

Cells in focus

# In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion

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## Abstract

Pancreatic beta-cells are unique neuroendocrine cells displaying the peculiar feature of responding to nutrients, principally glucose, as primary stimulus. This requires translation of a metabolic substrate into intracellular messengers recognized by the exocytotic machinery. Central to this signal transduction mechanism, mitochondria integrate and generate metabolic signals, thereby coupling glucose recognition to insulin secretion. In response to a glucose rise, nucleotides and metabolites are generated by mitochondria and participate, together with cytosolic calcium, to the stimulation of insulin exocytosis. This review describes the mitochondrion-dependent pathways of regulated insulin secretion. In particular, importance of cataplerotic and anaplerotic processes is discussed, with special attention to the mitochondrial enzyme glutamate dehydrogenase. Mitochondrial defects, such as mutations and reactive oxygen species production, are presented in the context of beta-cell failure in the course of type 2 diabetes.

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## 1. Metabolism secretion coupling

Pancreatic beta-cells function as glucose sensors with the crucial task of perfectly adjusting insulin release to blood glucose levels. In the consensus model of glucose-stimulated insulin secretion (Fig. 1), glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase initiating glycolysis (Matschinsky, 1996).

*Abbreviations:* AGC, aspartate/glutamate carrier; BCH, beta-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GLP-1, glucagon-like peptide 1;  $K_{ATP}$ -channel, ATP-sensitive  $K^+$  channel; LCPT1, liver carnitine palmitoyltransferase I; ROS, reactive oxygen species; TCA, tricarboxylic acid; UCP2, uncoupling protein 2

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Subsequently, mitochondrial metabolism generates ATP, which promotes the closure of ATP-sensitive  $K^+$  channels ( $K_{ATP}$ -channel) and, as a consequence, depolarization of the plasma membrane (Rorsman, 1997). This leads to  $Ca^{2+}$  influx through voltage-gated  $Ca^{2+}$  channels and a rise in cytosolic  $Ca^{2+}$  concentrations triggering insulin exocytosis (Lang, 1999; Rorsman, 1997). Additional signals are necessary to reproduce the sustained secretion elicited by glucose. They participate in the amplifying pathway (Henquin, 2000), formerly referred to as the  $K_{ATP}$ -channel independent stimulation of insulin secretion. Efficient coupling of glucose recognition to insulin secretion is ensured by the mitochondrion, an organelle that integrates and generates metabolic signals. This crucial role goes far beyond the sole generation of ATP necessary for the elevation of cytosolic  $Ca^{2+}$  (Maechler, Kennedy, Pozzan, & Wollheim, 1997).

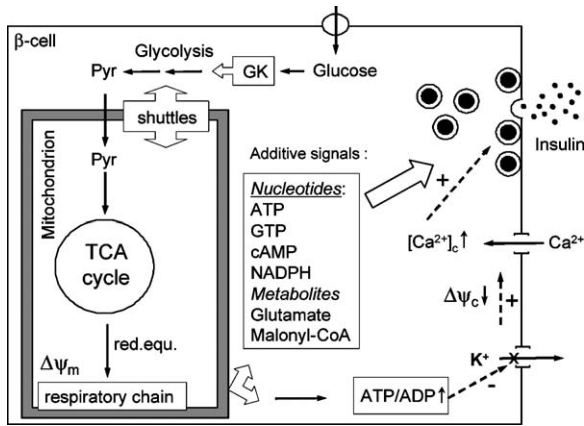
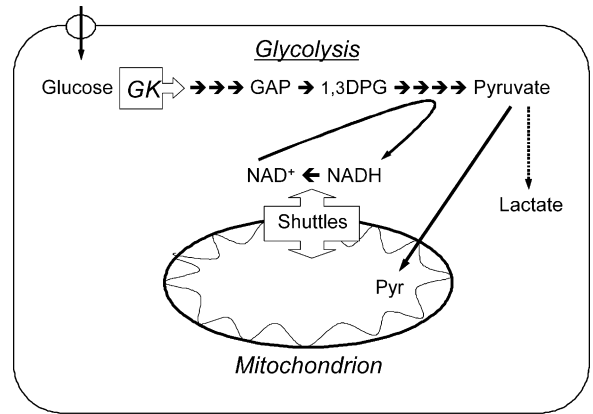


Fig. 1. Model for coupling of glucose metabolism to insulin secretion in the beta-cell. Glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase (GK). Further, glycolysis produces pyruvate (Pyr), which preferentially enters the mitochondria and is metabolised by the TCA cycle. The TCA cycle generates reducing equivalents (red. equ.), which are transferred to the electron transport chain, leading to hyperpolarisation of the mitochondrial membrane ( $\Delta\psi_m$ ) and generation of ATP. ATP is then transferred to the cytosol, raising the ATP/ADP ratio. Subsequently, closure of  $K_{ATP}$ -channels depolarises the cell membrane ( $\Delta\psi_c$ ). This opens voltage-dependent  $Ca^{2+}$  channels, increasing cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_c$ ), which triggers insulin exocytosis. Additive signals participate to the amplifying pathway of metabolism secretion coupling.

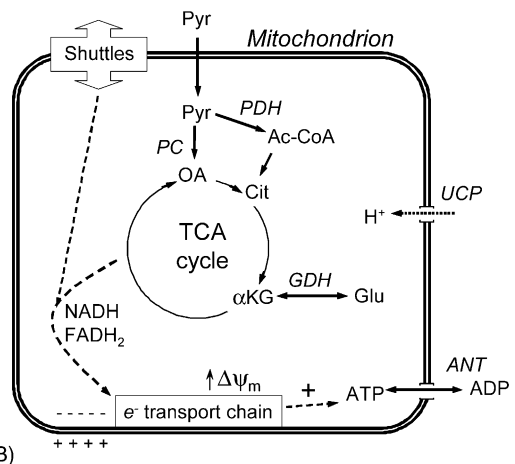
## 2. Mitochondrial NADH shuttles

In pancreatic beta-cells, mitochondria serve as integrator and generator of metabolic signals. In the course of glycolysis, i.e. upstream of pyruvate production, mitochondria are already implicated in the necessary reoxidation of  $NADH + H^+$  to  $NAD^+$ , thereby enabling maintenance of glycolytic flux. In most tissues, lactate dehydrogenase ensures NADH oxidation to avoid inhibition of glycolysis secondary to the lack of  $NAD^+$ . In beta-cells, according to low lactate dehydrogenase activity, high rates of glycolysis are maintained through the activity of mitochondrial NADH shuttles (Fig. 2A). These glycolysis-derived electrons carried by NADH are mostly transferred to mitochondria through the two main NADH shuttle systems, i.e. the glycerophosphate and the malate–aspartate shuttles (Eto et al., 1999; MacDonald, 1981; Rubi, del Arco, Bartley, Satrustegui, & Maechler, 2004). Therefore, NADH shuttles couple glycolysis to activation of mitochondrial energy metabolism, leading to insulin secretion. Lowering of NADH levels in beta-cells affects glucose-stimulated insulin secretion (Malaisse, Hutton, Kawazu, & Sener, 1978; Sekine et al., 1994).

In beta-cells, the NADH shuttle system is composed essentially of the glycerophosphate and the malate–



(A)



(B)

Fig. 2. (A) Glycolysis is initiated by glucokinase (GK) dependent phosphorylation of glucose, followed by isomerisation and further phosphorylation, resulting into the three-carbon molecules dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate (GAP). The next step generates 1,3diphosphoglycerate (1,3DPG) plus NADH. Mitochondrial NADH shuttles are required in order to ensure  $NAD^+$  regeneration, compensating for low lactate formation. As a consequence, pyruvate (Pyr) is the major end product of glycolysis in the beta-cell. (B) In the mitochondria, pyruvate (Pyr) is a substrate both for pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC), forming respectively acetyl-CoA (Ac-CoA) and oxaloacetate (OA). Condensation of Ac-CoA with OA generates citrate (Cit) that is either processed by the TCA cycle or exported out of the mitochondrion as a precursor for malonyl-CoA synthesis. Both the mitochondrial shuttles and the TCA cycle generate reducing equivalents in the form of NADH and  $FADH_2$ , which are transferred to the electron transport chain resulting in hyperpolarisation of the mitochondrial membrane ( $\Delta\psi_m$ ). The thus formed ATP is exchanged for cytosolic ADP by the adenine nucleotide translocator (ANT). Upon glucose stimulation, glutamate (Glu) can be produced from  $\alpha$ -ketoglutarate ( $\alpha$ KG) by glutamate dehydrogenase (GDH). The uncoupling protein (UCP) diminishes the proton gradient.

aspartate shuttles (MacDonald, 1982), with its respective key members mitochondrial glycerol phosphate dehydrogenase and aspartate/glutamate carrier—also referred to as Aralar. Mice lacking mitochondrial glyc-

erol phosphate dehydrogenase exhibit a normal phenotype (Eto et al., 1999), whereas general abrogation of Aralar results in severe growth retardation (Jalil et al., 2005). Pancreatic islets of the latter knockout mice have not yet been studied although the observed impaired central nervous system function might, at least partly, contribute to the general phenotype (Jalil et al., 2005). Islets isolated from mitochondrial glycerol phosphate dehydrogenase knockout mice respond normally to glucose regarding metabolic parameters and insulin secretion (Eto et al., 1999). However, additional inhibition of the malate–aspartate shuttle in these islets with aminooxyacetate strongly impairs the secretory response to glucose (Eto et al., 1999). The respective importance of these shuttles is indicated in islets of mice with abrogation of NADH shuttle activities, pointing to the malate–aspartate shuttle as essential for both mitochondrial metabolism and cytosolic redox state.

Aralar1 (or aspartate/glutamate carrier 1, AGC1) is a  $\text{Ca}^{2+}$  sensitive member of the malate–aspartate shuttle (del Arco & Satrustegui, 1998). Aralar1 and citrin are members of the subfamily of  $\text{Ca}^{2+}$ -binding mitochondrial carriers and correspond to two isoforms of the mitochondrial aspartate/glutamate carrier. These proteins are activated by  $\text{Ca}^{2+}$ , acting on the external side of the inner mitochondrial membrane (del Arco, Agudo, & Satrustegui, 2000; del Arco & Satrustegui, 1998; Palmieri et al., 2001). We recently reported that adenoviral-mediated overexpression of Aralar1 in insulin-secreting cells increases glucose-induced mitochondrial activation and secretory response (Rubi et al., 2004). This is accompanied by enhanced glucose oxidation and reduced lactate production. Therefore, aspartate/glutamate carrier capacity appears to set a limit for NADH shuttle function and mitochondrial metabolism. The importance of the NADH shuttle system also illustrates the tight coupling between glucose metabolism and the control of insulin secretion.

### 3. Pyruvate and the mitochondria

Downstream of the NADH shuttles, pyruvate produced by glycolysis is preferentially transferred to mitochondria through recently identified mitochondrial pyruvate carrier (Hildyard & Halestrap, 2003), although the corresponding mammalian orthologue has not yet been described (Sugden & Holness, 2003). Pyruvate import into mitochondria is associated with a futile cycle (Jezek & Borecky, 1998) that transiently depolarizes the mitochondrial membrane (de Andrade, Casimir, & Maechler, 2004). After its entry into the mitochondria, pyruvate is converted to acetyl-CoA by

pyruvate dehydrogenase or to oxaloacetate by pyruvate carboxylase (Fig. 2B). The pyruvate carboxylase pathway ensures the provision of carbon skeleton (i.e. anaplerosis) to the tricarboxylic acid (TCA) cycle, a key pathway in beta-cells (Brennan et al., 2002; Lu et al., 2002; Schuit et al., 1997). This high anaplerotic activity suggests the loss of TCA cycle intermediates (i.e. cataplerosis), compensated for by oxaloacetate. In the control of glucose-stimulated insulin secretion, such TCA cycle derivatives might potentially operate as mitochondrion derived coupling factors, such as citrate and  $\alpha$ -ketoglutarate for the respective formation of the proposed coupling factors malonyl-CoA (Prentki et al., 1992) and glutamate (Maechler & Wollheim, 1999).

Beside of its importance for ATP generation, the mitochondrion in general, and the TCA cycle in particular, is the key metabolic crossroad enabling fuel oxidation as well as provision of building blocks, or cataplerosis, for lipids and proteins (Owen, Kalhan, & Hanson, 2002). In beta-cells, approximately 50% of pyruvate is oxidised to acetyl-CoA by pyruvate dehydrogenase (Schuit et al., 1997). Pyruvate dehydrogenase is an important site of regulation as, among other effectors, the enzyme is activated by elevation of mitochondrial  $\text{Ca}^{2+}$  (McCormack, Halestrap, & Denton, 1990; Rutter et al., 1996) and, conversely, its activity is reduced upon exposures to either excess fatty acids (Randle, Priestman, Mistry, & Halsall, 1994) or chronic high glucose (Liu, Moibi, & Leahy, 2004). Oxaloacetate, produced by the anaplerotic enzyme pyruvate carboxylase, condenses with acetyl-CoA forming citrate, which undergoes stepwise oxidation and decarboxylation yielding  $\alpha$ -ketoglutarate. The TCA cycle is completed *via* succinate, fumarate and malate, in turn producing oxaloacetate. The fate of  $\alpha$ -ketoglutarate is influenced by the redox state of mitochondria. Low NADH to  $\text{NAD}^+$  ratio would favour further oxidative decarboxylation to succinyl-CoA as  $\text{NAD}^+$  is required as co-factor for this pathway. Conversely, high NADH to  $\text{NAD}^+$  ratio would promote NADH-dependent reductive transamination forming glutamate, a spin-off product of the TCA cycle (Owen et al., 2002). The latter situation, i.e. high NADH to  $\text{NAD}^+$  ratio, is observed following glucose stimulation.

Although the TCA cycle oxidises also fatty acids and amino acids, carbohydrates are the most important fuel under physiological conditions for the beta-cell. Upon glucose exposure, mitochondrial NADH elevations reach a plateau after approximately 2 min (Rocheleau, Head, & Piston, 2004). In order to maintain pyruvate input into the TCA cycle, this new redox steady state requires continuous reoxidation of mitochondrial NADH to  $\text{NAD}^+$ , primarily by complex I on the electron

transport chain. However, as complex I activity is limited by the inherent thermodynamic constraints of proton gradient formation (Antinozzi, Ishihara, Newgard, & Wollheim, 2002), additional NADH contributed by this high TCA cycle activity must be reoxidised by other dehydrogenases, i.e. through cataplerotic functions. Significant cataplerotic function in beta-cells was suggested by the quantitative importance of anaplerotic pathway through pyruvate carboxylase (Schuit et al., 1997), as confirmed recently by use of NMR spectroscopy (Brennan et al., 2002; Cline, Lepine, Papas, Kibbey, & Shulman, 2004; Lu et al., 2002).

#### 4. Glutamate dehydrogenase

Glutamate dehydrogenase (GDH), encoded by *GLUD1* (Michaelidis, Tzimagiorgis, Moschonas, & Papamatheakis, 1993), is a homohexamer located in the mitochondrial matrix. GDH catalyses the reversible reaction  $\alpha$ -ketoglutarate + NH<sub>3</sub> + NAD(P)H  $\leftrightarrow$  glutamate + NAD(P)<sup>+</sup>. The enzyme is allosterically regulated by leucine, pyridine, adenine and guanine nucleotides (Fisher, 1985; Smith, Peterson, Schmidt, Fang, & Stanley, 2001); e.g. inhibited by GTP and activated by L-leucine (Hudson & Daniel, 1993). In the brain, GDH is highly expressed in astrocytes (Aoki et al., 1987), ensuring efficient clearance and catabolism of glutamate released from neurons (Anderson & Swanson, 2000; Schousboe, Svenneby, & Hertz, 1977). Human brain expresses a GDH resistant to GTP-mediated inhibition and encoded by *GLUD2* (Plaitakis, Spanaki, Mastorodemos, & Zaganas, 2003), an isoform proposed to originate by retroposition from *GLUD1* in the hominoid (Burki & Kaessmann, 2004). Such *GLUD2* encoded GDH might confer to astrocytes higher capacity of glutamate catabolism following glutamate release as neurotransmitter (Burki & Kaessmann, 2004), thereby preventing glutamate induced neurotoxicity (Duchen, 2004). GDH also plays a major role in ammonia metabolism and detoxification, mainly in the liver and the kidney (Nissim, 1999).

In pancreatic beta-cells, the importance of GDH as a key enzyme in the control of insulin secretion has been recognized long ago (Sener & Malaisse, 1980). However, its specific role is still debated, essentially concerning the preferential flux direction in beta-cells. Is it an anaplerotic role forming  $\alpha$ -ketoglutarate from glutamate or a cataplerotic role generating glutamate at the expense of  $\alpha$ -ketoglutarate? The confusion might be related to the fact that most of the reports investigated GDH in beta-cells by means of increased activity of the enzyme. Numerous studies have used the GDH

allosteric activator L-leucine or its non-metabolized analogue beta-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) to question the role of GDH in the control of insulin secretion (Fahien, MacDonald, Kmietek, Mertz, & Fahien, 1988; Panten, Zielmann, Langer, Zunkler, & Lenzen, 1984; Sener & Malaisse, 1980; Sener, Malaisse-Lagae, & Malaisse, 1981; Sener, Owen, Malaisse-Lagae, & Malaisse, 1982). Alternatively, one can increase GDH activity by means of overexpression, an approach that we combined with allosteric activation of the enzyme (Carobbio et al., 2004). Another line of studies in beta-cells looked at activating mutations of GDH that have been associated with a hyperinsulinism syndrome (Stanley et al., 1998; Yorifuji, Muroi, Uematsu, Hiramatsu, & Momoi, 1999). Reduced GTP-mediated inhibition of the enzyme was associated with most of GDH mutations linked to the hyperinsulinism syndrome (Stanley et al., 2000).

Beta-cells forced to express such activating mutations became glutamine responsive in terms of insulin secretion, both in cell and mouse models (Kelly, Li, Gao, Stanley, & Matschinsky, 2002; Tanizawa et al., 2002). Unlike glucose, glutamine is not efficiently processed through oxidative metabolism in beta-cells (Malaisse et al., 1980; Panten et al., 1984). However, glutamine oxidation can be prompted by allosteric activation of GDH, an effect correlating with stimulation of insulin secretion (Panten et al., 1984; Sener et al., 1981). Alternatively, we have shown that overexpression of GDH in isolated islets promotes glutamine metabolism associated with insulin secretion (Carobbio et al., 2004). Taken together, these gain-of-function approaches demonstrated that glutamine can be turned into a secretagogue under conditions of increased GDH activity, although glucose-stimulated insulin secretion is not, or very modestly, modified (Carobbio et al., 2004; Sener et al., 1982; Tanizawa et al., 2002). Hence, in specific activating conditions, either experimental or pathological, it seems clear that GDH exhibits an anaplerotic function.

In only a very limited number of studies, GDH activity was reduced by the use of inhibitors in pancreatic islets (Bryla, Michalik, Nelson, & Erecinska, 1994; Yang et al., 2003). Upon glucose stimulation, GDH inhibition results in both lower insulin release and cellular glutamate levels (Lee et al., 2004; Yang et al., 2003). In order to verify if reduced GDH activity would affect glucose-stimulated insulin release, we lowered GDH expression in rat clonal INS-1E beta-cells by means of antisense approach. We constructed an adenovirus, essentially as described before (Carobbio et al., 2004), with antisense GDH insert containing the 1–583 bp fragment of human GDH cDNA, which is 90% homologous to the rat

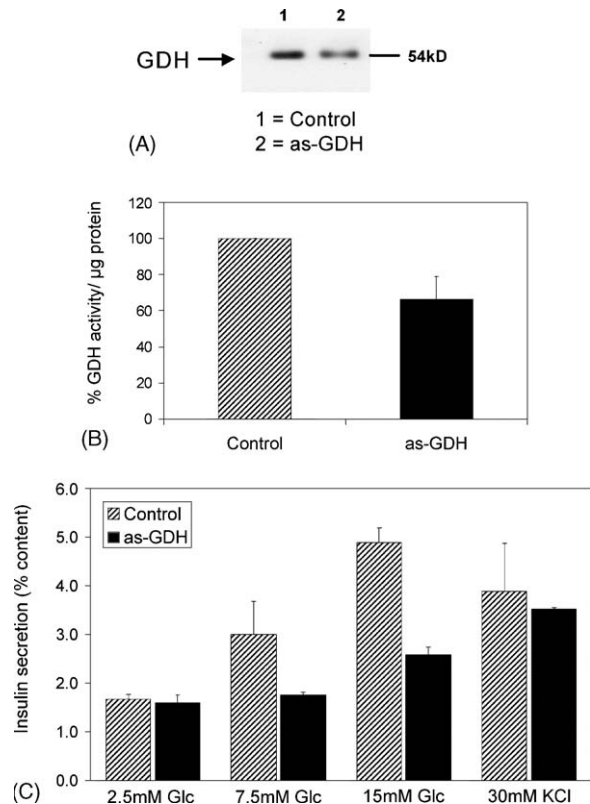


Fig. 3. The effects of reduced expression of GDH were tested in INS-1E beta-cells transduced with an adenovirus carrying antisense GDH. INS-1E cells were transduced two times with the adenovirus containing the antisense GDH fragment (as-GDH), as described in the text, before analysing GDH at the protein level by immunoblotting (A) and measuring GDH activity (B). Following the same culture conditions, insulin secretion was measured over a 30 min incubation period at different glucose (Glc) concentrations: basal 2.5 mM, intermediate 7.5 mM, and high 15 mM Glc. Insulin release was also stimulated by non-nutrient calcium-raising concentration of KCl (30 mM) (C). See text for statistics.

GDH sequence. INS-1E cells seeded in 24-well plates were transduced two times with the adenovirus AdCA-asGDH for 90 min at days 1 and 4, and then used at day 6 for experiments performed as described (Carobbio et al., 2004). Immunoblotting revealed that GDH expression was reduced in cells treated with antisense AdCA-asGDH (Fig. 3A) and that corresponding enzyme activity was inhibited by 34% (Fig. 3B). Basal insulin release at 2.5 mM glucose was not affected by GDH antisense treatment (Fig. 3C). On the contrary, in response to 15 mM glucose, the secretory response observed in control cells (2.9-fold,  $p < 0.001$ ) was inhibited by 47% ( $p < 0.005$ ) in INS-1E cells with GDH knockdown. Non-nutrient stimulation of insulin secretion induced by the  $\text{Ca}^{2+}$  raising agent KCl (30 mM) was not inhibited.

These data show that reduced GDH expression and activity impair glucose-stimulated insulin secretion.

Taken together, results on GDH activation and inhibition in beta-cells would favour a cataplerotic function of GDH in the course of glucose-evoked insulin release. One recent NMR study specifically questioned the relative contributions of pyruvate dehydrogenase plus pyruvate carboxylase versus glutamate dehydrogenase for anaplerotic pathway in insulin-secreting cells (Cline et al., 2004). Authors concluded that upon glucose stimulation, the flux of glucose carbons into the TCA cycle is essentially through both pyruvate dehydrogenase plus pyruvate carboxylase, arguing against an anaplerotic role for GDH in beta-cells. Therefore, the flux direction of GDH might depend on the redox state of mitochondria (discussed above), the provision of substrates (glucose versus amino acids) and potential mutations of the enzyme. The importance of GDH, and the related glutamate pathway in general, is also highlighted by the putative role of glutamate as an intracellular messenger in glucose-stimulated insulin exocytosis (Bertrand, Ishiyama, Nenquin, Ravier, & Henquin, 2002; Hoy et al., 2002; Liu et al., 2003; MacDonald & Fahien, 2000; Maechler & Wollheim, 1999; Rubi, Ishihara, Hegardt, Wollheim, & Maechler, 2001). Therefore, GDH might have a dual role in glutamate handling (Maechler, 2002). A cataplerotic role upon normal glucose stimulation when glutamate is produced as a derived factor or an anaplerotic role when glutamate might serve as a mitochondrial substrate feeding the TCA cycle (Broca, Brennan, Petit, Newsholme, & Maechler, 2003). Taken together, these modes of action point to GDH as a major regulatory enzyme for the control of insulin secretion, with preferential cataplerotic function upon stimulation with the main secretagogue glucose.

## 5. The electron transport chain and ATP generation

Through activation of the TCA cycle, electrons are transferred to the respiratory chain resulting in hyperpolarisation of the mitochondrial membrane and generation of ATP. The electrons are transferred by the pyridine nucleotide  $\text{NAD}^+$  and the flavin adenine nucleotide FAD. In the mitochondrial matrix, NADH is formed by several dehydrogenases, some of which being activated by  $\text{Ca}^{2+}$  (Duchen, 1999; McCormack et al., 1990), and  $\text{FADH}_2$  is generated in the succinate dehydrogenase reaction.

Electron transport chain activity results in proton export from the mitochondrial matrix across the inner membrane, and thereby establishes a strong mitochondrial membrane potential, negative inside. The respira-

tory chain comprises five complexes, the subunits of which are encoded by both the nuclear and mitochondrial genomes (Wallace, 1999). Complex I is the only acceptor of electrons from NADH in the inner mitochondrial membrane and its blockade abolishes glucose-induced insulin secretion (Antinozzi et al., 2002; Newgard & McGarry, 1995). Complex II (succinate dehydrogenase) transfers electrons to coenzyme-Q from FADH<sub>2</sub>, the latter being generated both by the oxidative activity of the TCA cycle and the glycerophosphate shuttle. Complex V (ATP synthase) promotes ATP formation from ADP and inorganic phosphate. The synthesised ATP is translocated to the cytosol in exchange for ADP by the adenine nucleotide translocator (ANT). Thus, the work of the separate complexes of the electron transport chain and the adenine nucleotide translocator couples respiration to ATP supply.

NADH electrons are transferred to the electron transport chain, which in turn supplies the energy necessary to create a proton electrochemical gradient that drives ATP synthesis. In addition to ATP generation, mitochondrial membrane potential drives the transport of metabolites between mitochondrial and cytosolic compartments, including the transfer of mitochondrial factors participating in insulin secretion. Hyperpolarization of the mitochondrial membrane relates to the proton export from the mitochondrial matrix and directly correlates with insulin secretion stimulated by different secretagogues (Antinozzi et al., 2002). Accordingly, potentiation of glucose-stimulated insulin secretion by enhanced mitochondrial NADH generation is accompanied by increased glucose metabolism and mitochondrial hyperpolarization (Rubi et al., 2004).

## 6. The amplifying pathway of insulin secretion

The Ca<sup>2+</sup> signal in the cytosol is necessary but not sufficient for the full development of biphasic insulin secretion. Nutrient secretagogues, in particular glucose, evoke a long-lasting second phase of insulin secretion. In contrast to the transient secretion induced by Ca<sup>2+</sup>-raising agents, the sustained insulin release depends on the generation of metabolic factors. The elevation of cytosolic Ca<sup>2+</sup> is a prerequisite also for this phase of secretion, as evidenced among others by the inhibitory action of voltage-sensitive Ca<sup>2+</sup> channel blockers. Glucose evokes K<sub>ATP</sub>-channel independent stimulation of insulin secretion, or amplifying pathway (Henquin, 2000), which is unmasked by glucose stimulation when cytosolic Ca<sup>2+</sup> is clamped at permissive levels (Gembal, Gilon, & Henquin, 1992; Panten, Schwanstecher, Wallasch, & Lenzen, 1988; Sato, Aizawa, Komatsu, Okada, &

Yamada, 1992). This suggests the existence of metabolic coupling factors generated by glucose.

## 7. Mitochondrion-dependent nucleotides and the control of insulin exocytosis

ATP is a primary metabolic factor implicated in K<sub>ATP</sub>-channel regulation (Miki, Nagashima, & Seino, 1999), secretory granule movement (Varadi, Ainscow, Allan, & Rutter, 2002; Yu et al., 2000), and the process of insulin exocytosis (Rorsman et al., 2000; Vallar, Biden, & Wollheim, 1987). In a recent study, newly identified mitochondrial phosphatase was shown to be involved in the regulation of ATP production in insulin producing cells (Pagliarini et al., 2005). Such dual-specific protein tyrosine phosphatase localized to the mitochondrion might play a novel role in the control of glucose-evoked insulin secretion.

Among other putative nucleotide messengers, NADH and NADPH are generated by glucose metabolism (Prentki, 1996). Single beta-cell measurements of NAD(P)H fluorescence have demonstrated that the rise in pyridine nucleotides precedes the rise in cytosolic Ca<sup>2+</sup> concentrations (Pralong, Bartley, & Wollheim, 1990) and that the elevation in the cytosol is reached more rapidly than in the mitochondria (Patterson, Knobel, Arkhammar, Thastrup, & Piston, 2000). Cytosolic NADPH is generated by glucose metabolism via the pentosephosphate shunt (Verspohl, Handel, & Ammon, 1979) and by mitochondrial shuttles (Farfari, Schulz, Corkey, & Prentki, 2000). Using toadfish islets, NADPH was proposed as a coupling factor in glucose-stimulated insulin secretion (Watkins, Cooperstein, Dixit, & Lazarow, 1968; Watkins, Cooperstein, & Lazarow, 1971). A direct effect of NADPH was reported on the release of insulin from isolated secretory granules (Watkins, 1972), NADPH being possibly bound or taken up by granules (Watkins & Moore, 1977). The mitochondrial pyruvate/malate shuttle was shown to be the main contributor of cytosolic NADPH, with regulatory role on glucose-induced insulin secretion (MacDonald, 1995). Recently, the putative role of NADPH, as a signalling molecule in beta-cells, has been substantiated by experiments showing direct stimulation of insulin exocytosis upon intracellular addition of NADPH (Ivarsson et al., 2005).

Glucose also promotes the elevation of GTP (Detimary, Van den Berghe, & Henquin, 1996), which could trigger insulin exocytosis via GTPases (Lang, 1999; Vallar et al., 1987). In the cytosol, GTP is mainly formed through the action of nucleoside diphosphate kinase from GDP and ATP. In contrast to ATP, GTP

is capable of inducing insulin exocytosis in a  $\text{Ca}^{2+}$ -independent manner (Vallar et al., 1987).

The universal second messenger cAMP, generated at the plasma membrane from ATP, potentiates glucose-stimulated insulin secretion (Ahren, 2000). Many neurotransmitters and hormones, including glucagon as well as the intestinal hormones glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide, increase cAMP levels in the beta-cell by activating adenyl cyclase (Schuit, Huypens, Heimberg, & Pipeleers, 2001). Although glucose itself is inefficient in producing cAMP, permissive levels of cAMP are necessary for normal responsiveness of secretion (Huypens, Ling, Pipeleers, & Schuit, 2000). Moreover, GLP-1 might preserve beta-cell mass, both by induction of cell proliferation and inhibition of apoptosis (Drucker, 2003a). According to all these actions, GLP-1 and biologically active related molecules are of interest for the treatment of diabetes (Drucker, 2003b; Gromada, Brock, Schmitz, & Rorsman, 2004).

## 8. Mitochondrion-dependent metabolites and the control of insulin exocytosis

In mitochondria, glucose and lipid products compete for their catabolism and oxidation. Carnitine palmitoyltransferase I, which is expressed in the pancreas as the liver isoform (LCPTI), catalyzes the rate-limiting step in the transport of fatty acids into the mitochondria for their oxidation. In glucose-stimulated beta-cells, citrate exported from the mitochondria to the cytosol reacts with coenzyme-A (CoA) to form malonyl-CoA. Then, malonyl-CoA derived from glucose metabolism regulates fatty acid oxidation by inhibiting LCPTI. The malonyl-CoA/long-chain acyl-CoA hypothesis of glucose-stimulated insulin release postulates that malonyl-CoA derived from glucose metabolism inhibits fatty acid oxidation, thereby increasing the availability of long-chain acyl-CoA for lipid signals implicated in exocytosis. In the cytosol, this cataplerotic process promotes the accumulation of long chain acyl-CoAs such as palmitoyl-CoA (Liang & Matschinsky, 1991; Prentki et al., 1992), which enhances  $\text{Ca}^{2+}$ -evoked insulin exocytosis (Deeney et al., 2000).

In agreement with the malonyl-CoA/long-chain acyl-CoA model, overexpression of native LCPTI in clonal INS-1E beta-cells was shown to increase  $\beta$ -oxidation of fatty acids and to decrease insulin secretion at high glucose (Rubi et al., 2002), although glucose-derived malonyl-CoA was still able to inhibit LCPTI in these conditions. When the malonyl-CoA/CPTI interaction is altered in cells expressing a malonyl-CoA-insensitive

CPTI, glucose-induced insulin release is impaired (Herrero et al., 2005). In disagreement with malonyl-CoA/long-chain acyl-CoA model, abrogation of malonyl-CoA accumulation during glucose stimulation did not attenuate the secretory response (Antinozzi, Segall, Prentki, McGarry, & Newgard, 1998). In a recent study, overexpressing malonyl-CoA decarboxylase in the cytosol, in the presence of exogenous free fatty acids but not in their absence, reduced glucose-stimulated insulin release in beta-cells (Roduit et al., 2004). The role of long chain acyl-CoA derivatives remains a matter of debate, although several studies indicate that malonyl-CoA could act as a coupling factor regulating the partitioning of fatty acids into effector molecules in the insulin secretory pathway (Prentki, Joly, El-Assaad, & Roduit, 2002).

Acetyl-CoA carboxylase catalyzes the formation of malonyl-CoA, a precursor in the biosynthesis of long-chain fatty acids. Interestingly, glutamate-sensitive protein phosphatase 2A-like protein activates acetyl-CoA carboxylase in beta-cells (Kowluru, Chen, Modrick, & Stefanelli, 2001). This observation might link two metabolites proposed to participate in the control of insulin secretion. Indeed, the amino acid glutamate is another discussed metabolic factor, generated in mitochondria (Maechler, Antinozzi, & Wollheim, 2000), proposed to participate in the amplifying pathway (Hoy et al., 2002; Maechler & Wollheim, 1999; Maechler & Wollheim, 2000). Glutamate can be produced from the TCA cycle intermediate  $\alpha$ -ketoglutarate or by transamination reactions (Newsholme, Brennan, Rubi, & Maechler, 2005; Nissim, 1999). During glucose stimulation total cellular glutamate levels have been reported to increase in human, mouse and rat islets as well as in clonal beta-cells (Bertrand et al., 2002; Brennan et al., 2002; Broca et al., 2003; Carobbio et al., 2004; Lehtihet, Honkanen, & Sjoholm, 2005; Maechler & Wollheim, 1999; Rubi et al., 2001), whereas in other studies no change was detected (Danielsson, Hellman, & Idahl, 1970; MacDonald & Fahien, 2000).

The finding that mitochondrial activation in permeabilised beta-cells directly stimulates insulin exocytosis (Maechler et al., 1997) initiated investigations that identified glutamate as a putative intracellular messenger (Maechler & Wollheim, 1999). In order to challenge the glutamate hypothesis, we overexpressed glutamate decarboxylase (GAD65) in INS-1E cells and rat islets to reduce cytosolic glutamate levels (Rubi et al., 2001). In control cells, stimulatory glucose concentrations increased glutamate concentrations, whereas the glutamate response was significantly reduced in GAD overexpressing cells. GAD overexpression also

inhibited secretory responses to high glucose in INS-1E cells as well as rat islets. In perfused islets, GAD overexpression inhibited glucose-stimulated insulin release by 31%, eventually indicating the contribution of glutamate in the sustained secretory response (Rubi et al., 2001). In the *in situ* pancreatic perfusion, increased provision of glutamate using a cell permeant precursor results in augmentation of the sustained phase of insulin release (Maechler, Gjinovci, & Wollheim, 2002).

The use of selective inhibitors led to a model where glutamate, downstream of mitochondria, would be taken up by secretory granules, thereby promoting  $\text{Ca}^{2+}$ -dependent exocytosis (Hoy et al., 2002; Maechler & Wollheim, 1999). Such a model was strengthened by demonstration that clonal beta-cells express two vesicular glutamate transporters (VGLUT1 and VGLUT2) and that glutamate transport characteristics are similar to neuronal transporters (Bai, Zhang, & Ghishan, 2003). The mechanism of action inside the granule could possibly be explained by glutamate-induced pH changes, as observed in secretory vesicles from pancreatic beta-cells (Eto et al., 2003). Another alternative or additive mechanism of action would be activation of acetyl-CoA carboxylase (Kowluru et al., 2001) as mentioned above. Moreover, another action of glutamate has been proposed. In insulin-secreting cells, rapidly reversible protein phosphorylation/dephosphorylation cycles have been shown to play a role in the rate of insulin exocytosis (Jones & Persaud, 1998). It has recently been reported that glutamate, generated upon glucose stimulation, might sustain glucose-induced insulin secretion through inhibition of protein phosphatase enzymatic activities (Lehtihet et al., 2005).

To date, several mechanisms of action have been proposed for glutamate as a metabolic factor playing a role in the control of insulin secretion. Further studies should dissect these complex pathways that might be either additive or cooperative.

Finally, succinate should be mentioned as a mitochondrial metabolite proposed to control insulin production. Indeed, it was reported that succinate and/or succinyl-CoA are metabolic stimulus-coupling factors for glucose-induced proinsulin biosynthesis translation (Alarcon, Wicksteed, Prentki, Corkey, & Rhodes, 2002). Specifically, glucose caused a 40% increase in islet intracellular succinate levels and succinic acid monomethyl ester specifically stimulated proinsulin biosynthesis (Alarcon et al., 2002). More recently, an alternative mechanism has been postulated regarding succinate stimulation of insulin production. Authors showed that such stimulation was dependent on succinate metabolism *via* succinate dehydrogenase, rather

than being the consequence of a direct effect of succinate itself (Leibowitz et al., 2005).

## 9. Mitochondrial defects and beta-cell function

Mitochondria are the principal source of reactive oxygen species (ROS) and ROS generation increases with age (Beckman & Ames, 1998). The weak expression of natural enzymatic defences, e.g. catalase and superoxide dismutase (Tiedge, Lortz, Drinkgern, & Lenzen, 1997), renders beta-cells particularly susceptible to ROS (Maechler, Jornot, & Wollheim, 1999). Moreover, it was shown that beta-cell production of ROS was linked to mitochondrial metabolism and that ROS content in isolated islets of Zucker diabetic fatty rats is higher in resting conditions (Bindokas et al., 2003). ROS can also elicit beta-cell apoptosis leading to decreased beta-cell mass (Mandrup-Poulsen, 2001). In a model of intrauterine growth retardation in the rat that leads to diabetes in adulthood (Ogata, Bussey, & Finley, 1986; Simmons, Templeton, & Gertz, 2001), investigators recently reported increased production of ROS (Simmons, Suponitsky-Kroyter, & Selak, 2005). This was associated with damaged mitochondrial DNA, causing further production of ROS, ultimately leading to progressive loss of beta-cell function and development of type 2 diabetes in the adult (Simmons et al., 2005). Taken together, these observations indicate that ROS may participate in the impairment of glucose-induced insulin secretion observed in association with ageing and type 2 diabetes (Coordt, Ruhe, & McDonald, 1995). One can speculate that antioxidant therapeutic interventions might contribute preserving beta-cell function (Robertson, Harmon, Tran, Tanaka, & Takahashi, 2003). However, the direct contribution of oxidative stress to the aetiology and complications of diabetes remains poorly documented (Scott & King, 2004).

In type 2 diabetes, beta-cell dysfunction might be secondary to chronic increased exposure to both glucose and fatty acids, a situation usually associated with obesity (Poitout & Robertson, 2002; Prentki et al., 2002; Unger, 1995). Such glucolipotoxicity (Prentki et al., 2002) might implicate the uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein that diminishes the proton gradient generated by the respiratory chain. Overexpression of UCP2 in beta-cells attenuates ATP synthesis and insulin secretion in response to glucose (Chan et al., 2001), whereas islets isolated from UCP2 deficient mice exhibit enhanced ATP generation and insulin secretion upon glucose exposure (Zhang et al., 2001). In a high-fat diet-induced type 2 diabetes model, the lack of UCP2 improves blood glu-

cose levels and insulin secretory capacity (Joseph et al., 2002). In the INS-1 beta-cell model, chronic exposure to fatty acids decreased the secretory response to glucose along with UCP2 gene induction and partial mitochondrial uncoupling (Lameloise, Muzzin, Prentki, & Assimakopoulos-Jeannet, 2001). Taken together, these findings led to the notion that UCP2 might play an important role for beta-cell dysfunction in the pathogenesis of type 2 diabetes and that UCP2 inhibitors could be used to prevent or treat type 2 diabetes (Lowell & Shulman, 2005). However, such attractive view on UCP2, associated with potential drug target for the prevention of type 2 diabetes, might be tempered by other observations. Overexpression of peroxisome proliferator-activated receptor alpha, a fatty acid activated transcription factor, in INS-1E beta-cells increased UCP2 expression without affecting mitochondrial membrane potential but enhancing glucose-stimulated insulin secretion (Ravnskjaer et al., 2005). Moreover, it was observed in clonal beta-cells that UCP2 gene expression is induced as a defence mechanism against oxidative stress (Li, Skorpen, Egeberg, Jorgensen, & Grill, 2001) and that induction of UCP2 prevents cell death in response to cytokines by decreasing the production of ROS (Zengaffinen et al., 2005). Therefore, restricting UCP2 activity to deleterious effects in beta-cells might not reflect complex equilibriums of energy states and nutrient recognition in these cells.

Mutations in the mitochondrial genome are another cause of mitochondrial defects and have been associated with diabetes (Maassen, van Essen, van den Ouweland, & Lemkes, 2001; Maechler & Wollheim, 2001). Transgenic mice lacking expression of the mitochondrial genome specifically in the beta-cells have been generated and characterized (Silva et al., 2000). These mice are diabetic and their islets poorly release insulin in response to glucose. Mitochondrial DNA-deficient cellular models are also glucose unresponsive and are defective in mitochondrial function, although they still exhibit secretory responses to  $\text{Ca}^{2+}$  raising agents (Kennedy, Maechler, & Wollheim, 1998; Soejima et al., 1996; Tsuruzoe et al., 1998). At the clinical level, the putative impact of mitochondrial DNA mutations on insulin secretion was examined in diabetic patients carrying such mutations. They exhibit marked reduction in insulin release upon intravenous glucose tolerance tests and hyperglycemic clamps compared to non-carriers (Brandle, Lehmann, Maly, Schmid, & Spinass, 2001; Maassen et al., 2004; Velho et al., 1996). The consequences of such mutations on the beta-cell emphasizes once more the importance of the mitochondria in the control of insulin secretion.

For other causes of beta-cell dysfunction, such as lipotoxicity (Unger, 2002), the reader is referred to specialized reviews.

## 10. Conclusions

Mitochondria are key organelles that generate the bulk part of cellular ATP and represent the central cross-road of metabolic pathways. Recent molecular tools available for cell biology studies shed light on new mechanisms regarding the contribution mitochondrial metabolism in the coupling of glucose recognition to insulin exocytosis. Delineation of signals coming in and out beta-cell mitochondria will certainly be instrumental in the development of drugs for the treatment of diabetes. To date, beta-cell mitochondria were tested as a drug target only in a limited number of studies. For instance, inhibition of the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in beta-cells was proposed as a novel therapeutic approach, as it increases oxidative metabolism as well as glucose-stimulated insulin secretion [Lee et al., 2003, p. 401]. Current investigations are building bridges between basic science and clinical observations. The ultimate goal of these research works will be reached once patients could directly benefit from new findings.

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