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Control by Ca2+ of mitochondrial structure and function in pancreatic β-cells

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Abstract:	Mitochondria play a central role in glucose metabolism and the stimulation of insulin secretion from pancreatic b-cells. In this review, we discuss firstly the regulation and roles of mitochondrial Ca2+ transport in glucose-regulated insulin secretion, and the molecular machinery involved. Next, we discuss the evidence that mitochondrial dysfunction in b-cells is associated with type 2 diabetes, from a genetic, functional and structural point of view, and then the possibility that these changes may in part be mediated by dysregulation of cytosolic Ca2+. Finally, we review the importance of preserved mitochondrial structure and dynamics for mitochondrial gene expression and their possible relevance to the pathogenesis of type 2 diabetes.
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13th July, 2020
Dr Paula Pizza, Dr Shmuel Muallem
c/o
Cell Calcum

Dear Paula, Dear Shmuel,

As per our recent email exchanges, I would like to submit here our review entitled:

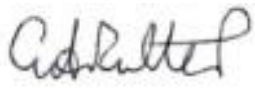
Control by Ca^{2+} of mitochondrial structure and function in pancreatic β-cells

by Dr Eleni Georgiadou and myself for consideration for publication in the Mitochondrial Function special addition of *Cell Calcium.*

Our review is focused on the pancreatic islet β cell as promised, and we hope it may be of interest.

Potential reviewers might include Dr Gregory Ku (Gregory.Ku@ucsf.edu), Dr Hindrik Mulder (hindrik.mulder@med.lu.se), Dr Gyorgy Hajnoczky (Gyorgy.Hajnoczky@jefferson.edu) and Professor Patrick Chinnery (pfc25@cam.ac.uk).

Yours sincerely,

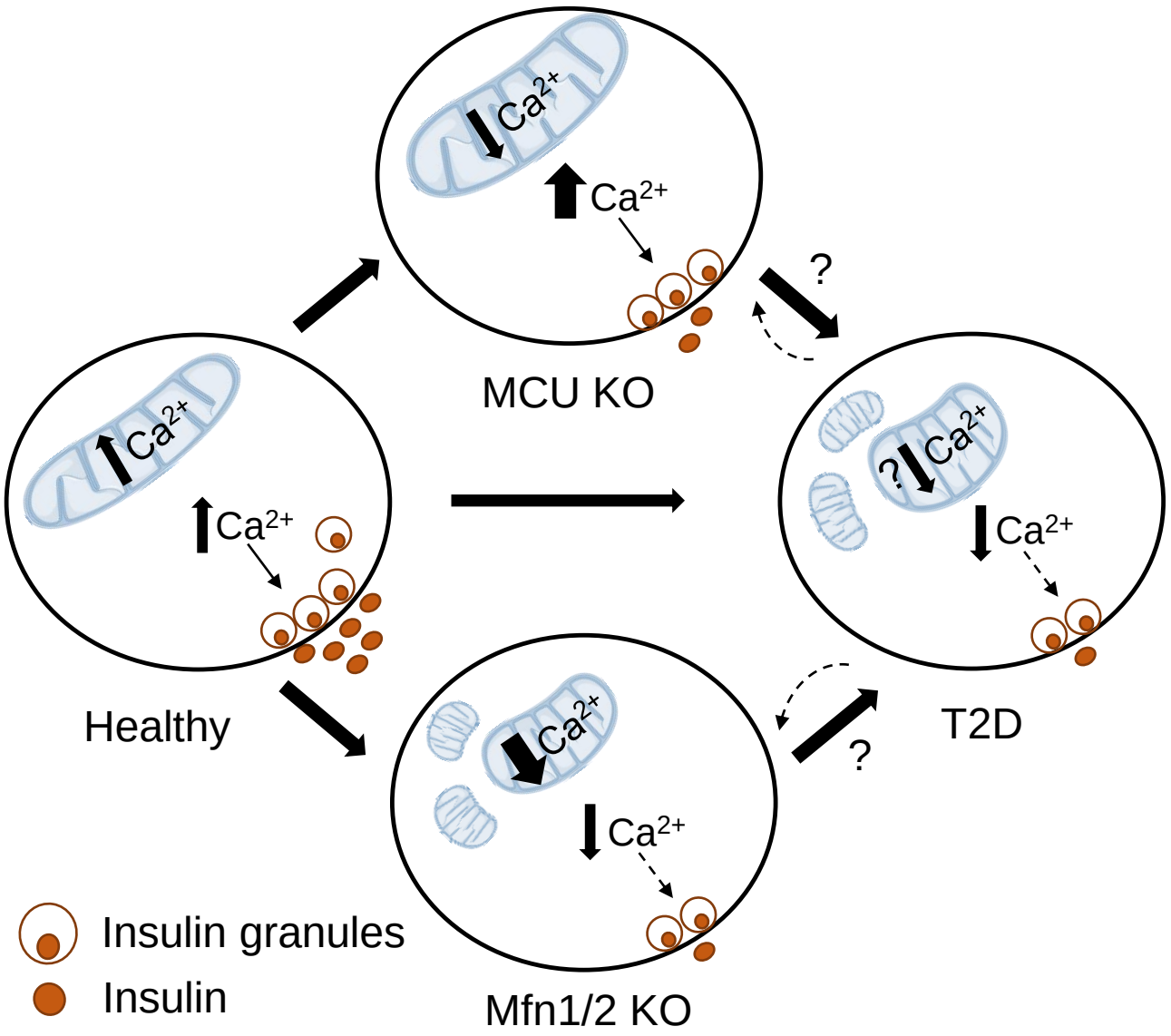


Guy A Rutter PhD

Highlights

- Mitochondrial Ca^{2+} uptake is essential for normal β-cell function and insulin secretion
- Changes in mitochondrial ultrastructure are observed in type 2 diabetes and may be driven by altered Ca^{2+} homeostasis.
- mtDNA mutations lead to impaired GSIS and diabetes in rodents and humans.

Graphical abstract



Control by Ca^{2+} of mitochondrial structure and function in pancreatic β-cells

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Highlights

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Abstract

Mitochondria play a central role in glucose metabolism and the stimulation of insulin secretion from pancreatic β-cells. In this review, we discuss firstly the regulation and roles of mitochondrial Ca^{2+} transport in glucose-regulated insulin secretion, and the molecular machinery involved. Next, we discuss the evidence that mitochondrial dysfunction in β-cells is associated with type 2 diabetes, from a genetic, functional and structural point of view, and then the possibility that these changes may in part be mediated by dysregulation of cytosolic Ca^{2+}. Finally, we review the importance of preserved mitochondrial structure and dynamics for mitochondrial gene expression and their possible relevance to the pathogenesis of type 2 diabetes.

Graphical abstract

A reduction in mitochondrial Ca^{2+} uptake through altered *Mcu* expression or impairment of the mitochondrial fusion and fission machinery may be potential pathways towards β-cell failure in T2D.

Keywords: Type 2 diabetes, mitochondria, Ca^{2+}, β-cells, insulin, secretion.

Abbreviations: $[Ca^{2+}]_{cyt}$,($[Ca^{2+}]_{mt}$, free cytosolic and intramitochondial Ca^{2+} respectively, IMM, OMM inner and outer mitochondrial membrane, mitochondrial membrane potential ($\Delta\psi_m$), glucose-stimulated insulin secretion (GSIS), ATP-sensitive potassium (K_{ATP}) channels, voltage-dependent-calcium channels (VDCCs), mitochondrial Ca^{2+} uniporter (MCU), mitochondrial permeability transition pore (mPTP), mitochondrial Na^+/Ca^{2+} exchanger (NCLX), mitochondrial DNA (mtDNA), type 2 diabetes (T2D).

1. Introduction

Diabetes now affects more than 450 million people worldwide, a number expected to grow to >700 million by 2045 [1]. Whereas type 1 diabetes (T1D) involves immune-mediated destruction of insulin-secreting pancreatic islet β-cells [2], type 2 diabetes (T2D) involves both impaired insulin secretion and, in most cases, insulin resistance [3]. The pathological changes which occur in the β-cell are still poorly understood but are likely to include alterations to mitochondrial structure and function [4]. Here, we focus on the role of Ca^{2+} regulation of mitochondrial function in both the healthy and the diseased β-cell.

Glucose uptake and metabolism, and a consequent increase in ATP production, are central to the activation of insulin secretion from β-cells as concentrations of the sugar increase in the blood following a meal [5]. This leads to an increase in cytosolic ATP/ADP ratio and closure of ATP-sensitive potassium (K_{ATP}) channels in the plasma membrane. By favouring a net influx of positive charge, K_{ATP} closure promotes plasma membrane depolarization and the opening of the voltage-dependent calcium channels (VDCCs) [6]. The resultant influx of Ca^{2+} then triggers the exocytotic release of insulin-containing secretory granules by activating Ca^{2+}-sensitive granule-resident proteins such as synaptotagmin-7 [7]. Mobilization of intracellular Ca^{2+} from intracellular stores including the endoplasmic reticulum (ER) [8] is important for the potentiation of glucose-stimulated secretion by G_q-coupled metabotropic receptor agonists such as acetyl choline which generate intracellular inositol (1,4,5) *tris* phosphate. Although ryanodine receptors are expressed at low levels in β-cells [9] the role of Ca^{2+}-induced Ca^{2+} release [10] in the response to glucose and other secretagogues is still unclear [11]. Nevertheless, acidic Ca^{2+} stores, including endosomes [12] and secretory granules [13] may also generate local Ca^{2+} signals that may be relevant for exocytosis or transfer of Ca^{2+} to other organelles including mitochondria.

“Amplifying” pathways for glucose-stimulated insulin secretion (GSIS) [4, 14], further enhance hormone release in the absence of detectable additional increases in cytosolic Ca^{2+} and are thought to contribute to the second, sustained phase of insulin secretion [15].

Mitochondrial metabolism is central to both signalling pathways (and hence the two phases of glucose-stimulated secretion), as demonstrated by (a) the ability of mitochondrial inhibitors to suppress secretion, (b) the fact that fuel secretagogues which are metabolised largely or exclusively by mitochondria (e.g. leucine, 2-ketoisocaproate, methylpyruvate) [16] are efficient substances to induce secretion [5, 17] and (c) the association of penetrant mutations in the mitochondrial genome with some forms of diabetes, including maternally-inherited diabetes and deafness (MIDD) [18]. Freidrich's ataxia [19] and the inheritance of common variants in the *TFB1M* gene encoding transcription factor B1 mitochondria [20], are also associated with increased disease risk.

Further emphasising the likely importance of mitochondrial oxidative metabolism for the stimulation of insulin secretion, weak expression in β-cells of both lactate dehydrogenase and the lactate/pyruvate (monocarboxylate) transporter MCT-1 (*Slc16a1*) [21] – both members of the so-called "disallowed" gene group in these cells [22] – means that glycolytically-derived pyruvate is preferentially metabolised by mitochondria [23], ensuring that > 85% of glucose carbon is oxidised fully to CO_2 and H_2O. Supporting the importance of diverting pyruvate towards mitochondrial oxidation, overexpression of LDHA [24, 25] inhibits secretion in clonal β-cells, though secretion was not affected from rat primary islets [26]. Of note, low levels of LDHA and MCT-1 also ensure that muscle-derived pyruvate and lactate do not cause an inappropriate secretion of insulin during exercise [26, 27]. Nevertheless, these data point towards the critical importance in the control of secretion of both mitochondrial production of ATP, as well as of potential coupling factors involved in the amplification process such as glutamate [28, 29], isocitrate [30] and certain lipid species [31].

1.1 Roles of intramitochondrial Ca^{2+} in the control of insulin secretion

Accumulation of Ca^{2+} by β-cell mitochondria in response to glucose, depolarisation or agonist-induced cytosolic Ca^{2+} increases was first described using recombinant, genetically-encoded and organelle-targeted aequorin (a bioluminescent Ca^{2+} sensor from the jelly fish *Aequoria victoria*) in the mid 1990s [32, 33]. Increased cytosolic

Ca^{2+} would naturally impose a drive towards lowered cytosolic ATP levels through increased consumption for ion pumping, granule movement etc. Ca^{2+} transport into mitochondria may also compromise ATP synthesis by lowering the inner mitochondrial membrane (IMM) potential ($\Delta\psi_m$). Indeed, some studies have demonstrated decreased cytosolic (sub-plasma membrane) ATP in response to depolarisation-induced Ca^{2+} influx [34]. However, these ATP-consuming processes are countered, firstly, by the activation of three intramitochondrial Ca^{2+}-sensitive dehydrogenases: pyruvate, isocitrate and 2-oxoglutarate dehydrogenases [35]. Activation of mitochondrial ATP synthesis [36] is likely further to complement the enhanced provision of NADH and FADH2 to the respiratory chain. Finally, activation of the Ca^{2+}-stimulated glycerol phosphate dehydrogenase located in the inter-membrane space of mitochondria (and thus regulated by cytosolic Ca^{2+} [37]) is likely also to enhance mitochondrial respiratory chain activity in βcells [38], where this enzyme is strongly expressed [21]. Stimulation of the malate-aspartate exchange by cytosolic Ca^{2+} may also be involved [39], though the contribution of this process to the control of pyruvate oxidation has only been examined under conditions where the pyruvate dehydrogenase complex is likely to be fully active, and thus insensitive to further control by Ca^{2+} [40]. The role of this process in β-cells has not been explored yet.

In summary, cytosolic ATP levels (or rather ATP/ADP ratio) are thus likely to reflect the minute-to-minute balance of enhanced mitochondrial oxidation versus enhanced consumption, with Ca^{2+} influencing both processes.

1.2 Regulators of mitochondrial Ca^{2+} transport in pancreatic β-cells

Given the above observations, enhanced mitochondrial Ca^{2+} uptake in pancreatic βcells might thus provide an innovative and promising strategy to improve insulin secretion in patients with T2D. Accumulation of Ca^{2+} by mitochondria depends on the mitochondrial membrane potential ($\Delta\psi_m$), and is mediated by the mitochondrial Ca^{2+} uniporter (MCU) and its associated regulatory subunits [41-44]. The uniporter complex consists of multiple subunits, namely the regulatory scaffolds MCU Regulator 1 (MCUR1), the Essential MCU Regulator Element (EMRE) and the Ca^{2+} sensors Mitochondrial Calcium Uptake proteins 1, 2, and 3 (MICU1, MICU2 and MICU3) [41, 42, 45-48]. The identification of these components, as well as of the

mitochondrial Na^+-Ca^{2+} exchanger (NCLX) [49] in molecular terms over the last decade has given rise to the possibility that they might provide targets for therapeutics which may modulate insulin secretion.

In 2012, Tarasov *et al*. [44] reported, using simultaneous, multiparametric measurements of Ca^{2+} and ATP/ADP dynamics in the same, patch-clamped single β-cell (Fig. 1A), the existence of two phases of change in the response to glucose (Fig. 1B). In the first, an initial glucose-induced increase in cytosolic [ATP/ADP] occurred independently of any increase in cytosolic ($[Ca^{2+}]_{cyt}$) or of mitochondrial ($[Ca^{2+}]_{mt}$) Ca^{2+} concentration. In the second phase, the elevation of $[Ca^{2+}]_{cyt}$ resulting from K_{ATP} channel closure led to a gradual increase in $[Ca^{2+}]_{mt}$. This, in turn, was likely to activate intramitochondrial dehydrogenases [50] (and perhaps the F_1F_0 ATP synthase) [51], stimulating respiratory chain activity and hence mitochondrial ATP production (see the description above). Importantly, Ca^{2+} changes in mitochondria were damped with respect to those in cytosol (Fig. 1C) [52], such that low frequency pulses of cytosolic Ca^{2+} resulted in minimal increases in mitochondrial Ca^{2+}, i.e. changes in frequency, as imposed by physiologically glucose, are decoded by mitochondria as modifications in the ATP/ADP increase, providing a further rational for the existence of kinetically-controlled Ca^{2+} transport systems across the IMM.

Importantly, shRNA-mediated silencing [44] demonstrated that MCU was critical for mitochondrial Ca^{2+} accumulation in primary mouse pancreatic β-cells in response to depolarisation-induced Ca^{2+} increases. Thus, the initial rapid glucose-induced increase in [ATP/ADP] was not affected by MCU silencing whereas the second phase of [ATP/ADP] increase was essentially eliminated. Mitochondrial Ca^{2+} accumulation, catalysed by MCU, was thus revealed in these studies to be essential for the second phase of glucose-induced ATP synthesis by glucose [44] and for insulin secretion as assessed by the release of co-stored Zn^{2+} (Fig. 1A).

Complementing these findings, Graier and colleagues [53] used a pancreatic β– cell line (INS-1 (832/13) cells) to demonstrate that transient silencing of MICU1 or MCU resulted in impaired mitochondrial Ca^{2+} uptake in response to both Ca^{2+} influx across the plasma membrane and release from the ER, without affecting $[Ca^{2+}]_{cyt}$ [53].

Silencing of either protein reduced cytosolic Ca^{2+} oscillations and insulin secretion in response to high glucose.

MICU1 is essential for MCU regulation, binding both MCU and EMRE, and its expression correlates with tissue-dependent differences in mitochondrial Ca^{2+} uptake [45, 54-57]. Additionally, reduced mitochondrial Ca^{2+} sequestration in MICU1- or MCU-silenced cells led to impaired respiration rates, decreased ATP production and reduced glucose-stimulated insulin secretion (GSIS). These findings indicate that the stimulatory effects of Ca^{2+} on metabolism dominate over the inhibitory effect expected from the depolarization that occurs when Ca^{2+} enters through the uniporter [53].

Moreover, a recent study focusing on MICU1 [58] revealed that the latter is essential for proper proteomic organization of the Mitochondrial Contact Site and Cristae Organizing System (MICOS) complex and that its ablation in cultured HEK293T cells and mouse embryonic fibroblasts resulted in altered cristae organization and mitochondrial ultrastructure. The authors hypothesized that MICU1 can serve as a MICOS Ca^{2+} sensor, since perturbing MICU1 lead to cristae disorganisation and cytochrome c release independently of mitochondrial Ca^{2+} uptake across the IMM. The latter study thus provided the first experimental evidence suggesting that MICU1 regulates cellular functions independently of mitochondrial Ca^{2+} uptake and may serve additionally as a critical mediator of Ca^{2+}-dependent signalling to modulate mitochondrial membrane dynamics and cristae organization [58].

As noted above, extrusion of Ca^{2+} from the mitochondrial matrix is catalysed chiefly by NCLX. NCLX exchanges one Ca^{2+} ion for three Na^+, with the latter balanced in turn by a Na^+/H^+ exchanger to maintain a polarised $\Delta\psi_m$ [49]. Several studies have demonstrated that silencing of NCLX using siRNA in mouse β–cells lead to $[Ca^{2+}]_{mt}$ overload with glucose-induced $[Ca^{2+}]_{cyt}$ increases, ATP production and the first phase of insulin secretion all strongly impaired [49, 59].

1.3 Inactivation of MCU in mouse β-cells

Even though changes in $[Ca^{2+}]_{mt}$ were found to be critical for increases in cytosolic ATP/ADP ratio in the aforementioned *in vitro* studies, it remained unknown whether

mitochondrial Ca^{2+} transport may be required for GSIS *in vivo.* Since MCU represents the main route for Ca^{2+} uptake in mitochondria, our group selectively inactivated the uniporter in mouse β–cells to provide a better understanding of its importance in insulin secretion and glucose homeostasis in the living animal [60].

By developing a conditional *Mcu* allele that allowed deletion of all isoforms, and using a highly β-cell-selective *Cre* driver line (INS1Cre) [61], these studies [60] revealed that mitochondrial Ca^{2+} uptake (Fig. 2A-B) was essential for both phases of glucose-stimulated ATP synthesis and insulin secretion in isolated islets from β*Mcu*-null mice (Fig. 2C-D), as well as for the maintenance of normal β-cell mass. Measured in isolated islets, mitochondria within knock-out (KO) β-cells were also more polarised than those in islets from wild-type (WT) animals, suggesting an impaired entrance of positively charged ions (such as Ca^{2+}), necessary for oxidative phosphorylation and ATP production. On the other hand, and despite experiencing larger increases in $[Ca^{2+}]_{cyt}$ in response to high glucose in intact KO mouse islets, GSIS was severely inhibited *in vitro* (Fig. 2C-D). These experiments are thus in line with earlier findings in which gene inactivation was achieved *in vitro* and implies important roles for Ca^{2+}-activated mitochondrial oxidative metabolism (i.e. ATP production) and conceivably for the production of coupling molecules such as glutamate, citrate/malate or others.

Extending these findings to the *in vivo* phenotype, β*Mcu*-KO mice showed impaired glucose tolerance 15 min. (Fig. 2E) and insulin secretion 5 min. (Fig. 2F) post-intra-peritoneal glucose injection, while no differences between WT and KO mice were observed at other time points, pointing to the existence of currently undefined compensatory mechanisms. One intriguing possibility could be that in the absence of MCU, mitochondrial NCLX operates in the reverse mode at low $\Delta\psi_m$ and allows Ca^{2+} influx. Another observation made during this study was that insulin secretion post-oral glucose gavage was elevated in KO mice at 5 min. This paradoxical result was not due to altered release or action of gut-derived incretins, since islets from KO mice displayed impaired sensitivity to incretin hormones *in vitro*. Measurements of the expression of NCLX, as well as of other genes which may contribute to these apparent compensatory mechanisms, will be required in the future. Nevertheless, these results are in line with previous findings where global *Mcu*-null mice displayed

relatively minor phenotypes in cardiac and skeletal muscle function, and no overt glycaemic abnormalities [62, 63].

In conclusion, these findings, which provide only the second description of a conditional tissue-restricted *Mcu*-KO mouse (the first being described in [44]) revealed a critical role for MCU expression in the control of insulin secretion and the potential for a role in some forms of diabetes.

2. Mitochondrial dynamics

Mitochondria continuously undergo fission and fusion cycles in a dynamic process that is influenced by nutrient supply or demand, as well as by extra- or intracellular factors that are critical to cell survival [64]. As well as influencing mitochondrial turnover (mitochondrial fragmentation is required for mitophagy and hence the replacement of defective mitochondria [65]), these dynamics, along with motor-protein-driven mitochondrial translocation by dynein and kinesin family members [11] control the localisation of the organelles vis-à-vis other intracellular organelles and the plasma membrane. These changes may, as a result, control localised changes in Ca^{2+} and ATP generation [66].

Mitochondrial fusion is controlled by the outer mitochondrial membrane dynamin related protein GTPases mitofusin 1 (MFN1) and mitofusin 2 (MFN2) [67], as well as an inner mitochondrial membrane GTPase protein optic atrophy protein 1 (OPA1) [68]. This process is essential as it involves exchange of proteins, metabolites, and mitochondrial DNA (mtDNA) and thus allows the maintenance of functional mitochondria, which may otherwise have become depolarized by intracellular or extracellular stress [69]. Mitochondrial fission is regulated by the transmembrane protein fission 1 (FIS1) [70], the outer membrane protein mitochondrial fission factor (MFF) [71], as well as the GTPase dynamin-related protein 1 (DRP1).The latter is recruited to the mitochondria from the cytosol by FIS1 and MFF to initiate fission events [72]. Mitochondrial fission favours the isolation of dysfunctional or damaged mitochondria, which can either be repaired through the fusion cycle or degraded via the mitophagy/autophagy machinery [73]. Alterations of the balance of mitochondrial dynamics, by gain or loss of function of the fission or fusion proteins mentioned

above, have been shown to lead to impairments in mitochondrial structure, function or intracellular signalling events in β-cells [69, 74].

2.1 Role of Ca^{2+} in mitochondrial dynamics

It is widely accepted that Ca^{2+} regulates mitochondrial oxidative metabolism [35]. Several studies have demonstrated that there is also a close association between $[Ca^{2+}]_{mt}$ and mitochondrial morphology [75-78]. Direct evidence that Ca^{2+} is a critical regulator of mitochondrial dynamics comes from the fact that mitochondrial Ca^{2+} overload, triggering mitochondrial permeability transition pore (mPTP) opening [79], can lead to mitochondrial fragmentation whereas mitochondrial Ca^{2+} depletion results in mitochondrial hyperfusion in a number of cell types [80].

In non-pancreatic cells, such as cardiomyocytes and hepatocytes, increases in $[Ca^{2+}]_{cyt}$ promote the translocation of DRP1 to mitochondria and induce mitochondrial fission [81, 82]. In addition, mitochondrial Ca^{2+} overload through voltage-dependent anion channels (VDACs) and MCU can stimulate rapid mitochondrial fission by activating DRP1 in cultured neuronal cells [83]. On the other hand, muscle fibres in Drp1 KO mice show increased expression of MCU, mitochondrial Ca^{2+} overload, opening of mPTP, mitochondrial swelling and ultimately, myofiber weakness or death [84, 85]. Lablanche S. *et al*., demonstrated that the Ca^{2+}-dependent mPTP opening in INS-1 cells led to cell death under high levels of glucose or fructose but did not investigate mitochondrial structure changes [86]. Hence, the potential role of these processes in controlling mitochondrial structure in pancreatic β-cells, and its role in diabetes aetiology, remains poorly explored.

Intracellular mitochondrial motility is another pathway regulated by Ca^{2+} and allows appropriate distribution of organelles for local ATP supply and Ca^{2+} buffering near perinuclear or cytoplasmic membrane clusters [87]. Thus, Ca^{2+} binds to the mitochondria-associated GTPase Miro through a pair of EF-hand Ca^{2+}-binding motifs on the OMM complex and regulates Ca^{2+}-dependent mitochondrial motility [88]. For example, motility of astrocytic mitochondria is dependent on intracellular Ca^{2+} concentrations since elevated $[Ca^{2+}]$ immobilizes astrocytic mitochondria, whereas low $[Ca^{2+}]$ under resting conditions causes mitochondria to be highly mobile [89]. Similar behaviour is observed in neurons [90].

Finally, participation by Ca^{2+} in the control of mitochondrial dynamics may also include mitophagy, a highly specialized process that removes dysfunctional mitochondria [65]. This has been observed in mammalian cells and animal models where Ca^{2+} dysregulation and reactive oxygen species (ROS) production are potential mitophagy inducers [91, 92]. More details on the role of mitophagy and its control by Ca^{2+} can be found in [87, 92].

2.2 Ca^{2+} regulation of mitochondrial dynamics in β-cells

Abnormalities in mitochondrial morphology, function and dynamics are observed in diabetic rat models [93, 94], β-cell-derived lines [95], as well as in islets isolated from people with T2D. Whether and how defects in Ca^{2+} homeostasis, known to occur in the β-cell in diabetes [96] contribute to these structural changes, is presently unclear and is an important area for exploration.

β-cells from patients with T2D and both diabetic Goto Kakizaki (GK) and Zucker Diabetic Fatty (ZDF) rats develop mitochondrial fragmentation suggesting an impairment of mitochondrial dynamics under high circulating glucose conditions [64, 93]. Molina *et al*. [69] demonstrated that mice β-cells treated with free fatty acids (FFAs) – circulating levels of which are increased in T2D - displayed dramatic and rapid mitochondrial fragmentation. The authors associated this observation with defective mitochondrial fusion since MFN2 expression levels decreased. Men *et al*., on the other hand, noted that the increased mitochondrial fission observed under conditions of glucotoxicity in GK rats was linked to increased expression of DRP1 in β-cells [97]. Nevertheless, the role of mitochondrial fragmentation in β-cells under high nutrient conditions remains unclear.

Defects in mitochondrial ultrastructure, including swelling and altered cristae structure, were observed in diabetic rat models [93] and were associated with reduced insulin secretion, lower ATP/ADP ratio and depolarisation of the IMM (i.e. impaired ability to sustain highly negatively charged membrane potential) [98]. Mitochondrial volume density was also significantly increased in comparison with WT animals, and $\Delta\psi_m$ was more positive in diabetic versus normoglycemic rats. Hence,

a possible explanation could be that mitochondrial Ca^{2+} overload followed by opening of the mPTP [99] are linked to mitochondrial impairment, insulin secretory failure and, ultimately, hyperglycaemia [100].

Are these changes in structure functionally relevant? Recent results from Ku and colleagues [101], and ourselves [102] have sought to answer this question by imposing forced changes in the expression of key regulators of mitochondrial fission or fusion and demonstrate that the balance between these processes is critical for normal β-cell fuel sensing. Deletion or silencing of one or more of these factors (e.g. *Drp1* or *Mfn1* plus *Mfn2*) [101-103] exerted profound effects on insulin secretion and glucose homeostasis in mice, despite opposing effects on mitochondrial fragmentation. Thus, deletion of *Drp1* led to aggregation of mitochondria near the nucleus and inhomogeneity in glucose-induced NAD(P)H increases across the cell versus WT controls [101]. Although overall O_2 consumption was normal, the period of cytosolic Ca^{2+} oscillations was increased in *Drp1* KO islets, demonstrating a requirement for mitochondrial localisation and/or fragmentation or normal responses: of note the expression of a number of mitochondria-associated genes was decreased (e.g. *Ndufb8, Sdhb, Mtco1 or* ETC complexes I, II and IV respectively) [101].

On the other hand, deletion of *Opa1*, responsible for fusion of the IMM in β-cells, resulted in respiratory chain defects and impaired insulin secretion [74]. Our own study [102] of the effects of combined *Mfn1* and *Mfn2* inactivation revealed that simultaneous ablation of both mitofusins selectively in β-cells in adult mice had catastrophic effects on β-cell function, prompting sharp reductions in glucose-induced cytosolic and mitochondrial Ca^{2+} increases and impaired insulin secretion both *in vivo* and *in vitro*. Mitochondrial membrane potential was highly depolarised and β-cell mass was significantly reduced in β*Mfn1/2* KO animals demonstrating the importance of maintained mitochondrial ultrastructure for β-cell survival. Nonetheless, the role and regulation of mitochondrial fission and fusion factors in the β-cell in diabetes mellitus still remain to be fully elucidated. Whether Mfn1/2 KO in β-cells influences ER-mitochondria contacts as proposed by Scorrano and colleagues [104] but disputed [105], is unknown.

An interesting recent report from Montemurro C. *et al.* [106] revealed changes in mitochondrial structure and function, coincident with cytosolic and mitochondrial Ca^{2+} at different stages of the cell cycle (Fig. 3). Thus, in the INS1(832/13) insulinoma line, fragmented mitochondria and phosphorylated Drp1 were observed in S and G2/M phases of the cell cycle during which DNA/lipid synthesis and mitosis occur and was associated with increase basal O_2 consumption rates. A rise in cytosolic (and reduction in mitochondrial) Ca^{2+} uptake was observed at G1/S and was consistent with the requirement for Ca^{2+} to initiate centrosome duplication at early G1/S. On the other hand, intramitochondrial Ca^{2+} was elevated in G2/M, providing a possible explanation for increased respiration (through activation of mitochondrial Ca^{2+}-dependent dehydrogenases of the tricarboxylic acid (TCA) cycle). Taken together, this study confirmed the link between mitochondrial network and Ca^{2+} compartmentalization as β-cells transition through cell cycle.

3. mtDNA mutations and diabetes

mtDNA integrity and stability are necessary for normal β-cell function and may play crucial roles in the development of hyperglycemia [107]. Among the most convincing links between mitochondrial dysfunction and diabetes are mtDNA mutations which occur either as single deletions, which usually manifest as *de novo* mutations, or multiple mtDNA deletions, which occur somatically and accumulate with age (Fig. 4). Both forms can result in mitochondrial respiratory chain deficiency and disease [108]. For example, in Kearns-Sayre syndrome (KSS), which is associated with a single deletion myopathy, patients can develop impaired GSIS, reduced β-cell mass, and dysmorphic islet architecture [109]. Another example is the A3243G mutation in mitochondrial tRNA-Leu, which also causes mitochondrial encephalomyelopathy and lactic acidosis [18], and is associated with MIDD. Sufferers of MIDD show reduction in β-cell mass and subsequent insulin deficiency without reduced insulin sensitivity in peripheral tissues [107]. Hence, MIDD mutations were among the first evidence of a role for energetic failure in GSIS.

Mitochondrial function in the β-cell can also be modulated by the extent of mitochondrial gene transcription. Stability, transcriptional activity and copy number of mtDNA is predominantly controlled by a nuclear-encoded factor, the mitochondrial

transcription factor A (TFAM) and the mitochondrial transcription factor B2 (TFB2M) [110] [111]. Wiederkehr *et al*., demonstrated that suppression of the transcription factor pancreatic duodenum homeobox-1 (PDX1), required for the expression of insulin in mouse islets, decreased TFAM expression which coincided with hyperglycaemia, attenuation of mitochondrial signals and failure to raise cytosolic ATP levels [110]. These results confirmed that TFAM was a direct target of PDX1 and implicate TFAM in the preservation of mitochondrial function [110]. On the other hand, TFAM overexpression in *Pdx1* deficient (*Pdx1*$^{-/-}$) mice restored both glucose-dependent ATP synthesis and insulin secretion by increasing the expression of mtDNA-encoded genes. Additionally, the authors were able to restore the mitochondrial Ca^{2+} signal in *Pdx1*$^{-/-}$ islets using a mitochondrial Na^{+}/Ca^{2+} exchanger inhibitor which resulted in improved ATP production and insulin secretion [112]. These findings thus demonstrate that an increase in $[Ca^{2+}]_{mt}$ can compensate for reduced levels of mtDNA encoded respiratory chain complex subunits.

While the role of TFAM in pancreatic β-cells has been well characterised, it is only recently that the significance of TFB2M in mitochondrial and cellular function of pancreatic β-cells has been elucidated. β-cell-specific knockout of *Tfb2m* in a mouse model, as well as in INS1 832/13 cells [113], resulted in decreased expression of mitochondrial-encoded genes and lowered mtDNA content. This led to severe mitochondrial dysfunction, characterised by diminished hyperpolarisation of the IMM, impaired O_2 consumption and reduced ATP production. Nicholas *et al*., also observed increased level of autophagy and mitophagy suggesting an unforeseen critical role for this mitochondrial transcription factor [113].

Mitochondrial transcription factor B1 (TFB1M) on the other hand, is believed to not be an authentic transcription factor. In fact, it functions as a methyl transferase which, if lost, leads to ribosome destabilization and an abrogation of protein synthesis. Koeck *et al*. identified a common variant in the human *TFB1M* gene [114] associated with reduced insulin secretion, elevated postprandial glucose levels and future risk of T2D in female patients. Carriers of the risk allele also exhibited decreased complex I activity and protein level in pancreatic islets [114]. Similarly, heterozygous *Tfb1m* expression in mouse models induced lower levels of TFB1M in

islets, impaired mitochondrial function and lower GSIS *in vivo* and *in vitro* [114]. Moreover, silencing of TFB1M in clonal insulin-secreting cells impaired complexes of the mitochondrial OXPHOS system leading to impaired ATP synthesis and GSIS while ROS levels in response to cellular stress increased. These authors also found signs of apoptosis and necrosis in *Tfb1m* $^{-/-}$ islets which may also be relevant for human T2D, where (some) β-cell loss and apoptosis in islets have been reported [115]. Hence, these findings demonstrated that *Tfb1m* deficiency in β-cells can induce mitochondrial dysfunction and subsequently diabetes owing to combined loss of β-cell function and mass (Fig. 4). The effects of these changes on cytosolic or mitochondrial Ca^{2+} are, however, presently undefined.

Lastly, normal mitochondrial dynamics are essential to control mitochondrial content mixing and the maintenance of mtDNA. Thus, and for as yet unknown reasons, severe impairment of mitochondrial fusion drastically affects mtDNA copy number, in pancreatic and extra-pancreatic cells. For example, mtDNA copy number in mouse β-cells lacking *Opa1* is unchanged, but the activity of complex IV is significantly decreased, perturbing glucose-stimulated ATP production and GSIS [74]. Additionally, since glucose-unresponsive cells (INS1-832/2) exhibit poorer mitochondrial dynamics than glucose-responsive cells (INS1-832/13), Schultz *et al*. decided to investigate mitochondrial morphology by overexpressing *Fis1* in glucose-unresponsive cells and demonstrated that increased fission could restore GSIS [116]. Conversely, Silva Ramos *et al*. demonstrated that loss of OMM, but not IMM fusion, lead to nucleoid clustering in mouse cardiomyocytes [117]. Assessment of mitochondrial transcription in tissue homogenates revealed that abolition of OMM fusion did not affect transcription or mutagenesis of mtDNA but instead, mitochondrial fusion was necessary to maintain the stoichiometry of the protein components of the mtDNA replisome in cardiac cells [117]. Whether mitochondrial fusion is similarly important in β-cells remains to be explored.

4. Conclusions and perspectives

Mitochondria represent the targets for fluctuations in intracellular Ca^{2+} in β-cells, with the ions acting both within the mitochondrial matrix (on oxidative metabolism) and on targets on the mitochondrial intermembrane space or the outer membrane

(translocation, fission). Abnormal Ca^{2+} dynamics in the diseased β-cell, including both elevated basal Ca^{2+} and impaired increases in response to high glucose, may thus contribute to altered mitochondrial function, stability and structure, leading to a vicious circle in which these changes further impair stimulus-secretion coupling and β-cell viability. Whether they also impact the β-cell in the context of T1D, for example by affecting ER stress pathways or antigen presentation, is unknown. Nonetheless, therapeutics which mitigate or suppress these alterations, for example by activating MCU [118], may have utility for the treatment of some forms of diabetes.

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Conflict of Interest

G.A.R. has received grant funding from Sun Pharmaceuticals and from Les Laboratoires Servier, and is a consultant for Sun Pharmaceuticals.

Figure legends

Figure 1. Exploring relationships between cytosolic, mitochondrial Ca^{2+}, metabolism and insulin secretion in single primary β-cells. (**A**) Measurements of cytosolic Ca^{2+} (e.g. with the trappable Ca^{2+} indicator fura red) mitochondrial Ca^{2+} or cytosolic ATP/ADP ratio with the indicators noted are performed in perforated patch-clamped β-cells. The cell-attached Zn^{2+} sensor ZIMIR (Zinc indicator for monitoring induced exocytotic release) can be used to monitor the release of co-secreted insulin. (**B**) Increases in glucose concentration over the physiological range prompt a biphasic increase in ATP (blue); note the transient decrease in ATP/ADP upon the induction of action potential firing a Ca^{2+} influx (red) which gradually recovers. Current injection (arrow) provokes a further small increase in Ca^{2+} channel opening (repolarisation) and collapse upon addition of the uncoupler carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone (FCCP). (**C**) Mitochondrial Ca^{2+} uptake (green) depends on the frequency of cytosolic Ca^{2+} (red) oscillations, imposed here artificially through current injection via the patch pipette. Adapted from [44] and [52] with permission.

Figure 2. Deletion of the MCU channel in pancreatic β-cells impairs mitochondrial Ca^{2+} uptake and insulin secretion. (**A**) Each snapshot of isolated WT (i–iv) and KO-derived (v–viii) islets was taken during the time points indicated by the respective arrow in (**B**). Scale bars: 50 μm. (**B**) $[Ca^{2+}]_{mito}$ changes in response to 17mmol/l glucose (17G) (with or without diazoxide) and 20 mmol/l KCl + diazoxide were assessed in WT or KO islets following R-GECO (Ca^{2+} indicator in mitochondria) infection. Traces represent mean normalised fluorescence intensity over time (F/F_{min}). The corresponding AUC are also shown ($n = 3$ mice per genotype). (**C**) Insulin secretion from islets isolated from WT and KO mice during perifusion and (**D**) serial incubations of islets in batches, at 3 mmol/l glucose (3G) or 17 mmol/l glucose (17G). (**E**) Blood glucose and (**F**) glucose-induced insulin secretion (intraperitoneal injection of 3 g/kg body weight of glucose) were assessed in WT and KO mice ($n = 6–9$ mice per genotype). Blue, WT mice; red, KO mice. Data are presented as mean ± SEM. *$p < 0.05$; **$p<0.01$; ***$p < 0.001$. Adapted from [60] with permission.

Figure 3. Schematic representation of the metabolic cellular changes in replicating INS 832/13 cells synchronized at G1/S, S and G2/M. At G1/S the mitochondrial network is interconnected and fused which progressively fragments during the S and G2/M phases to secure mitochondrial inheritance during division. Glucose uptake and mitochondrial fusion at G1/S likely co-ordinately act to maximize ATP generation prior to the high energy requiring S and G2/M phases. While ATP levels are lowest during S phase, this suggests a high ATP consumption that physiologically occurs during DNA replication. Cytosolic and mitochondrial Ca^{2+} concentrations are elevated at the G1/S transition and further rise during G2/M to secure membrane formation during mitosis. Adapted from [106].

Figure 4. Potential mechanisms and roles of β-cell mitochondrial dysfunction in T2D. Changes in mitochondrial structure or function in pancreatic β-cells that lead to reduced insulin secretion and T2D may be induced by several processes, alone or in combination. A number of mutations in mtDNA can cause dysfunction of mitochondria in pancreatic β-cells as can the inheritance of identified risk alleles for T2D, e.g. *TFB1M* that is required for translation of proteins in mitochondria. Chronic metabolic changes such as elevations of circulating lipids and/or glucose, known as lipo- and glucotoxicity, respectively, may also interact with gene variants and affect negatively mitochondrial function in β-cells. Mitochondrial dysfunction can be reflected by an impairment in Ca^{2+} uptake, bioenergetics and ATP production, as well as in biogenesis and apoptosis. These processes may impair the production and release of insulin and may potentially lead to mitochondrial fragmentation and β-cell death. Adapted from [119].

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Graphical abstract

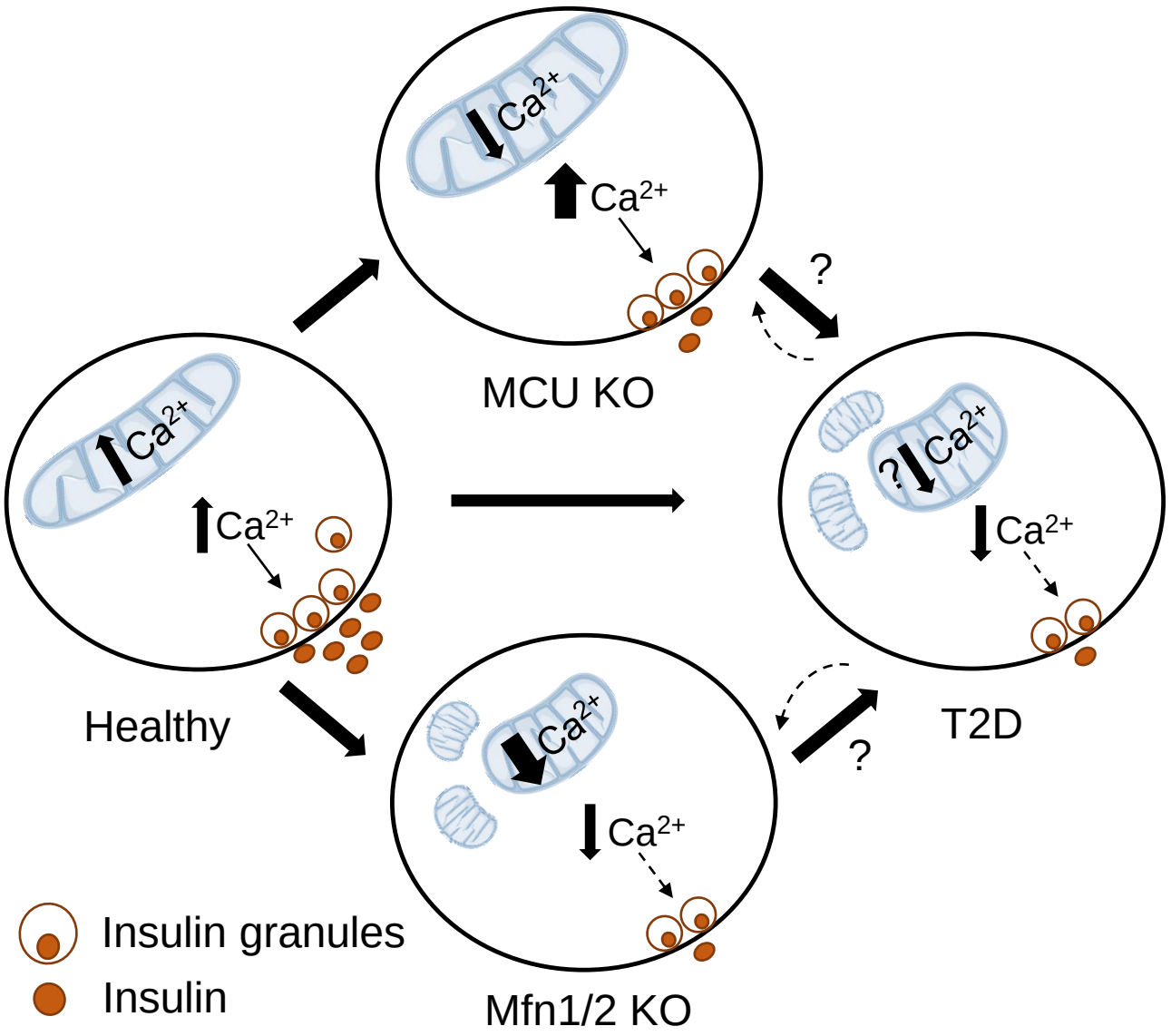


Figure 1

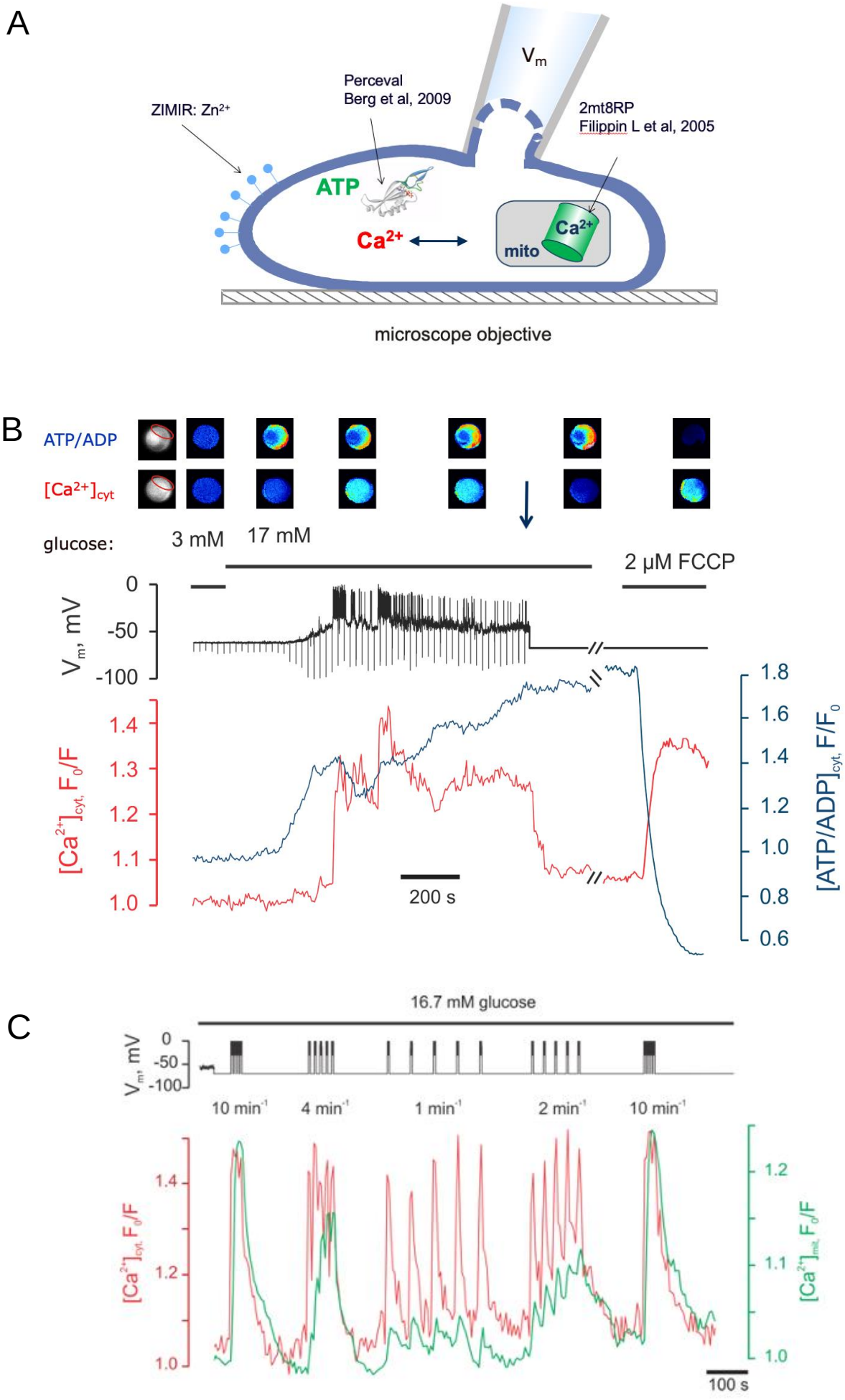

A
Vm
Perceval
Berg et al, 2009
ZIMIR: Zn2+
2mt8RP
Filippin L et al, 2005
ATP
Ca2+
mito
Ca2+
microscope objective
B
ATP/ADP
[Ca2+]cyt
glucose:
3 mM
17 mM
2 μM FCCP
Vm, mV
0
-50
-100
[Ca2+]cyt, F0/F
1.4
1.3
1.2
1.1
1.0
200 s
[ATP/ADP]cyt, F/F0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
C
16.7 mM glucose
Vm, mV
0
-50
-100
10 min-1
4 min-1
1 min-1
2 min-1
10 min-1
[Ca2+]cyt, F0/F
1.4
1.2
1.0
[Ca2+]mit, F0/F
1.2
1.1
1.0
100 s

Figure 2

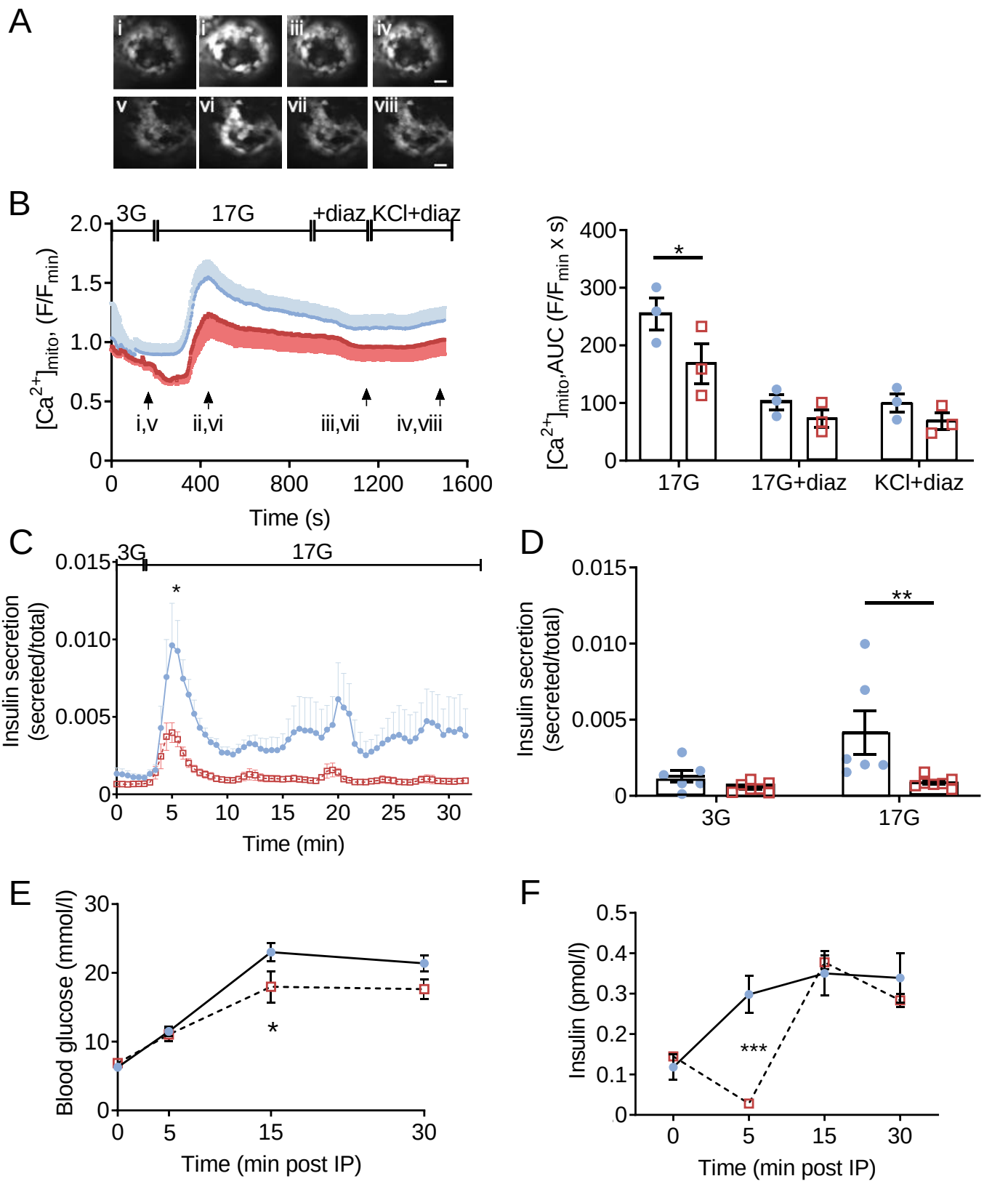

A
i
ii
iii
iv
v
vi
vii
viii
B
3G
17G
+diaz
KCl+diaz
2.0
1.5
1.0
0.5
0
[Ca2+]mito, (F/Fmin)
i,v
ii,vi
iii,vii
iv,viii
0
400
800
1200
1600
Time (s)
400
300
200
100
0
[Ca2+]mito,AUC (F/Fmin x s)
*
17G
17G+diaz
KCl+diaz
C
3G
17G
0.015
0.010
0.005
0
Insulin secretion (secreted/total)
*
0
5
10
15
20
25
30
Time (min)
D
0.015
0.010
0.005
0
Insulin secretion (secreted/total)
**
3G
17G
E
30
20
10
0
Blood glucose (mmol/l)
*
0
5
15
30
Time (min post IP)
F
0.5
0.4
0.3
0.2
0.1
0
Insulin (pmol/l)
***
0
5
15
30
Time (min post IP)

Figure 3

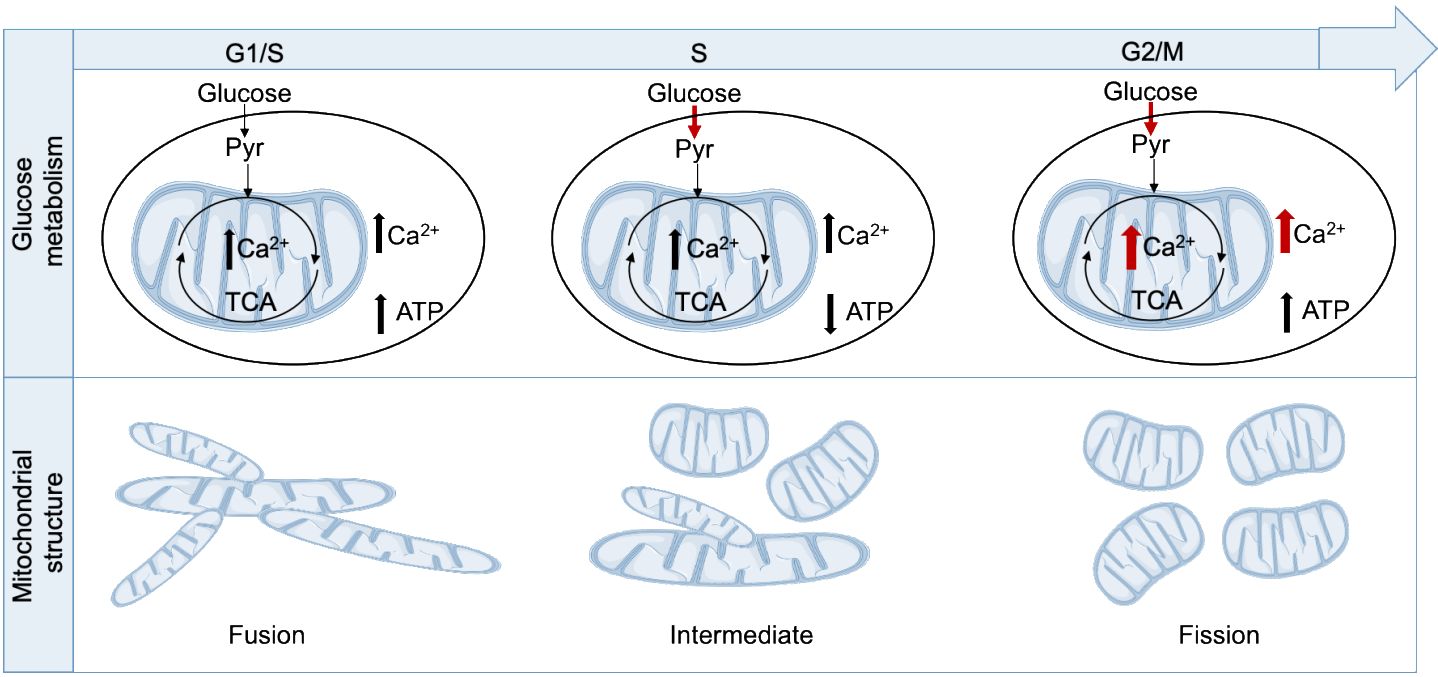
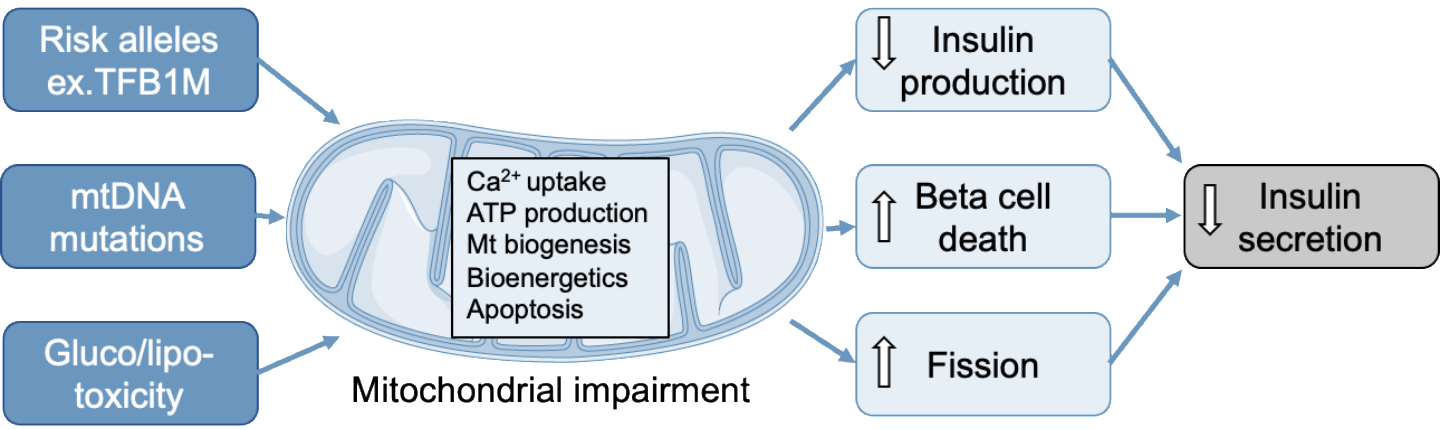

Glucose metabolism
G1/S
S
G2/M
Glucose
Pyr
Ca2+
TCA
ATP
Mitochondrial structure
Fusion
Intermediate
Fission

Figure 4


Risk alleles ex.TFB1M
mtDNA mutations
Gluco/lipo-toxicity
Ca2+ uptake
ATP production
Mt biogenesis
Bioenergetics
Apoptosis
Mitochondrial impairment
⇩ Insulin production
⇧ Beta cell death
⇧ Fission
⇩ Insulin secretion

Conflict of Interest

G.A.R. has received grant funding from Sun Pharmaceuticals and from Les Laboratoires Servier, and is a consultant for Sun Pharmaceuticals.