Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion

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MacDonald, Michael J., Leonard A. Fahien, Laura J. Brown, Noaman M. Hasan, Julian D. Buss, and Mindy A. Kendrick. Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. Am J Physiol Endocrinol Metab 288: E1–E15, 2005; doi:10.1152/ajpendo.00218.2004.—The importance of mitochondrial biosynthesis in stimulus secretion coupling in the insulin-producing β-cell probably equals that of ATP production. In glucose-induced insulin secretion, the rate of pyruvate carboxylation is very high and correlates more strongly with the glucose concentration the β-cell is exposed to (and thus with insulin release) than does pyruvate decarboxylation, which produces acetyl-CoA for metabolism in the citric acid cycle to produce ATP. The carboxylation pathway can increase the levels of citric acid cycle intermediates, and this indicates that anaplerosis, the net synthesis of cycle intermediates, is important for insulin secretion. Increased cycle intermediates will alter mitochondrial processes, and, therefore, the synthesized intermediates must be exported from mitochondria to the cytosol (cataplerosis). This further suggests that these intermediates have roles in signaling insulin secretion. Although evidence is quite good that all physiological fuel secretagogues stimulate insulin secretion via anaplerosis, evidence is just emerging about the possible extramitochondrial roles of exported citric acid cycle intermediates. This article speculates on their potential roles as signaling molecules themselves and as exporters of equivalents of NADPH, acetyl-CoA and malonyl-CoA, as well as α-ketoglutarate as a substrate for hydroxylases. We also discuss the “succinate mechanism,” which hypothesizes that insulin secretagogues produce both NADPH and mevalonate. Finally, we discuss the role of mitochondria in causing oscillations in β-cell citrate levels. These parallel oscillations in ATP and NAD(P)H. Oscillations in β-cell plasma membrane electrical potential, ATP/ADP and NAD(P)/NAD(P)H ratios, and glycolytic flux are known to correlate with pulsatile insulin release. Citrate oscillations might synchronize oscillations of individual mitochondria with one another and mitochondrial oscillations with oscillations in glycolysis and, therefore, with flux of pyruvate into mitochondria. Thus citrate oscillations may synchronize mitochondrial ATP production and anaplerosis with other cellular oscillations.

citrate oscillations; anaplerosis; insulin release; NADPH; pyruvate carboxylase; cataplerosis; succinate mechanism
between their possible direct initiating vs. supporting roles, we will not attempt to distinguish between the two concepts.

**EVIDENCE FOR MITOCHONDRIAL FACTORS IN ADDITION TO ATP**

ATP is the only mitochondrial factor known with certainty to couple metabolism to insulin exocytosis. An increase in the ATP-to-ADP (ATP/ADP) ratio acting on the sulfonylurea receptor (1) and the ATP-dependent potassium channel (K\textsubscript{ATP}) in the β-cell plasma membrane causes membrane depolarization. This opens a voltage-sensitive calcium channel in the plasma membrane, and the resulting influx of calcium increases cytosolic calcium, which promotes the exocytosis of insulin granules (5) (Fig. 1). However, as will be discussed, it is clear that more than the K\textsubscript{ATP} channel is involved in insulin secretion. The work of Henquin et al. (39) and others (reviewed in Ref. 108) showed that fuel secretagogues can stimulate insulin release independently of the K\textsubscript{ATP} channel. Although ATP may participate in the K\textsubscript{ATP} channel-independent pathway, there is evidence that mitochondrial factors in addition to ATP (inter)act concomitantly with ATP to stimulate or enhance insulin secretion. The evidence for this idea comes from the fact that the β-cell has a tremendous capacity for anaplerosis (58, 59, 61).

There are two phases of insulin secretion. The first phase starts within seconds of an increased level of a fuel secretagogue making contact with the β-cell and has been called the triggering phase. In this phase, a sharp peak of insulin is released, probably from insulin granules stored immediately next to the plasma membrane, and is believed to be due to an increase in cellular calcium. In contrast, the second phase of insulin release is of much longer duration and is qualitatively the more important phase of insulin secretion. The second phase also requires calcium and ATP and has been called the amplification phase (39). Insulin secretagogues that do not affect metabolism, such as high potassium or arginine, can trigger the first phase of insulin release and raise intracellular calcium but cannot cause the second phase of insulin secretion. Because only fuel secretagogues can cause both the first and second phases of insulin secretion, this has been taken as evidence that the second phase is dependent on more products of fuel metabolism than just ATP (39, 108).

**PYRUVATE CARBOXYLATION DRIVES THE HIGH RATE OF ANAPLEROSIS IN THE GLUCOSE-STIMULATED β-CELL AND DEMONSTRATES THAT MITOCHONDRIA PRODUCE MORE THAN ATP**

The β-cell possesses a high level of the anaplerotic mitochondrial enzyme pyruvate carboxylase (13, 21, 54, 61, 62, 69, 76, 80, 81, 103), about equal to that in the gluconeogenic organs, liver and kidney (61). However, unlike these tissues, the β-cell cannot carry out gluconeogenesis from the oxaloacetate generated by pyruvate carboxylase because it lacks the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (38, 68, 80) and fructose biphosphatase (M. J. MacDonald, unpublished data). This suggests a novel and interesting purpose for anaplerosis in the β-cell. We know that anaplerosis is important for insulin secretion for several reasons. It has been demonstrated by using the 14CO\textsubscript{2} ratios method that ~50% of glucose-derived pyruvate enters mitochondrial metabolism via carboxylation catalyzed by pyruvate carboxylase (46, 58, 59, 60, 61). The other one-half of the glucose-derived pyruvate enters mitochondrial metabolism via decarboxylation to acetyl-CoA catalyzed by the pyruvate dehydrogenase complex (46, 58, 59, 60, 61). We have shown that the rate of pyruvate carboxylation correlates better than the rate of its decarboxylation with the concentration of glucose that the β-cell is exposed to and thus also with the rate of insulin secretion (59).
In addition, it has been shown that a large amount of glucose-derived pyruvate cycles through the pyruvate carboxylase reaction and that this cycling is proportional to the glucose responsiveness of insulin secretion from various INS-1 cell lines (55).

If ATP production alone is sufficient for stimulating insulin secretion, pyruvate carboxylase would not need to be present in the β-cell, because the decarboxylation pathway alone should be sufficient to stimulate insulin secretion. (Many metabolically active tissues, such as cardiac muscle, possess little or no pyruvate carboxylase.) The metabolism of acetyl-CoA in the citric acid cycle provides >95% of the ATP that a cell produces. Acetyl-CoA metabolism produces mitochondrial NADH, which is oxidized by the mitochondrial respiratory chain to supply the energy for ATP formation (Figs. 2 and 3). The fact that the carboxylation pathway is so active in the β-cell suggests that anaplerosis (the net synthesis of citric acid cycle intermediates and not simply their formation and utilization in the citric acid cycle) is important for insulin secretion.

In each turn of the citric acid cycle, carbon entering the cycle as the two carbon acetate units of acetyl-CoA is balanced by the release of two molecules of CO₂. Thus, with pyruvate decarboxylation and oxidation of acetyl-CoA, the cycle intermediates act somewhat like catalysts for the oxidation of acetyl units, and their levels should remain relatively constant. In contrast, the carboxylation of pyruvate can increase the intra-

Fig. 2. Mitochondrial schematic showing respiration, the citric acid cycle, and transporters of metabolites across the mitochondrial inner membrane. Respiration via the oxidation of NADH and flavoproteins pumps protons (H⁺) out of mitochondria. The diffusion of protons back across the inner mitochondrial membrane powers ATP synthesis. Metabolism of substrates in the TCA cycle reduces NAD to NADH and reduces flavoproteins, e.g., succinate dehydrogenase. The mitochondrial inner membrane is impermeable to many metabolites, and, therefore, transporters have evolved to carry metabolites across the inner membrane, frequently in exchange for another metabolite. Some carriers are capable of transporting more than one metabolite, and some metabolites are transported on more than one carrier. The red Xs at top right indicate that oxaloacetate (OAA), NAD(P)(H), and acetyl-CoA (Ac-CoA) are not transported as such across the inner membrane and must be transported either as parts of other molecules (oxaloacetate and acetyl-CoA) or as equivalents in the form of reduced or oxidized metabolites [NAD(P)(H)].
mitochondrial level of citric acid cycle intermediates. Because most citric acid cycle intermediates directly inhibit or activate various enzymes of the cycle (examples are discussed in Ref. 73), the potential for increases in their concentrations means that cycle function would be altered if excess amounts of intermediates were not exported to the cytosol (catablerosis). In addition, increased levels of many intermediates would alter the mitochondrial NAD-to-NADH (NAD/NADH) ratio, and this would also alter the flux through the cycle. Experimental studies have confirmed that cycle intermediates are exported from β-cell mitochondria (57, 61, 64, 65). Because a cell cannot afford to waste energy by synthesizing unutilized intermediates, this suggests that the metabolites are exported from mitochondria for specific purposes, such as signaling and supporting insulin secretion.

All fuel insulin secretagogues are capable of anaplerosis (Fig. 4). It seems that, in order for a fuel to be a secretagogue, it must be capable of forming either pyruvate or α-ketoglutarate and, in addition, fulfill other requirements. Fuels that cannot form pyruvate directly, such as methyl esters of succinate, cannot be anaplerotic via their conversion to malate in the mitochondrion and the export of malate to the cytosol where it is converted to pyruvate by malic enzyme. Pyruvate is then taken up into the mitochondrion where it can be carboxylated to oxaloacetate or decarboxylated to acetyl-CoA (Fig. 4, reactions 21, 8, and 9 followed by reactions 12, 1, and 2). Oxaloacetate can be converted to malate, fumarate, succinate, and succinyl-CoA in the reverse of the reactions of the citric acid cycle (Fig. 4, reactions 10, 9, 8, and 7). Fuels that increase α-ketoglutarate directly (Fig. 4, reactions 19, 20, and 18) can be converted to the other intermediates of the cycle in the forward reactions of the cycle (Fig. 4, reactions 6–10). In addition, α-ketoglutarate can be converted to isocitrate because the mitochondrial NADP-linked isocitrate dehydrogenase reaction is reversible in the β-cell (MacDonald MJ, unpublished data), as in some other cells (17, 24). Isocitrate can then be converted to citrate in the aconitase reaction, which is freely reversible (Fig. 4, reactions 5 and 4). Thus all citric acid cycle intermediates can be synthesized from α-ketoglutarate but perhaps not as efficiently as from pyruvate. Leucine and α-ketoisocaproate (α-ketoisocaproic acid; KIC) can be metabolized directly to acetyl-CoA through a series of steps without conversion to pyruvate (Fig. 4, reaction 19 and the 5 reactions indicated by no. 25). The acetyl-CoA can combine with oxaloacetate for the synthesis of citrate and any citric acid cycle intermediate (Fig. 4, reactions 3–10).

INITIAL STAGES OF METABOLISM OF FUEL SECRETAGOGUES

Surprisingly, there are only a few fuel secretagogues, and only the initial reactions of fuel secretagogue metabolism in the β-cell are understood. Briefly, the current consensus combined with some of our own thoughts about their metabolism is as follows.

Glucose. Glucose is the most potent physiological insulin secretagogue, and it stimulates insulin release by its metabolism via aerobic glycolysis. It is metabolized to pyruvate, the terminal product of aerobic glycolysis, from which all citric acid cycle intermediates can be formed. Both decarboxylation and carboxylation of pyruvate are necessary for glucose-induced insulin secretion (Fig. 4, reactions 1 and 2), as was discussed above.

Leucine. Leucine is about one-third as potent an insulin secretagogue as glucose (28). Leucine allosterically activates glutamate dehydrogenase, thus enhancing conversion of glutamate to α-ketoglutarate (Fig. 4, reaction 18), which can be metabolized in part of the cycle (Fig. 4, reactions 6–10) to form ATP, but less than can be obtained with metabolism of pyruvate-derived acetyl-CoA in a complete turn of the cycle. As mentioned in the previous section, α-ketoglutarate can be converted in the reverse direction of the cycle to isocitrate and citrate (Fig. 4, reactions 4 and 5). In addition to enhancing endogenous glutamate metabolism, leucine can be converted to KIC, which can be metabolized to hydroxymethylglutaryl-CoA.
and acetyl-CoA (Fig. 4, reaction 19 and the 5 reactions indicated by no. 25) (31, 33, 79).

Glutamine. Glutamine by itself is not an insulin secretagogue. However, the combination of glutamine and leucine is about as potent as glucose in stimulating insulin release (28, 72, 87). This is most likely due to the activation of glutamate dehydrogenase by leucine in the presence of a surfeit of glutamine-derived glutamate. In addition, it is possible that the metabolism of leucine combines with that of the glutamate to create a synergistic stimulus on insulin secretion.

2-Aminobicyclo[2.2.1]heptane-2-carboxylic acid. Although 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) is a nonmetabolizable leucine analog that is as potent as leucine in activating glutamate dehydrogenase by leucine in the presence of a surfeit of glutamine-derived glutamate. In addition, it is possible that the metabolism of leucine combines with that of the glutamate to create a synergistic stimulus on insulin secretion. 2-Aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) is a nonmetabolizable leucine analog that is as potent as leucine in activating glutamate dehydrogenase by leucine in the presence of a surfeit of glutamine-derived glutamate. In addition, it is possible that the metabolism of leucine combines with that of the glutamate to create a synergistic stimulus on insulin secretion.

KIC. Interestingly, KIC, the first metabolite of leucine, is much more potent an insulin secretagogue than leucine and is about as potent a secretagogue as glucose. KIC probably stimulates insulin secretion via its transamination with endogenous glutamate to form leucine and α-ketoglutarate (31, 51) (Fig. 4, reaction 20) and via α-ketoglutarate’s metabolism, as well as by the conversion of KIC to acetyl-CoA, (Fig. 4, reaction 19 plus the 5 reactions indicated by no. 25). The leucine derived from transamination can enhance glutamate metabolism by activation of glutamate dehydrogenase, as mentioned above. The reason KIC is a much more potent secretagogue than leucine might be because metabolism of KIC supplies acetocetate and acetyl-CoA at a faster rate than does metabolism of leucine.

Methyl esters of succinate. Methyl esters of succinate are about one-third as potent as glucose as insulin secretagogues and are about equal to leucine in potency (28, 34, 58, 59, 70, 71, 74, 75, 85, 86, 121) in fresh rat pancreatic islets. Methyl esters of succinate probably stimulate insulin release by producing both oxaloacetate and acetyl-CoA. After hydrolysis of the ester to succinate and conversion of the succinate to fumarate and then malate in the malic enzyme reaction [Fig. 4, reactions 21, 8, 9 (or 22), and 12], Pyruvate can then be taken up into the mitochondrion and carboxylated to oxaloacetate (Fig. 4, reaction 2) or decarboxylated to acetyl-CoA (Fig. 4, reaction 1). The carboxylation route of conversion of malate to oxaloacetate (Fig. 4, reactions 12 and 2) generates cytosolic NADPH and, unlike the direct

Fig. 4. Pathways of secretagogue metabolism and anaplerosis/cataplerosis in the β-cell. Individual reactions are referred to by their nos. when mentioned in the text. At bottom left, the 5 steps of α-ketoisocaproic acid (KIC) metabolism indicated by no. 25 are its conversion to isovaleryl-CoA, methylcrotonyl-CoA, methylglutaconyl-CoA, hydroxymethylglutaryl-CoA, and acetyl-CoA plus acetoacetate catalyzed by, respectively, the branched-chain ketoacid dehydrogenase complex, isovaleryl-CoA dehydrogenase, methylcrotonyl-CoA carboxylase, methylglutaconyl-CoA hydratase, and hydroxymethylglutaryl-CoA lyase.
conversion of malate to oxaloacetate (Fig. 4, reaction 10), does not generate mitochondrial NADH. Succinate-derived oxaloacetate can then condense with acetyl-CoA derived from the pyruvate (Fig. 4, reaction 3) to form any citric acid cycle intermediate (58, 63, 64, 86). Interestingly, esters of other citric acid cycle intermediates do not stimulate insulin release (75).

A possible explanation for this fact is that succinate is ideally situated among metabolic pathways to supply both NADPH and malonate as discussed below (see Succinate Mechanism).

Fatty acids. The consensus about the effect of fatty acids such as palmitate on insulin release is that they potentiate glucose-induced insulin secretion when added acutely to islets, but they cannot stimulate insulin release by themselves (18, 34, 96). The long-term effect of incubation of islets with palmitate is a reduction in glucose-induced insulin release (the lipotoxicity hypothesis) (9, 34, 92, 114). Fatty acids are metabolized to acetyl-CoA (Fig. 2), which can be metabolized in the citric acid cycle, but acetyl-CoA cannot be anaplerotic unless there is a source of oxaloacetate to combine with it. Perhaps this is why fatty acids do not, by themselves, stimulate insulin release.

EXPORT OF CITRIC ACID CYCLE INTERMEDIATES
FROM MITOCHONDRIA

When pyruvate is supplied to suspensions of β-cell mitochondria, the export of malate is increased markedly (64, 65), and the export of other citric acid cycle intermediates increases to various extents. Although the export of citrate is not increased as much as malate, evidence from intact cell experiments suggests that anaplerosis of citrate is necessary for insulin secretion (19, 29, 30, 97, 102). We observed that most of the citrate and malate are extramitochondrial in suspensions of β-cell mitochondria (61), and others have previously made the same observation with mitochondria from other tissues (44, 113). The extramitochondrial location of citrate may enable it to act as a communicator among individual mitochondria to synchronize their metabolism with one another and to synchronize mitochondrial processes with other cellular processes (73), as discussed in the last section of this perspective.

The high rate of carboxylation of pyruvate in the β-cell indicates that it is possible that each citric acid cycle intermediate is synthesized in amounts that exceed its consumption in the citric acid cycle, and the excess is exported from the mitochondria to the cytosol, where it has a role in stimulating or supporting insulin secretion. Obviously, some intermediates cannot cross the mitochondrial inner membrane and are exported in the form of another intermediate and are converted back to the same intermediate outside the mitochondria. For example, oxaloacetate cannot cross the mitochondrial inner membrane but can be exported as malate, fumarate, citrate, or isocitrate (Figs. 2 and 4, reactions 10, 9, 22, 12, 13, and 26), and acetyl-CoA is also not transported as such but can be transported as citrate (Figs. 2 and 4, reaction 13.) The following sections discuss the possible extramitochondrial roles for each cycle intermediate. Less will be said about well-studied extramitochondrial mechanisms and more will be said about possible actions for which evidence is emerging.

Malate. The conversion of oxaloacetate to malate and the export of malate to the cytosol form a pyruvate-malate shuttle (61), permitting the export of NADPH equivalents to the cytosol. Malate and NADP are converted to NADPH and pyruvate by malic enzyme. Pyruvate can then reenter mitochondrial pools (Fig. 4, reactions 10, 12, 1, and 2). The potential roles of NADPH are depicted (see Fig. 6) and discussed below.

Citrate and isocitrate. The citrate-pyruvate shuttle (21, 29, 30, 102, 103) utilizes portions of the malate-pyruvate shuttle and additional pathways to export both NAD equivalents and NADPH equivalents from mitochondria. Exported citrate is cleaved to acetyl-CoA and oxaloacetate by ATP citrate lyase in the cytosol. Cytosolic malate dehydrogenase catalyzes the conversion of exported oxaloacetate and cytosolic NADH to malate and NAD, thus exporting NAD equivalents from the mitochondria. The malate can be converted to pyruvate by malic enzyme, thus exporting NADPH equivalents to the cytosol (Fig. 4, reactions 13, 11, and 12). The acetyl-CoA can be carboxylated to malonyl-CoA, which can be used in the synthesis of lipids. Malonyl-CoA itself is believed also to have a signaling role (12, 18, 23, 29, 30, 102, 103) because it inhibits carnitine palmitoyl-CoA transferase-1, an enzyme that is required for the transport of long-chain acyl-CoAs into mitochondria, where they are metabolized. Inhibition of this enzyme should increase the level of long-chain acyl-CoAs in the cytosol, where these molecules are believed to have numerous signaling activities (Fig. 5). The proposed signaling roles of long-chain acyl-CoAs include influences on the K_ATP channel, glucokinase, ATPases, and vesicular trafficking (18). The malonyl-CoA hypothesis, originally championed by the late Dennis McGarry (Chen et al., Ref. 15) and by Corkey and coworkers (18, 19) and Prentki et al. (97), is one of the more popular and well-studied hypotheses about the role of anaplerotic products in insulin secretion. Evidence in support of this hypothesis abounds, and, as with all popular hypotheses, there is some evidence against it (18, 39, 90). Many excellent reviews of this hypothesis have been published (18, 23, 96).

α-Ketoglutarate. α-Ketoglutarate, when it is exported from mitochondria, can act as a transporter of oxidizing equivalents of NAD out of mitochondria in the malate-aspartate shuttle (Fig. 4, reactions 10, 11, 23, and 16) and of NADP into mitochondria in the isocitrate shuttle, which transports NADP out of mitochondria (Fig. 4, reactions 5, 15, 23, 11, 10, and 16). There are no fuel secretagogues that cannot produce α-ketoglutarate, as described below. α-Ketoglutarate almost certainly has a fuel function, but evidence is emerging that α-ketoglutarate has signaling roles beyond its acting as a metabolite in the citric acid cycle and as a precursor for anaplerosis of other cycle intermediates.

α-Ketoglutarate is a substrate for α-ketoglutarate hydroxylases (dioxygenases). These enzymes use ferrous ion, molecular oxygen, and a reducing agent such as ascorbate to catalyze hydroxylation of prolyl, lysyl, or aspartyl (and asparagine) residues in proteins. Phytanoyl-CoA hydroxylase is also a member of this enzyme family.

By using RT-PCR, we have detected transcripts for propane and lysine hydroxylases as well as phytanoyl-CoA hydroxylase in human and rat pancreatic islets and INS-1 cells. We have also found enzyme activity and immunoreactivity for prolyl hydroxylases in human and rat pancreatic islets and INS-1 cells. On the basis of our own RT-PCR studies and from histochemical studies of the pancreas performed by J. Dinchuk (personal communication), very little or no aspartyl hydroxylase is present in the β-cell. Although the α-ketoglutarate...
hydroxylases are known to participate in chronic processes, such as modification of collagen, or nonacute regulatory functions, such as the modification by prolyl hydroxylases of the transcription of a number of genes regulated by the hypoxia-inducible factor (HIF) system, we have evidence that these hydroxylases participate in acute processes in the β-cell. Inhibitors of these enzymes decrease insulin release from islets in proportion to their ability to inhibit the hydroxylation of endogenous substrates as well as of generic peptides by extracts of β-cells. These data suggest that hydroxylation of β-cell proteins is occurring during fuel-induced insulin secretion.

LESSONS LEARNED FROM MOUSE ISLETS

Differences between mouse and rat pancreatic islets in their responses to esters of succinate provide additional evidence that anaplerosis of α-ketoglutarate or citrate and isocitrate is necessary for insulin secretion. Succinic acid methyl esters are potent insulin secretagogues in rat pancreatic islets, but not in mouse pancreatic islets, which lack malic enzyme and cannot form pyruvate from the succinate hydrolyzed from these esters (63). Thus, in the mouse islet, succinate metabolism can produce fumarate, malate, and oxaloacetate in the forward direction of the citric acid cycle but not acetyl-CoA, from which citrate, isocitrate, and α-ketoglutarate can be formed (reactions 8 and 9 in Fig. 4 can occur, but reaction 12 plus reactions 1–6 cannot occur). Because the succinate thio kinase reaction is reversible, succinate can be converted to succinyl-CoA in the reverse direction of the citric acid cycle (Fig. 4, reaction 6). However, the irreversibility of the α-ketoglutarate dehydrogenase reaction (Fig. 4, reaction 6) prevents succinyl-CoA from being converted to α-ketoglutarate, isocitrate, or citrate. Glucose and any secretagogue that augments α-ketoglutarate production are equally potent in mouse islets and rat islets. Isocitrate and citrate can be formed from α-ketoglutarate in the reverse of the isocitrate dehydrogenase and aconitate reactions (Fig. 4, reactions 5 and 4 or 15 and 26). Therefore, anaplerosis of one or all of the intermediates (citrate, isocitrate, and α-ketoglutarate) must be necessary for insulin secretion.

IS GLUTAMATE A MESSENGER IN THE β-CELL?

It has been proposed that glutamate formed from theamination of α-ketoglutarate in the reverse direction of the glutamate dehydrogenase reaction signals insulin secretion, and elegant imaging methods have shown that glutamate might have stimulatory effects on insulin secretory granules (84). However, almost all studies of glutamate indicate that glutamate is a fuel secretagogue from which other metabolites can be formed and not an anaplerotic product in the β-cell. Our opinion is that, if glutamate is a messenger in insulin secretion, it must be recruited from preexisting intracellular stores because the preponderance of evidence indicates that β-cell glutamate is not increased by insulin secretagogues. Several laboratories have been unable to show that glucose, the most potent insulin secretagogue, significantly increases glutamate in islets or INS-1 cells (8, 64, 65, 72, 87). In addition, the basal level of glutamate is very high in the islet (64, 72), as in many tissues, and it seems unlikely that increasing it to even higher levels would create a signal. Perhaps the strongest evidence against glutamate being a signal is that glutamine by itself cannot stimulate insulin release from islets, even though it increases islet glutamate levels up to 10-fold (53, 64, 65, 72, 87).

Consideration of the kinetic properties of glutamate dehydrogenase also suggests that net synthesis of glutamate is unlikely. α-Ketoglutarate is inhibitory to glutamate dehydrogenase, indicating that flux through the reaction catalyzed by the enzyme should be in the direction of α-ketoglutarate and its removal by metabolism. In addition, several human patients with hyperinsulinism, hyperammonemia, and hypoglycemia have been reported to have a mutation in the GTP-binding region of glutamate dehydrogenase (106, 107), which results in severely decreased allosteric inhibition of glutamate dehydrogenase by GTP. This results in constitutive activation of islet glutamate dehydrogenase and confirms the important role of the enzyme in insulin secretion. Maintaining normal islets in the presence of glucose inhibits leucine-induced insulin release (31, 51, 74, 79), probably by increasing GTP (106, 107) and inhibition of glutamate dehydrogenase. Glucose would not stimulate insulin secretion via glutamate dehydrogenase if it
caused inhibition of the enzyme. This is consistent with evidence obtained from our studies of β-cell mitochondria that indicates that flux through glutamate dehydrogenase is quiescent during glucose-induced insulin secretion (72).

**NAD(H) SHUTTLES**

The activities of the mitochondrial NAD(H) shuttles are particularly high in the β-cell, indicating they are important for insulin secretion, and have been studied extensively. The glycerol-phosphate shuttle (Fig. 3) and the malate-aspartate shuttle (Fig. 4, reactions 10, 16, 11, and 23) oxidize NADH formed in the cytosol by glycolysis and export NAD equivalents back to the cytosol (10, 11, 26, 56, 57, 77, 78, 101). The malate-aspartate shuttle partially overlaps or intersects with several anaplerotic pathways (Fig. 4), such as glutamate metabolism and the citrate and isocitrate shuttles, but the glycerol-phosphate shuttle does not.

**NADPH**

The production of NADPH equivalents in mitochondria might be especially important in the β-cell because many studies have shown that, in rat and mouse pancreatic islets, very little glucose is metabolized through the pentose phosphate shunt (see Ref. 59 and references therein), the pathway that forms a large amount of NADPH in the cytosol in many cells. NADPH equivalents can be exported to the cytosol as malate (Fig. 4, reactions 2, 10, and 12) and citrate (Fig. 4, reactions 13, 11, 12, 1, 2, and 3) in human and rat islets and as isocitrate (Fig. 4, reactions 1, 2, 3, 4, 15, 23, 11, and 10) in islets of the mouse, rat, and human. In 1972, Ammon and Steinke (3) showed that giving 6-aminonicotinamide (6-AN), a compound that forms a metabolically inactive analog of NADPH in vivo, caused hyperglycemia in rats. Furthermore, glucose-induced (3) and amino acid-induced insulin releases (67) were inhibited in islets isolated from the 6-AN-treated rats. In addition, in 1977, Watkins and Moore (115) showed that NADPH is taken up by insulin granules of the toadfish islet and stimulates insulin release. Because of these findings, the potential roles of NADPH in insulin secretion are summarized (Fig. 6).

NADPH is a substrate for glutathione reductase, which is plentiful in the pancreatic islet (4, 48). Whether this enzyme plays a role in signaling insulin secretion or is present in the β-cell only to preserve the redox state of cellular thiols is unknown. In most cells, the enzyme is present in the cytosol, mitochondria, and endoplasmic reticulum, where its products very likely interact with the thioredoxin and thioredoxin reductase reactions also using NADPH as a cofactor. Numerous enzymes are likely regulated by the redox state of protein thiol groups. These include many of the glycolytic enzymes, which are more active when the ratio of reduced glutathione to oxidized glutathione is high (42, 45, 66).

N-ethylmaleimide (NEM) inactivates thiol groups. The fact that NEM-sensitive factor (NSF), a regulator of soluble NSF attachment protein receptors (SNAREs), is required for vesicular transport in many eukaryotic cells, including β-cells, suggests that thiol status could regulate movement of insulin granules. Glutathione also interacts with glutaredoxin, and the ratio of reduced to oxidized glutathione (GSH/GSSG) influences the ratio of reduced to oxidized glutaredoxin. Evidence is emerging that glutathionylation reversibly influences activities of enzymes. For example, glutathionylation inactivates α-ketoglutarate dehydrogenase, and glutaredoxin facilitates the GSH-dependent recovery of the enzyme activity (94). The GSH/GSSG ratio also influences the activity of protein disulfide isomerase (PDI) (6). PDI is known to be plentiful in rat β-cells, and we have detected transcripts for thioredoxin, thioredoxin reductase, and glutaredoxin in mouse islets with DNA microarrays (Brown LJ, unpublished data).

PDI has a number of functions, including influencing protein folding and acting as a chaperone for proteins synthesized in the endoplasmic reticulum. Protein folding in the endoplasmic reticulum is necessary for packaging of insulin into secretory granules. PDI is also the β-subunit for the prolyl hydroxylase family of enzymes that use α-ketoglutarate as a substrate, as mentioned above.

Interestingly, indirect evidence is emerging to suggest a role for thioredoxin in insulin secretion. High glucose is known to blunt the responsiveness of the β-cell to fuel secretagogues. Shalev et al. (105) found that transcripts for thioredoxin-interacting protein (Txnip), a protein that binds thioredoxin and lowers its activity, are induced 11-fold in human islets after prolonged incubation in the presence of high glucose. In addition, Hui et al. (40) have shown that mice deficient in Txnip have increased plasma insulin levels due to increased insulin secretion and low blood glucose levels during brief starvation. These inverse correlations between Txnip and insulin levels are consistent with the idea that thioredoxin activates or enhances insulin secretion.

NADPH is required for elongation of fatty acids, and evidence is emerging that fatty acid synthesis occurs in β-cells, as mentioned below. NADPH is a substrate for stearoyl-CoA desaturases, and mRNA transcripts for these enzymes have been found in islets (30). Our laboratory (48) has found their enzyme activity in islets, and Ramanadham et al. (99) have identified glycerolphospholipid products of these desaturases in INS-1 cells and islets (Fig. 6).

NADPH is a substrate for 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, an enzyme in the pathway of mevalonate formation that is needed for protein isoprenylation. Kowluw and coworkers (2, 47, 89) have found evidence for protein isoprenylation in islets. Mevalonate is believed to have additional unknown signaling properties, as discussed in sucinate mechanism (Fig. 7).

NADPH is also a substrate for quinone reductases and aldose/aldehyde reductases. These enzyme activities are very high in the islet, but they are not likely directly involved in insulin secretion, because potent inhibitors of these enzymes do not inhibit insulin release (48). It is possible these enzymes are present in islets to protect the cell against damaging peroxides. However, interestingly, voltage-gated K⁺ channel β-subunits belong to the aldehyde reductase family of proteins and are also present in the β-cell. Like the aldose and aldehyde reductases, these K⁺ channel β-subunits are ~30,000 Da in size and possess an oxidoreductase-binding site similar to that for NADPH in aldehyde reductases. We have found transcripts and immunoreactivity for these proteins in both human and rat pancreatic islets and in INS-1 cells (16, 48). Gulbis et al. (35) have crystallized these β-subunits and studied them by X-ray diffraction and observed NADPH to be present within the
native protein. This suggests the pyridine dinucleotide phosphate has the potential to act as a redox switch to regulate channel activity. Although the β-subunits have never been shown to possess aldehyde reductase enzyme activity per se, Bahring et al. (7) have mutated the substrate-binding site of the protein and shown that this alters the gating activity of the β-subunit (pore) of the channel. In the β-cell, these channels repolarize the plasma membrane after it has been depolarized by closure of the K<sub>ATP</sub> channel. Therefore, inactivating the voltage-gated channels should prolong the repolarization phase of the plasma membrane current and potentiate insulin secretion. Interestingly, it was recently shown that increasing the NADPH/NADP ratio in pancreatic β-cells prolongs the inactivation phase of these channels (82) (Fig. 6).

Finally, nitric oxide synthases use NADPH and are present in islets. Recent evidence suggests that nitric oxide synthases are directly involved in insulin secretion (91).

LIPOID SYNTHESIS

As mentioned above, citrate exported from mitochondria is a source of acetyl-CoA and malonyl-CoA used in lipid synthesis (Fig. 4, reactions 13 and 14, and Fig. 5). Until recently, traditional dogma held that lipid synthesis does not occur to a great extent in the islet. However, indirect evidence is emerging to indicate that it does occur (9, 30, 102, 114). Flamez et al. (30), using microarrays, found that glucose increases the level of many mRNAs, such as those for the sterol regulatory element-binding protein-1c (SREBP-1c), and multiple lipogenic enzymes, including those for the mevalonate pathway. β-cell SREBP-1c is increased in type 2 diabetes, and overexpression of it increases lipid synthesis, implicating the protein in the pathogenesis of β-cell lipotoxicity that occurs in type 2 diabetes (114). Others have found evidence for acylation of proteins involved with exocytosis, such as G proteins (47, 89,
and other proteins in the \( \beta \)-cell (109, 118). Recently, Itoh et al. (41) showed that free fatty acids, acting as a ligand and without covalent attachment, can alter G protein activity. Exactly how protein acylation affects insulin secretion is largely unknown (Fig. 5).

Fuel-derived synthesis of cholesterol (95) and phospholipids (100), including sphingolipids (50), may promote insulin exocytosis by stimulating insulin secretory granule packaging, assembly, and movements as well as fusion of the granules with the plasma membrane and extrusion of granular contents into the circulation. Although the apparent necessity for anaplerosis in the \( \beta \)-cell might suggest that synthesis of these molecules is directly from the added fuel, it is currently not known whether their formation is direct or whether the fuel stimulates their synthesis and release from preexisting stores of precursors. Membrane fluidity is regulated by the ratio of cholesterol to phospholipids and the ratio of saturated to unsaturated fatty acids incorporated into phospholipids (110). Alteration of this ratio has been implicated in various diseases, including type 2 diabetes (98). Stearoyl-CoA desaturases may make membranes more fluid by increasing their content of unsaturated fatty acids (98, 99), thus facilitating granule movement and fusion with the plasma membrane (Fig. 6).

**SUCCINATE MECHANISM**

We recently proposed the “succinate mechanism” of insulin release (27). According to this mechanism, when mevalonate is synthesized in islets, it or one of its metabolic products plays a major role in triggering and/or supporting insulin release. This concept is supported by experiments demonstrating that metabolites that are insulin secretagogues can also readily supply HMG-CoA reductase with its substrate NADPH and precursors for its other substrate, HMG-CoA (27, 28, 48, 51, 52, 61, 63, 64, 72) (Fig. 7).

Methyl esters of succinate are insulinotropic because they enter the cell and are converted into succinate. The succinate mechanism hypothesizes that all fuel secretagogues form NADPH and mevalonate. Individual reactions are referred to by their nos. when mentioned in the text.
succinate, unlike other esters of citric acid cycle intermediates or compounds structurally similar to succinate, are insulinotropic (28, 70, 71).

As was mentioned in previous sections, α-ketoglutarate can be formed from all fuel secretagogues. However, most metabolites that increase α-ketoglutarate production are not insulinotropic. The succinate mechanism proposes that, to be insulinotropic, the product metabolite must be capable of increasing both HMG-CoA and NADPH production. This may explain why enhancing the oxidative deamination of glutamate by glutamate dehydrogenase results in enhanced insulin release (28, 31, 36, 72, 89, 105), because both NADPH and α-ketoglutarate are produced in the glutamate dehydrogenase reaction. Thus leucine or leucine plus glutamine are insulinotropic because leucine allosterically activates glutamate dehydrogenase, and glutamine provides glutamate, a substrate of glutamate dehydrogenase. On the other hand, increasing the rate of pyruvate carboxylation, respectively, of pyruvate would be utilized to produce NADPH plus α-ketoglutarate in the combined citrate synthase, aconitase, and NADPH isocitrate dehydrogenase reactions (Fig. 4, reactions 3 and 4 or 26 plus 15).

**CITRATE OSCILLATIONS AS A SYNCHRONIZER OF MITOCHONDRIA AND A COORDINATOR OF MITOCHONDRIAL AND CELLULAR METABOLISM**

It is well known that β-cell glycolysis, plasma membrane activity, and ATP/ADP ratios oscillate in the β-cell and that these processes are likely in synchrony with oscillations of insulin release (20, 22, 32, 43, 93). If mitochondrial factors influence cellular metabolism, such as the ATP/ADP ratio modifying the activity of the KATP channel and the activities of contractile proteins that propel insulin granules to the plasma membrane, then ATP production and other signaling processes, including anaplerosis, need to be synchronized.

Recent work in our laboratory (73) has shown that citrate oscillates in suspensions of mitochondria from liver, pancreatic islets, and INS-1 cells supplied with pyruvate and in intact INS-1 cells supplied with glucose. Citrate oscillations were synchronous with oscillations in ATP and NAD (73). Interestingly, in suspensions of mitochondria from tissues as diverse as liver (113), pancreatic islets (61), and heart (49), most of the citrate is extramitochondrial. The extramitochondrial location of citrate suggests that citrate itself can establish communication among mitochondria within a cell (Fig. 8). Because citrate inhibits its own synthesis by inhibiting citrate synthase (Fig. 4,
and this makes the study of cataplerosis in the pancreatic islet cells a valuable research topic.

In conclusion, evidence overwhelmingly supports the idea that anaplerosis is important for insulin secretion and that the formation and export of anaplerotic metabolites from mitochondria correlate more with insulin secretion than does energy production (46, 55, 59, 61, 64). However, very little is known about the extramitochondrial actions of these products, and this makes the study of cataplerosis in the β-cell an exciting and fruitful area for future research. Recent exciting work even demonstrates unexpected direct signaling roles for two citric acid cycle intermediates, α-ketoglutarate and succinate, in the kidney. At <100 μM concentrations, these two metabolites act as ligands for kidney G protein-coupled receptors. In addition, infusions of succinate into mice raise their blood pressure via the renin-angiotensin system (37). Clearly, there remains a lot to be learned about the role of the citric acid cycle intermediates as intracellular and possibly even intercellular messengers.

ACKNOWLEDGMENTS

We thank Robert J. Gordon for excellent graphics work.

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This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-28348 and the Oscar C. Rennebohm Foundation.


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