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Metabolic Cycles and Signals for Insulin Secretion

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Abstract

In this perspective we focus on recent developments in our understanding of nutrient-induced insulin secretion that challenge a key aspect of the "canonical" model, in which an oxidative phosphorylation-driven rise in ATP production closes K_{ATP} channels. We discuss the importance of intrinsic β-cell metabolic oscillations, the phasic alignment of relevant metabolic cycles, shuttles and shunts, and how their temporal and compartmental relationships align with the triggering phase or the secretory phase of pulsatile insulin secretion. Metabolic signalling components are assigned regulatory, effectory and/or homeostatic roles vis-à-vis their contribution to glucose sensing, signal transmission, and resetting the system. Taken together, these functions provide a framework for understanding how allostery, anaplerosis, and oxidative metabolism are integrated into the oscillatory behavior of the secretory pathway. By incorporating these temporal as well as newly-discovered spatial aspects of β-cell metabolism, we propose a much-refined $Mito_{Cat}$-$Mito_{Ox}$ model of the signaling process for the field to evaluate.

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In this perspective Merrins *et al*. discuss recent developments in our understanding of nutrient stimulated insulin secretion, with a focus on oscillatory metabolic signaling. By integrating the temporal as well as newly-identified spatial aspects of β-cell metabolism, the authors propose a revised model of pulsatile insulin secretion.

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DECLARATION OF INTERESTS
The authors declare no competing interests.

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Introduction

The year 2021 celebrated the 100th anniversary of the discovery of insulin and its administration to the first patient with diabetes. The names of Frederick Banting and Charles Best in this landmark discovery in medicine are widely known, and the key roles played by John MacLeod and Cecil Collip are being increasingly recognized. Insulin is considered the most important anabolic hormone in glucose and energy homeostasis, and a rise in blood glucose itself promotes the secretion of insulin by the pancreatic β-cell. Initial *in vivo* evidence that glucose promotes insulin secretion came from Grafe and Meythaler (Grafe and Meythaler, 1927a, 1927b), who injected glucose in the femoral versus the pancreatic duodenal vein of dogs and found that only the latter caused hypoglycemia. Zunz and La Barre joined two dogs by a pancreatic-jugular anastomosis and observed that pancreatic blood of the donor lowered the blood sugar of the recipient animal (Zunz and La Barre, 1927). In an elegant *in vivo* experiment, London and Kotschneff placed an angiostomy cannula in the pancreato-duodenal vein and determined the rise in glucose and insulin content of blood from the pancreas after a glucose meal (London and Kotschneff, 1934).

A decade later came key evidence for glucose-stimulated insulin secretion (GSIS) *ex vivo*. Anderson and Long (Anderson and Long, 1947) established a perfusion system that incorporated the stomach, duodenum and pancreas, and using a bioassay for insulin found that glucose promotes insulin release. Then came the well-known study of Grodsky and colleagues (Grodsky et al., 1963) where a perfused pancreas system was established to demonstrate by immunoassay that glucose dose-dependently promotes insulin secretion from β-cells. Importantly, this study also provided evidence that glucose must be metabolized by the β-cell to cause insulin release.

Much effort and major progress have identified key components of the β-cell metabolic signaling machinery for GSIS. Yet, there is still a fragmented understanding of the relationships among the different metabolic pathways and signaling metabolites of the apparatus. There is a need to better define those signals, traditionally named metabolic coupling factors (MCF), which are either *regulatory* of key metabolic pathways in the cascade leading to exocytosis, or are *effectory* as they target key late components of the secretory apparatus, or *homeostatic* as they bring metabolic signals and secretion back to the ground state. Neither is the temporal assembly that coordinates GSIS mechanistically well-defined. Understanding this metabolic machinery is of importance not only for the islet-cell field but for understanding cellular nutrient sensing at large.

The biochemical components of insulin secretion promoted by the intracellular metabolism of fuel stimuli like glucose, fatty acids, and amino acids have been extensively reviewed (Campbell and Newgard, 2021; Prentki et al., 2013; Rorsman and Braun, 2013; Rutter et al., 2015). To date, they have been interpreted largely vis-à-vis the “canonical” model where oxidative phosphorylation (OxPhos) raises ATP/ADP to trigger K_{ATP} channel closure, Ca^{2+} influx and insulin release. However, more recent discoveries of *spatially* and *temporally* compartmentalized metabolism have encouraged a re-examination of this model, which has reached a level of dogma that we believe should be revised.

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The canonical model of GSIS should be reconsidered

In 1968 glucokinase (GK) was discovered as a ‘glucose-sensing’ enzyme in the β-cell that regulates glycolytic flux (Matschinsky and Ellerman, 1968). Endowed with a K_m for the sugar in its physiological concentration range, around 7–8 mM, GK traps glucose by producing glucose 6-phosphate (G6P). G6P is generated continuously because GK lacks product inhibition unlike the other hexokinases. In 1976, a reconstituted system for insulin secretion from cod islet plasma membrane provided evidence that Ca^{2+} as well as the metabolites G6P and phosphoenolpyruvate (PEP) play a role in secretion (Davis and Lazarus, 1976; Lazarus et al., 1976), and pyruvate kinase (PK) activity was identified in rat islet lysates soon after (Sugden and Ashcroft, 1977). These early studies were quickly overshadowed by the subsequent discovery of the β-cell ATP-sensitive K^+ (K_{ATP}) channel (Cook and Hales, 1984), which provided a mechanism to explain how the plasma membrane responds to glucose metabolism and controls the timing of Ca^{2+} influx and insulin secretion (Ashcroft et al., 1984; Misler et al., 1986; Rorsman and Trube, 1985). Since this discovery, and with the recognition that K_{ATP} channels also respond to the corresponding fall in Mg-ADP (Dunne and Petersen, 1986; Kakei et al., 1986), the “canonical” model inferred the intermediary signaling between GK and K_{ATP} with a postulate where substrate-driven mitochondrial oxidative phosphorylation (OxPhos) raises cytosolic ATP/ADP (ATP/ADP_c) to close K_{ATP} channels and activate Ca^{2+} influx to induce insulin secretion (Figure 1A). It is especially this ‘intermediary signaling’ mechanism that should be reconsidered.

Pros and cons of the canonical model related to mitochondrial ATP-dependent K_{ATP} closure

An intuitive part of the canonical model is that OxPhos, being an efficient ATP generator (up to 31 ATP/glucose vs. 2 ATP from glycolysis alone), should generate the bulk of the ATP/ADP rise to close K_{ATP} channels. Below we summarize the key evidence that has been identified in support of the canonical model and discuss the caveats of each argument.

In support of the canonical model, a rise in *NAD(P)H fluorescence* and substantial *O_2 consumption* occurs prior to Ca^{2+} influx following a step increase in glucose (Jung et al., 2000; Kennedy et al., 2002; Luciani et al., 2006). Also, *ATP/ADP_c and total ATP levels* correlate with GSIS in many studies. In addition, imaging tools demonstrate that cytosolic and plasma membrane ATP both rise prior to Ca^{2+} influx (Ainscow and Rutter, 2002; Kennedy et al., 1999; Li et al., 2013) (Figure 1A). However, the common protocol used in these studies was to rescue β-cell ATP/ADP_c from a fuel-starved state (2–3 mM glucose) to fuel saturation (11–16 mM glucose, or even higher). This may have little to do with physiological stimulus-secretion coupling, where β-cell oscillations in metabolism and insulin secretion are observed *in vitro* and *in vivo* (discussed in detail below). Thus, when metabolic signaling was examined during such steady-state oscillations at ~9–11 mM glucose, multiple groups using different technologies observed that the rise in O_2 consumption and the fall in mitochondrial membrane potential (Ψ_m) occur *after* K_{ATP} closure (Jung et al., 2000; Kennedy et al., 2002; Krippeit-Drews et al., 2000; Lewandowski et al., 2020) (Figure 1B).

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β-cells have a unique metabolism that allows ATP/ADP_c to change in response to substrate availability, a feature that is not present in most tissues (Nicholls, 2016). A rigorous bioenergetic approach to understanding the β-cell electron transport chain (ETC), termed *metabolic control analysis (MCA),* has identified stronger substrate control of ATP/ADP_c in β-cells than in muscle due to a much higher proton leak (Affourtit and Brand, 2006). Furthermore, many β-cell studies show that glucose increases mitochondrial ATP synthesis (Affourtit et al., 2018), including in the presence of other physiological fuels (Mugabo et al., 2017) (Figure 1B). However, MCA measures only O_2 consumption to estimate ATP production by mitochondria. Therefore, MCA is blind to anaplerotic cycles, and other sources of *local ATP production* that may be key for signaling, in particular the anaplerotic PEP cycle (Foster et al., 2022; Stark et al., 2009). In this cycle, mitochondria convert pyruvate to PEP, which leaves the mitochondria and augments the glycolytic PK reaction that produces plasma membrane ATP/ADP (ATP/ADP_{pm}) at the K_{ATP} channel (Foster et al., 2022; Lewandowski et al., 2020) (Figure 1B). Noteworthy, the high proton leak in β-cells identified in respirometry experiments (Affourtit and Brand, 2009; Gerencser, 2015; Gerencser et al., 2017, 2016; Taddeo et al., 2020; Wikstrom et al., 2012) facilitates flux through PK and detracts from the ability of mitochondria to generate ATP.

To date, *mathematical models* have universally started with the assumption that OxPhos drives K_{ATP} closure, and have successfully modeled oscillatory glucose metabolism (Bertram et al., 2018; Fridlyand et al., 2005, 2003; Merrins et al., 2016, 2012, 2010). Indeed, the most updated models faithfully reproduce the glycolytic and mitochondrial phase relationships observed in β-cells by incorporating the feedback of Ca^{2+} on metabolism (Marinelli et al., 2021; McKenna et al., 2016). While these models are compatible with the canonical model, they do not demonstrate causality. Furthermore, like MCA, a significant drawback of these kinetic models is that they lack anaplerosis-dependent cycles known to play key roles in GSIS and lack spatially compartmentalized metabolism that plays a key role in signaling (Foster et al., 2022; Lewandowski et al., 2020) (Figure 1b).

Often-cited evidence for the canonical model is that so-called *mitochondrial fuels* (e.g., glutamine/leucine, α-ketoisocaproate, methyl-pyruvate, as well as pyruvate in β-cell lines) increase O_2 consumption and ATP/ADP_c levels, close K_{ATP} channels, and stimulate insulin secretion (Prentki et al., 2013). In this review we integrate the roles of anaplerosis, PK and the PEP cycle into a model of GSIS. Suffice is to say here, although these so-called "pure" mitochondrial fuels provide mitochondrial acetyl-CoA, they also induce anaplerosis by activating pyruvate carboxylase (PC) or glutamate dehydrogenase (GDH) and generate mitochondrial PEP. Therefore these non-glucose fuels do not bypass PK and the PEP cycle (Foster et al., 2022; Stark et al., 2009). Insulin stimulation by membrane permeant methyl-pyruvate was taken as another argument for the canonical model (Mertz et al., 1996), but it also directly closes K_{ATP} channels independently of metabolism (Düfer et al., 2002).

Mitochondrial poisons (rotenone, oligomycin, etc.) prevent K_{ATP} closure, demonstrating that mitochondria are essential to this process (Dukes et al., 1994; Gregg et al., 2019; Kiranadi et al., 1991). However, this does not mean that ETC-derived ATP is responsible for closing K_{ATP} channels. An alternative explanation is that because β-cells do not express plasma membrane pyruvate/lactate transporters and consequently lack the Pasteur effect (Pullen et

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al., 2012; Schuit et al., 1997), mitochondrial poisons will also stall glycolytic flux through PK, which is present on the plasma membrane and generates sufficient ATP/ADP_{pm} to close K_{ATP} channels (Foster et al., 2022; Lewandowski et al., 2020).

Although the direct *ETC electron donor* TMPD/ascorbate closes K_{ATP} channels, and this was taken as evidence that mitochondrially-derived ATP is sufficient to close K_{ATP} channels (Duchen et al., 1993), this treatment also reduces cytosolic ATP (Prentki, unpublished observations). Notably, the complex II fuel methyl-succinate does not stimulate insulin secretion in mouse islets despite raising NADH and ATP (MacDonald, 2002), arguing that mitochondrial ATP is, in fact, insufficient to close K_{ATP} channels. Indeed, mitochondrial fuels (amino acids including glutamine/leucine) that raise ATP/ADP_c do not close K_{ATP} channels in the absence of PK activity (Foster et al., 2022).

Overall, we believe that the evidence for the canonical model where glucose and other fuels “push” OxPhos to induce mitochondrial ATP production that closes K_{ATP} channels is circumstantial, and has significant caveats that are incompatible with recent and historical data showing the importance of spatial and temporal metabolic signaling.

Spatially and temporally compartmentalized glycolysis controls OxPhos and K_{ATP} closure

Glucose-induced insulin secretion is associated with a rise in Ca^{2+}, which increases workload (ATP hydrolysis) from ion pumps and elevates ADP. In response, the mitochondrial ETC will “pull” nutrients from the surrounding milieu to be oxidized (Chance and Williams, 1955). It remains possible that the elevated glucose and downstream pyruvate (a metabolic “push”) will benefit mitochondrial ATP generation and insulin secretion *after* K_{ATP} closure, when Ca^{2+} and ADP are high. But what about *before* K_{ATP} closure, which is dependent upon a rise in ATP/ADP and therefore occurs in the setting of low Ca^{2+} and ADP levels? As the ATP/ADP ratio rises, and ADP levels fall, OxPhos is less and less able to generate ATP, as evidenced by the fall in O_2 consumption and the rise in Ψ_m that precede each Ca^{2+} pulse during stead-state oscillations (Jung et al., 2000; Kennedy et al., 2002; Krippeit-Drews et al., 2000; Lewandowski et al., 2020). These time-resolved measurements, while not exclusive of the canonical model, indicate that mitochondrial ATP production is at its lowest at the time of K_{ATP} closure. This motivated the search for an alternative source of ATP production for K_{ATP} channel closure that functions prior to membrane depolarization.

There is now strong evidence that glycolytic, rather mitochondrially-generated ATP, closes K_{ATP} channels to initiate insulin secretion, and that this process involves the PEP cycle (Figure 1B). First, the *timing* of PK activity can explain membrane depolarization. During glucose-estimulated Ca^{2+} oscillations, allosteric activation of PK by fructose 1,6-bisphosphate (F16BP) increases *prior* to K_{ATP} closure (Lewandowski et al., 2020; McKenna et al., 2016; Merrins et al., 2013), whereas Ψ_m decreases and O_2 consumption occurs *after* K_{ATP} closure (Jung et al., 2000; Kennedy et al., 2002; Krippeit-Drews et al., 2000; Lewandowski et al., 2020). In fact, respiration in permeabilized β-cells is actually inhibited by PEP (Lewandowski et al., 2020), and in intact islets by pharmacologic PK activation (Regeenes et al., 2022). Second, PEP is a strong candidate for a signaling molecule. Among all cellular metabolites, PEP has the highest free energy bond, twice that of ATP, and is present at high (~1 mM) levels (Sugden and Ashcroft, 1977). About 40% of PEP derives

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from the mitochondrial PEP cycle that returns pyruvate from the PK reaction to the cytosol as PEP (Abulizi et al., 2020; Alves et al., 2015; Foster et al., 2022; Lewandowski et al., 2020; Stark et al., 2014, 2009). Third, the substrates for PK, PEP and ADP, are sufficient to stimulate biphasic insulin release in permeabilized β-cells (Pizarro-Delgado et al., 2016), and pharmacologic PK activators stimulate GSIS in mouse, rat, and human islets (Abulizi et al., 2020; Foster et al., 2022; Lewandowski et al., 2020). Fourth, PK is localized to the β-cell plasma membrane, where it is sufficient to raise ATP/ADP_{pm} and close K_{ATP} channels (Foster et al., 2022; Lewandowski et al., 2020). These data match prior observations in cardiac myocytes (Dhar-Chowdhury et al., 2007, 2005; Weiss and Lamp, 1989, 1987). Fifth, although the OxPhos-dependent rise in ATP/ADP_c may help buffer this plasma membrane-compartmentalized effect, mitochondrial fuels (amino acids) that raise ATP/ADP_c were shown to be insufficient to close K_{ATP} channels in the absence of PK activity (Foster et al., 2022). Sixth, not only glucose but also amino acids promote PK- and PEP cycle-dependent K_{ATP} closure, as shown by whole-body and β-cell deletion of mitochondrial PEPCK (PCK2) (Abulizi et al., 2020; Foster et al., 2022). Hence, the emergent evidence indicates that glucose and other nutrients promote K_{ATP} channel closure primarily via PK and the PEP cycle.

In the sections below, we propose an oscillatory model of glucose signaling in the β-cell that we wish to name the $Mito_{Cat}$-$Mito_{Ox}$ model (Figure 1B). This novel model surmises that during $Mito_{Cat}$, PK raises ATP/ADP_{pm} to close K_{ATP} channels, whereas during the $Mito_{Ox}$ phase, OxPhos responds to ADP rise to produce ATP that sustains the energy demands of insulin secretion and ion pumping. Looking at the bigger picture, the $Mito_{Cat}$-$Mito_{Ox}$ model recognizes that multiple metabolic and ionic processes accelerate and decelerate with time, coincident with the electrically-silent triggering phase ($Mito_{Cat}$) or the electrically-active secretory phase ($Mito_{Ox}$) of insulin secretory oscillations. Synchronized oscillations (e.g., in ADP and Ca^{2+}) strongly dictate which metabolic and signalling processes are active at any given time. While we do not yet know with precision the relative phase relationship of the many oscillating processes, this model describes the causal relationships among key pathways that collectively influence GSIS.

Metabolic oscillations separate β-cell metabolism into two states: $Mito_{Cat}$ and $Mito_{Ox}$

Excess insulin causes hypoglycemia and in extreme cases death; appropriately turning insulin secretion *on* and *off* is therefore key to survival. *In vivo,* insulin secretion is pulsatile even in the basal state (Pørksen, 2002). The primary effect of glucose elevation is to increase the amplitude of the pulses (Goodner et al., 1977; Lang et al., 1979), in part by recruiting more cells to respond (Heart et al., 2006). Insulin oscillations are lost early in the progression to diabetes (Lang et al., 1981; O'Rahilly et al., 1988; Polonsky et al., 1988). Disrupted islet pulsatility is associated with glucose intolerance (Head et al., 2012), and the increased efficacy of insulin pulses at target tissues, relative to continuous delivery, further demonstrates their physiologic relevance (Matthews et al., 1983; Matveyenko et al., 2012).

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Pulsatility is equally important for the β cell itself – by ensuring the coordination and integration of metabolism. Proportionate only to a rise in glucose, the GK reaction continually pushes carbon into the system (Matschinsky and Ellerman, 1968). Virtually every subsequent step of central carbon metabolism is oscillatory (for reviews see (Bertram et al., 2018; Henquin, 2009; Idevall-Hagren and Tengholm, 2020)). Energetic and redox metabolic pairs, such as ATP/ADP and NAD(P)H/NAD(P)$^+$, are used by multiple reactions across the entire metabolic network. For instance, as the ATP/ADP ratio increases, all ADP-dependent reactions begin to slow and thermodynamically favor ATP hydrolysis.

There is an oscillatory release of insulin not only when pancreas or isolated islets are perifused with a fixed level of glucose, but also in single β-cells, indicating that the oscillator is cell-intrinsic (Ainscow and Rutter, 2002; Civelek et al., 1997, 1996; Detimary et al., 1998; Jung et al., 2000; Juntti-Berggren et al., 2003; Kennedy et al., 2002; Kindmark et al., 2001; Luciani et al., 2006). The reasonably short period (~5 minutes) of oscillatory processes (e.g., insulin secretion, membrane potential, Ca^{2+}) (Gilon et al., 1993; Kindmark et al., 2001), plasma membrane ATP (Li et al., 2013) and cAMP (Dyachok et al., 2006), as well as NAD(P)H fluorescence (Luciani et al., 2006)) largely excludes oscillatory transcriptional and translational control mechanisms. The evidence further indicates that super-connected subpopulations of β-cells within the islet (including hubs, leaders, and wave initiators) drive Ca^{2+} dynamics and pulsatile insulin secretion (Benninger and Kravets, 2021).

Glucose-driven metabolic and Ca^{2+} oscillations are complex (Bertram et al., 2007; Dean and Matthews, 1968), with fast electrical oscillations (lasting tens of seconds) superimposed on slower metabolically-driven oscillations (~5 min). The slow oscillations better reflect the timing of Ca^{2+} and insulin oscillations observed in living animals (Adams et al., 2021; Lang et al., 1979; Nunemaker et al., 2006, 2005). Although Ca^{2+} can reinforce metabolic oscillations, they can exist independently of oscillations in membrane potential or Ca^{2+} (Merrins et al., 2010). Substantial evidence implicates activation of glycolytic phosphofructokinase-1 (PFK1) by its product fructose 1,6-bisphosphate (F1,6BP) as the mechanism that initiates slow metabolic oscillations in β-cells (Tornheim, 1997; Merrins et al., 2012, 2013; Bertram et al., 2018). PFK1 is also activated by fructose 2,6-bisphosphate (F2,6BP), generated from fructose 6-phosphate (F6P) by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) (Merrins et al., 2012). While glucose concentration determines GK flux, the PFK1 reaction separates glycolysis into two segments (upper and lower glycolysis) (Figure 2A). As PFK1 accelerates and F1,6BP accumulates, G6P and F6P are consumed. The near-equilibrium enzymes of lower glycolysis from aldolase to enolase propagate the F1,6BP signal to PK (Wilson and Matschinsky, 2021). A second node is found at the PK step, after which pyruvate/lactate levels fall as mitochondria consume pyruvate faster than it is generated (Civelek et al., 1997; Lewandowski et al., 2020; Sdao et al., 2021). Consequently, the lower glycolytic intermediates between PFK1 and PK oscillate in phase (Figure 2A) and are reinforced by the positive allosteric control of PFK1 by F1,6BP and F2,6BP, ultimately creating synchronous oscillations in ATP/ADP produced by phosphoglycerate kinase and PK and NADH/NAD$^+$ produced by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Due to the PFK1 and

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PK nodes in glycolysis, the upper glycolytic intermediates and the post PK metabolites oscillate roughly in phase.

Glycolytic oscillations propagate into the mitochondria driving two opposed “metabolic states” that are separated in time – *$Mito_{Ox}$* and *$Mito_{Cat}$*. $Mito_{Ox}$ is named for the dominant activity of OxPhos and is predominantly controlled by ADP and supported by Ca^{2+}. $Mito_{Cat}$ is named for the matched processes of anaplerosis (i.e., the net expansion of the TCA cycle metabolite pool) and cataplerosis (i.e., the egress of TCA cycle intermediates into other pathways). Figure 2B outlines the $Mito_{Cat}$-$Mito_{Ox}$ of oscillatory β-cell metabolism proposed by Lewandowski et al. (Lewandowski et al., 2020) where the progressive closure of K_{ATP} channels occurs during the electrically-silent triggering phase of metabolic signaling (during $Mito_{Cat}$), and secretion during the electrically-active phase delineated by membrane depolarization (during $Mito_{Ox}$):

> **$Mito_{Cat}$**. The electrically quiet period leading up to K_{ATP} channel closure and β-cell depolarization, $Mito_{Cat}$, is dominated by the progressive lowering of cytosolic ADP by PK thus creating an ADP-starved “state-4-like” mitochondria with low ETC flow but high Ψ_m. In the cytosol, rising $NADH/NAD^+$ due to flux through GAPDH slows glycolysis and the ensuing buildup of F1,6BP activates PFK1 in a positive feedback loop, generating the upstroke of an oscillation. F1,6BP also feeds forward to allosterically activate PK, lowering its K_m for PEP and also raising its V_{max}. Pyruvate-mediated elevations in mitochondrial $NADH/NAD^+$ and acetyl-CoA then activate pyruvate carboxylase (PC) to generate OAA that is converted to PEP in the mitochondria via PEP carboxykinase (PEPCK, or PCK2). Mitochondrial PEP then exits the mitochondrion, thus feeding cataplerosis to further augment the ADP-lowering power of PK which produces ATP. $Mito_{Cat}$ concludes after plasma membrane-associated PK raises ATP/ADP sufficiently to close K_{ATP} channels, depolarizing the cell. Although the PEP cycle is ‘futile’, it spatially and temporally redistributes ATP, and is therefore essential for β-cell signaling. This occurs when the PEP produced from pyruvate within the mitochondria, having burned 1 ATP (at PC) and 1 GTP (at PCK2), is delivered following its cataplerotic output to a plasma membrane-associated PK that locally lowers ADP and produces 1 ATP in the close vicinity of K_{ATP} channels.
>
> **$Mito_{Ox}$.** Following K_{ATP} channel closure and β-cell depolarization, Ca^{2+} influx triggers exocytosis. The resulting increase in workload (i.e. ATP hydrolysis) – due to the energy requirement for exocytosis, ion pumps, etc. – elevates ADP to stimulate OxPhos in this “state-3-like” phase with active glycolytic and TCA cycle fluxes. Eventually ADP rises sufficiently to reopen K_{ATP} channels and repolarize the plasma membrane.

The $Mito_{Cat}$-$Mito_{Ox}$ model describes β-cell metabolism at intermediate levels of glucose that support oscillatory behavior. Unlike the plasma membrane potential, which has a square wave pattern, $Mito_{Cat}$ and $Mito_{Ox}$ are not binary, all-or-none processes. Indeed the ETC also functions during $Mito_{Cat}$ although at a lower pace than $Mito_{Ox}$. Of note, an additional step not included in the model is needed to describe a step elevation in glucose from starvation (2–3 mM). In this case, $Mito_{Cat}$ is preceded by massive O_2 consumption that raises ATP/

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ADP_c to restore the cellular energy state; then, as described above, PK raises ATP/ADP_{pm} to close K_{ATP} channels. As this situation never occurs in vivo, we will not discuss it further. At very high glucose (16–20 mM) when Ca^{2+} reaches a plateau, both $Mito_{Cat}$ and $Mito_{Ox}$ metabolic fluxes are maximal, oscillations in metabolism and insulin secretion do not occur (Kjems et al., 2002).

“Metabolic coupling factors” propel the β-cell through $Mito_{Cat}$ and $Mito_{Ox}$

The temporal separation inherent to oscillatory behavior is conducive to specific metabolic cycles and signals that sequentially propel the β-cell through the electrically-silent ($Mito_{Cat}$) and electrically-active energy consuming ($Mito_{Ox}$) phases of GSIS. These signals, termed “metabolic coupling factors” (MCFs), link the intracellular metabolism of fuel stimuli in the β-cell to insulin exocytosis (Prentki et al., 2013). Figure 3 presents a reorganized model of β-cell metabolic signaling by aligning MCFs into one of three categories: ‘regulatory’, ‘effectory,’ and ‘homeostatic.’ The latter category is a new designation and includes the important need of a strong “off switch” of metabolic signaling and insulin secretion to reset the system after depolarization. Thus, “regulatory MCFs” (e.g., F1,6BP, ATP/ADP, mitochondrial GTP, citrate, malonyl-CoA, etc.) facilitate switching between metabolic pathways and cycles to modulate the effectory networks. “Effectory MCFs” (e.g. ATP/ADP, Ca^{2+}, reactive oxygen species (ROS), monoacylglycerol (MAG)) directly trigger and/or amplify GSIS via late effectors (e.g., K_{ATP} channels, Ca^{2+} channels, exocytotic proteins). Note that effectory MCFs are *sufficient* signals for insulin secretion, and are necessarily *transient*. They must be counterbalanced by even stronger homeostatic signal removers that prevent hyperinsulinemia by resetting the system between oscillations (e.g., ATP, by Ca^{2+}-ATPases; cAMP, by phosphodiesterases; MAG, by ABHD6; ROS and S-S proteins, by NADPH, GSH/GSSG, catalase, and superoxide dismutase).

A key limitation in the field is that the oscillatory behaviors of many metabolites and MCFs are not fully characterized in the intact β-cell. Furthermore, even if a metabolite is oscillatory, its assignment to $Mito_{Cat}$ or $Mito_{Ox}$ is often lacking. Aside from ATP/ADP (Ainscow and Rutter, 2002; Lewandowski et al., 2020; Li et al., 2013; Merrins et al., 2016), F1,6BP (Merrins et al., 2013), glutamate (Lewandowski et al., 2020), lactate (Sdao et al., 2021), citrate (Gregg et al., 2019), mitochondrial pH and Ψ_m (Krippeit-Drews et al., 2000; Lewandowski et al., 2020; Li et al., 2013), there is limited clarity on whether other metabolites and MCFs oscillate or remain static (e.g. cytosolic GSH/GSSG (Santos et al., 2017)) as part of their regulatory contribution. Cytosolic ATP/ADP, F1,6BP-dependent PK activation, citrate, and mitochondrial matrix alkalization all positively associate with the $Mito_{Cat}$ phase (Gregg et al., 2019; Lewandowski et al., 2020). Mitochondrial and cytosolic Ca^{2+}, lactate, and O_2 consumption are positively associated with the $Mito_{Ox}$ phase (Jung et al., 2000; Kennedy et al., 2002; Lewandowski et al., 2020; Sdao et al., 2021). Citrate, in particular, is a long-established regulator of multiple key enzymes involved in GSIS, such as PFK1 and acetyl-CoA carboxylase, and together with Ca^{2+} it may reinforce oscillatory metabolism (MacDonald et al., 2003). These lists are certainly incomplete and some components may be found to belong to multiple classes. Finally, it should be emphasized that ADP plays a critical role in GSIS regulation. ADP acts as both as key effectory and regulatory signal (Figure 3), since in the cytosol it controls, together with ATP, K_{ATP} channel

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closure and is the main regulator of PFK1 that controls oscillations in glycolytic flux. High ADP in cytosol resets the balance between $Mito_{Cat}$ and $Mito_{Ox}$ and elevated mitochondrial ADP is central to the regulation of the TCA cycle, ETC activity, and ATP production. In the next section, we provide a rationale for assigning mitochondrial cycles and signals to either $Mito_{Cat}$ or $Mito_{Ox}$.

What regulates different states of mitochondria?

Oscillations of metabolic networks are largely coordinated by allosteric regulations that are evolutionarily hard-wired into the tertiary and quaternary structures of enzymes. These prevent metabolic traffic jams that would otherwise render signaling sluggish and unresponsive. Oscillations dynamically encode nutrient signaling into frequency and amplitude domains, rather than just relying on static variations in metabolite concentrations (Henquin, 2009). An equally important attribute of allosteric regulation and oscillatory signaling is that they provides a fast mechanism to turn *off* insulin secretion to prevent hypoglycemia. It is important to point out that the functional contribution to allosteric regulations in intact β-cells has not been formally evaluated in most cases. Therefore, a collective analysis of allosteric control is used inferentially here to disentangle phasic metabolism.

Allosteric regulation during $Mito_{Cat}$

Allosteric regulation of mitochondrial metabolism during $Mito_{Cat}$ is more complex than that during $Mito_{Ox}$ since the TCA cycle during this phase is fragmented (Figure 4A). When ATP demand is low because insulin secretion is also low, the energy level of the β-cell increases. This is indicated by the elevated cytosolic and mitochondrial ATP/ADP and $NADH/NAD^+$ ratios and Ψ_m all moving toward their apices (Krippeit-Drews et al., 2000; Lewandowski et al., 2020). At the same time cytosolic Ca^{2+} is lowered (to ~50 nM relative to ~250 nM during $Mito_{Ox}$), with mitochondrial Ca^{2+} following suit. ETC activity and O_2 consumption rates during $Mito_{Cat}$ approach their nadir (Kennedy et al., 2002). The slowing of the ETC and consistent “pressure” from the glucose-driven $NADH/NAD^+$ rise increases Ψ_m and ROS generation. During this time, inhibitory allostery remodels mitochondrial metabolism. The enzymatic targets are the proximal, NAD^+-dependent dehydrogenases. For instance, ATP inhibits PDH (via PDH kinase), IDH1, IDH3, and αKGDH in addition to citrate synthase. NADH, in turn, inhibits IDH3 and αKGDH (Rafalowska et al., 1974; Seelig and Colman, 1978). Succinyl-CoA inhibits αKGDH. This inhibitory allostery tends to be K_m raising for several enzymes and as such, raises substrate concentrations and redirects metabolites out of the TCA cycle. Thus, the inhibition of the matrix dehydrogenases during $Mito_{Cat}$ disconnects the sequential enzymatic activities of the TCA cycle downstream of $IDH_{2/3}$, and this favors anaplerosis/cataplerosis. Indeed, this fragmented TCA cycle accumulates matrix oxaloacetate and citrate whose carbons can then flow out of the matrix into the cytosol as PEP and citrate. Both of these provide additional routes, besides oxidation to CO_2, to clear pyruvate. Accumulation of acetyl-CoA also promotes anaplerosis/cataplerosis as it is a potent allosteric activator of PC. Thus, activated PC anaplerosis expands the TCA cycle with OAA. The increased OAA can be balanced by cataplerotic export of PEP as part of the GTP-dependent mitochondrial PEPCK reaction (PCK2) and

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the PEP cycle (discussed below). Once in the cytosol, citrate can form malonyl-CoA, an important regulatory MCF (Prentki et al., 1992).

Allosteric regulation during $Mito_{Ox}$

The more familiar allosteric regulation of metabolism during $Mito_{Ox}$ favors OxPhos capacity (Figure 4B). With the exception of uncoupling, the main control point for OxPhos is via ADP as substrate for complex V (ATP synthase), which through the consumption of the proton motive force promotes ETC activity and O_2 consumption. ADP itself is also a substrate for the ATP-specific isoform of succinyl-CoA synthase (SUCLA2) in the TCA cycle for OxPhos-independent synthesis of ATP in the mitochondrial matrix. In contrast to its substrate role, through allostery ADP lowers the substrate K_m of many TCA cycle enzymes, especially the NADH dehydrogenases. ADP is an allosteric activator of multiple mitochondrial enzymes, including pyruvate dehydrogenase (PDH) (via PDH kinase), citrate synthase, isocitrate dehydrogenase 3 (IDH3), and α-ketoglutarate dehydrogenase (αKGDH) (Sankaran et al., 1996). Citrate also can feed forward to allosterically activate IDH3 to promote isocitrate lowering. Likewise, Ca^{2+} favors substrate lowering for the dehydrogenases of the proximal TCA cycle including PDH (via PDH phosphatase), all three IDH isoforms, and αKGDH (Glancy and Balaban, 2012; Strumiło, 1995). Transport across membranes is also regulated by allostery during $Mito_{Ox}$ (Figure 4D). Thus, a rise in cytosolic Ca^{2+} activates components of redox shuttles such as the aspartate-glutamate carrier (AGC1, ARALAR/SLC25A12) and mitochondrial Gro3P dehydrogenase (GPD2) (addressed later). These reactions favor the activity of both the malate/asparate and glycerol-3-phosphate (Gro3P) shuttles that transfer cytosolic NADH electrons into the ETC, and regenerate NAD^+ to facilitate glycolysis. Importantly, cytosolic rather than mitochondrial Ca^{2+} controls the pyruvate supply for OxPhos through these shuttles and transporters (Mármol et al., 2009; Szibor et al., 2020). Worth emphasis, the allosteric roles of ADP and Ca^{2+} to promote fuel oxidation and ATP synthesis are likely secondary in control strength to the regulation of ATP synthase by ADP (Glancy and Balaban, 2012).

Mitochondrial shuttles and shunts in nutrient signaling

The chemical potential stored in the covalent bonds of glucose and other nutrients are distributed via metabolism into energy generation, signaling, and/or homeostatic processes in both the cytosol and mitochondria. This distribution occurs through the transfer of electrons from fuel stimuli into redox intermediates such as NAD^+, $NADP^+$, and FAD. Biochemical products of these electron exchanges are diverse and include molecules such as ATP, ROS, CoA esters, and GSH. Electron transfer across membranous cellular compartments does not occur directly but is coupled mostly via shuttles and shunts. *Shuttles* are defined as mass-balanced cycles that transfer reducing equivalents or metabolites in and out of the mitochondria. A *shunt*, in contrast, is an offshoot of the main pathways (e.g., glycolysis or the TCA cycle) that can either divert metabolites to another pathway, or allow them to rejoin the main pathway at a later point. During β-cell glucose stimulation the pathways and destinations of the electrons are not constant and change temporally with the oscillations of $Mito_{Ox}$ and $Mito_{Cat}$. Mitochondrial shuttles and shunts are key components

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of GSIS in the β-cell and we will discuss below those which are implicated during the oscillatory $Mito_{Cat}$ and $Mito_{Ox}$ states.

Re-oxidation of cytosolic NADH and acetyl-CoA export during $Mito_{Cat}$

Sustaining glycolysis during $Mito_{Cat}$ requires reoxidation of cytosolic NADH. Four electron sinks, allowing glycolytic flux through GAPDH, are considered during $Mito_{Cat}$ (Figure 4C).

Glycerol shunt.—The recently characterized enzyme in the β-cell, glycerol 3-phosphate phosphatase (G3PP) (Mugabo et al., 2016), provides a pathway for electron disposal/NAD^+ regeneration during $Mito_{Cat}$. In this shunt NADH is oxidized to NAD^+ by cytosolic glycerol-3-phosphate dehydrogenase (GPD1) generating Gro3P from dihydroxyacetone-phosphate (DHAP). But instead of regenerating DHAP as in the Gro3P shuttle discussed below, the *glycerol shunt* utilizes G3PP to directly generate glycerol from Gro3P, bypassing lipogenesis and lipolysis. In the absence of β-cell glycerol kinase (Prentki et al., 2013), glycerol is lost from the cell via aquaporins, thus lowering the maximal yield of ATP/glucose to about 12.5. The high K_m of G3PP for Gro3P (Mugabo et al., 2016) make this pathway ideal for the β-cell to manage metabolic excess via carbon spillage to glycerol at the higher end of the glucose range (Possik et al., 2021). That said, peaks of glycolytic oscillations may be sufficiently close to the K_m to spill over into this pathway during physiologic glucose. β-cell specific G3PP KO mice show enhanced GSIS in association with increased anaplerosis and mitochondrial metabolism, and are more susceptible to glucotoxicity. Interestingly, activation of this pathway protects from glucotoxicity and fat deposition and promotes healthspan in *C.elegans* (Possik et al., 2022).

Glycerolipid shunt.—This shunt is part of the *glycerolipid/free fatty acid (FFA) cycle* (not pictured in Figure 4C) and is implicated in the amplification arm of GSIS and fuel excess detoxification (Poursharifi et al., 2020; Prentki et al., 2020a). An additional NAD^+ regeneration pathway occurs via the incorporation of Gro3P into complex lipids via Gro3P acyltransferase. Stimulatory glucose increases incorporation of Gro3P into triglyceride 2-fold and into phospholipids by 5-fold (Berne, 1975) and impacts signaling by long chain acyl-CoA (LC-CoA) and complex lipids as well as supporting cycles of triglyceride synthesis and breakdown (Prentki et al., 2013). The initial steps of the glycerolipid/FFA cycle (Prentki and Madiraju, 2012) are to esterify FFA into glycerolipids followed by lipolysis into FFA and glycerol. The energy requirement is significant and includes phosphorylation of glucose for the Gro3P and the activation of three FFA to acyl-CoAs with a net cost of 7 ATP per turn. Because this cycle is energetically costly and removes the glucose carbons available for ATP production, it may therefore be more important for signaling or fuel excess than the reoxidation of NADH.

Aspartate-Glutamate shuttle.—Reversed glutamate dehydrogenase (GDH) flux from αKG to glutamate was proposed as a regulatory/effectory MCF (Maechler and Wollheim, 1999). However, this is much debated (MacDonald and Fahien, 2000; Prentki et al., 2013), and the genetic evidence goes against this view (Stanley et al., 1998). Recent evidence suggests that glutamate may be more important for incretin/cAMP signaling but not for GSIS *per se* (Gheni et al., 2014). In the absence of stimulatory cytosolic Ca^{2+}, AGC1 a is

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not activated and limits the full *malate-aspartate shuttle* described below. Thus, we refer to the AGC1-independent half of the *malate-aspartate shuttle* as the *aspartate-glutamate shuttle* (Figure 4C). In this mechanism, a high cytosolic NADH/NAD^+ ratio pulls aspartate into malate via glutamate/oxaloacetate transaminase (GOT1) and MDH1, and malate enters the mitochondria in exchange for αKG. Without net cellular uptake of aspartate, the aspartate-glutamate exchange is a time-limited electron sink into glutamate. Notably, its relevance aligns well with the long known marked drop in aspartate levels and the small increase in cellular glutamate that occurs with glucose stimulation (Alves et al., 2015; Lamontagne et al., 2017; Li et al., 2003; Mugabo et al., 2017), and the rise in cytosolic glutamate that occurs during the $Mito_{Cat}$ phase of oscillations (Lewandowski et al., 2020).

Acetyl-CoA shuttle.—In this pathway, mitochondrial acetyl-CoA and oxaloacetate are incorporated into citrate using citrate synthase. Citrate is then transported via the CIC to the cytosol where acetyl-CoA plus oxaloacetate is re-generated by ATP citrate lyase (ACLY). A high cytosolic NADH/NAD^+ favors the transfer of NADH electrons into oxaloacetate to form malate, regenerating NAD^+ (Palmieri, 2004). This shuttle functions to short circuit the otherwise inhibited TCA cycle during $Mito_{Cat}$. Importantly, malate returns to the matrix in coupled exchange for citrate via the CIC and stoichiometrically balances the shuttle. Therefore, we distinguish this from the so-called "pyruvate-citrate cycle" (Farfari et al., 2000; Jensen et al., 2008), because of a required exchange of citrate for malate as well as independence from PC anaplerosis. Importantly, the malate-citrate exchange via CIC rules out the possibility of malate decarboxylation by cytosolic malic enzyme 1 (ME1). In addition to regeneration of cytosolic NAD^+, important signaling benefits of this shuttle are supporting glycolytic flux during $Mito_{Cat}$, and malonyl-CoA generation. In other words, transformation of pyruvate into cytosolic acetyl-CoA allows glycolysis to continue despite a stalled TCA cycle. As such, the *acetyl-CoA shuttle* supports continued PEP metabolism by PK to support ATP production for K_{ATP} closure (El Azzouny et al., 2016; Guay et al., 2007; Joseph et al., 2007). Malonyl-CoA formed from cytosolic acetyl-CoA also blocks lipid oxidation via carnitine palmitoyl-transferase 1 inhibition to favor pyruvate metabolism and the *PEP cycle*, and to support the generation of other lipid MCF signals such as MAG (Prentki et al., 2020a).

Further research is needed to clarify which of the NADH reoxidation systems are quantitatively important during $Mito_{Cat.}$ However, the glycerolipid shunt and the glycerol shunt because of their high energy cost, as discussed above, are unlikely to play prominent roles. The *acetyl-CoA shuttle* together with the *glutamate-aspartate shuttle* likely accounts for the majority of the cytosolic NADH reoxidation during $Mito_{Cat}$.

Re-oxidation of cytosolic NADH and mitochondrial transfer of reducing equivalents during $Mito_{Ox}$

During $Mito_{Ox}$, when OxPhos is the dominant activity, the main goal of metabolism is to move the electrons of glucose into the ETC to generate ATP. Once pyruvate, with its 10 disposable electrons, is transported into the mitochondria, the mitochondrial dehydrogenases use NADH and $FADH_2$ to transfer electrons into the ETC. It is the 4 electrons per glucose (2 NADH) accumulated in the cytosol by the GAPDH reaction that have a more complicated

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pathway to reach the ETC. To prevent a slowing of glycolysis at the GAPDH step from a high cytosolic NADH/NAD^+ ratio, several shuttles, shunts and/or exchanges re-oxidize cytosolic NADH with varying efficiency (Figure 4D). Of these, the *malate-aspartate shuttle*, the *Gro3P shuttle*, and the *pyruvate/lactate exchange* are likely to be more active during $Mito_{Ox}$.

Malate-aspartate shuttle.—This is the most efficient shuttle in term of ATP production that transfers electrons to site 1 of the ETC and plays key role in GSIS, although it is not essential due to redundancy with the Gro3P shuttle discussed below (Ravier et al., 2000). It uses the two isozymes of malate dehydrogenase (MDH1 and MDH2) for electron transfer from the cytosol to the matrix, yielding up to 31 ATP per glucose. The two aspartate transaminase isoforms (GOT1 and GOT2) repackage oxaloacetate as aspartate, and glutamate as αKG, to be compatible for mitochondrial transport via inner membrane antiporters. The energetic cost of balancing this shuttle is one co-transported proton used by AGC1. Mitochondrial NADH then transfers its electrons into complex I to eventually translocate 10 protons out of the matrix via the ETC for a net gain of 9 protons available to ATP synthase.

Gro3P shuttle.—This slightly less efficient shuttle (up to 29 ATP/glucose) uses the two isoforms of Gro3P dehydrogenase (GPD1 and GPD2) to move an electron pair first into coenzyme Q and then into complex III for the net translocation of 6 protons out of the matrix. β-cells are endowed with very high levels of mitochondrial bound Gro3P dehydrogenase (GPD2) pointing to an important role of this shuttle in GSIS (MacDonald, 1981). However, the *Gro3P shuttle* is not essential as β-cell specific deletion of GPD2 does not impair GSIS, due to compensation by the *malate-aparate shuttle* (Ravier et al., 2000). Worth emphasizing, in the β-cell the AGC1 and mitochondrial GPD2 used by the *malate-aspartate* and *Gro3P shuttles*, respectively, require Ca^{2+} activation (Civelek et al., 1996; Rutter et al., 1992, p.). Ca^{2+} activation of both shuttles assigns their dominant roles as redox equivalent transfer pathways to the ETC during $Mito_{Ox}$ after depolarization when β-cell cytosolic Ca^{2+} is elevated.

Pyruvate-lactate exchange.—In contrast to the general belief that β-cells lack lactate dehydrogenase (LDH), only one of the four isoforms, LDHA, is a ‘repressed’ gene (Pullen et al., 2010). The LDHA/B/D proteins are expressed at significant levels (Mitok et al., 2018), and in response to glucose, catalyze the reversible accumulation of lactate during $Mito_{Ox}$ (Sdao et al., 2021). Lactate synthesis, as an electron sink to regenerate NAD^+, is both temporary and limited because the dead-end metabolite is trapped in the cytosol due to the lack of MCT1 plasma membrane monocarboxylate transporters, explaining the low level of glucose-dependent lactate output from β-cells (Pullen et al., 2012; Sekine et al., 1994). Eventually during $Mito_{Ox}$, this transient sink reverses to regenerate pyruvate and NADH and pyruvate enters the mitochondria to be metabolized. However, this exchange can only provide short-term NADH/NAD^+ buffering and plays a minor role in NADH reoxidation.

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The mitochondrial GTP cycle and spatially compartmentalized PEP cycling

As discussed above, PEP metabolism by PK controls a key regulatory switch from $Mito_{Ox}$ to $Mito_{Cat}$. PEP is generated either from glycolysis or in the mitochondria, in the *PEP cycle* which requires the *mitochondrial GTP cycle* for its capacity to generate GTP for the mitochondrial PEPCK (PCK2) reaction (Abulizi et al., 2020; Foster et al., 2022; Jesinkey et al., 2019; Lewandowski et al., 2020; Stark et al., 2014). In the *PEP cycle* (Figure 4C), pyruvate, formed from cytosolic PEP by PK, undergoes ATP-dependent carboxylation to oxaloacetate via PC, followed by the GTP-dependent generation of PEP by PCK2. Mitochondrial PEP^{3-} is then transported out of the mitochondria in exchange for another di- or trivalent anion. Since β-cell CIC deletion has no effect on GSIS (Bauchle et al., 2021), PEP transport may be mediated by the ANT as occurs in the heart (Sul et al., 1976). Once mitochondrial PEP arrives in the cytosol, cytosolic PK regenerates pyruvate to further lower ADP, completing the PEP cycle. It should be pointed out that, upon glucose stimulation, glycolytic PEP is sufficient to close K_{ATP} channels without the PEP cycle; however, the PEP cycle accelerates cytosolic PEP production because pyruvate is recycled to PEP itself (Foster et al., 2022). Importantly, the PEP cycle has two different compartmentalized effects. Cytosolic PK reduces ADP to inhibit mitochondrial respiration (Lewandowski et al., 2020; Regeenes et al., 2022), while plasma membrane-associated PK raises ATP/ADP to close K_{ATP} channels (Foster et al., 2022; Lewandowski et al., 2020). Spatially compartmentalized PEP cycles use the favorable energetics from hydrolysis of one mitochondrial ATP at the PC step and one mitochondrial GTP to form one cytosolic ATP. This sustains the low ADP of $Mito_{Cat}$ until ADP levels are increased by the cellular work during $Mito_{Ox}$ that follows depolarization.

Crucial to the *PEP cycle* is the generation of mitochondrial GTP. Since there is no GTP transport activity in the inner mitochondrial membrane (McKee et al., 2000), the primary source of mitochondrial GTP, then, is the GTP-specific subunit of succinyl-CoA synthase (SUCLG2) (Kibbey et al., 2007). Mitochondrial nucleotide diphosphokinase (NDPK), despite the initial suggestion of its matrix localization (Kowluru et al., 2002), is in the intermembrane space and unavailable to convert ATP to GTP in the matrix (Lacombe et al., 2009). Mitochondrial GTP can be made either during $Mito_{Ox}$ or $Mito_{Cat}$. During $Mito_{Ox}$, mitochondrial GTP synthesis is produced in proportion to the fraction of the TCA cycle that flows through SUCLG2 relative to SUCLA2, the ATP-specific subunit of succinyl-CoA synthase. The dynamics of this enzyme pair (SUCLA2 vs SUCLG2) changes dramatically during $Mito_{Cat}$ since: 1) the high ATP/ADP ratio reverses the direction of SUCLA2 towards formation of succinyl-CoA and ADP (Ottaway et al., 1981); 2) pyruvate anaplerosis makes OAA to support GTP hydrolysis by PCK2 during the PEP cycle further favoring the generation of succinate by SUCLG2 (Figure 4C, panel named *PEP cycle/SUCLA2-ATP*); 3) during $Mito_{Cat}$ the succinyl-CoA synthetase reaction is bookended by the inhibited αKG dehydrogenase and succinate dehydrogenase (Zeyelmaker and Slater, 1967) (Figure 4A) that together limit net loss or gain of succinyl-CoA or succinate. As such, in the *mitochondrial GTP cycle* SUCLG2 and SUCLA2 use the high ATP/ADP ratio to favor GTP synthesis with GDP regenerated by PCK2 which is a component of both the *PEP cycle* and the *mitochondrial GTP cycle* (Figure 4C). In this manner the two isoforms of succinyl CoA

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synthase (A2 and G2) work antiparallel akin to NDPK but instead cycling succinyl-CoA and succinate.

In light of mutations of the fat oxidizing enzyme hydroxy-acyl dehydrogenase (HADH, also named SCHAD) causing hyperinsulinemia (see also Figure 6) it is worth considering it as another source of succinyl-CoA to support mitochondrial GTP synthesis (Figure 4C, panel named *PEP cycle/OXCT1-AcAcCoA*). The mitochondrial enzyme 3-oxoacid CoA transferase (OXCT1, also named succinyl-CoA:3-ketoacid coenzyme A transferase 1, SCOT1) catalyzes the reversible transfer of the CoA moiety from succinate to acetoacetate during mitochondrial acetoacetate metabolism. This aligns with a reported role for ketone bodies in a pathway implicating acetoacetyl-CoA in the regulation of GSIS (Hasan et al., 2010). Thus, it may be speculated that besides a role in the activation of GDH to favor glutamine anaplerosis (Li et al., 2010) and fat oxidation inhibition causing the accumulation of LC-CoA or acyl-carntine compounds (Pepin et al., 2010), HADH/SCHAD mutations causing hyperinsulinemia also implicates the PEP cycle (Figure 6).

Hence, during $Mito_{Cat}$ a stalled and fragmented TCA cycle – but a highly energized mitochondria – transfers its phosphorylation potential to the cytosol where PEP metabolism via PK restricts OxPhos via ADP privation, while plasma membrane PK metabolizes PEP to increase the ATP/ADP ratio for K_{ATP} channel closure followed by a rise in cytosolic Ca^{2+} that promotes insulin secretion (Figure 2B). Recent work indicates that while the PEP cycle is important for K_{ATP} channel closure during $Mito_{Cat}$, it is equally important during $Mito_{Ox}$ for switching insulin secretion off by mechanisms that remain to be established (Foster et al., 2022).

Turning “off” the signals for secretion: cytosolic NADPH-generating shuttles and shunts

Which NADPH generating pathway is most important for insulin secretion?

When a metabolic process is essential to the function of a cell, nature often provides redundancy. For instance, cytosolic $NADH/NAD^+$ ratio is maintained highly *oxidized* in the cytosol through the above-mentioned shuttle and exchange pathways to ensure dynamic glycolysis. Likewise, the cytosolic $NADPH/NADP^+$ ratio is maintained highly *reduced* in the cytosol through supernumerary pathways that utilize combinations of the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose phosphate pathway, as well as IDH1 and ME1 to generate NADPH. In the setting of very powerful “on signals” for secretion and substantial metabolic oxidative toxicities, a highly reduced cytosolic NADPH pool is likely essential as an “off switch” to reset the system during each oscillation. In addition to improving control strength to prevent hyperinsulinemia, NADPH is consumed to restore redox and to modify proteins via reduced thiols (Ferdaoussi et al., 2015; Santos et al., 2017), and for synthetic functions (e.g., cholesterol and fatty acid synthesis) to maintain cellular homeostasis. One exception may be the NADPH oxidase (NOX) family of enzymes that uses NADPH to generate ROS and especially the constitutively active NOX4 (Figure 5) which generates H_2O_2 from NADPH, albeit independent of variations in the $NADPH/NADP^+$ ratio that do not regulate the enzyme

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(Ježek et al., 2021; Nisimoto et al., 2010). As such, for an essential homeostatic metabolite, any knockout, drug, or nutrient condition that lowers the NADPH level is predicted to impair the function and health of the β-cell and thus insulin secretion. The relative importance of the different NADPH synthesizing pathways has been the subject of much debate. Some studies have suggested involvement of the pentose phosphate pathway (Spégel et al., 2013), whereas other did not (Schuit et al., 1997). Likewise, metabolic flux through IDH1 and ME1 are of variable impact on GSIS (Guay et al., 2013; MacDonald, 2002, 1995; Pongratz et al., 2007; Ronnebaum et al., 2008, 2006). Much of this variability could be due to species specific differences in isozyme expression. Given such redundancy it is not surprising that it has been difficult finding consensus for the supremacy of one pathway over another related to cytosolic NADPH production by observed gain or loss of function following knockout, overexpression, and/or inhibition (Campbell and Newgard, 2021; Prentki et al., 2013). This is not easily settled as the specific contributions of each individual pathway are likely dependent upon cell line, species (e.g., mouse vs. rat vs. human), and condition (e.g., the presence of fatty acids or not or presence of high pyruvate levels that scavenge ROS) (MacDonald et al., 2009; Prentki et al., 2013).

In addition to the pentose phosphate pathway, malic enzyme and IDH1, cytosolic NADPH is generated by moving carbons and/or electrons out of the mitochondrial matrix. Multiple potential mitochondrial pathways to generate cytosolic NADPH have been proposed for the β-cell (Prentki et al., 2013), but plausible mechanisms require balanced translocation of charged metabolites across the inner mitochondrial membrane. Here we critically re-evaluate the generation of cytosolic NADPH with attention to energetics, membrane transport and stoichiometry. Per our nomenclature, *NADPH shuttles* are defined as mass-balanced cycles that are independent of the TCA cycle activity *per se*. A *NADPH shunt*, in contrast, is a balanced extramitochondrial excursion that depends on TCA cycle flux. NADPH shuttles involve carboxylation-decarboxylation cycles of either pyruvate/malate or αKG/isocitrate. An essential player is nicotinamide nucleotide transhydrogenase (NNT) that generates mitochondrial NADPH and NAD^+ at the expense of a proton translocated to the mitochondria matrix. We focus on the three mitochondrial shuttles and one shunt where balanced transport across the mitochondria inner membrane occurs (Figure 4, *green boxes*) and provide a rationale to assign them to $Mito_{Ox}$ or $Mito_{Cat}$. While we strongly agree that anaplerotic/cataplerotic metabolism is important for β-cell function, we are unable to firmly identify any direct pathway linking PC anaplerosis and cytosolic NADPH synthesis via IDH1 despite suggestions otherwise (Ferdaoussi et al., 2015; MacDonald et al., 2005; Prentki et al., 2013). The reason for this is explained below.

Is cytosolic NADPH a regulatory or effectory on-switch, or a homeostatic off-switch?

There is abundant evidence that NADPH is absolutely required for numerous cellular functions and signaling cascades but essentiality does not distinguish the regulatory, effectory, or homeostatic nature of their function. For instance, complete washout of essential molecules during a patch clamp experiment (including GSH and NADPH) reduces vesicle fusion, and replacing them correspondingly improves vesicular fusion (Ferdaoussi et al., 2015). As this example shows, it is challenging to distinguish the essentiality from effectory, regulatory, or homeostatic control especially without assessing the dose

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dependency within the physiologic realm. Even at low glucose concentration, the cytosolic NADPH/NADP$^+$ ratio is highly reduced and in response to 20 mM glucose the free cytosolic NADPH only increases by ~2% (Nicholls, 2016). Thus, a regulatory or effectory processes would have to discriminate between a very high NADPH/NADP$^+$ ratio and a slightly higher ratio upon glucose stimulation. At the time of this writing, such a molecular target of NADPH with these biochemical features remains to be identified. At the very least, NADPH is an homeostatic cofactor supporting multiple reactions in the mitochondria and cytosol. As such, we do not dispute that NADPH-dependent processes (e.g., sumoylation of SENP-1) are *essential* for GSIS (Ferdaoussi et al., 2015; Lin et al., 2021), nevertheless, additional studies are required to determine if these and others are also regulatory or effectory.

Does reductive carboxylation of mitochondrial αKG to isocitrate and the IDH shuttle regulate insulin secretion?

Mitochondrial enzymes are generally considered in terms of their activity in the oxidative direction. One exception are the IDH isozymes, which can catalyze antiparallel reactions within and across the inner mitochondrial membrane (reductive carboxylation and oxidative decarboxylation). In the late eighties the Boquist lab first provided evidence in islet lysates that reductive carboxylation of cytosolic αKG to isocitrate via IDH1 (Boquist, 1987; Boquist and Alehagen, 1986; Boquist and Lorentzon, 1986). To distinguish directionality of flux through IDH isozymes in an intact system, there are many caveats due to the multiple isoforms (3 altogether) variably working in antiparallel fashion in multiple compartments. Especially challenging to tracer methods is that the IDH reaction lies close to its biochemical equilibrium, so the *same isoform* can reversibly transfer isotopic label in the retrograde and anterograde direction (i.e., an exchange flux) even without a net flux in either direction. Taking this reversibility into account, Alves et al. (Alves et al., 2015) used quantitative isotopomeric tracer studies, and documented that neither the IDH reversible exchange flux nor the net reductive IDH flux (αKG to isocitrate) correlated with β-cell GSIS. In this study, reductive fluxes were at most about 15–20% of the net forward IDH reaction. Later knockout and inhibitor studies suggested that targeting individual isoforms can change stable isotope labeling patterns, but unfortunately the directionality, magnitude, and isoform specificity of these changes were not determined (G.-F. Zhang et al., 2021). Thus, quantitative measurements show that reductive carboxylation of αKG to isocitrate is small relative to the TCA cycle flux and likely has only limited importance to generate either an NADPH "on"- or "off"-switch for GSIS.

Most cytosolic NADPH generation likely occurs during MitoOx

The pentose phosphate pathway that occurs in the cytosol is inhibited by ATP ($Mito_{Cat}$ phase), suggesting a role of this pathway in sustaining the NADPH/NADP$^+$ during $Mito_{Ox}$. Likewise, cytosolic NADPH-producing ME1 and IDH1 are inhibited by ATP (Boquist, 1987; Chang et al., 1991). Cytosolic acetyl-CoA, likely high during $Mito_{Cat}$, also inhibits ME1 (Bartholomé et al., 1972). Thus, the bulk of cytosolic NADPH synthesis likely occurs during $Mito_{Ox}$. This may serve to minimize mitochondrial ROS toxicity during this active phase of secretion and refill the cytosolic NADPH pool.

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Cytosolic NADPH synthesis during $Mito_{Cat}$

Pyruvate-malate shuttle.—In the *pyruvate-malate shuttle* (Figure 4C), anaplerotic PC makes oxaloacetate from pyruvate. A high mitochondrial matrix NADH/NAD^+ is required to reverse ME2 to generate malate that enters the cytosol via the DIC. There ME1 generates NADPH and pyruvate from malate in the cytosol. Shuttle completion follows pyruvate return to the matrix via the mitochondrial pyruvate carrier (MPC). This shuttle, also referred in the literature as pyruvate/malate cycling, is likely the only mitochondrial NADPH shuttle to carboxylate pyruvate that is using PC. Driven by high matrix acetyl-CoA/CoA, NADH/NAD^+, and ATP/ADP ratios paired with ATP hydrolysis via PC makes it the most likely of the cytosolic NADPH-generating processes coincident with $Mito_{Cat}$. Some studies have proposed a role of this shuttle in GSIS and NADPH reduction (Huypens et al., 2011; Pongratz et al., 2007). However, the role of this shuttle is in mice debated (Prentki et al., 2013) as ME1 is poorly expressed in mouse islets (MacDonald, 2002) and some studies did not support a role of this shuttle in GSIS in normal mouse islet (Heart et al., 2009). Also, it competes with the PEP cycle for PC flux (Jesinkey et al., 2019). However, in this study a decrease in the *pyruvate-malate shuttle* relative to the *PEP cycle* was associated with increased GSIS, suggesting that the *pyruvate-malate shuttle* is less important for GSIS than the *PEP cycle*.

Cytosolic NADPH synthesis during $Mito_{Ox}$

Malic enzyme shuttle.—An alternative version of the *pyruvate-malate shuttle* is the PC-independent *ME shuttle* (Figure 4D) where proton coupled pyruvate uptake in the mitochondrion via the transporter MPC is followed by conversion via ME2 to malate coupled to the high mitochondrial NAD(P)H/NAD(P)$^+$ ratios. Malate is then exported into the cytosol in electroneutral exchange for phosphate via the DIC (Huypens et al., 2011). Once in the cytosol, ME1 decarboxylates malate back into pyruvate making cytosolic NADPH. The role of ME1 in GSIS as discussed above is debated (MacDonald et al., 2009; Prentki et al., 2013) and therefore the significance of this shuttle in GSIS is uncertain.

Isocitrate dehydrogenase shuttle.—Like the ME shuttle, the IDH shuttle (Figure 4D) uses antiparallel operation of cytosolic IDH1 and mitochondrial IDH2 that is driven by the NNT to reduce cytosolic $NADP^+$ (Palmieri, 2004). Balanced translocation of isocitrate out of and αKG into the matrix is achieved via two carriers, the CIC and the OGC, by cycling malate as counterion for both. There is no anaplerotic PC involvement. Consistent with the view that this shuttle is related to an *off*-signal for secretion via NADPH production, knockdown of IDH1 in the β-cell resulted in enhanced GSIS in association with increased isocitrate, $NADP^+$, and ATP levels (Guay et al., 2013).

Isocitrate dehydrogenase shunt.—Unlike the *IDH shuttle*, the *IDH shunt* (Figure 4D) is essentially a transient extramitochondrial extension of the TCA cycle, which assigns its activity to $Mito_{Ox}$. Here cytosolic NADPH is generated (like in the *IDH shuttle*) after isocitrate exits the matrix to be converted into α-ketoglutarate by IDH1 in the cytosol. Once returned to the matrix, αKG re-enters the oxidative TCA cycle. It is important to emphasize that the *IDH shunt* is simply a redirected loop of the TCA cycle out of then into mitochondria that has complete independence from anaplerotic pyruvate metabolism via PC,

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and like the IDH shuttle, for the reasons discussed above, is unlikely to contribute to GSIS but may be an *off*-pathway that reduces ROS and perhaps also acts as a signal remover for insulin secretion (Prentki et al., 2013).

Unlikely NADPH shuttles and pyruvate cycles.—Given the importance of both PC and NADPH to β-cell function, it has been compelling to try to link pyruvate anaplerosis to cytosolic NADPH generation through a pathway utilizing citrate synthase. As described above, the *acetyl-CoA shuttle*, the *IDH shuttle*, and the *IDH shunt* all are completely independent of pyruvate anaplerosis. Since citrate efflux needs malate as a counter ion, we are unaware of a balanced stoichiometric pathway that can link pyruvate anaplerosis to NADPH from citrate or isocitrate. In the absence of such a connection, we recommend that proposed constructs such as the "pyruvate-citrate cycle" and the "pyruvate-isocitrate cycle" no longer be used. Thus, we now favor the view that inhibition of GSIS following knockdown or pharmacological inhibition of ACLY (Guay et al., 2007) may *not* be due to an altered putative pyruvate/citrate cycle, but rather to reduced activity of the acetyl-CoA shuttle that provides malonyl-CoA and reoxidizes NAD^+ in the cytosol.

Phasic metabolism and exocytosis also implicate oscillatory ROS, lipid signals, and cAMP

Reactive oxygen species

β-cell ROS is a critical participant in insulin secretion and likely oscillate during phasic glucose metabolism and insulin secretion (Leloup et al., 2009). Glucose dose-dependently increases H_2O_2 in rat islets that plateaus between 10–16 mM (about 7-fold rise above 4 mM glucose) (Mugabo et al., 2017). A consequence of elevated Ψ_m and mitochondrial $NADH/NAD^+$ during $Mito_{Cat}$ is increased ROS production by the ETC (Figure 5). During this time, ROS sensitizes K_{ATP} channels to closure by ATP/ADP (Plecitá-Hlavatá et al., 2020b). In contrast, when the proton motive force is fully engaged with the production of ATP, then Ψ_m and relative ROS production fall during $Mito_{Ox}$ (Plecitá-Hlavatá et al., 2020a). It has been proposed that the NADPH oxidase NOX4 plays a significant role in ROS production, K_{ATP} closure and GSIS (Plecitá-Hlavatá et al., 2020b). This potentially important signaling role of NOX4 requires clarification as to how this enzyme is activated upon glucose stimulation. Although H_2O_2 is made by NOX4 which uses NADPH as a substrate (Plecitá-Hlavatá et al., 2020b), this enzyme may not be limited by the cellular levels of NADPH (Nisimoto et al., 2010), and thus variations in NADPH levels upon metabolic oscillations (if they even occur) should not modulate its activity. The potency of ROS signaling in the β-cell has been attributed to low antioxidant enzyme expression (Lenzen et al., 1996). However, like any strong "on-switch", ROS needs a powerful "off-switch", given its potential toxicity. Phasic production of ROS during $Mito_{Cat}$ (when Ψ_m production is high) and scavenging of ROS during $Mito_{Ox}$ (when NADPH production is high) provide a mechanism to generate and remove pulses of ROS that could otherwise be toxic if sustained (Figure 5).

Overall, we favor the view that ROS levels oscillate during GSIS, and that H_2O_2 produced by the ETC and possibly by NADPH-dependent (but not NADPH regulated) NOX4 is

an important signal for GSIS during $Mito_{Cat}$. How NOX4 is activated, and the ROS targets related to exocytosis remain to be identified. Furthermore, we believe that NADPH production by the IDH shuttle/shunt during $Mito_{Ox}$ negatively impacts secretion both by promoting a reduced state of proteins linked to exocytosis and also via ROS removal (Figure 5).

Glycerolipid/FFA cycle signals

There is very strong evidence that a GL/FFA cycle, driven by both glucose and FFA, plays an essential regulatory role in GSIS, and that β-cell lipolysis-derived MAG is the mediator of this pathway via its binding to the exocytosis coordinator Munc13–1. As this has been extensively reviewed by us and others (Campbell and Newgard, 2021; Prentki et al., 2020a) we will not discuss it further. Whether oscillations in β-cell MAG and other lipid signals occur in response to fuels remains to be shown.

cAMP

Glucose induces oscillatory cAMP near the β-cell plasma membrane (Dyachok et al., 2008, 2006). However, the spatial bias of cAMP signaling, well-known to other fields, has not yet been thoroughly examined in β-cells. One striking example of such compartmentation is that cAMP and Ca^{2+} oscillations in INS-1 cells are in-phase in the microdomain of voltage-dependent Ca^{2+} channels tethered by A-kinase anchoring protein 79 (AKAP79) scaffolds to the plasma membrane, and out-of-phase in the general plasma membrane domain (Tenner et al., 2020). Also, cAMP may regulate K_{ATP} channel trafficking (Cochrane et al., 2021) and K_{ATP} activity via the cAMP effector EPAC2 (Kang et al., 2008; Shuai et al., 2021) in response to glucagon-like peptide-1 receptor activation (Holz et al., 1993). New optical tools for compartmentalized cAMP signaling (J.-F. Zhang et al., 2021), may be helpful to assess signaling on the timescale of $Mito_{Cat}$ and $Mito_{Ox}$.

Inborn errors of metabolism and insulin secretion

Inborn errors of metabolism that alter islet function provide profoundly informative insights into key mechanistic components of the β-cell glucose sensing in humans. These include those causing familial hyperinsulinemic hypoglycemia (HHF) and those associated with maturity onset diabetes of the young (MODY).

Glucokinase (HHF3) and SUR1/K_{ATP} (HHF1/2).

Mutations in the earliest steps of glucose signaling (GK/HHF3/MODY2) and late steps (SUR/K_{ATP} channels/MODY12/13/HHF1/HHF2) offer clear human evidence mechanistically connecting the beginning steps of glucose metabolism to plasma membrane depolarization needed for insulin secretion (Snider et al., 2013).

Monocarboxylate transporter 1 (MCT1/HHF7).—Exercise-induced hyperinsulinemic hypoglycemia (HHF7) is a consequence of inappropriate expression of the monocarboxylate lactate transporter, MCT1/SLC16A1, in the plasma membrane of β-cells (Otonkoski et al., 2007; Pullen et al., 2012). Normally, β-cells are impermeable to lactate (Pullen et al., 2012; Sdao et al., 2021; Sekine et al., 1994). Hypoglycemia follows excess insulin

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secretion caused by plasma lactate generated during exercise in individuals via ectopic β-cell expression of SLC16A1. While LDH activity in primary β-cell extracts is low relative to most tissues, it is sufficient to enable HHF *in vivo* when the cell membrane is permeable to lactate. This fits well with the view that mitochondria-dependent signals are important for GSIS.

Glutamate dehydrogenase (GDH/HHF6).—Gain-of-function mutations in glutamate dehydrogenase (GLUD1/HHF3) increase anaplerosis due to loss of mitochondrial GTP inhibition of glutamate deamination (Figure 6). In humans, this causes protein-meal-induced HHF as well as asymptomatic hyperammonemia (Kelly et al., 2002). Unopposed oxidation of the anaplerotic amino acid glutamate by this mutation expands the entry of αKG to TCA metabolism. This pathway generates NAD(P)H and $FADH_2$, but also will make mitochondrial GTP and oxaloacetate to support cataplerotic PEP generation via the *mitochondrial GTP cycle* (Kibbey et al., 2014, 2007). The glutamate-derived PEP then leaves the mitochondria to potently stimulate insulin release by augmenting the PEP cycle (Abulizi et al., 2020; Jesinkey et al., 2019; Lewandowski et al., 2020; Stark et al., 2009). This form of HHF supports the view that anaplerosis and the *PEP cycle* are central to β-cell activation.

Short-chain acyl-CoA dehydrogenase (SCHAD/HADH/HHF4).—Loss of function of hydroxyacyl-CoA dehydrogenase short chain (HADH, a.k.a., short-chain acyl-CoA dehydrogenase; SCHAD) causes fasting HHF and hyperammonemia (HHF4) possibly via multiple mechanisms. First, via interaction with GDH, HADH inhibits GDH (GLUD1) through a protein-protein complex (Li et al., 2010) and thus, loss of HADH augments glutamine-derived anaplerosis to stimulate the TCA cycle, cataplerosis, and fasting insulin release. In light of the phenotypic differences with HHF3 (GK) and HHF6 (GDH) , it is worth considering alternative explanations (Figure 6). Second, during $Mito_{Cat}$, when acetyl-CoA levels peak, acetoacetyl-CoA (AcAc-CoA) generation by mitochondrial ACAT1 offloads pyruvate oxidation. Since $NADH/NAD^+$ is high during $Mito_{Cat}$, AcAc-CoA gets converted by HADH/SCHAD to β-hydroxybutyrate-CoA. With the loss of HADH, AcAc-CoA is pushed towards acetoacetate by SCOT1 (a.k.a., OXCT1) with succinyl-CoA as a byproduct. Thus loss of HADH augments the ability of acetyl-CoA to drive the production of mitochondrial GTP to support the PEP cycle. SCOT1 deficiency also impairs insulin secretion (Hasan et al., 2010), supporting the functional involvement of this enzymatic step. Third, HADH loss could reduce β-oxidation to potentiate MAG synthesis and insulin secretion via the GL/FFA cycle (Prentki et al., 2020a). Altogether, HHF3, HHF4 and HHF6 point towards a role for anaplerosis and the PEP cycle in the regulation of GSIS.

Disallowed/repressed metabolic genes in the β-cell point to key pathways of insulin secretion

Several metabolic enzymes or transporters are under-represented in the β-cell relative to other tissues and are referred to as “disallowed” or “repressed” genes (Lemaire et al., 2016; Rutter et al., 2020) (Figure 6). This likely prevents metabolic interference with GSIS.

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Low K_m hexokinases.

β-cells preferentially express the high K_m and high V_{max} hexokinase 4 isozyme GK, but low levels of the other low K_m isoforms. This permits GK to act as a glucose sensor to control glycolytic flux and GSIS according to blood glucose levels.

Lactate/pyruvate transporter (MCT1) and lactate dehydrogenase A (LDHA).

Both the lactate/pyruvate plasma membrane transporter (MCT1/SLC16A1) and the LDHA isoform have low mRNA expression levels (Ghiasi and Rutter, 2021). However, three isoforms of LDH (A, B, and D) are detectable at the protein level (Mitok et al., 2018), and glucose elevation is accompanied by lactate production in intact mouse islet β-cells (Sdao et al., 2021), indicating that LDH is a viable short-term NAD^+ generator. Human β-cells must also express sufficient LDH activity for ectopic SLC16A1 to cause hyperinsulinemic hypoglycemia (HHF7). Low MCT1 in the β-cell (Pullen et al., 2012; Sdao et al., 2021) is appropriate as it spares glucose derived carbons for the TCA cycle and cataplerotic processes linked to insulin secretion.

Adenylate kinase 3 and guanine deaminase.

Two other disallowed enzyme, adenylate kinase 3 (AK3, that can interconvert GDP and GTP) and guanine deaminase (GDA, which converts guanine to xanthine to limit the guanine pool) potentially oppose the mitochondrial GTP cycle and their reduced level should favor mitochondrial GTP production and the *PEP cycle*.

Ornithine transaminase 1.

Similarly, significant expression of ornithine transaminase 1 (OAT1) would restrict the anaplerotic glutamate pool by diversion of the metabolite glutamate-5-semialdehyde into the ornithine/urea cycle. Disallowance of this gene favors amino acid induced secretion by glutamine, glutamate, and leucine (which activates GDH).

Monoacylglycerol lipase.

Disallowed genes in lipid metabolism point towards interference with LC-CoA and MAG signaling. Limited MAG lipase (*Mgll* gene) expression permits the accumulation of MAG to activate the exocytotic coordinator Munc13–1 (Zhao et al., 2014). Likewise, signaling MAG is specifically degraded by the hydrolase ABHD6 which is located at the plasma membrane (Poursharifi et al., 2017). In a mouse study to identify plasma biomarkers of β-cell deficits on low and high fat diets, the genes (mRNA levels) that best correlated were first ABHD6 and second glucokinase (Sánchez-Archidona et al., 2021).

Acyl-CoA Thioesterase 7.

Another enzyme opposing LC-CoA accumulation and the glycerolipid/FFA cycle is acyl-CoA thioesterase 7 (ACOT7). Its overexpression reduced GSIS *in vitro* and *in vivo* (Martinez-Sanchez et al., 2016, p. 7) and lowered ATP due to increased synthesis and breakdown of acyl-CoA which consumes ATP. This provide genetic evidence that LC-CoA or its derived signals are key to GSIS.

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Conclusion

Contrary to what is often believed, the glucose-induced signaling process in β-cells has not been largely solved and the current model implicating an initial rise in mitochondrially-derived ATP driving K_{ATP} channel closure and Ca^{2+} influx is incomplete and possibly wrong. The main reasons are: 1) it does not account for mitochondrial thermodynamics, which govern the way by which mitochondrial ATP generation is accelerated *following* enhanced workload via a rise in ADP, and not via a rise in NADH and $FADH_2$ that would drive ROS as well as ATP production when ADP is limiting prior to membrane depolarization; 2) it does not account for metabolic compartmentation, including the ability of PK to switch off oxidative phosphorylation, and the sufficiency and essentiality of plasma membrane PK to close K_{ATP} channels (Foster et al., 2022; Lewandowski et al., 2020); 3) it does not account for temporal compartmentation, which is provided by oscillatory metabolism. However, very significant recent progress in the area of metabolic signaling may allow the field to reconcile potential caveats and contrasting reports. In this review article, we integrated many aspects of β-cell metabolic signal transduction that have not been previously covered together with new knowledge in the field. From this analysis we developed a testable model of glucose, amino acid, and FFA induced secretion (Figure 7). This model with four main metabolic cycles incorporates the following: spatial and temporal aspects of metabolic signaling (i.e. $Mito_{Cat}$ and $Mito_{Ox}$); the relationship between cellular compartments, including the mitochondrion, cytosol and the area beneath the plasma membrane; "on-switch" signals for secretion but also "off-signals" that permit oscillations of metabolism and insulin secretion; and the redundancy among some pathways, in particular redox components that may explain diverging views in the literature.

This area of research, although not new, addresses a central question in metabolism at large: how do calorigenic nutrients activate a cell? This is of importance for the islet field but also for many other cell types such as the fuel-sensitive cells in the gut, the portal vein and the brain. In fact discoveries in β-cell metabolism often foresee findings in other related fields and cell types. Thus, we believe that research on nutrient signaling in the β-cell has exciting years of research and discoveries ahead.

Some timely key questions can be identified for future research. Do all regulatory, effectory and homeostatic MCF, or post-translational modifications, oscillate upon glucose stimulation? Are there oscillations in metabolism when a β-cell is stimulated with FFA or some amino acids? What are the MCFs at various glucose concentrations as they may not be necessarily the same or of the same importance? What are the late effectory targets of MCFs besides ATP and MAG, in particular ROS? What are the similarities and differences between β-cell signaling in mice, rats, and humans? How is basal secretion regulated? How is metabolic signaling enhanced in obesity (compensation) and how does it fail precisely in diabetes (decompensation)? Does the integrated model with interlinked metabolic cycles apply to other glucose-sensitive cell types? These are only a few questions and undoubtedly as the field evolves others will be formulated to enhance basic knowledge but also to uncover translational aspects related to the prevention of "nutri-stress" (Prentki et al., 2020b) in various cell types and the treatment of metabolic disorders.

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ABBREVIATIONS

αKG	α-ketoglutarate
αKGDH	α-ketoglutarate dehydrogenase
Ψ_m	mitochondrial membrane potential
ABHD6	monoacylglycerol lipase ABHD6
Ac-CoA	acetyl-CoA
AcAc-CoA	acetoacetyl-CoA
ACLY	ATP citrate lyase
ACOT7	acyl-CoA thioesterase 7
AGC1	aspartate-glutamate carrier SLC25A12/ARALAR
AK3	Adenylate kinase 3
AKAP79	A-kinase anchoring protein 79
CIC	citrate-isocitrate carrier SLC25A1
EPAC2	exchange factor directly activated by cAMP/RAPGEF4
ETC	electron transport chain
F6P	fructose 6-phosphate
F1,6BP	fructose 1,6-bisphosphate
F2,6BP	fructose 2,6-bisphosphate
FFA	free fatty acid
G3PP	glycerol 3-phosphate phosphatase
G6P	glucose 6-phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDA	guanine deaminase

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GDH	glutamate dehydrogenase/GLUD1
GK	glucokinase
GLP-1	glucagon-like peptide 1
GPD2	mitochondrial glycerol-3-phosphate dehydrogenase
Gro3P	glycerol 3-phosphate
GSIS	glucose-stimulated insulin secretion
GSH	glutathione, reduced
GSSG	glutathione, oxidized
HADH	short-chain acyl-CoA dehydrogenase/SCHAD/HHF4
HHF	familial hyperinsulinemic hypoglycemia
IDH	isocitrate dehydrogenase
K_{ATP}	ATP-sensitive K^+ channel
LC-CoA	long chain acyl-CoA
LDH	lactate dehydrogenase
MAG	monoacylglycerol
MCT1	Monocarboxylate transporter 1
MDH	malate dehydrogenase
ME1	cytosolic malic enzyme 1
ME2	mitochondrial malic enzyme 2
MODY	maturity onset diabetes of the young
NNT	nicotinic nucleotide transhydrogenase
NOX4	NADPH oxidase 4
OAA	oxaloacetate
OXCT1	3-oxoacid CoA transferase/SCOT1
OxPhos	oxidative phosphorylation
PC	pyruvate carboxylase
PCK2	mitochondrial phosphoenolpyruvate carboxykinase/PEPCK-M
PDH	pyruvate dehydrogenase
PEP	phosphoenolpyruvate

PEPCK	phosphoenolpyruvate carboxykinase
PFK1	phosphofructokinase-1
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PK	pyruvate kinase
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SUCLA2	ATP-generating succinyl-CoA synthase/SCS^{ATP}
SUCLG2	GTP-generating succinyl-CoA synthase/SCS^{GTP}
TCA cycle	tricarboxylic acid cycle

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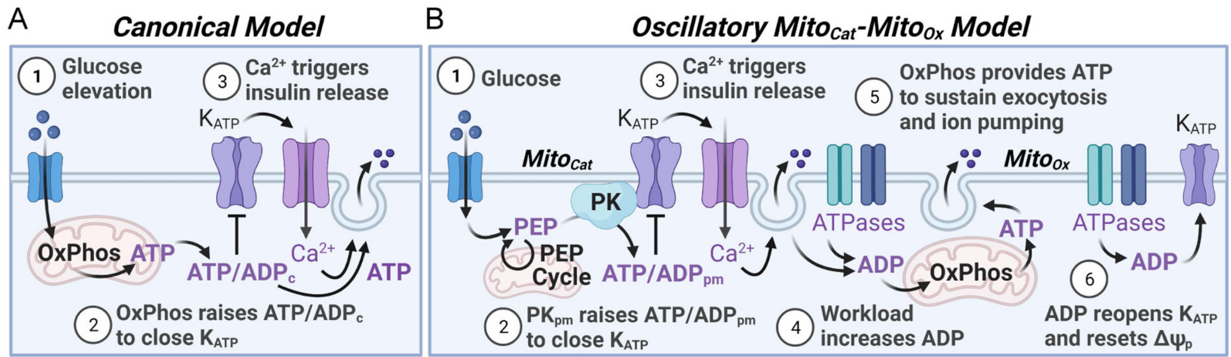

Figure 1. Reconsidering the canonical model of glucose-stimulated insulin secretion.

(A) In the canonical model, glucose metabolism drives an initial rise in ATP/ADP ratio via oxidative phosphorylation (OxPhos) that plays the dominant role in both K_{ATP} channel closure and ATP production to sustain exocytosis. **(B)** A new spatially and temporally compartmentalized model of stimulus-secretion coupling, termed the $Mito_{Cat}$-$Mito_{Ox}$ model, is motivated by the recent discovery that plasma membrane-associated pyruvate kinase (PK_{pm}), rather than OxPhos, locally generates the rise in ATP/ADP to close K_{ATP} channels and initiate insulin secretion. OxPhos has been repositioned after K_{ATP} closure, membrane depolarization and the rise in cytosolic Ca^{2+} to highlight its complimentary role in generating ATP to sustain secretion. While the figure shows that $Mito_{Cat}$ and $Mito_{Ox}$ are separated by the onset of membrane depolarization, they are not all or none processes. It remains possible that OxPhos may help to sustain K_{ATP} channel closure after the channel is initially closed by PK and the PEP cycle. *Abbreviations:* ATP/ADP_c, cytosolic ATP/ADP ratio; ATP/ADP_{pm}, plasma membrane ATP/ADP ratio; Ψ_p, plasma membrane potential.

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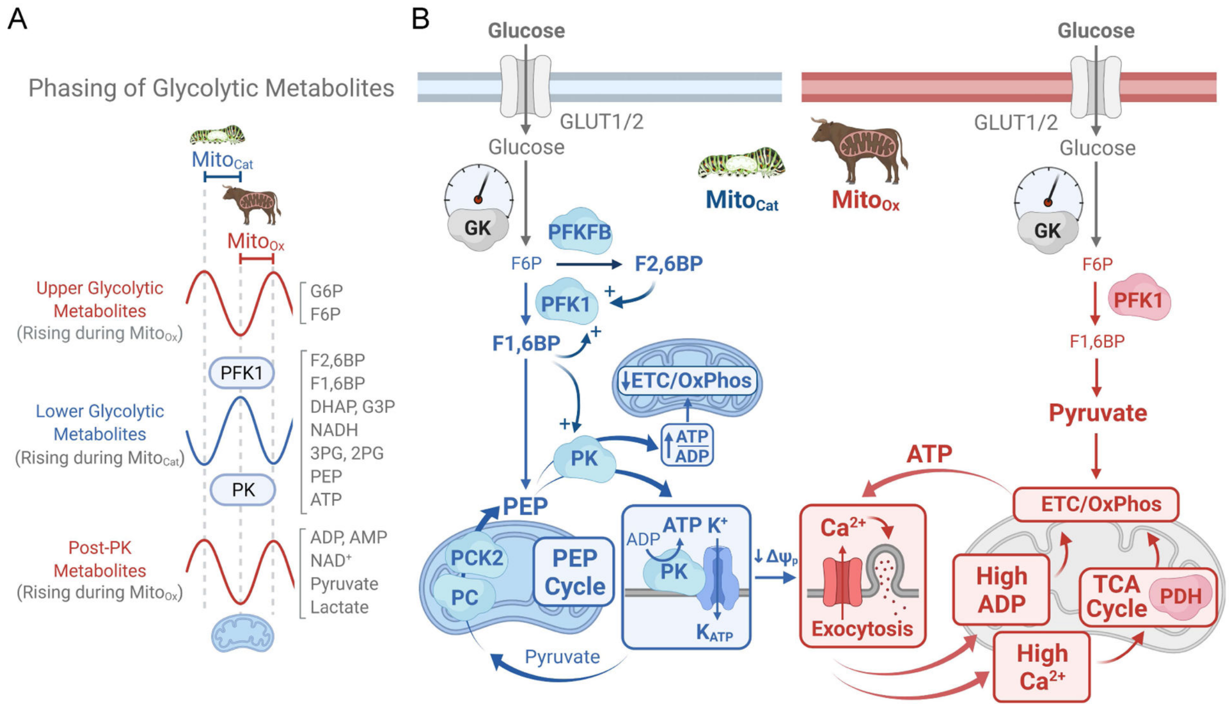

Figure 2. Allosteric regulation of glycolysis and oscillations of β-cell metabolism and insulin secretion during Mito$_{Cat}$ and Mito$_{Ox}$.

(A) Phosphofructokinase-1 (PFK1), responding to activation by its product fructose 1,6-bisphosphate (F1,6BP), generates glycolytic oscillations that separate the phases of upper glycolysis (*red*) and lower glycolysis (*blue*), while pyruvate kinase (PK) controls glycolytic efflux. During Mito$_{Cat}$ the lower glycolytic metabolite levels rise, as the flux through PFK1 increases while PK is slowed by rising ATP/ADP; oscillations in post-PK metabolites (*red*) are out of phase with lower glycolysis due to rapid metabolism of pyruvate by mitochondria. During Mito$_{Ox}$ the fall in ATP/ADP stalls PFK1 and maximizes flux through PK, causing a crash in the lower glycolytic metabolite levels. **(B)** Oscillations in glycolysis and anaplerosis-cataplerosis during Mito$_{Cat}$ (*blue*) are matched by antiphase oscillations in Ca^{2+}, ADP, TCA cycle activity and OxPhos during Mito$_{Ox}$ (*red*). Importantly, PK is localized to both the mitochondrial and plasma membranes. This compartmentation of PK is central to β-cell oscillatory metabolism and insulin secretion. Before plasma membrane depolarization, during Mito$_{Cat}$, PFK1 generates F1,6BP to activate PK, which lowers ADP at the inner mitochondrial membrane, reducing flux through the adenine nucleotide translocator (ANT), slowing the ETC (which becomes ADP-starved and state 4-like) and therefore TCA cycle, while activating anaplerosis/cataplerosis and the phosphoenolpyruvate (PEP) cycle. During the PEP cycle, anaplerosis (filling of TCA cycle intermediates) is due to the PC reaction that carboxylates pyruvate to oxaloacetate, whereas cataplerosis (egress of the TCA cycle intermediates to the cytosol) results from the exit of mitochondrial PEP to the cytosol following the mitochondrial PEP carboxykinase (PCK2) reaction. PEP then exits the mitochondrion to feed mitochondrial and plasma membrane PK. While PK reinforces the PEP cycle, plasma membrane compartmentalized PK drives a rise in ATP/ADP that closes K_{ATP} channels. Following membrane depolarization and the rise in Ca^{2+}, during Mito$_{Ox}$, the high workload (i.e. ATP hydrolysis) associated with

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ion pumping and insulin secretion restores ADP and increases flux through the ETC (which is now ADP replete and state 3-like), the TCA cycle, and lower glycolysis. *Abbreviations:* DHAP, dihydroxyacetone phosphate; ETC, electron transport chain; F6P, fructose 6-phosphate; F2,6BP, fructose 2,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate, GK, glucokinase; G6P, glucose 6-phosphate; PC, pyruvate carboxylase; PCK2, phosphoenolpyruvate kinase 2; PDH, pyruvate dehydrogenase; PFKFB, phosphofructo-2-kinase/fructose 2,6-bisphosphatase.

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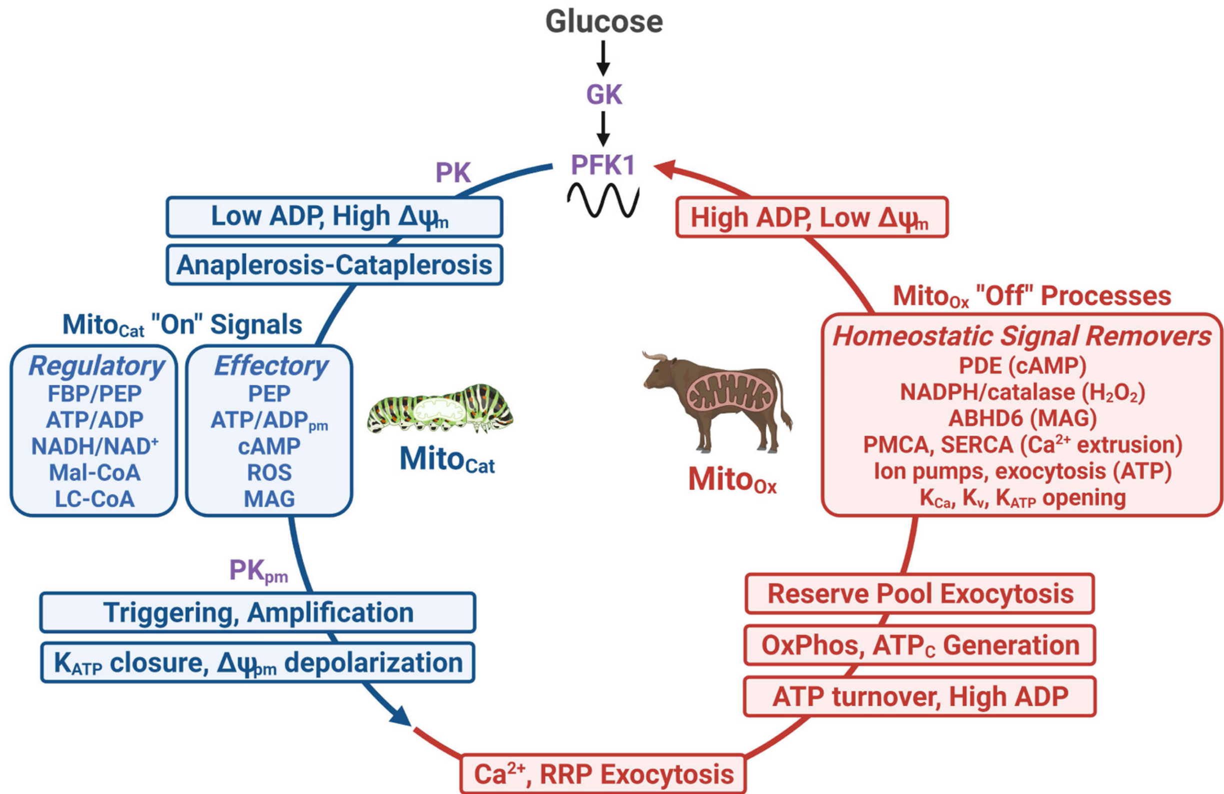

Figure 3. Metabolic coupling factors and homeostatic signal removers are temporally compartmentalized.

Glucose metabolism via phosphofructokinase-1 (PFK1) creates two metabolic states separated in time: an initial increase in mitochondrial anaplerotic/cataplerotic fluxes (termed $Mito_{Cat}$), followed by enhanced OxPhos ($Mito_{Ox}$) (see Fig. 2). Viewed sequentially, the net result of glycolysis during $Mito_{Cat}$ is a pyruvate kinase (PK)-driven reduction in ADP that is sensed by the mitochondrial adenine nucleotide translocase and therefore ATP synthase, causing increased voltage across the mitochondrial inner membrane (Ψ_m), slowing NADH consumption by the electron transport chain, and increasing signals for secretion. These $Mito_{Cat}$ "on" signals (*blue boxes*) include both regulatory and effectory metabolic coupling factors. Several $Mito_{Cat}$ signals, such as PEP, plasma membrane ATP/ADP (ATP/ADP_{pm}) and reactive oxygen species (ROS), participate in both the triggering and amplification arms of glucose-stimulated insulin secretion. Following K_{ATP} closure by plasma membrane PK (PK_{pm})-driven ATP/ADP_{pm}, membrane depolarization and Ca^{2+} influx terminates the $Mito_{Cat}$ phase by initiating a cascade of $Mito_{Ox}$ processes that consume ATP, including ion pumping and exocytosis. The ensuing rise in ADP stimulates respiration, increasing cytosolic ATP (ATP_c) that provides energy to sustain secretion until homeostatic signal removers (*inset red box*) reset the membrane potential. A rise in cytosolic ADP plays key role in this resetting as it will activate PFK1 and PK, which control oscillations in glycolysis. *Abbreviations:* ABHD6, α/β-hydrolase domain containing 6; K_{ATP}, ATP-sensitive K^+ channels; K_{Ca}, Ca^{2+}-activated K^+ channels; K_v, voltage-dependent K^+ channels; LC-CoA long chain acyl-CoA; MAG, monoacylglycerol; Mal-CoA, malonyl-CoA; PDE, phosphodiesterase; PMCA, plasma membrane Ca^{2+} ATPase;

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RRP, readily-releasable pool; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; Ψ_{pm}, plasma membrane potential.

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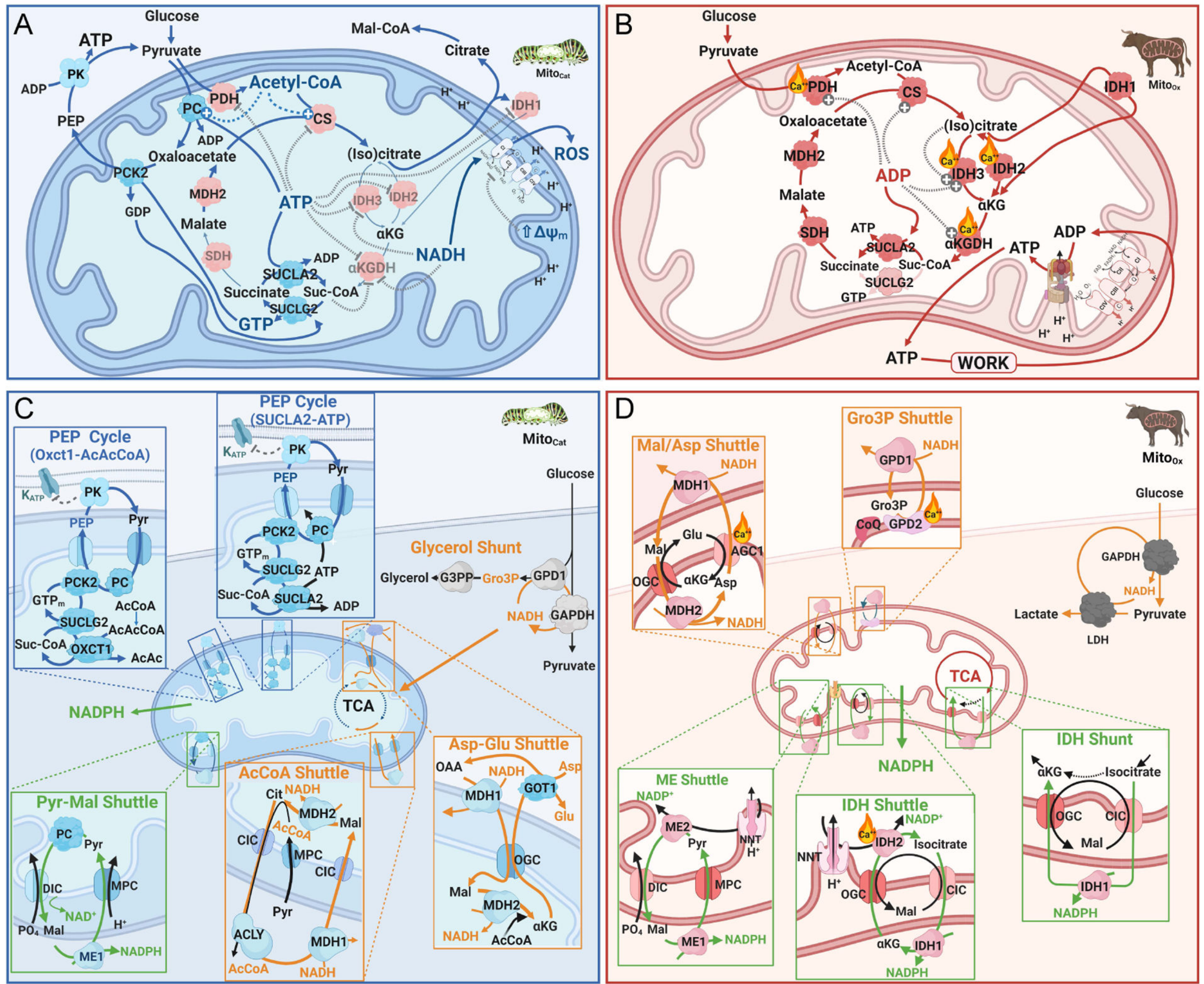

Figure 4. Allosteric and Ca^{2+} regulation of mitochondrial enzymes dictates the mitochondrial cycles, shuttles, and shunts during $Mito_{Cat}$ and $Mito_{Ox}$.

(**A**) $Mito_{Cat}$ is a high-energy mitochondrial state-4-like condition that exerts strong allosteric control over the PEP cycle, the TCA cycle, and the ETC through increased ATP/ADP, $NADH/NAD^{+}$, Ac-CoA/CoA and Suc-CoA/CoA ratios. Key to $Mito_{Cat}$ is the blockade of isocitrate dehydrogenase (IDH2 and IDH3), and α-ketoglutarate dehydrogenase (αKGDH), an energy level sensor in the mitochondrial matrix that supports the following events through partial TCA cycle blockade: anaplerosis and increased matrix oxaloacetate levels via allosteric activation of PC stimulated by a rise in acetyl-CoA; cataplerotic output of citrate and the formation of cytosolic acetyl-CoA and malonyl-CoA (Mal-CoA), which is also favored by cytosolic IDH1 inhibition; enhanced PCK2 flux and PEP cycle activity promoted via oxaloacetate production. The accompanying rise in NADH drives reactivate oxygen species (ROS) production by the ETC in this low ADP state. (**B**) During $Mito_{Ox}$, ADP activates OxPhos, reduces Ψ_{m}, and fully engages the TCA cycle. Ca^{2+}-activated dehydrogenases are critical to support an NADH burst for OxPhos, and ADP activation of IDH2 and IDH3 reinforces citrate commitment to the TCA cycle for ATP production to cope with the workload of insulin secretion. (**C, D**) Putative assignments of metabolic

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cycles (*blue boxes*), as well as NADH and NADPH shuttles and shunts (*orange and green boxes*, respectively), and are shown for $Mito_{Cat}$ (C) and $Mito_{Ox}$ (D). GAPDH during glycolysis reduces NAD^+ in the cytosol that has to be reoxidized during $Mito_{Cat}$ to maintain glycolytic flux. This occurs through the *acetyl-CoA shuttle,* the *aspartate-glutamate exchange,* as well as the *glycerol shunt* and the *glycerolipid/fatty acid cycle* (not shown). During $Mito_{Ox}$, NAD^+ is reoxidized via the *malate-aspartate shuttle*, the *Gro3P shuttle*, or *pyruvate lactate exchange* performed by LDH. During $Mito_{Cat}$, succinyl-CoA (Suc-CoA) that supports mitochondrial GTP synthesis for the PCK2 reaction during the *PEP cycle* is generated from succinate by SUCLA2 in reverse mode that uses ATP. The increased Suc-CoA can then drive GTP synthesis by SUCLG2 (*PEP cycle SUCLA2-ATP*). Alternatively, in a variant *PEP cycle (OXCT1-AcAcCoA*), Suc-CoA and mitochondrial GTP are generated from AcAc-CoA via the action of OXCT1. However, during $Mito_{Ox}$, Suc-CoA is directly generated by αKGDH in the TCA cycle (not shown). NADPH can be generated in the cytosol by the pentose phosphate pathway (not shown) or the *pyruvate-malate shuttle* during $Mito_{Cat}$ or from electron transfer from the mitochondria to the cytosol through the *malic enzyme (ME) shuttle*, the *isocitrate dehydrogenase (IDH) shuttle*, or the *IDH shunt* during $Mito_{Ox}$. *Abbreviations:* AcCoA, acetyl-CoA; AcAcCoA, aceto-acetyl-CoA; DIC, dicarboxylate carrier; GPD1, cytosolic glycerol 3-phosphate dehydrogenase; GPD2, mitochondrial membrane associated glycerol 3-phosphate dehydrogenase; GOT1, glutamic-oxaloacetic transaminase; Gro3P, glycerol 3-phosphate; GTP_m, mitochondrial GTP; OGC, oxoglutarate carrier; ME, malic enzyme; MDH, malate dehydrogenase; MPC, mitochondrial pyruvate carrier; OXCT1, 3-oxoacid CoA transferase, also called SCOT1; Pyr-Mal shuttle, pyruvate-malate shuttle; SUCLA2, ATP-specific succinyl-CoA synthase; SUCLG2, GTP specific succinyl-CoA synthase; ACLY, ATP citrate lyase; NNT, nicotinic nucleotide transhydrogenase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase, PC, pyruvate carboxylase; PCK2, mitochondrial PEP carboxykinase.

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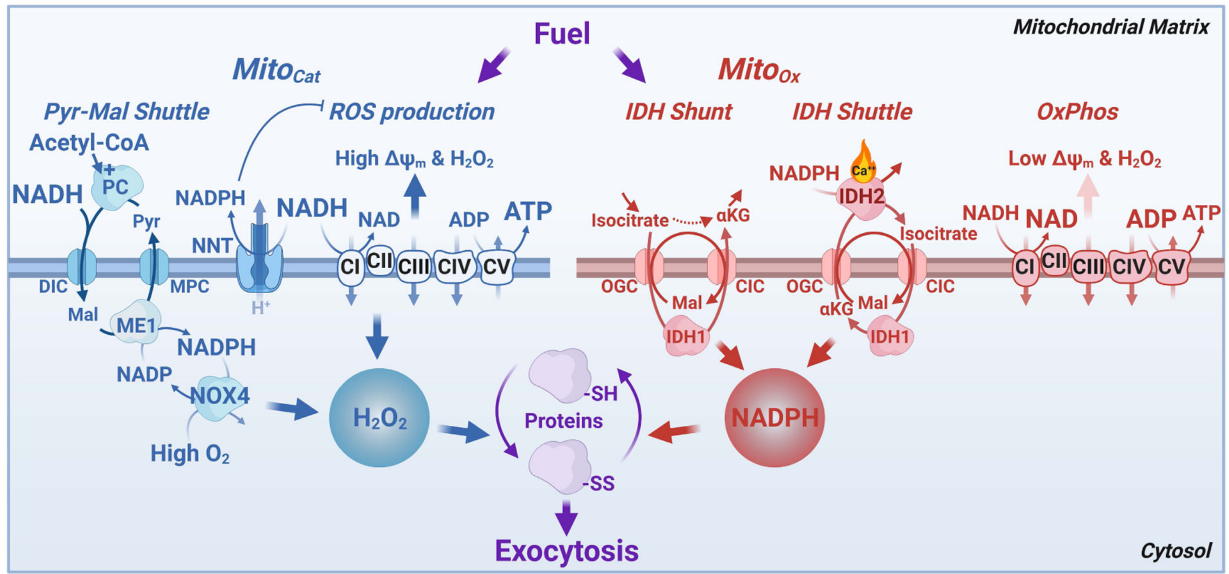

Figure 5. Model illustrating the redox cycles and the roles of ROS and NADPH in controlling insulin exocytosis and recovery.

Cellular redox is communicated differently between mitochondria and cytosol during Mito_{Cat} and Mito_{Ox}. During Mito_{Cat} (*left*) mitochondrial NADH is elevated leading to ROS production via the ETC, NNT activation, and efflux of malate to the cytosol via the *pyruvate-malate shuttle*. This leads to cytosolic NADPH formation by malic enzyme (ME1) allowing *ROS production* in part via NOX4. ROS efflux from the mitochondria and generation via NOX4 modifies reactive cysteines in susceptible proteins involved in exocytosis that primarily favor insulin secretion. During Mito_{Ox} (*right*), NADH is consumed to support respiration and cytosolic NADPH is formed as a result of isocitrate transport to the cytosol as part of the *IDH shunt* and *IDH shuttle,* the latter associated with Ca^{2+}-dependent IDH2 activity in reverse mode, in the direction of α-ketoglutarate to isocitrate. During metabolic oscillations both mitochondrial and cytosolic NADPH help convert H_2O_2 to H_2O and restore ROS to basal levels. Cytosolic NADPH has dual effects on insulin exocytosis. During Mito_{Cat} NADPH-dependent NOX4 generates H_2O_2 which promotes exocytosis, whereas NADPH produced during Mito_{Ox} inhibits exocytosis, thus preventing excessive secretion by reducing the activity of thiol redox sensitive exocytosis effector(s). *Abbreviations*: IDH, isocitrate dehydrogenase; αKG, α-ketoglutarate; NOX4, NADPH oxidase 4; NNT, nicotinic nucleotide transhydrogenase; Px, peroxidase.

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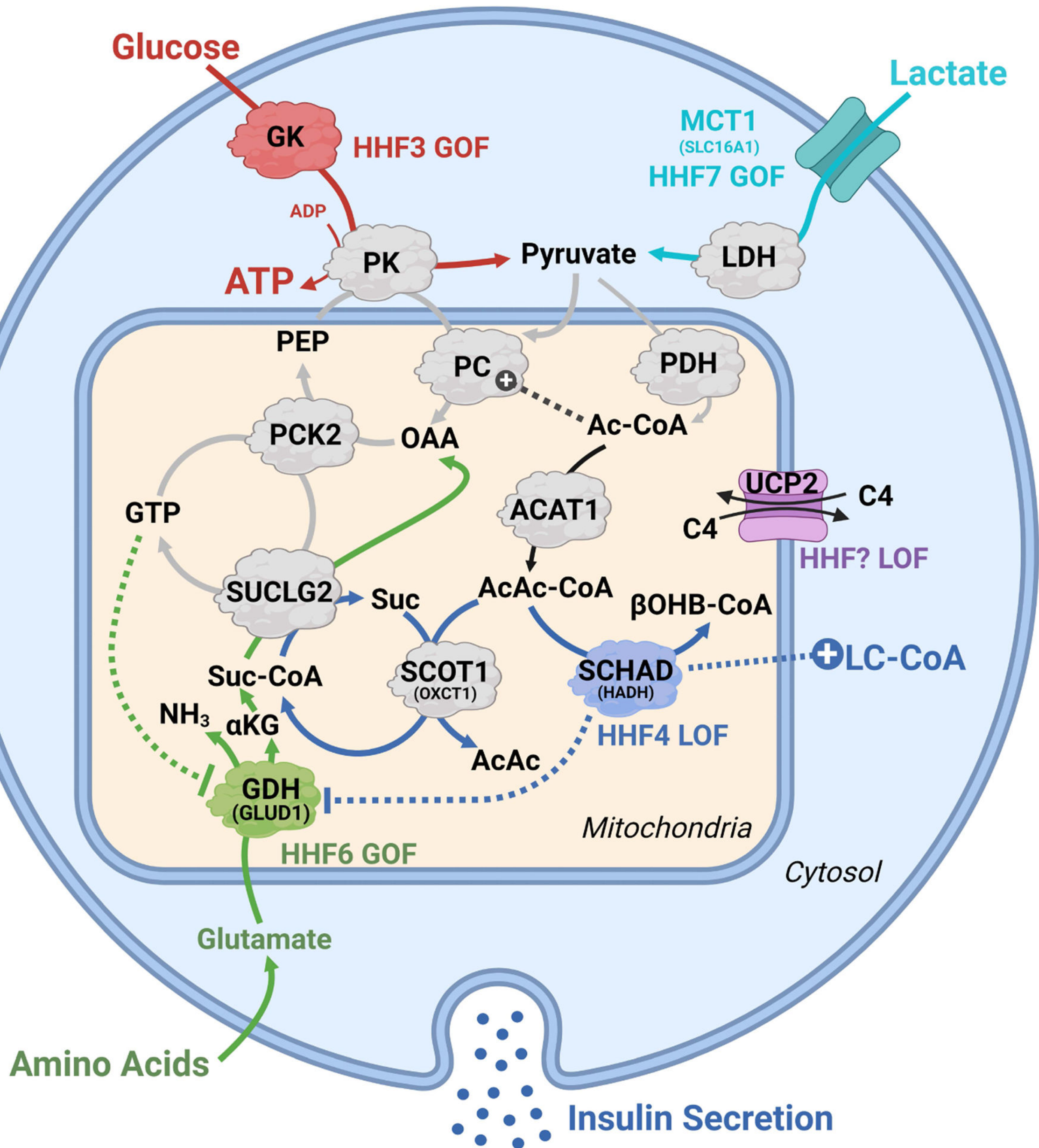

Figure 6. Familial hyperinsulinemic hypoglycemia (HHF): human genetic clues to the metabolic mechanisms of insulin secretion.

Several inborn errors of metabolism associated with congenital hyperinsulinemia intersect with pathways that inappropriately increase insulin release. Gain-of-function (GOF) mutations in glucokinase (*red*, HHF3) and ectopic expression of the lactate transporter (*teal*, MCT1/HHF7) increase pyruvate delivery to the mitochondria. GOF in glutamate dehydrogenase (*green*, GLUD1/HHF6), from loss of mitochondrial GTP inhibition, increases glutamate anaplerosis by providing α-ketoglutarate (αKG), mitochondrial GTP and mitochondrial PEP in the setting of a protein rich meal. Loss-of-function (LOF) of SCHAD (*blue*, HADH/HHF4) promotes hyperinsulinemia via three potential mechanisms: restriction of AcAc-CoA clearance to β-hydroxybutryate-CoA (βOHB-CoA) to favor succinyl-CoA generation by SCOT1/OXCT1, promoting activity of the PEP cycle; the

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clearance of LC-CoAs to AcCoA, thus favoring LC-CoA accumulation; disinhibition of GDH to favor anaplerosis via provision of αKG. A novel HHF has been proposed from LOF of the four carbon (C4) carboxylate exchanger (*purple*, UCP2), which may promote hyperinsulinemia by altering anaplerosis-cataplerosis and/or Ψ_m. *Abbreviations:* ACAT1, acetyl-CoA acetyltransferase 1; Ac-CoA, acetyl-CoA; AcAcCoA, acetoacetyl-CoA; GDH, glutamate dehydrogenase; GK, glucokinase; αKG, α-ketoglutarate; LDH, lactate dehydrogenase; LC-CoA, long chain acyl-CoA; MCT1, lactate/pyruvate transporter; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PC, pyruvate carboxylase; PCK2, mitochondrial PEP carboxykinase; PDH, pyruvate dehydrogenase; PK, pyruvate kinase; SUCLG2, GTP specific succinyl-CoA synthase; SCHAD, short-chain acyl-CoA dehydrogenase/HADH; SCOT1, succinyl-CoA:3-ketoacid coenzyme A transferase/OXCT1; UCP2, uncoupling protein 2.

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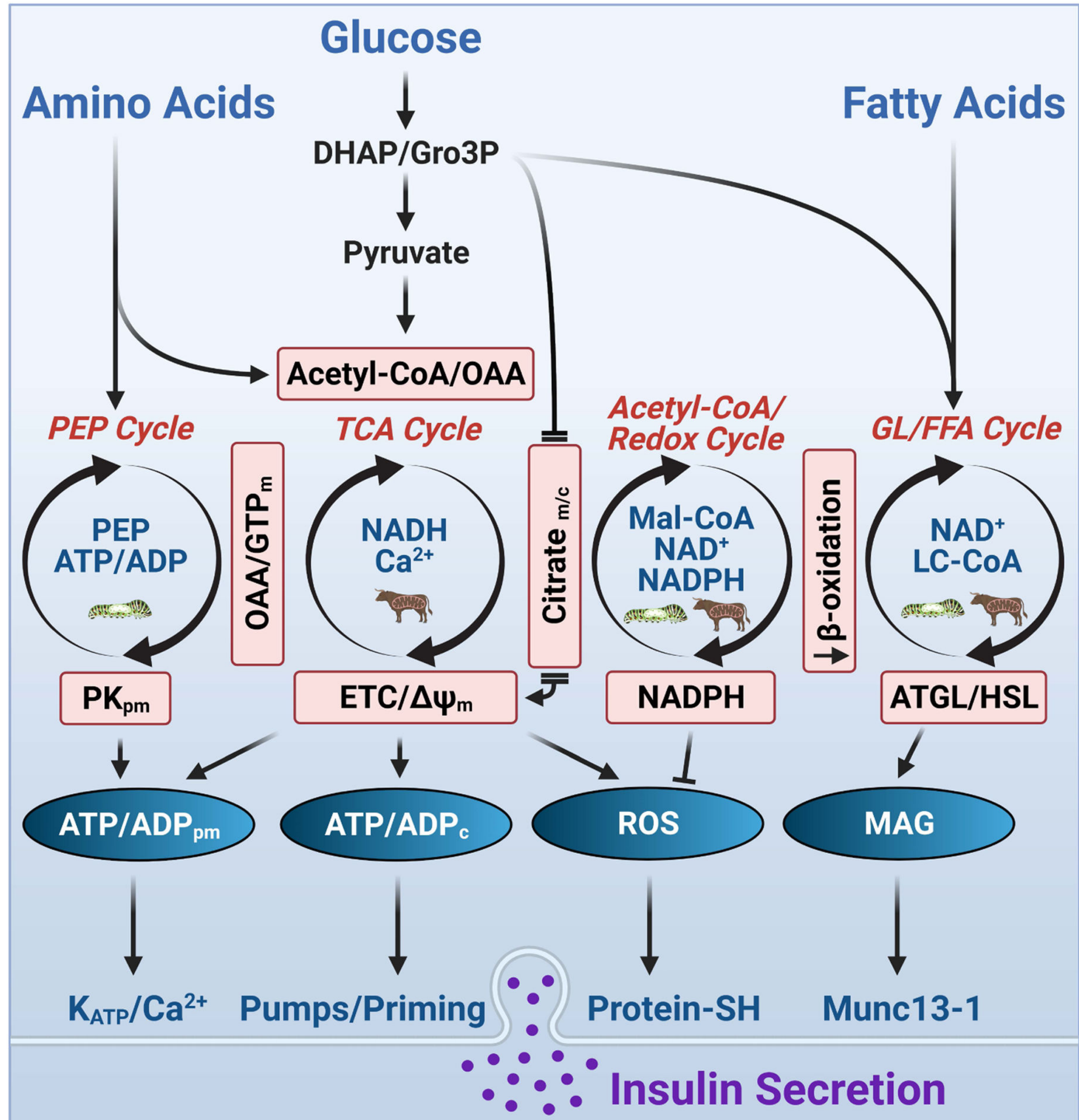

Figure 7. Integrated metabolic cycles of nutrient induced insulin secretion.

Glucose metabolism in the β-cell drives the oscillatory activity of four interconnected metabolic cycles (PEP, TCA, Acetyl-CoA/Redox, and GL/FFA) that produce regulatory metabolic coupling factors (*blue text within the cycles*) and effectory metabolic coupling factors (*white text within ovals*) controlling the distal steps of insulin secretion. The relationships of each of these cycles with the highest activities of $Mito_{Cat}$ and $Mito_{Ox}$ are shown within the cycles. Some amino acids, like alanine and glutamine, feed the Krebs cycle via the production of Ac-CoA, OAA, and αKG. The TCA and PEP cycles are interlinked by OAA and mitochondrial GTP; the TCA and Ac-CoA/redox cycles are interlinked by citrate and other TCA cycle intermediates; the Ac-CoA/redox and GL/FFA cycles are interlinked by citrate-derived malonyl-CoA, which inhibit fat oxidation, thus

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preventing the removal of GL/FFA cycle intermediates. FFA promotes insulin secretion via lipolysis derived MAG, which activates the exocytotic effector Munc13–1, and also via the plasma membrane receptor FFAR1 (*not shown*). NADPH prevents excessive ROS accumulation and possibly excessive insulin secretion as well. *Abbreviations:* ATGL, adipose triglyceride lipase; DHAP, dihydroxyacetone phosphate; GTP_m, mitochondrial GTP; Gro3P, glycerol 3-phosphate; GL/FFA, glycerolipd/free fatty acid; HSL, hormone sensitive lipase; LC-CoA, long-chain acyl-CoA; Mal-CoA, malonyl-CoA; MAG, monoacylglycerol; OAA, oxaloacetate; PEP, phosphoenolpyruvate.