
</tr>
<tr>
<td>PDK1</td>
<td>Forward: CGG TGC CCC TGG CTG GAT TT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCA TCC GTC CCG TAG CCC TC</td>
</tr>
<tr>
<td>PDK2</td>
<td>Forward: GCT GTC CAT GAA GCA GTT TCT AGA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGG AGG AAG GTG AAT GAA GTT TT</td>
</tr>
<tr>
<td>PDK3</td>
<td>Forward: TGA CCT AGG TGG TGG AGT CCC A</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACC AAA TCC AGC CAA GGG AGC A</td>
</tr>
<tr>
<td>PDK4</td>
<td>Forward: TCT AAC GTC GCC AGA ATT AAA GC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAA CGT ACA CGA TGT GGA TTG G</td>
</tr>
</tbody>
</table>

22. siRNA transfection

INS-1E cells were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 353047) at a density of 7.5 x 10⁴ cells/well in antibiotic-free INS medium. Cells reached about 50% confluency the next day. At this time the medium was removed and antibiotic-free INS medium containing 30 nM siRNA, 1.4 µl/ml DharmaFECT 1 transfection reagent (Thermo Fisher Scientific, Lafayette, CO, USA, Cat. No. T-2001-03) was added. Dilution of siRNAs and preparation of transfection reagent/siRNA complexes were performed according to the manufacturer’s protocol. 24 h after transfection the medium was changed to regular INS medium. Decreased expression of target genes was confirmed by quantitative RT-PCR 96 h post-transfection. siRNAs were purchased from Ambion (Austin, TX, USA). siRNA sense strand sequences (5' → 3') are:

PDK1: GAA CUG UUC AAG AAC GCA ATT
PDK2: AGA ACA UCC AGU ACU UUU UTT
PDK3: GAU CCA CGC GUU UUA GAU ATT
As a negative control Negative Control siRNA #2 (Ambion) was used.

23. Amplification of adenoviruses in HEK293 cells

The restriction enzyme PacI was used to linearize pAdeno-S100A1 DNA (18 µg). Following digestion the DNA was precipitated using ethanol, NH₄OAc and glycogen as described in Adeno-X™ Tet-Off® & Tet-On® Expression System 1 User Manual PT3496-1(can be found at http://www.clontech.com). 14 µg of linearized DNA was used to transfect one 6 cm diameter plate (Cat. No. 353004, Becton Dickinson, Franklin Lakes, NJ, USA) of HEK293 cells (passage number 5-25). Cells were plated at a density of 1 x 10⁶ cells/plate 24 h before transfection. For transfection the calcium-phosphate method was used (CalPhos™ Mammalian Transfection Kit, Clontech Laboratories, Cat. No. 631312). At 4 days post-transfection cells remained intact but started to round up and detach from the plate in “grape-like” clusters (cytopathic effect). Cells were collected and transferred into a 50 ml Falcon tube. Attached cells were washed off the plate with PBS without Ca²⁺ and Mg²⁺ and transferred into the same tube. The cells were centrifuged at 65 g for 5-10 min and the cell pellet was resuspended in 300 µl PBS. The cells were lysed by 4 freeze-thaw cycles with vortexing between cycles. The cell lysates were centrifuged at 65 g for 5 min. The cleared supernatants were used for additional rounds of infection or stored at -80°C. After several rounds of amplification a stock lysate was prepared. HEK293 cells were plated in tissue culture plates (147.8 cm² growth area, TPP, Switzerland, Cat. No. 93150) at a density of 6 x 10⁶ cells/plate. Per virus 4-6 plates were used. The HEK293 cells were infected 24 h after plating using 5-20 µl of stock lysate. When approximately 50% of the cells were detached, the cells were collected and pelleted by centrifugation at 1100 g for 10 min at room temperature. Cells were resuspended in 2.5 ml of hypotonic Tris-HCl buffer (10 mM; pH 8.0) and lysed by freeze and thaw cycles. The lysates were frozen in liquid nitrogen and stored at -80°C.

24. Purification of adenoviruses

The cell lysate obtained from 4-6 plates of HEK293 cells (see chapter 23) was diluted to the volume of 5 ml with 10 mM Tris-HCl (pH 8.0) and centrifuged at 1100 g for 10 min at room temperature to remove cell debris. The supernatant was diluted to 6 ml with 10 mM Tris-HCl
This lysate was layered on top of a 1.43 g/ml CsCl (Applichem, Cat. No. A1098) solution (5 ml) in transparent tubes (Beckman 9/16 x 3.5, Cat. No. 344059) and centrifuged at 25000 rpm for 1 h at 4°C in SW41Ti rotor in the ultracentrifuge (Beckman L8-70M). Following centrifugation three distinct bands were observed. The upper band contained lipids and was removed and discarded. A pink band containing the bulk of the proteins was also discarded. A fainter greyish band containing virus particles was collected in ~2ml of volume. It was mixed with 8 ml of 1.34 g/ml CsCl, transferred into the transparent tube (Beckman 9/16 x 3.5, Cat. No. 344059) and centrifuged at 30000 rpm for 19-20 h at 4°C in SW41Ti rotor in the ultracentrifuge (Beckman L8-70M). After centrifugation a sharp band containing virus particles was formed. It was recuperated in 1-2 ml. The virus fraction was further purified using gel filtration. First, gel filtration columns (PD-10 Sephadex, GE Healthcare, Cat. No. 17-0851-01) were equilibrated with 25-30 ml of PBS without Ca²⁺ and Mg²⁺. Then virus was added and the effluent was collected in Eppendorf tubes (8 drops/tube). 10 µl from each tube was applied on the nitrocellulose sheet, which was stained with Ponceau red to determine the fraction with the highest protein concentration (corresponding to the highest concentration of virus particles). Several fractions were combined and sterile glycerol was added to a final concentration of 10%. This virus stock was frozen in liquid nitrogen and stored at -80°C.

25. Adenovirus titer determination

To determine the virus titer Adeno-X Rapid Titer Kit (Clontech Laboratories, Cat. No. 631028) was used according to the manufacturer's protocol. Briefly, HEK293 cells were plated in 12-well plates (Cat. No. 353225, Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5 x 10⁵ cells/well. The next day, 10-fold serial dilutions of the virus stock (from 10⁻² to 10⁻⁷) were prepared and used to infect HEK293 cells (1 dilution per well). At 48 h post infection, the cells were fixed and stained with an antibody raised against the adenovirus hexon protein (provided with the kit). Signal is detected after a secondary antibody conjugated with horseradish peroxidase (HRP) amplifies the signal of the antihexon antibody. HRP positive cells turn brown upon addition of metal-enhanced DAB substrate. The titer of the stock was determined by counting the number of brown cells in a given area. Each stained cell corresponds to a single infection unit (ifu). The virus titer was expressed as ifu/ml.
26. Mitochondria isolation and purification from INS-1E cells

26.1. Materials and equipment

Percoll, Amersham Biosciences, Cat. No. 17-0891-01
Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Cat. No. 04 693 132 001
Tissue culture dishes (147.8 cm$^2$ growth area), TPP, Switzerland, Cat. No. 93150
50 ml conical Falcon tubes, 11.5 x 3 cm, TPP, Switzerland, Cat. No. 91050
14 ml Greiner PP-tubes, round-bottom, 9.5 x 1.8 cm, Greiner Bio-One, Cat. No. 187 261
1.7 ml Eppendorf tubes
Plastic cell scrapers, TPP, Switzerland, Cat. No. 99010
Syringes 1 ml, BD Plastipak, Ref. 300013
Needles 0.8 x 40 mm 21G 1.5 inch Nr.2, BD Microlance, Cat. No. 304432
Glass potter (10.5 x 1.5 cm) with Teflon pestle and motor-driven homogenizer
Refrigerated centrifuge accepting 9.5 x 1.8 cm tubes and capable of up to 31000g centrifugation (Beckman J2-21 with JA-20 rotor)
Refrigerated centrifuge accepting 50 ml conical tubes (Hettich Rotanta TR)
Refrigerated bench-top centrifuge accepting 1.7 ml eppendorf tubes (Eppendorf 5415 R)

This protocol describes the procedure of obtaining ~200 µg of purified mitochondria. If different amount of mitochondria is required the amounts of starting cell material and buffers should be adjusted accordingly.

26.2. Preparation of starting material and reagents

3-4 days before the experiment, INS-1E cells were plated in 20 tissue culture plates (147.8 cm$^2$ growth area) at a density of 8-9 x 10$^6$ cells/plate. Experiments were started when the cells reached 80-90% confluency. Before the experiment the centrifuges were pre-cooled to 4°C.

The following solutions were prepared and kept on ice during the procedure:
500 ml of PBS without Ca$^{2+}$ and Mg$^{2+}$
300 ml of isolation buffer (IB). 260 ml were kept separately for collecting cells, the rest – for subsequent steps.
15 ml of IB containing 40% (vol/vol) Percoll,
10 ml of IB containing 23% (vol/vol) Percoll
5 ml of IB containing 15% (vol/vol) Percoll,
See Tables 4-7 for the recipes.
Table 4. Preparation of isolation buffer

<table>
<thead>
<tr>
<th>Isolation buffer (IB)</th>
<th>Amount of stock solution necessary for mitochondria purification from 20 tissue culture plates (147.8 cm²) of INS-1E cells</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solutions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1M sucrose</td>
<td>75 ml</td>
<td>0.25 M</td>
</tr>
<tr>
<td>1M Tris-HCl pH7.4</td>
<td>6 ml</td>
<td>20 mM</td>
</tr>
<tr>
<td>0.1M EGTA-KOH pH7.4</td>
<td>6 ml</td>
<td>2 mM</td>
</tr>
<tr>
<td>500 mM NaF *</td>
<td>6 ml</td>
<td>10 mM</td>
</tr>
<tr>
<td>100 mM Na₃VO₄ * (activated)</td>
<td>6 ml</td>
<td>2 mM</td>
</tr>
<tr>
<td>100 mM PMSF (in propanol-2)</td>
<td>300 µl</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Roche protease inhibitor cocktail 25x solution</td>
<td>12 ml (6tablets)</td>
<td>1x</td>
</tr>
<tr>
<td>pH to 7.4 with 1M HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>300 ml</td>
<td></td>
</tr>
</tbody>
</table>

* NaF and Na₃VO₄ are used to inhibit Ser/Thr- and Tyr-protein phosphatases, respectively. They can be omitted if protein phosphorylation is not going to be analyzed.
Table 5. Preparation of isolation buffer containing 40% (v/v) Percoll

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Amount of stock solution necessary for mitochondria purification from 20 tissue culture plates (147.8 cm²) of INS-1E cells</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll</td>
<td>6 ml</td>
<td>40%</td>
</tr>
<tr>
<td>1M Sucrose</td>
<td>3.75 ml</td>
<td>0.25 M</td>
</tr>
<tr>
<td>1M Tris-HCl pH7.4</td>
<td>0.3 ml</td>
<td>20 mM</td>
</tr>
<tr>
<td>0.1M EGTA-KOH pH7.4</td>
<td>0.3 ml</td>
<td>2 mM</td>
</tr>
<tr>
<td>dd H₂O</td>
<td>4.65 ml</td>
<td></td>
</tr>
<tr>
<td><strong>pH to 7.4 with 0.1M HCl</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>15 ml</strong></td>
<td></td>
</tr>
</tbody>
</table>

To avoid dilution of IB components, the 15% and 23% Percoll solutions were prepared by mixing IB containing 40% Percoll (see Table 5) and IB as shown in the tables below.

Table 6. Preparation of isolation buffer containing 23% (v/v) Percoll

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Amount of stock solution necessary for mitochondria purification from 20 tissue culture plates (147.8 cm²) of INS-1E cells</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll 40%</td>
<td>5.75 ml</td>
<td>23%</td>
</tr>
<tr>
<td>Isolation buffer</td>
<td>4.25 ml</td>
<td>1 x</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10 ml</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Preparation of isolation buffer containing 15% (v/v) Percoll

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Amount of stock solution necessary for mitochondria purification from 20 tissue culture plates (147.8 cm²) of INS-1E cells</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll 40%</td>
<td>1.875 ml</td>
<td>15%</td>
</tr>
<tr>
<td>Isolation buffer</td>
<td>3.125 ml</td>
<td>1 x</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>5 ml</strong></td>
<td></td>
</tr>
</tbody>
</table>
26.3. Mitochondria purification protocol

All steps of the mitochondrial isolation procedure were performed on ice.

Each plate was placed on ice, the growth medium was aspirated and the cells were washed twice with 10 ml of ice-cold PBS without Ca\(^{2+}\) and Mg\(^{2+}\). 7 ml of isolation buffer (IB) was added, the cells were scraped off the plate using a cell scraper and transferred into a 50 ml Falcon tube. Then the remaining cells were washed off the plate with 5 ml of IB and transferred into the same Falcon tube. In the end there were five Falcon tubes, each containing about 50 ml of cell suspension. The tubes were centrifuged for 5 min at 1700 rpm (~500 g) at 4°C. The supernatant was discarded. The cells were resuspended in 6 ml of IB using 1ml pipette tip, followed by addition of another 6 ml of IB (the resulting suspension is ~12 ml). The cell suspension was homogenized in glass potter using rotating Teflon pestle (500 rpm, 25 strokes) on ice. The homogenate was transferred into two 14 ml Greiner tube (labeled #1). The tubes were centrifuged for 8 min at 3000 rpm (~1000 g) at 4°C. The supernatant was transferred into two Greiner tubes (labeled #2). The pellet was resuspended in 4 ml of IB and homogenized at 500 rpm (16 strokes). The homogenate was transferred into Greiner tubes. These tubes were centrifuged for 8 min at 3000 rpm (~1000 g) at 4°C. The supernatant was transferred into the Greiner tubes #2. The pellet (nuclei, cell debris) was discarded. Tubes #2 were centrifuged for 8 min at 3000 rpm (~1000 g) at 4°C. Supernatant was collected into Greiner tubes, the pellet was discarded. These tubes were centrifuged for 10 min at 9000 rpm (~9000 g) at 4°C. Supernatant (cytosolic fraction) was collected and kept at -80°C for the future analysis. The pellet (crude mitochondrial fraction) was resuspended in 250 μl of IB. It was stirred well with the plastic pipette tip, pipetted up/down several times to remove clumps and transferred into an Eppendorf tube. Several μl were kept at -80°C for the future analysis. The rest mitochondria were centrifuged for 9 min at 11500 rpm (~11000 g) at 4°C (bench-top centrifuge). The supernatant was discarded and pelleted mitochondria were kept.

A discontinuous Percoll gradient was prepared in two Greiner tubes. First, 2 ml of 23% Percoll solution was placed in the tube. Then, 2 ml of 40% Percoll was carefully layered to the bottom of the tube using a syringe with 21G needle. A sharp interface between 23% and 40% Percoll was visible. Mitochondrial pellet was resuspended in 1 ml of 15% Percoll. 0.5 ml of this suspension was layered on top of the Percoll gradient in each tube. These tubes were centrifuged for 20 min at 16000 rpm (~31000 g) at 4°C. The upper fraction (#1) was collected in ~1 ml. The fraction formed in the interphase of two Percoll layers (mitochondria-enriched fraction) was collected in ~1.25 ml and transferred into Greiner tube. This fraction was diluted
to 12 ml with IB and centrifuged for 10 min at 12000 rpm (~17000 g) at 4°C. Supernatant was carefully removed leaving loose pellet and a few millimeters of liquid at the bottom of the tube. The pellet (mitochondria) was resuspended in 1 ml of IB and transferred into an Eppendorf tube. The tube was centrifuged for 10 min at 11500 rpm (~11000 g) at 4°C (bench-top centrifuge). The supernatant was discarded. The pellet (purified mitochondria) was kept at -80°C.

27. Detection of phosphorylated proteins

27.1. Protein sample preparation

Mitochondria were isolated from INS-1E cells as described above. Pelleted mitochondria (100-120 μg of total protein) were thawed in 37°C waterbath and resuspended in 100 μl of 100 mM Tris-HCl (pH 7.4) containing 1% (v/v) SDS. Mitochondria were solubilized by heating for 5 min at 95°C, followed by 5 min cooling on ice. Mitochondrial proteins were precipitated by chloroform/methanol to remove lipids and salts. This was done by adding 400 μl methanol, 100 μl chloroform and 300 μl ddH₂O. The sample was vortexed after each addition. The mixture was centrifuged at 13000 rpm (~14000 g) for 5 min at 4°C. The upper phase was discarded and white precipitated disc (proteins) at the interphase was kept. The protein pellet was washed with 300 μl of methanol followed by centrifugation at 13000 rpm (~14000 g) for 5 min at 4°C. Methanol was removed and precipitated proteins were air-dried in a vacuum centrifuge (SpeedVac) for 5 min.
For one-dimensional SDS-PAGE, the dry protein pellets were resuspended in 1x sample buffer: 62.5 mM Tris-HCl, 2% (w/v) SDS, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and heated for 5 min at 100°C. The molecular weight marker PeppermintStick™ (Invitrogen, Cat. No. P27167) was used in one-dimensional SDS-PAGE (Figure 1 and 2).
For 2D PAGE protein pellets were resuspended in 8 M urea, 4% CHAPS, 65 mM dithioerythritol (DTE), 2% Resolyte 4–8, containing trace amounts of bromophenol blue.
27.2. Alkaline phosphatase treatment of mitochondrial protein samples

INS-1E cells were plated in tissue culture plates (147.8 cm² growth area, TPP, Switzerland, Cat. No. 93150) at a density of 8-9 x 10⁶ cells/plate. Cells were used 3-4 days after plating. For the experiment shown in Figure 1, mitochondria were obtained by 9000 g centrifugation of INS-1E cell homogenates prepared from two 147.8 cm² plates (“crude mitochondrial fraction” as described above). The mitochondrial pellet (200 μg total protein) was washed twice by resuspending it in 400 μl of 250 mM sucrose, 20 mM Tris-HCl followed by centrifugation at 10000 rpm (~8500 g) for 4 min in a pre-cooled (4°C) bench-top centrifuge. Washed mitochondria were resuspended in 400 μl of 250 mM sucrose, 20 mM Tris-HCl and separated into two 200 μl aliquots. The tubes were centrifuged at 10000 rpm (~8500 g) for 4 min at 4°C.

One aliquot (100 μg of total protein) was used to prepare mitochondrial lysates. To this end, pelleted mitochondria were resuspended in 350 μl of lysis buffer (250 mM sucrose, 20 mM Tris-HCl, 1% (v/v) SDS) and sonicated 5 times, 3 sec. each. Then 350 μl of lysis buffer + 77.8 μl of 10x NEB Buffer 3 (New England Biolabs; 500 mM Tris-HCl, 100 mM MgCl₂, 1000 mM NaCl, 10 mM dithiothreitol, pH7.9) were added. The suspension was split to two 388 μl aliquots (A and B). Aliquot A received 50 units of calf intestinal alkaline phosphatase (New England Biolabs, Cat. No. M0290S, 10 units/μl). Aliquot B received 5 μl of lysis buffer.

Mitochondria in the other aliquot (100 μg of total protein) were left intact. They were resuspended in 700 μl of 250 mM sucrose, 20 mM Tris-HCl + 77.7 μl of 10x NEB Buffer 3. The suspension was split to two 388 μl aliquots (C and D). Aliquot C was treated with alkaline phosphatase, while aliquot D was left untreated.

Aliquots A, B, C and D were then incubated for 50 min in a 37°C waterbath. Then tubes were shifted for 10 min to 96°C to heat-inactivate alkaline phosphatase.

To dissolve mitochondria from aliquots C and D, 10% SDS solution was added (to bring SDS concentration to 1%) followed by 10 min heating at 100°C.

Proteins from aliquots A, B, C and D were precipitated in chloroform/methanol and separated on SDS polyacrylamide gel.

For the experiment shown in Figure 5 mitochondria were isolated by Percoll gradient centrifugation (as described above). The frozen mitochondrial pellet (160 μg of total protein) was thawed and immediately resuspended in 300 μl of 40 mM Tris-HCl containing a protease inhibitor cocktail (Roche Diagnostics, Cat. No. 04 693 132 001), 1% (v/v) SDS and sonicated...
5 times, 3 sec. each. Then 400 μl of the same solution + 78 μl of 10x NEB Buffer 3 were added. The suspension was split to two 390 μl aliquots (80 μg total protein each). The first aliquot received 76 units of calf intestinal alkaline phosphatase. The second aliquot received 8 μl of 500 mM NaF (inhibitor of Ser/Thr protein phosphatases) and 8 μl of 100 mM Na₃VO₄ (inhibitor of protein tyrosine phosphatases). Both aliquots were then incubated for 50 min in a 37°C waterbath. Then tubes were shifted for 10 min to 96°C to heat-inactivate alkaline phosphatase. Proteins were precipitated in chloroform/methanol and separated on 2D polyacrylamide gel.

27.3. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and protein identification

2D PAGE and protein identification by mass spectrometry were performed at the Proteomics Core Facility of the Faculty of Medicine by the facility personnel. For 2D PAGE, proteins were first separated on the gel in which a pH gradient has been established (immobilized pH gradient gel (IPG) Immobiline Drystrip pH 3-10 NL 7 cm, GE Healthcare Bio-Sciences, Cat. No. 17-6001-12). Electrophoresis was performed using Ettan IPGphor II System electrophoresis unit (GE Healthcare Bio-Sciences) at 15 °C with the following program:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Voltage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>300 V</td>
<td>1 min</td>
</tr>
<tr>
<td>Stage 2</td>
<td>3500 V</td>
<td>30 min</td>
</tr>
<tr>
<td>Stage 3</td>
<td>3500 V</td>
<td>90 min</td>
</tr>
</tbody>
</table>

The IPG strip with separated proteins was attached to the 12.5% polyacrylamide gel using solution containing 0.5% (w/v) agarose, 25 mM Tris, 198 mM glycine and 0.1% w/v SDS (pH 8.3) heated to ~70°C. Proteins were separated by electrophoresis in a direction perpendicular to the first separation using SE260 Mighty Small II electrophoresis unit (Hoefer, Holliston, MA, USA, Cat. No. SE26010A75) at 100V for 3 h at room temperature. The gels were fixed in a solution of 50% methanol and 10% acetic acid. Protein identification was performed using peptide fragmentation sequencing by Maldi-Tof-Tof and NanoLC-ESI MS/MS.
27.4. Phosphoprotein staining protocol

Important: Polypropylene containers should be used for all incubations. Glass dishes should not be used. All fixation, staining and washing steps were performed with gentle agitation (on an orbital shaker 50-60 rpm) at room temperature.

Mitochondrial proteins were separated on 10% polyacrylamide gels. Gels were left for 40 min in a solution of 50% methanol and 10% acetic acid. Then fresh solution was added and gels were fixed overnight. Gels were rinsed 5-6 times with ddH₂O followed by three 15 min washes in 100 ml of water. Next, gels were stained with Pro-Q Diamond solution (Invitrogen, Cat. No. P33301, ~40 ml for 6 x 8 cm minigel) for 90 min. During the staining and subsequent wash steps gel containers were covered with aluminum foil to protect them from light.

After staining the Pro-Q Diamond solution was removed and gels were washed three times (30 min each) in 100 ml of destain solution: 50 mM sodium acetate, 20% acetonitrile. The gels were then washed four times (5 min each) in 100 ml of ddH₂O.

Gels were scanned on a Typhoon 9400 scanner using 532 nm excitation filter and 580 nm emission filter (580 BP30).

27.5. Total protein staining protocol

For subsequent total protein staining, gels were washed in ddH₂O and incubated overnight with Sypro Ruby (Invitrogen, Cat. No. S12001), ~40 ml for 6 x 8 cm minigel. Then gels were washed twice (20 min each) in 100 ml of solution of 10% methanol, 7% acetic acid. Next, gels were washed three times in 100 ml of ddH₂O. Gels were scanned in a Typhoon 9400 instrument using 532 nm excitation filter and 610 nm emission filter (610 BP30).

Gels were kept in 10% ethanol at 4°C for later excision of protein spots and analysis by mass spectrometry.

28. Cell lysate preparation and Western blotting

After incubations in KRBH buffer containing different additives, INS-1E cells were washed once with ice-cold PBS without Ca²⁺ and Mg²⁺. Lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 10 mM NaF, 0.04% (v/v) β-mercaptoethanol, 1% (v/v) Triton X-100) was added and the cells were scraped off the plate. For complete lysis, the suspensions
were sonicated and mixed with 4x sample buffer (250 mM Tris-HCl, 8% (w/v) SDS, 0.02% (w/v) bromophenol blue, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol) and heated for 5 min at 96°C. Protein concentrations were determined using the Bradford assay with bovine serum albumin (BSA) as a standard. Samples were separated on 10% SDS polyacrylamide gels and transferred electrophoretically to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Cat. No. RPN303D). Membranes were blocked in 4% fat-free powdered milk in TBS (25 mM Tris, 150 mM NaCl, 2.68 mM KCl, pH 7.4) containing 0.1% (v/v) Tween-20 for 50 min at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies (see Table 8) in same blocking solution (unless otherwise indicated).

Next, the membranes were washed once in TBS for 10 min, followed by one wash in TBS with 0.1% (v/v) Tween-20 for 15 min and three 10 min washes in TBS. Then membranes were incubated with horseradish peroxidase-labeled secondary antibodies (see Table 9) for 90 min at room temperature. The membranes were washed as described above. The bands were detected using chemiluminescence (ECL Western blotting detection reagents, Amersham, Cat. No. RPN 2106) as described in the manufacturer’s protocol. Images were obtained using a luminescent image analyzer (LAS-4000 mini, Fujifilm).

Table 8. Primary antibodies used for Western blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Produced in</th>
<th>Clonality</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calnexin (90 kDa)</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:5000 in 5% milk-TBS-T</td>
<td>Stressgen, Cat. No. SPA-860</td>
</tr>
<tr>
<td>GAPDH (36 kDa)</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:5000 (0.2 µg/ml) in 5% milk-TBS-T</td>
<td>Sigma, Cat. No. G9545</td>
</tr>
<tr>
<td>COX subunit IV (19.6 kDa)</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:1000 in 3% milk-TBS-T</td>
<td>Molecular Probes, Cat. No. A-21348</td>
</tr>
<tr>
<td>S100A1 (10 kDa)</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:400 (0.5 µg/ml) in 4% milk-TBS-T</td>
<td>Acris Antibodies, Cat. No. SP5355P</td>
</tr>
<tr>
<td>PDH E1α subunit phosphorylated on site 1 (Ser 293) (43 kDa)</td>
<td>Sheep</td>
<td>Polyclonal</td>
<td>1:1670 (1 µg/ml) in 3% milk-TBS-T</td>
<td>provided by Dr. Pilegaard, University of Copenhagen</td>
</tr>
<tr>
<td>PDH E1α subunit phosphorylated on site 2 (Ser 300) (43 kDa)</td>
<td>Sheep</td>
<td>Polyclonal</td>
<td>1:1000 (1 µg/ml) in 3% milk-TBS-T</td>
<td>provided by Dr. Pilegaard, University of Copenhagen</td>
</tr>
<tr>
<td>PDH E1α subunit (43 kDa)</td>
<td>Sheep</td>
<td>Polyclonal</td>
<td>1:1460 (1 µg/ml) in 3% milk-TBS-T</td>
<td>provided by Dr. Pilegaard, University of Copenhagen</td>
</tr>
</tbody>
</table>
Table 9. Secondary antibodies used for Western blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Produced in</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Goat</td>
<td>1:3000 in 5% milk-TBS-T</td>
<td>Sigma, Cat. No. A8275</td>
</tr>
<tr>
<td>Mouse</td>
<td>Goat</td>
<td>1:1000 in 5% milk-TBS-T</td>
<td>Sigma, Cat. No. A5278</td>
</tr>
<tr>
<td>Sheep</td>
<td>Rabbit</td>
<td>1:3000 (0.43 µg/ml) in 3% milk-TBS-T</td>
<td>DAKO Cytomation, Glostrup, Denmark, Cat. No. P 0163</td>
</tr>
</tbody>
</table>

To re-probe a membrane, the bound antibodies were removed by heating in TBS containing 1% (v/v) SDS, 5% (w/v) fat-free powdered milk, 3% (v/v) β-mercaptoethanol, 0.1% (v/v) Tween-20. The membranes were rinsed with water, blocked with 4% milk TBS-T and incubated with primary antibody as described above.

29. Immunofluorescence

INS-1E cells were plated on glass coverslips (Menzel-Gläser, 12 mm diameter, #1, Menzel GmbH, Braunschweig, Germany) in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 353047) at a density of 1 x 10⁵ cells/well. Prior to plating cells, coverslips were autoclaved, placed in the 24-well culture plate and treated with 10 µg/ml of polyornithin overnight at 37°C. Then polyornithin was removed, the plate was washed twice with sterile ddH₂O and once with INS medium. 48 h after plating, the cells were infected with Ad-S100A1 and Tet-ON viruses for 2 h. Then medium was replaced with regular INS medium containing 1 µg/ml of doxycycline. 48 h post-infection the growth medium was removed and cells were fixed in 4% paraformaldehyde in 57.7 mM Na₂HPO₄, 42.3 mM NaH₂PO₄, 50 mM sucrose, 0.4 mM CaCl₂ for 30 min at 37°C. The coverslips were washed three times with PBS pH 7.2. Then coverslips were removed from the plate, placed on a sheet of parafilm with the cells facing up, and incubated with blocking solution: 68.4 mM Na₂HPO₄, 31.6 mM NaH₂PO₄, 0.1% BSA, 1% FCS, 50 mM glycine, 0.2% saponine for 30 min at room temperature. For the detection of mitoS100A1, the blocking solution contained 0.1% of Triton-X100. Then coverslips were incubated with S100A1 antibody diluted 1:400 (0.5 µg/ml) in blocking solution for 2 h at room temperature. Next, coverslips were washed three times with blocking solution to remove unbound antibodies. Samples were incubated with Alexa 568-labeled goat anti-rabbit antibody diluted 1:300 in blocking solution for 1 h at room temperature. Coverslips were washed three times with blocking solution and once with
PBS pH 7.2. Coverslips were dried and mounted on glass microscope slides (Menzel Gläser, 76 x 26 mm) using fluorescent mounting medium (Dako Cytomation, Cat. No. S3023). Samples were observed under Axiophot 1 microscope (Carl Zeiss), and images were captured with an Axiocam color CCD camera (Carl Zeiss).

30. Measurements of oxygen consumption of mitochondrial suspensions

Measurements of oxygen consumption were performed using Clark-type electrode (Digital model 10, Rank Brothers Ltd, Cambridge, UK) as described in the manufacturer’s manual (can be found at http://www.rankbrothers.co.uk). Before measurements, the water-jacketed sample chamber was preheated to 37°C. This temperature was maintained throughout the experiment. Then, 410 µl of respiration buffer (200 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 1 mM MgCl₂, 5 mM KH₂PO₄, 6.66 mM CaCl₂, 10 mM EGTA, pH 7.0 (free Ca²⁺ concentration ~500 nM)) was added to the sample chamber. Next, mitochondria (80-120 µg of protein) isolated from INS-1E cells by differential centrifugation (9000 g pellet, see chapter 26) were injected into the chamber in small volume of respiration buffer (~40 µl). After 5-10 min of measurements succinate was added (25 µl of 100 mM solution, final concentration 5 mM), followed by the addition of ADP (25 µl of 2 mM solution, final concentration 100 µM). Measurements were recorded using Oxymeter program.

31. MTT assay

The MTT assay is a colorimetric assay to measure cell metabolic activity. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt, which in living cells is reduced to insoluble purple formazan by mitochondrial reductases. MTT assay has been successfully used to measure metabolic activity of INS-1 cells, RINm5F cells and rat pancreatic islets (Janjic and Wollheim, 1992).

INS-1E cells were plated in 96-well plates (Becton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 353072) at a density of 1.5 x 10⁴ cells/well. 24 h after plating, the cells were infected with Ad-S100A1, Tet-ON and Ad-RIP-Luciferase viruses as described earlier. 48 h post-infection the growth medium was removed and wells were washed once with KRBH without glucose (200 µl/well) and then left in the same buffer for 40 min at 37°C. This preincubation medium was removed and KRBH containing 11.2 mM glucose and 0.5 mg/ml MTT was added (100
µl/well). The plates were wrapped in aluminium foil and incubated for 90 min at 37°C. The solution was removed and 100 µl/well of DMSO was added to solubilize formazan crystals. The plates were incubated on the shaking platform for 20 min at room temperature. Absorbance was measured at 570 nm using an automatic spectrophotometer.

32. Cell death assay

INS-1E cells were plated in 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 353047) at a density of 7.5 x 10^4 cells/well. The next day, cells were transfected with siRNAs. 48 h post-transfection, the medium was changed to regular INS medium or INS medium containing 30 mM glucose. Cell death assays were performed 96 h after transfection using Cell Death Detection ELISA PLUS kit (Roche Diagnostics GmbH, Mannheim, Germany, Cat. No. 11 774 425 001) as described in the manufacturer’s protocol. The cell death measurement is based on the detection of nucleosomes in the cytoplasmic fraction of cell lysates. Briefly, growth media were removed, lysis buffer was added (0.8 ml/well) and the plate was left for 30 min on a rotating platform (100 rpm). The plates were centrifuged at ~200 g for 10 min at room temperature. From each well 20 µl of lysate was transferred to the 96-well assay plate (provided with the kit). The antibody mix was added (80 µl/well) and the plate was incubated for 2 h at room temperature on a shaking platform (50 rpm). Then plate was washed three times with incubation buffer (200 µl/well) before 100 µl of substrate was added and left for an additional 3 min on the shaking platform (100 rpm). Absorbance was measured at 405 and 490 nm on spectrophotometer accepting 96-well microplates (Thermomax microplate reader, Molecular Devices, Hayward, CA, USA).

33. PDH activity assay

PDH activity was measured using the PDH Enzyme Activity Microplate Assay Kit (MitoSciences, Eugene, OR, USA, Cat. No. MSP18) according to the manufacturer's protocol with the following modifications. After static incubations INS-1E cells were washed once with ice-cold PBS without Ca^{2+} and Mg^{2+} to lower extracellular calcium. To avoid proteolytic degradation of PDH and preserve its phosphorylation state the following components were included during cell lysis and PDH immunocapture: 1 mM PMSF, protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany, Cat. No. 04 693 132 001), 1 mM EDTA,
20 mM NaF (general inhibitor of Ser/Thr phosphatases), 5 mM DCA (general inhibitor of PDKs). 1 mg of total cell lysate was loaded into each well of the microplate coated with the anti-PDH antibody. Activity of PDH was determined using an absorbance assay as described in the manufacturer’s protocol. Measurements were performed on FLUOstar OPTIMA microplate reader (BMG Labtechnologies, Offenburg, Germany). The reaction was followed for 1 h at 25°C. To determine the maximal activity of fully-dephosphorylated PDH parallel samples were treated for 15 min at 30°C with pyruvate dehydrogenase phosphatase PDP1 (MitoSciences, Eugene, OR, USA, Cat. No. MSP45) in the presence of 0.4 mM CaCl₂. The percentage of active PDH was calculated as activity without phosphatase treatment divided by activity after phosphatase treatment x 100.

34. INS-1E cell static incubations and insulin secretion assay

INS-1E cells were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 353047) at a density of 7.5 x 10⁴ cells/well. Before plating cells, plates were incubated with 10 µg/ml of polyornithin overnight at 37°C. Polyornithin was removed and plates were washed twice with ddH₂O and once with INS medium without antibiotics. Next day after plating, cells were transfected with siRNAs as described (see chapter 22). Insulin secretion assays were performed 96 h post-transfection. Before the experiment, cells were switched for 2 h to RPMI-1640 medium containing 4 mM glucose and 1% (v/v) FCS. Cells were washed three times with KRBH containing 0.1% BSA, 0 mM glucose. The cells were then preincubated for 30 min in KRBH 0.1% BSA, 2.5 mM glucose. Following this preincubation, the cells were washed twice with KRBH 0.1% BSA, 0 mM glucose followed by 30 min incubation in KRBH 0.1% BSA, containing 2.5 or 16.7 mM glucose. Plates were placed on ice and supernatants were saved for the measurement of secreted insulin. Insulin content was extracted by acid-ethanol (10% acetic acid in ethanol) overnight at -20°C. Insulin concentrations were measured using an enzyme immunoassay (SPI bio, France).

35. ATP kinetics measurements

INS-1E cells (1 x 10⁵ cells) were plated on 15 mm diameter Thermanox coverslips (Nalge Nunc, Rochester, NY, USA, Cat. No. 174969) placed in 4-well tissue culture plate (Nunc, Cat. No. 176740). Prior to cell plating, the coverslips were coated with polyornithin as
described (see chapter 34). The day after plating, the cells were infected with adenovirus expressing luciferase under control of the rat insulin promoter (Ad-RIP-Luciferase), Ad-S100A1 and Tet-ON viruses for 2 h at 37°C (as described earlier). 48 h post-infection, the cells were transferred for 2 h to RPMI-1640 medium containing 4 mM glucose and 1% (v/v) FCS. Then cells were placed in pre-warmed (37°C) chamber protected from light and perifused at a rate of 1 ml/min with KRBH containing 2.5 mM or 16.7 mM glucose and 10 µM luciferin (Cat. No. E1601, Promega, Madison, WI, USA). Emitted photons were counted with a photomultiplier apparatus (Thorn-EMI Electron Tubes, UK).

36. Mitochondrial calcium measurements

INS-1E cells (7.5 x 10^4 cells) were plated on polyornithin-treated 15 mm diameter Thermanox coverslips (Nalge Nunc, Rochester, NY, USA, Cat. No. 174969) in 4-well tissue culture plates (Nunc, Cat. No. 176740). The day after plating, cells were transfected with siRNAs as described (see chapter 22). 48 h post-transfection cells were infected for 2 h with adenovirus expressing mitochondrially-targeted aequorin under the control of the rat insulin promoter (Ad-RIP-mitoAequorin). 96 h post-transfection cells were transferred for 2 h to RPMI-1640 medium containing 4 mM glucose, 1% (v/v) FCS and 5 µM coelenterazine (Calbiochem, Cat. No. 233900). Then cells were perifused at a rate of 1 ml/min with KRBH containing different stimuli and emitted photons were counted with a photomultiplier apparatus (Thorn-EMI Electron Tubes, UK). At the end of the measurement cells were permeabilized with 0.1 mM digitonin and 10 mM CaCl_2. The aequorin luminescence signal produced during permeabilization was used to calculate calcium concentrations.

37. [1-14C] pyruvate oxidation measurements

INS-1E cells were plated in 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 353046) at a density of 0.5 x 10^6 cells/well. Throughout the experiment the volume of media and buffers used for incubations and washes was 3ml/well. Experiments were performed 48 h after plating the cells. Before the experiment, cells were switched for 2 h to RPMI-1640 medium containing 4 mM glucose and 1% (v/v) FCS and kept at 37°C in a cell culture incubator. Then cells were washed once with KRBH containing 2.5 mM glucose and left for 30 min at 37°C in a waterbath. Then cells were switched to KRBH containing 2.5 or
16.7 mM glucose plus 0.1 mM cold pyruvate and 0.02 nCi/µl of [1-\(^{14}\)C] pyruvate (American Radiolabeled Chemicals, Cat. No. ARC 0223, 55 mCi/mmol). Specific activity in the resulting solution was 0.208 mCi/mmol. In order to capture \(^{14}\)CO\(_2\), Whatman filters (6.5 x 2 cm) were soaked with 2M NaOH (400 µl) and attached using strips of adhesive tape to the inner side of the culture plate lid. The plates were sealed with parafilm and incubated for 1 h at 37°C on the shaking platform (50 rpm). For each experimental condition a separate plate was used to avoid the exchange of CO\(_2\) between the different conditions studied. Plate without cells was used as control in each experiment. Following incubations, each filter was collected in a scintillation vial (Wheaton, Millville, NJ, USA, 6 ml HDPE vial, Cat. No. 986644). Medium from two neighboring wells (6 ml) were pooled in 20 ml glass vial (PerkinElmer, Waltham, MA, USA, Econo Glass Vial, Cat. No. 6000097). Then each well was washed with 1.5 ml of PBS w/o Ca\(^{2+}\) and Mg\(^{2+}\) and this buffer was added to the same glass vial (total volume of liquid 9 ml). Each vial received an Eppendorf tube containing Whatman filter (3 x 1.5 cm) soaked with 2M NaOH (250 µl). Vials were closed with rubber caps (Precision Seal rubber septa, Sigma, Cat. No. Z553964). Then 400 µl of 5M HCl was injected into the medium using syringe with a needle. Following HCl injection, the vials were sealed with parafilm and incubated for 2 h at 37°C on the shaking platform (50 rpm). Then filters from each condition were combined in a scintillation vial and 4 ml of scintillation fluid (Packard, USA, Emulsifier Safe, Cat. No. 6013389) added. \(^{14}\)C counting was performed on LS6500 liquid scintillation counter (Beckman Instruments) 24-48 h after adding the scintillation fluid. Earlier measurements gave very large artefactual variations.
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