



## Review

# The mitochondrial isoform of phosphoenolpyruvate carboxykinase (PEPCK-M) and glucose homeostasis: Has it been overlooked? ☆



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## ABSTRACT

**Background:** Plasma glucose levels are tightly regulated within a narrow physiologic range. Insulin-mediated glucose uptake by tissues must be balanced by the appearance of glucose from nutritional sources, glycogen stores, or gluconeogenesis. In this regard, a common pathway regulating both glucose clearance and appearance has not been described. The metabolism of glucose to produce ATP is generally considered to be the primary stimulus for insulin release from beta-cells. Similarly, gluconeogenesis from phosphoenolpyruvate (PEP) is believed to be the primary pathway via the cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK-C). These models cannot adequately explain the regulation of insulin secretion or gluconeogenesis.

**Scope of review:** A metabolic sensing pathway involving mitochondrial GTP (mtGTP) and PEP synthesis by the mitochondrial isoform of PEPCK (PEPCK-M) is associated with glucose-stimulated insulin secretion from pancreatic beta-cells. Here we examine whether there is evidence for a similar mtGTP-dependent pathway involved in gluconeogenesis. In both islets and the liver, mtGTP is produced at the substrate level by the enzyme succinyl CoA synthetase (SCS-GTP) with a rate proportional to the TCA cycle. In the beta-cell PEPCK-M then hydrolyzes mtGTP in the production of PEP that, unlike mtGTP, can escape the mitochondria to generate a signal for insulin release. Similarly, PEPCK-M and mtGTP might also provide a significant source of PEP in gluconeogenic tissues for the production of glucose. This review will focus on the possibility that PEPCK-M, as a sensor for TCA cycle flux, is a key mechanism to regulate both insulin secretion and gluconeogenesis suggesting conservation of this biochemical mechanism in regulating multiple aspects of glucose homeostasis. Moreover, we propose that this mechanism may be important for regulating insulin secretion and gluconeogenesis compared to canonical nutrient sensing pathways.

**Major conclusions:** PEPCK-M, initially believed to be absent in islets, carries a substantial metabolic flux in beta-cells. This flux is intimately involved with the coupling of glucose-stimulated insulin secretion. PEPCK-M activity may have been similarly underestimated in glucose producing tissues and could potentially be an unappreciated but important source of gluconeogenesis.

**General significance:** The generation of PEP via PEPCK-M may occur via a metabolic sensing pathway important for regulating both insulin secretion and gluconeogenesis. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

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**Abbreviations:** AAT, aspartate aminotransferase; ANT, adenine nucleotide transporter; Asp, aspartate; Asp/Glu, aspartate glutamate transporter;  $\alpha$ KG, alpha-ketoglutarate;  $\alpha$ KGT, alpha-ketoglutarate transporter; CIC, citrate isocitrate transporter; cytAAT, cytosolic aspartate aminotransferase; cytGlyc-3-PDH, cytosolic glycerol-3-phosphate dehydrogenase; cytMDH, cytosolic malate dehydrogenase; DIC, dicarboxylate transporter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GSIS, glucose-stimulated insulin secretion; GTP, guanosine-5'-triphosphate; Glu, glutamate; Glyc-3-PDH, glycerol-3-phosphate dehydrogenase; IDP, inositol-1, 4-diphosphate; ITP, inositol 1,4,5-trisphosphate; LDH, lactate dehydrogenase; Mal, malate; MDH, malate dehydrogenase; mtAAT, mitochondrial aspartate aminotransferase; mtGlyc-3-PDH, mitochondrial glycerol-3-phosphate dehydrogenase; mtGTP, mitochondrial GTP; mtMDH, mitochondrial malate dehydrogenase; mtPEP, mitochondrial PEP; NDPK, nucleotide diphosphokinase; OAA, oxaloacetate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PEPCK-C, cytosolic PEPCK; PEPCK-M, mitochondrial PEPCK; PK, pyruvate kinase; SCS, succinyl coenzyme A synthetase; SCS-ATP, ATP-forming SCS; SCS-GTP, GTP-forming SCS

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## 1. Introduction

The body regulates blood glucose levels to maintain glucose homeostasis. Together the rates of glucose entry and clearance from the circulation establish blood glucose levels. In the absence of exogenous nutritional sources an organism preserves normal blood glucose levels through a combination of glycogenolysis (glycogen breakdown) and gluconeogenesis (de novo glucose production) [1]. An increase in glucose levels results in insulin secretion from the pancreas and clears blood glucose acutely by promoting tissue glucose uptake and suppressing glucose production [2,3] (Fig. 1).

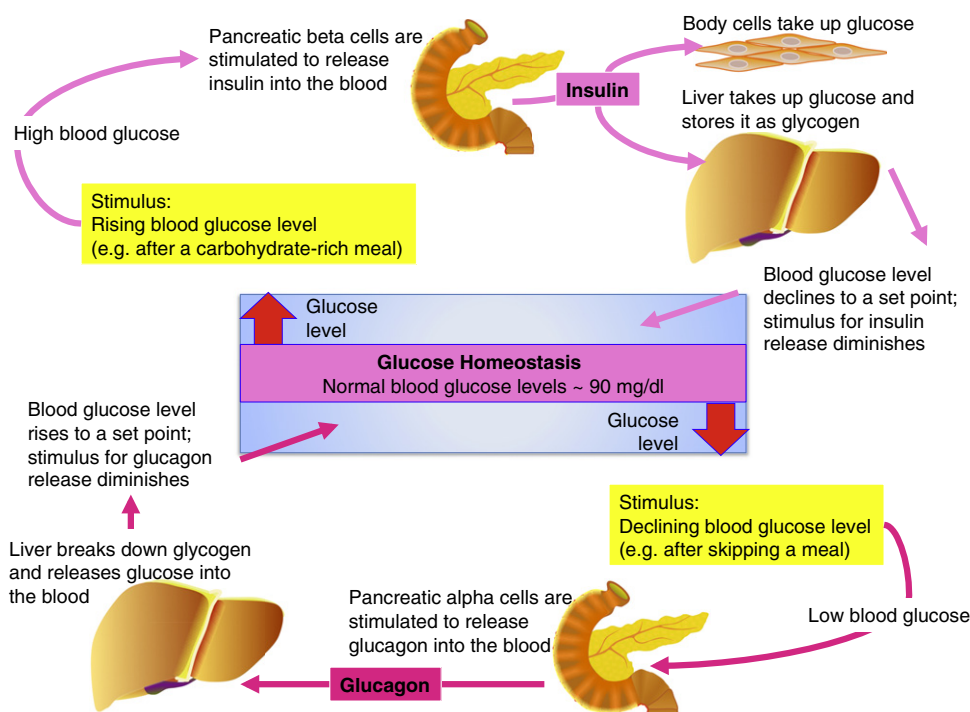
While glucose production and insulin secretion oppose each other, at a cellular level, these two distinct cellular processes share metabolic and biochemical features. Namely the biochemical reaction in which oxaloacetate (OAA) is decarboxylated to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK) (EC number 4.1.1.32) is central to both processes. This shared biochemistry leads to similarities in how these processes are regulated. This reaction requires GTP and is essential to support phosphoenolpyruvate (PEP) synthesis for insulin secretion as well as gluconeogenesis [4–7]. PEPCK exists in two isoforms: a cytosolic (PEPCK-C) and a mitochondrial (PEPCK-M) form [8]. Most research focused on the function and regulation of PEPCK-C, and glucose production is most commonly ascribed to this isoform. Curiously, the first function of PEPCK-M in mammals was found in glucose-stimulated insulin secretion (GSIS), a function on the diametrically opposite side of glucose production [7,9]. In the pancreatic  $\beta$ -cell, PEP synthesis by PEPCK-M couples mitochondrial metabolism to insulin release in a mechanism distinct from ATP production by oxidative phosphorylation. Mitochondrial PEP (mtPEP) synthesis is coupled to the TCA cycle via the production of mitochondrial GTP (mtGTP) that is produced by substrate-level synthesis via the enzyme succinyl-CoA synthetase (SCS) in the TCA cycle. Consequently, both glucose production and

glucose clearance could be determined, at least in part, by a common mitochondrial metabolic pathway.

Flux through this metabolic “tachometer” derived from mtGTP production by the TCA cycle followed by mtGTP hydrolysis by PEPCK-M may be a common reaction to both  $\beta$ -cells and gluconeogenic tissues. This review will focus on this potential duality of function where PEPCK-M senses TCA cycle flux through mtGTP production.

## 2. Glucose homeostasis

Many important functions of the body depend on glucose. Throughout the day plasma glucose concentrations remain within a relative narrow window from about 3 to 8 mM [10,11]. However, nutrition is variable and stored glucose (in the form of glycogen) is a finite supply that can be depleted. In order to maintain glucose homeostasis, glucose that is leaving the circulation must be balanced by the addition of glucose to the system (Fig. 1). Insulin is the hormone that largely determines the rate of clearance of glucose into peripheral tissues at rest. The source of the glucose, on the other hand varies. During meals, intestinal absorption is a major contributor but during fasting and exercise glucose comes primarily from two other pathways: gluconeogenesis (de novo glucose production in the kidney and liver) and glycogenolysis (glycogen breakdown in the muscle and liver) [1,12,13]. Only the liver and kidney can release glucose into the blood because they have the enzyme glucose-6-phosphatase [14]. Gluconeogenesis is a continuous process that supports both glycogen synthesis as well as endogenous glucose production. Interestingly, even in the immediate post-absorptive state when glycogen levels are high, gluconeogenesis still contributes about half of endogenous glucose production [15]. During a prolonged fast as glycogen stores become progressively depleted then the relative contribution of gluconeogenesis approaches 100%. It is particularly



**Fig. 1.** Glucose homeostasis. Plasma glucose levels are normally maintained within a relatively narrow range and are derived from three main sources: intestinal absorption, gluconeogenesis and glycogenolysis. Hormonal control is the most important mediator of plasma glucose. Acute glucoregulatory mechanisms that can affect plasma glucose levels within minutes involve insulin and glucagon. An increase in blood glucose levels provides the stimulus for insulin secretion. Insulin decreases blood glucose acutely by promoting tissue glucose uptake, followed by suppression of gluconeogenesis in both the liver and kidney as well as glycogenolysis in the liver. A decrease in blood glucose levels results in the secretion of glucagon. Glucagon only acts on liver and stimulates glucose release, by initiating glycogenolysis.

important to note that inappropriately elevated gluconeogenic flux is associated with and may be causal for diabetes mellitus [16,17].

Gluconeogenesis and glycolysis share many of the same enzymes that catalyze reversible reactions lying close to their equilibrium. However, three glycolytic reactions (hexokinase/glucokinase, phosphofructokinase, and pyruvate kinase) are exchanged with kinetically more favorable reactions (glucose-6-phosphatase, fructose-1,6-biphosphatase, and PEPCK, respectively) during gluconeogenesis. The reactions of gluconeogenesis occur predominantly in the cytosol with the exception of glucose-6-phosphatase (lumen of the ER) and PEPCK that can either take place in the cytosol (PEPCK-C) or the mitochondrial matrix (PEPCK-M). Interestingly, to some degree both gluconeogenesis and glycolysis occur simultaneously in the liver with significant cycling of plasma glucose into the mitochondria and back out [18–20].

The hormone insulin has a key role in normal glucose homeostasis opposing gluconeogenesis. Many of the features of the control of glucose homeostasis under basal conditions can be explained by a simple feedback loop between the liver and the pancreatic  $\beta$ -cell in the islets of Langerhans (Fig. 1). Endogenous glucose production supplies glucose through a combination of gluconeogenesis and glycogenolysis to support energy demand in peripheral tissues. If supply outstrips demand, then plasma glucose levels begin to rise.  $\beta$ -cells metabolically sense glucose as well as other nutrient levels and release insulin into the blood stream. Insulin decreases blood glucose acutely by promoting tissue glucose uptake, suppressing gluconeogenesis in both the liver and kidney as well as inhibiting glycogenolysis and promoting glycogen synthesis in the liver [2,3].

### 3. Sensing TCA cycle flux

Mitochondria are intimately involved in both processes of glucose sensing and gluconeogenesis. The TCA cycle is the “central wheel” of energy metabolism and is the final common pathway for the aerobic oxidation of fuel molecules. However, biosynthetic intermediates can leave the TCA cycle and be converted to products such as glucose, nucleotides, lipids, or non-essential amino acids [6]. The removal of TCA cycle intermediates is called *cataplerosis* (of Greek origin, *kata* = “downward” and *plerotikos* = “to fill”). If carbons are removed from the TCA cycle, they must be replaced. The replenishment of TCA cycle intermediates is termed *anaplerosis* (of Greek origin, *ana* = “up” and *plerotikos* = “to fill”) [6]. To maintain the appropriate balance of carbon flow in the TCA cycle for various metabolic processes, cataplerosis and anaplerosis must be maintained in equilibrium. If this does not occur, the cycle either accumulates or loses metabolites, either of which could impair TCA flux when excessive. Thus, anaplerosis and cataplerosis must be balanced so that during different physiologic conditions (e.g. fasting, feeding or exercise), the pool size of TCA intermediates remains consistent [21]. How, then, do mitochondria know how much is coming in and going out?

A key insight was obtained from the molecular characterization of a rare disease of familial hypoglycemia, known as Hyperinsulinemia–Hyperammonemia (HI/HA) syndrome. This autosomal dominant disease is associated with a gain-of-function mutation in the mitochondrial enzyme glutamate dehydrogenase (GDH) [22,23]. GDH is a key net entry point for glutamate into the TCA cycle. Glutamate is oxidatively deaminated to produce anaplerotic  $\alpha$ -ketoglutarate and free ammonia. Unrestrained glutamate entry into the TCA cycle is detrimental in the patients due to excessive amino acid catabolism that increases levels of ammonia as a toxic by-product. This process is normally tightly regulated by opposing allosteric signals: mtGTP (inhibitory) and leucine (activating). As long as there are adequate levels of TCA flux then mtGTP will prevent the excessive entry of anaplerotic glutamate by inhibiting GDH. Leucine is one of the essential amino acids and its levels generally only increase following a protein-rich meal. Activation of GDH by leucine provides a mechanism of redirecting the carbons from amino acids into other metabolic pathways during times of excess. The balance

of mtGTP and leucine regulation is lost in patients with HI/HA due to a mutation in GDH that impairs the ability of mtGTP to turn off the enzyme [22,23]. During protein-rich meals, leucine potentially over-activates the enzyme. Consequently, glutamate catabolism increases inappropriately and results in excessive insulin secretion with hypoglycemia. While it may not be surprising that excessive TCA flux in  $\beta$ -cells could promote insulin secretion, the characterization of these mutations identified mtGTP as a key metabolic regulatory signal in the mitochondria [9,22,23].

#### 3.1. Mitochondrial GTP

The synthesis of ATP in the mitochondria by oxidative phosphorylation is well characterized. Here TCA cycle metabolism provides reducing equivalents (e.g. NADH and FADH<sub>2</sub>) that donate electrons to the electron transport chain with molecular oxygen as the final acceptor at complex IV. Electron transport then pumps protons out of the matrix generating a proton motive force across the inner mitochondria. This provides the driving force for ATP synthesis as protons translocate back into the matrix through complex V along their electrochemical gradient. There are other pathways that allow the protons to “leak” back into the matrix so the efficiency of oxidative phosphorylation depends, in part, on the tightness of coupling of the proton motive force to ATP synthesis. ATP generated in the matrix is then rapidly and efficiently transported out into the cytosol in exchange for ADP.

Mitochondrial GTP (mtGTP) synthesis is quite different. First, mtGTP is formed by substrate-level phosphorylation of mitochondrial GDP via GTP-forming succinyl-CoA synthetase (SCS-GTP) [24]. Synthesis rates of mtGTP are directly related to flux through the TCA cycle (at least for the SCS step where approximately one molecule of mtGTP is built per one molecule of glucose) but independent of electron transport and oxidative phosphorylation. In contrast, the TCA cycle generates little mitochondrial ATP (mtATP) by the ATP forming isoform of SCS (SCS-ATP). Oxidative phosphorylation generates the majority of mtATP, which is controlled by the transmembrane mitochondrial potential ( $\Delta\Psi$ ). The  $\Delta\Psi$  controls important mitochondrial functions, such as protein import, heat formation, free-radical generation, ion transport, in addition to generating ATP. Consequently, the yield of mtATP from one glucose molecule can in theory vary between 1 and 29 [9]. This is particularly relevant in pancreatic beta-cells that are known to have a large proportion of the membrane potential uncoupled from oxidative phosphorylation [25]. In this manner, mtGTP is better poised to be an indicator of TCA cycle flux than ATP generation in a mechanism that is metaphorically similar to the tachometer of an engine [9].

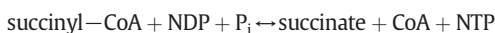
Considered within the framework of GDH regulation, if the intermediates of the TCA cycle are becoming depleted, then GTP levels will drop reducing the restraint on GDH. As noted above, this is evident in the disease Hyperinsulinemia Hyperammonemia syndrome, where mutations in GDH lacking the GTP “off-switch” become profoundly hypoglycemic from excessive amino acid induced insulin secretion. Similarly, glucose deprivation depletes TCA cycle flux and intermediates and reduces mtGTP, consequently relieving inhibition of GDH and enhances GDH activation by the amino acid leucine. In mice expressing the mutant human GDH, amino acids are much more potent insulin secretagogues. Even in normal mouse islets, the longer the duration of hypoglycemia in run down experiments leads to a greater responsiveness of insulin secretion to amino acids suggesting that this is a normal physiologic mechanism regulated by mtGTP [26]. If needed, glutamate carbons can replenish the TCA cycle, and in the process will generate more mtGTP leading to feedback inhibition of GDH. Such a feedback circuit limits amino acid catabolism via GDH as long as sufficient glucose is present to maintain levels of TCA cycle intermediates that permit adequate TCA cycle flux and mtGTP production. For amino acids to be oxidized, there must be net loss of the amino group as ammonia or urea. This mechanism may also indirectly favor glucose and fatty acids over amino acids as oxidative substrate since ammonialysis catalyzed by GDH is required not just for

anaplerosis, but also for complete amino acid oxidation. This preserves the amino acid pools and keeps ammonia levels low. Thus, GDH is a major regulatory site for ammonialysis, and mtGTP is a major regulator.

Another unique contrast with mitochondrial ATP is that transport of GTP or GDP into or out of the mitochondria is very slow [27,28]. Yeast lack a mechanism to synthesize mtGTP and therefore are dependent on a mitochondrial GTP transporter [29]. To date, no transporter has been identified in higher eukaryotes and any measurable transport activity is orders of magnitude slower than ATP [27,28]. This has important implications for mtGTP. First, since it is not in equilibrium with the cytosolic pool, then it can act as a mitochondrial metabolic sensor. Second, there must either be a mechanism to hydrolyze GTP back to GDP or another GDP-independent mechanism to metabolize succinyl-CoA to succinate. Otherwise, GDP would become rate-limiting for the TCA flux.

### 3.2. Regeneration of mitochondrial GDP by the mitochondrial GTP cycle

Mitochondrial GTP is synthesized in the TCA cycle via succinyl-CoA synthetase (SCS) [24]. This mitochondrial matrix enzyme catalyzes the reversible reaction: Succinyl-CoA to succinate and CoA with the generation of a purine triphosphate in the process:



Initially it was believed that, in contrast to the ADP-dependent reaction observed in single celled organisms, the reaction was entirely GDP-dependent in higher organisms. Later two isoforms of SCS were identified in the mitochondrial matrix—one ATP forming (SCS-ATP), the other GTP (SCS-GTP) forming [30]. Succinyl-CoA synthetase is a heterodimer, with a common  $\alpha$  subunit (SUCLG1) and two distinct  $\beta$  subunits encoded by separate nuclear genes that impart the GDP (SUCLG2) and ADP (SUCLA2) nucleotide specificity. The  $\beta$  subunits are highly homologous and the enzymatic characteristics of the two isoforms are similar differing primarily in their nucleotide specificity. Both isoforms work in parallel in the mitochondria with varying SCS-GTP/SCS-ATP ratios. There is a preponderance of the GDP-specific isoform in synthetic tissues (e.g. liver and kidney) while the ADP-specific isoform can be more commonly found in oxidative tissues (e.g. brain and skeletal muscle) [24,31]. As a consequence, the stoichiometry at which GTP is made by the TCA cycle is dependent on the ratio of SCS-GTP/SCS-ATP. If ADP and GDP are not limiting and there is no other source of GTP, then GTP production will be proportional to the TCA cycle [9]. At high SCS-GTP/SCS-ATP ratios this would approach a maximum of one GTP per turn of the TCA cycle. The “gain” of the signal will be reduced by increasing amounts of the ATP isoform. Thus, in principle, the mtGTP production rates can be tuned with respect to the TCA flux in a tissue-specific manner.

There may be additional advantages to having two parallel pathways for succinate synthesis besides just changing the GTP production rate. For instance, if GDP becomes rate-limiting during states of high metabolic flux, then a switch to the SCS-ATP isoform could maintain the TCA cycle flux since the adenine nucleotide transporter is very efficient. Secondly, since both isoforms catalyze reversible reactions, the phosphate potential of ATP/ADP and GTP/GDP could be distributed across both isoforms. For example, if GTP/GDP concentrations are high relative to ATP/ADP, then succinate + GTP + CoA may react in reverse via SCS-GTP to make succinyl-CoA + GDP +  $\text{P}_i$ . The succinyl-CoA formed by the reverse reaction may then contribute to the forward reaction through SCS-ATP to convert ADP to ATP. In this manner, the two isoforms may perform a function akin to the nucleotide diphosphokinase reaction (NDPK). Thus, if equilibration across the SCS isoforms is sufficiently fast relative to GTP consumption, then the ATP/ADP and GTP/GDP could be functionally linked. Additionally, metabolites such as succinate that enter distal in the TCA cycle to SCS are not known to directly generate mtGTP. However, reverse flux of SCS-ATP could generate succinyl-CoA without the

need for a pyruvate cycling pathway. Consequently, forward flux through SCS-GTP could then provide a source of mtGTP. Presently, there is no experimental evidence we are aware of to support bi-directional fluxes across SCS isoforms in intact cells.

Prior to the identification of the ATP and GTP isoforms of SCS in mammalian cells, a mitochondrial matrix NDPK was proposed as a mechanism to return mtGTP back to the GDP state by the transfer of the  $\gamma$ -phosphate to ADP forming ATP [32]. Indeed, initial data supported the presence of this activity in isolated mitochondria and it appeared to co-immunoprecipitate with SCS [33]. Subsequent studies now suggest that mitochondrial NDPK is primarily localized to the intermembrane space. Given that SCS-ATP and SCS-GTP together catalyze the NDPK reaction, then in the presence of contaminating levels of CoA and phosphate, the phosphate transfer might be falsely attributed to NDPK. The mitochondrial isoform of NDPK (nm23-H4) is homohexameric with two faces that bind to cardiolipin [34,35]. Recently it has been described as an enzyme that transfers cardiolipin between leaflets of the inner and outer mitochondrial membrane as well as performing an NDPK function [36]. Interestingly, it also associates with OPA-1, the intermembrane GTPase required for fusion of mitochondrial membranes and cristae remodeling. Here it may convert ATP from oxidative phosphorylation into GTP needed for these functions. Regardless, NDPK activity in mitochondria is two orders of magnitude lower than SCS that, even if it were in the matrix, it would not be likely to significantly alter the nucleotide ratios.

Neither NDPK nor the combined enzymatic activities of SCS-ATP and SCS-GTP adequately explain why mtGTP is synthesized in the first place. If there is already an ATP-specific SCS isoform then why go to the trouble of making GTP if it will be only used to make ATP? Identification of a significant matrix GTPase could potentially explain its purpose. PEPCK is a GTPase that decarboxylates OAA to form GDP and PEP. Hahn and Novak first proposed in 1975 that the cytosolic form of the enzyme might function as a GTPase to regenerate mtGDP in brown adipose [37]. Brown adipose, which has high activities of PEPCK, is the tissue for non-shivering thermogenesis and in a transmembrane, heat-generating futile cycle. They proposed that excessive mtGTP made by SCS-GTP would be transported out of mitochondria and hydrolyzed by PEPCK-C in the cycle. The cytosolic GDP would be then returned to the matrix to support further TCA flux. However, as mentioned above, lack of a mitochondrial GTP/GDP transporter makes this cycle less likely. There may be other mitochondrial processes besides PEP synthesis such as formation of iron sulfur complexes and mitochondrial protein synthesis that are dependent on mtGTP [38]. A few other mitochondrial GTPases of unknown function have been identified [39] suggesting that there may be other possible roles for this metabolic signal. In metabolically active cells like the liver and beta-cells, expressing high levels of SCS-GTP, these other GTPases may be quantitatively much less significant. For instance, as high as 30–40% of glycolysis flows into the PEPCK-M pathway in islets [7]. With the remaining glycolytic flux going into PDH, then virtually all of the predicted mtGTP synthesis would support the PEPCK-M reaction.

Drahota et al. identified very high specific activities of PEPCK-M in brown adipose [40]. In addition, they reported high rates of PEP export from mitochondria supplied with malate and alpha-ketoglutarate. They proposed that a PEP cycle involving pyruvate carboxylase (PC), PEPCK-M and pyruvate kinase might facilitate uncoupling by reducing the mitochondrial phosphate potential. Here, PEP would leave the mitochondria via the citrate isocitrate carrier (CIC) in exchange for malate and could be inhibited by 1,2,3-benzenetricarboxylate. In this PEP cycle, ATP consumed by PC and GTP used by PEPCK-M in the mitochondria would generate a single ATP in the cytosol. Such a cycle would help maintain high TCA flux rates by restoring the mitochondrial GDP pool for use by SCS-GTP. Lambeth et al. also proposed a cycle in which PEPCK-M and PEPCK-C collaborate to provide mtGDP for the succinyl-CoA synthetase step while transferring a high energy phosphate to the cytosol [24]. In this mechanism PEPCK-M consumes mtGTP from



SCS-GTP to make PEP. PEP is transported out of the mitochondria where it serves as a high-energy phosphate donor to generate GTP in the cytosol via PEPCK-C (working in the reverse direction). Cytosolic malate dehydrogenase (cytMDH) then reduces OAA to malate that can return to the matrix to be oxidized back to OAA by the mitochondrial isoform completing the cycle [24]. Such a PEP cycle would only be possible in tissues that contain PEPCK-C, an enzyme that is less widely expressed compared to PEPCK-M. With the exception of possibly brown fat, reversed flux (in the direction of OAA synthesis) by PEPCK-C would be counterproductive for gluconeogenesis and glyceroneogenesis.

Thus, a mitochondrial GTP cycle where SCS-GTP and PEPCK-M interact to generate and hydrolyze mtGTP respectively appears feasible. Possible functions of the cycle include 1) transferring the ATP and/or GTP phosphate potential to the cytosol, 2) regenerating GDP to maintain TCA flux rates, 3) energy consuming futile cycling, and 4) increasing cataplerotic flux. It is worth noting that tissues with high rates of anaplerosis (e.g. liver, kidney, islets and heart) express significant levels of PEPCK-M and SCS-GTP. In principle, linking mtPEP synthesis to mtGTP synthesis may secure an appropriate TCA cycle intermediate pool before draining carbon into cataplerotic pathways. Regulating TCA cycle pool size is important or it could grow indefinitely. The mtGTP sensing PEPCK-M-driven PEP cycle provides a cataplerotic pathway to balance anaplerosis. In general, these are all homeostatic functions not directly tied to the generation of a metabolic signal or product *per se*.

#### 4. Glucose “sensing” in the mitochondria of pancreatic $\beta$ -cells

The canonical model for glucose-stimulated insulin secretion is dependent on the metabolic generation of ATP. According to this model, glucose is metabolized by glycolysis and the TCA cycle to generate ATP that increases the cytosolic ATP/ADP ratio that in turn closes the  $K_{ATP}$  channel.  $K_{ATP}$  channel closure depolarizes the plasma membrane and activates voltage-gated calcium channels. As calcium floods into the cytoplasm, fusion of insulin granules with the plasma membrane is triggered so that insulin can be released. However, it has become increasingly more evident that this mechanism cannot explain the entirety of metabolism-coupled insulin secretion. Notably, mice that have the  $K_{ATP}$  channel knocked out still experience glucose-stimulated insulin secretion that is coupled to cytosolic calcium oscillations [41,42]. This, among other observations has lead a number of investigators to search for alternative mechanisms to link glucose metabolism with insulin release.

##### 4.1. Metabolic cycling in insulin secretion

Without other carbon additions (anaplerosis) or losses (cataplerosis), the TCA cycle consumes as much OAA as it produces. Pyruvate from glycolysis enters the TCA cycle either via pyruvate dehydrogenase (PDH) reaction or the pyruvate carboxylase (PC) reaction. While the oxidative PDH reaction generates acetyl-CoA, the ATP-dependent PC reaction is anaplerotic and consumes ATP to generate OAA. Considering the canonical view that mitochondrial ATP stimulates insulin secretion, it was somewhat surprising that the mitochondria of the pancreatic beta-cells express high levels of PC. This is because increased glycolytic carbon entry via PC would lead to ATP consumption rather than production [43–45]. In addition, since the fluxes of PC and PDH are roughly equivalent, then about half the carbon that could go towards oxidation would be diverted into an anaplerotic/cataplerotic cycle [46]. How then does PC flux increase insulin secretion if not through ATP production? NMR isotopomeric analysis of the flux of carbons in  $\beta$ -cell lines has demonstrated the strongest correlation of insulin secretion with pyruvate cycling and not with ATP [47–49]. A pyruvate cycle requires reactions that first carboxylate pyruvate followed by the decarboxylation back to pyruvate through a different reaction. Since  $\beta$ -cells do not express PEPCK-C [50], it was initially assumed that malic enzyme was the only metabolic pathway for the decarboxylation of malate into pyruvate. In that regard the anaplerotic

pyruvate cycling has been postulated from multiple laboratories to be a critical component of the mechanism regulating GSIS [48,49,51–58]. Several cycles (Fig. 2) have been proposed to account for the observed cycling of carbons to pyruvate including (for review, see Ref. [59]):

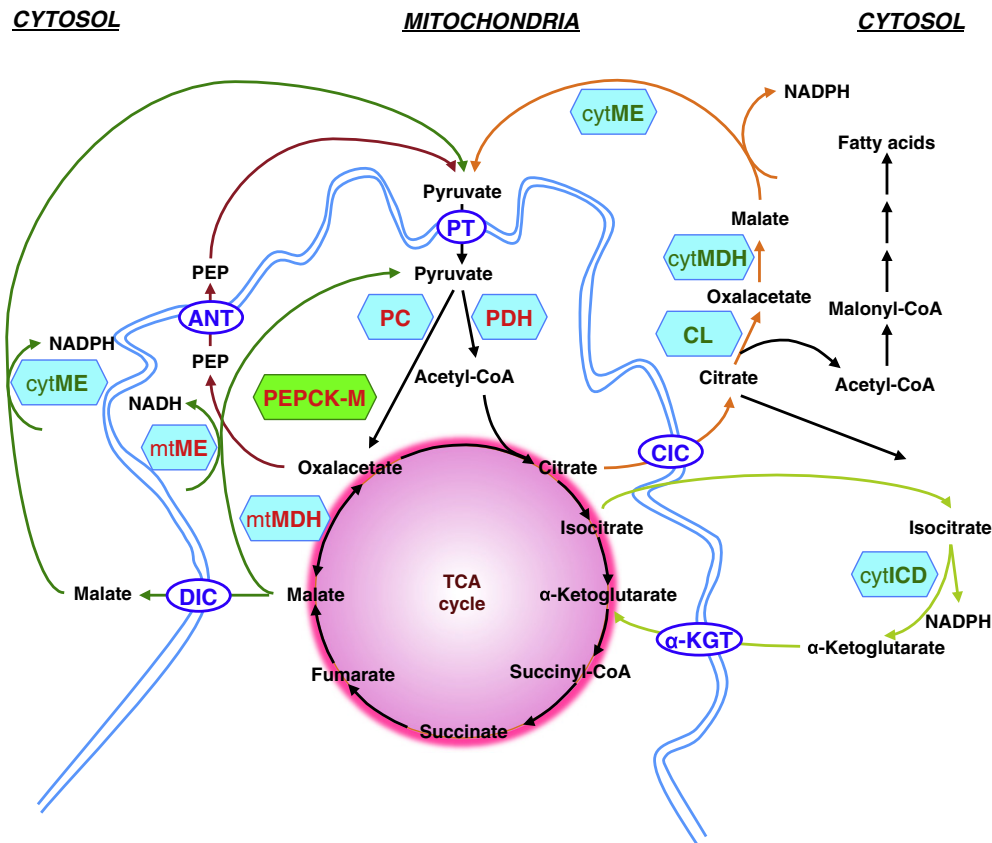
- 1) pyruvate-malate cycling
- 2) pyruvate-citrate cycling
- 3) pyruvate-isocitrate cycling (isocitrate- $\alpha$ -ketoglutarate shuttle)\*.

Each of these cycles increases cytosolic NADPH via the activity of either malic enzyme or isocitrate dehydrogenase. While an increase in the NADPH/NADP ratio may be important for glucose-stimulated insulin secretion, interestingly it does not appear to play a role in amino acid-stimulated insulin secretion [60]. \*Of note, the pyruvate-isocitrate cycle is not actually a true pyruvate cycle, but rather an extramitochondrial loop of the TCA cycle since it does not require net cycling through pyruvate. In this shuttle citrate or isocitrate exit the mitochondria and are oxidized by the cytosolic isoform of isocitrate dehydrogenase before returning to the TCA cycle as  $\alpha$ -ketoglutarate but not as pyruvate. The NMR methods used to identify pyruvate cycling require a net flux of carbon out of the mitochondria and back into pyruvate and therefore the pyruvate-isocitrate cycle would not be detected by this method. For this reason it has been more appropriately defined as an isocitrate- $\alpha$ -ketoglutarate shuttle [61]. This does not rule out a role for cytoplasmic metabolism of isocitrate in insulin secretion, but it does rule out a direct connection between the observed pyruvate cycling and this pathway. The shuttle could, however, occur in tandem with a true pyruvate cycle such as the pyruvate-malate or pyruvate-citrate cycle. Regardless, all these pyruvate cycles are all dependent upon malic enzyme whose role in insulin secretion remains unresolved [52,53,58].

It is particularly relevant to note, that at the time NMR isotopomer modeling techniques were developed to measure pyruvate cycling, malic enzyme was assumed to be the sole path back to pyruvate (i.e. PEPCK flux of carbons to PEP and then back to pyruvate were assumed to be absent) [49]. As will be discussed below, this is not the case. Thus, any estimate of pyruvate cycling by these techniques includes an indistinguishable component of PEP cycling. So, in addition to the pyruvate-malate and pyruvate-citrate cycle, the PEP cycle can also be explained by the high rates of PC flux in  $\beta$ -cells. It will be, therefore, important in future studies to distinguish the individual malic enzyme and PEPCK contributions when considering the relationship between pyruvate cycling and insulin secretion.

##### 4.2. The mitochondrial GTP cycle in energy sensing

As noted above, mtGTP was implicated in insulin secretion by human mutations in GDH. Insulin secreting cell lines were also noted to have both ATP and GTP-specific isoforms of SCS in comparable amounts [31]. The possibility that mtGTP synthesis rates might be directly linked to glucose-stimulated insulin secretion was first demonstrated by individually silencing the ATP- and GTP-specific isoforms of SCS in insulin secreting cell lines and rodent islets [9]. The hypothesis was that silencing one isoform would redirect metabolic flux through the other isoform. This would impact mitochondrial GTP synthesis rates primarily since ATP synthesis is predominantly performed by oxidative phosphorylation. Indeed, silencing the two isoforms had the predicted effects on SCS enzyme activities but did not lead to a reduction in oxygen consumption, NAD(P)H levels or mitochondrial membrane potential. However, there was a strong association between the rate of mtGTP synthesis and insulin secretion through a mechanism that involved cytosolic calcium. Surprisingly cellular mitochondrial ATP synthesis rates, ATP levels and ATP/ADP ratios did not correlate with GSIS. These data support the idea that  $\beta$ -cells sense glucose metabolism, at least in part, by the coupling of mtGTP production to the TCA flux rate. However, given that mtGTP is trapped in the mitochondrial matrix, another mechanism is needed to communicate the flux signal to the cytosol.



**Fig. 2.** Pyruvate and PEP cycling pathways. Pyruvate enters the mitochondrion via the pyruvate transporter (PT) and then enters the TCA cycle via the PDH-catalyzed reaction that forms acetyl-CoA or via an anaplerotic reaction catalyzed by PC. Several cycles have been proposed to account for the observed cycling of carbons to pyruvate which are following:

*Pyruvate–malate cycle (dark green):* OAA is converted to malate and converted back to pyruvate via mitochondrial NAD-dependent or cytosolic NADP-dependent malic enzyme (ME). Malate exits the mitochondrion via the dicarboxylate carrier (DIC).

*Pyruvate–citrate cycle (orange):* OAA is converted to citrate, which exits the mitochondrion via citrate/isocitrate carrier (CIC) and is converted to OAA and acetyl-CoA through citrate lyase (CL) reaction. Acetyl-CoA is converted to malonyl-CoA and long-chain acyl-CoA, while malate dehydrogenase (MDH) converts OAA into malate and further to pyruvate by cytosolic ME.

*Pyruvate–isocitrate cycle (light green):* Citrate can be converted to isocitrate and exits the mitochondrion via CIC. In the cytosol isocitrate is converted to α-ketoglutarate by NADP-dependent isocitrate dehydrogenase (ICDc) which can then re-enter mitochondrial metabolism by α-ketoglutarate transporter (α-KGT).

*PEP cycle (red):* OAA is converted to PEP by PEPCK-M and exits the mitochondrion via CIC or the adenine nucleotide transporter (ANT) (in exchange for ADP) in the inner mitochondrial membrane. PEP in the cytosol is converted to pyruvate by pyruvate kinase (PK).

#### 4.3. Anaplerosis meets the mtGTP cycle: The PEP cycle

The enzyme PEPCK-M lies at the intersection between anaplerosis and the mtGTP cycle. Since PEPCK-M consumes both anaplerotic OAA and mtGTP to produce PEP it could integrate TCA cycle and anaplerotic metabolism with insulin secretion. Because of its dependence upon mtGTP, maximal mitochondrial PEP cycle flux is limited to TCA cycle flux through SCS-GTP. This may in part explain the observation that the best correlation of “pyruvate” cycling with insulin secretion includes both PC and TCA flux [48]. The discovery of PEPCK-M in the pancreatic β-cell and identification of its crucial role in insulin secretion provided the first unique metabolic role for the enzyme [7]. Here, PEPCK-M plays an important part in the process of metabolic sensing and insulin secretion. Islet glucose metabolism generates mtGTP by SCS-GTP that is proportional to TCA cycle flux [9]. While mtGTP is trapped in the mitochondrial matrix due to the lack of a transporter [29], PEPCK-M can then utilize mtGTP to convert OAA into mtPEP. Mitochondrial PEP can then escape the mitochondria to cycle back into pyruvate completing a full PEP cycle (Fig. 2) [7]. Both cellular PEP concentrations as well as flux through PEPCK-M increase at rates proportional to the glucose concentration. It is a particularly high metabolic flux with rates as high as 40% of the glycolytic rate in islets. Silencing PEPCK-M prevents insulin secretion in response to glucose as well as reduces PEPCK-M flux.

Considering that glycolysis also produces PEP, it is still unclear how PEP derived from the mitochondria goes on to stimulate insulin release in the cytosol. One may speculate that mtPEP is the molecule that transfers the “energy-sensing” signal from the mitochondria into the cytosol where it can be used for other signaling pathways (by transferring its phospho group to generate ATP or GTP) or through other biosynthetic processes. Indeed, PEPCK-M synthesis of PEP in the mitochondria increases PEP export from the mitochondria [62]. This transport is carrier-dependent and occurs primarily by the CIC or in exchange for ADP by the adenine nucleotide transporter (ANT) in the inner mitochondrial membrane [63]. A PEP/pyruvate transporter has also been postulated [64]. Since the synthesis of mtGTP depends on substrate flux, biosynthetic processes in the cytosol using mitochondrial derived PEP would be also dependent on substrate availability. However, the export of mtPEP into the cytosol also affects the cellular energy balance via regulation of mitochondrial  $\text{Ca}^{2+}$  levels that control TCA cycle flux rates. Mitochondrial free  $\text{Ca}^{2+}$  activates PDH, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase [65]. Given that the mitochondrial matrix buffers intramitochondrial  $\text{Ca}^{2+}$  by forming an insoluble calcium-phosphate complex that depends on the accompanying  $\text{P}_i$  accumulation, the release of intramitochondrial free  $\text{Ca}^{2+}$  is a function of intramitochondrial  $\text{P}_i$  and ATP levels as well as PEP [66]. Studies show that PEP regulates both mitochondrial calcium transport and ATP/ADP exchange [57,66–73].

**Table 1**  
Summary of experimental procedures used to measure PEPCK activity with percentage of PEPCK-M activity to total PEPCK activity measured in rat liver.

	Wiese TJ et al. (1991) [174]	Söling H et al. (1970) [173]	MacDonald MJ and Chang CM (1985) [172]	Cornell NW et al. (1986) [171]	Nordlie RC and Lardy HA (1963) [8]	Ballard FJ and Hanson RW (1967) [85]	Usatenko M (1970) [80]	Heitzman R et al. (1972) [81]	Saggerson D and Evans CJ (1975) [79]	Horn D et al. (1997) [78]
% PEPCK-M	0	0	3	5	6	10	18	25	25	50
Direct/indirect	Difference	Difference	Per gram	Direct	Direct	Direct	Direct	Direct	Direct	Direct
pH	6.8	8	7.5	6.6	8	7	8.1	7.5	6.6	7.4
Ion	Mn <sup>2+</sup> Mg <sup>2+</sup>	Mn <sup>2+</sup> Mg <sup>2+</sup>	Mg <sup>2+</sup>	Mn <sup>2+</sup>	Mg <sup>2+</sup>	Mn <sup>2+</sup>	Mg <sup>2+</sup>	Mg <sup>2+</sup>	Mn <sup>2+</sup>	Mn <sup>2+</sup>
Nucleotide	dGDP	IDP	ITP	IDP	ITP	IDP	ITP	ITP	IDP	dGDP
Mitochondrial isolation	Deoxy-cholate	Deoxy-cholate	Freeze thaw	Digitonin	Freeze thaw	Lyophilisation	Freeze thaw	Freeze thaw	Sonication	No detergent
Leakage	Citrate synthase	Citrate synthase	No	Yes	No	Yes	forward	Forward	CO <sub>2</sub> fixation	Reverse
Reaction	Reverse	Reverse	Forward	CO <sub>2</sub> fixation	Forward	CO <sub>2</sub> fixation	KF	No	Rotenone	Rotenone
Inhibitor	NaF	No	No	No	NaF	No	Fed + fasted	Fed	Fed	Fast
Fed/fasted	Fed + fasted	Fed + fasted	Fed	Fed + fasted	Fed	No	Fed + fasted	Fed	Fed	Fast

There could be distinct advantages to having a metabolic signal dependent upon both anaplerotic and oxidative metabolism. For instance, a dependence on anaplerosis would prevent fatty acid oxidation by itself from contributing solely to insulin secretion. Dependence on mtGTP would limit the maximal mtPEP production rate to the TCA cycle rate and ensure ample ATP and TCA intermediates to maintain normal mitochondrial and cellular function. In the absence of activation of GDH by leucine from a protein meal, mtGTP would prevent glutamate, the most abundant intracellular amino acid, from stimulating insulin secretion inappropriately. Thus, substrate level synthesis of mtGTP by SCS-GTP provides energy for PEP synthesis in the mitochondrial matrix by PEPCK-M and thereby link PC flux with TCA cycle flux to stimulate insulin release [7].

## 5. PEPCK-M

PEPCK was first found in chicken liver (where only the mitochondrial isoform is present) and believed to fix carbon dioxide by the conversion of pyruvate into OAA. This reaction was called Wood–Werkman pathway [74]. The first published reference of PEPCK occurred in 1953 by Utter and Kurahashi [75] with the title, “Mechanism of Action of Oxalacetate Carboxylase from Liver.” Later the enzyme was given the name phosphoenolpyruvate carboxylase [76]. Interestingly, the first and only isolated isoform of PEPCK was PEPCK-M from chicken liver [77] but later most studies have focused upon PEPCK-C.

### 5.1. PEPCK-M expression and activity

Humans express PEPCK-M in most tissues but especially in the liver and kidney [78]. The pancreas also expresses PEPCK-M but unlike the liver and kidney, PEPCK-C is not expressed [50,79–81]. Much emphasis has been placed on the relative enzyme activities of the PEPCK isoforms as a reflection of their contribution to gluconeogenesis. Human livers show equal distribution of PEPCK activity, whereas in the liver of chickens almost all PEPCK activity is attributed to PEPCK-M but in the kidney of chickens both isoforms are equally active. Guinea pig liver and kidney has about 80% PEPCK-M activity. While perhaps the most cited references suggest that the rat and the mouse have a PEPCK-C/PEPCK-M ratio of 9 to 1 [76], some authors [82–85] detected more than 10% PEPCK-M activity in rodent liver. These discrepancies may be due to the differences in the enzymatic activity assay used (Table 1 and reviewed in [7]).

### 5.2. PEPCK gene expression at different stages of development

Interestingly, PEPCK-C and PEPCK-M show different expression profiles during development [86,87] and also gluconeogenic activity in mammalian liver. Gluconeogenesis is absent before birth as glucose is provided by the mother [88], and is thought to start with birth [89] as a result of decreasing plasma blood glucose levels and subsequent hormonal changes, namely a decrease in insulin and increase in cAMP, glucagon as well as epinephrine [90,91]. The prenatal mammalian liver does not express PEPCK-C, thus PEPCK-M is the only PEPCK isoform present before birth [92]. After birth and postnatal development the liver undergoes major metabolic alterations increasing PEPCK-C transcription and protein maturation [93–95]. In contrast to the liver, fetal kidney expresses PEPCK-C, which significantly rises after birth [96–98]. Glucocorticoids and acid/base status are also stimuli for postnatal PEPCK-C gene expression in the kidney [99].

### 5.3. The PEPCK gene

Mammals have two different nuclear genes for the two PEPCK isoforms. PCK1 encodes PEPCK-C and resides on human chromosome 20q13.31. PCK2 encodes PEPCK-M and resides on human chromosome 14q11.2 [100]. The human genes for PEPCK-M and PEPCK-C contain the

same genetic organization: 10 exons and 9 introns, whereas the length of PEPCK-C (5345 bp) is smaller than PEPCK-M (9839 bp), due to expanded intronic length in PEPCK-M [78,101]. However, the PEPCK-C and PEPCK-M proteins have a similar molecular weight (69,289 for rat PEPCK-C and 69,522 for chicken PEPCK-M). The human proteins are composed of 622 (PEPCK-C) and 640 (PEPCK-M) amino acids, respectively [76]. Both PEPCKs share 68% identity as well as 82% similarity in their sequence [76,78,100].

#### 5.4. The PEPCK protein

While GTP-dependent (in higher eukaryotic species) and ATP-dependent PEPCKs (in bacteria, yeast, C-4 plants) share little sequence homology (<20%), the active sites and structures are reasonably conserved and suggests an important similarity in function and regulation via enzyme confirmation [102]. Mammal GTP-dependent PEPCK isoforms have nearly identical protein folds [103]. The protein folds as a single domain with an N-terminal lobe and a C-terminal lobe forming an active site that carries out the decarboxylation/carboxylation and phosphoryl transfer half-reactions as well as stabilization of the enolate intermediate [102–109]. The active sites involve reactive cysteine residues located in a P-loop (phosphate-binding loop/kinase-1a motif) [103]. The specific modification of a single cysteine residue inactivates the enzyme [110,111], which is Cys288 in rat PEPCK-C [103,111] and the homologous cysteine in chicken PEPCK-M is Cys307 [107]. Oxidative state, cation redistribution, and/or pH influence PEPCK function via P-loop confirmation and therefore suggest that different metabolic states lead to loss or gain of function. Given that, PEPCK contains sulfhydryl groups and its activity depends on critical cysteine residues, PEPCK likely requires another protein to maintain its redox state [76]. A class of proteins termed ferroactivators is one possibility as they enable  $\text{Fe}^{2+}$  to stimulate PEPCK-C [112,113]. These ferroactivators were subsequently identified as catalase and glutathione peroxidase [114,115]. In bovine erythrocytes a similar ferroactivator protein, a green hemoprotein, was purified [116]. Another PEPCK-C stabilizing protein of 29,000 kDa, p29, was isolated and supports  $\text{Mg}^{2+}$ -induced activation. This protein was later identified as phosphoglycerate mutase and is highly expressed in the liver [117,118].

PEPCK-M binds a single divalent cation in tetrahedral coordination in the absence of substrates with  $\text{Mn}^{2+}$  being the most activating cation. In the presence of nucleotide an additional cation is bound in form of a cation–nucleotide complex [102]. In the fully ligated state both metal ions are octahedrally coordinated [103]. No large domain movements or conformational changes occur upon binding of GTP [103]. During the catalytic cycle, conformational changes occur at the active site. The most prevalent mobile feature is a 10-residue  $\Omega$ -loop domain, which functions like an active site lid. Closure of the lid reduces the active site region between the N- and C-terminal lobes and the P-loop, and shifts the location of the bound substrates [105]. The closed lid confirmation stabilizes and sequesters the reactive enolate intermediate, places the substrates in the correct position, minimizes non-specific reactions and ensures efficient PEPCK-mediated catalysis [102,104].

#### 5.5. The PEPCK enzyme—catalytic properties

PEPCK-C and PEPCK-M share similar kinetic characteristics and Michaelis constants for their substrates and products [119] as well as similar pH optima at around 7 [120]. Both PEPCKs are active as a monomer and catalyze the same reaction, namely decarboxylating OAA with the concomitant transfer of the  $\gamma$ -phosphate of GTP (or ITP):  $\text{OAA} + \text{GTP} \rightarrow \text{PEP} + \text{GDP} + \text{CO}_2$ . PEP formation is the preferred direction of both enzymes [76]. The only reported substrates for both PEPCKs are GTP (or GDP) and ITP (or IDP) [76,121]. Dissociation constants of PEPCK-C, measured by isothermal titration calorimetry, are 0.5  $\mu\text{M}$  for GTP, 4  $\mu\text{M}$  for ITP and for the other nucleotides > 100  $\mu\text{M}$  [103].

In addition, both isoforms require two divalent cations:  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$ . Other divalent metal ions such as  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  stimulate PEPCK, but to a lesser extent [107,122]. Noteworthy, PEP carboxylation by PEPCK-C absolutely requires  $\text{Mn}^{2+}$ , whereas  $\text{Mg}^{2+}$  can replace it for OAA decarboxylation [123]. Ash et al. [124] proposed that  $\text{Mg}^{2+}$  binds GTP and builds the metal nucleotide substrate, whereas  $\text{Mn}^{2+}$  associates with PEPCK-C serving as an activator, although  $\text{Mg}^{2+}$  can substitute. The  $\text{Mg}$ –GTP complex binds and activates PEPCK-C and then decarboxylates OAA ligated to the  $\text{Mn}^{2+}$  site of PEPCK-C. The  $\text{Mg}$ –GTP complex phosphorylates the enolate of pyruvate to PEP [124]. In contrast to PEPCK-C,  $\text{Mn}^{2+}$  is the best activator for PEPCK-M in chicken [125], whereas the selectivity of PEPCK-M for  $\text{Mn}^{2+}$  was 1000-fold greater than for  $\text{Mg}^{2+}$  [126]. Further  $\text{Mg}^{2+}$  has been noted to reduce PEPCK-M activity [80]. Both enzymes can randomly bind their substrate, although PEPCK-M usually binds GTP before OAA and has a slow off-rate [107,122]. Once GTP is bound, it has a high likelihood of progressing to product (PEP,  $\text{CO}_2$ , and GDP) [76]. Whether mitochondrial OAA or mtGTP are rate-determining for PEPCK-M is unclear, but the tight association between mtGTP synthesis and PEP production would suggest that mtGTP is rate-limiting [127].

#### 5.6. Regulation of PEPCK-M enzyme expression and activity

The two PEPCK isoforms are regulated differently. While PEPCK-C expression can be robustly stimulated by glucagon and suppressed by insulin, PEPCK-M is not known to be under such hormonal control and appears to be constitutively expressed [76,78]. Although sequence analysis of the PEPCK-M promoter region revealed several potential regulatory elements, including SREBP, CREB, C/EBP, AP-1, AP-2, and SRY elements [128]. Indeed, hormonal treatment of chicken hepatoma cells does not affect PEPCK-M mRNA expression [76,129]. Due to the mitochondrial location of PEPCK-M, it is a weaker candidate for rapid transcriptional regulation, and further, no post-translational modifications or allosteric effectors are known. Recently, posttranslational modification of PEPCK to regulate its activity has been suggested for PEPCK-C. Acetylation of PEPCK-C determines its stability as it targets the enzyme for ubiquitylation and subsequent proteasomal degradation, hence deactivates PEPCK-C [130–132]. The regulatory significance of acetylation of the enzyme remains to be established. Moreover, PEPCK-M mRNA half-life is relative long, namely ~50 h, while the half-life of PEPCK-C mRNA is short (~30 min) and transcriptional changes that affect PEPCK-C mRNA levels occur within 1–2 h [76]. During the shift to lactation, both PEPCK isoform activities increase in the mammary gland—a tissue with no known gluconeogenic activity, where they may support fat deposition (glyceroneogenesis) and subsequent milk production during lactation. During this time PEPCK-C activity (~11-fold) and expression increase, but in contrast to a dramatic increase in PEPCK-M activity (~43-fold) there are no changes in PEPCK-M expression [133,134]. Thus at least for PEPCK-M, activity levels do not necessarily parallel changes in gene expression. However, PEPCK-M expression may change under pathological conditions as observed in the diabetes model of the chronically glucose-infused rat [135]. Diabetic rats show elevated plasma insulin and glucagon levels as well as hepatic glucose production accompanied with raised PEPCK-M but decreased PEPCK-C expression. Whether PEPCK-M expression is altered by the combination of insulin and glucagon or some other signal remains to be clarified.

Recent work suggests a possible regulatory mechanism of PEPCK-M that operates at the enzyme level by transitioning between inactive tetrahedral and active octahedral coordination geometry, and represents a previously unappreciated mechanism of regulation [108]. Unlike PEPCK-C, the predominant conformation of the P-loop is the tetrahedral coordination of the reactive cysteine residue (Cys307) to  $\text{Mn}^{2+}$ . This loop confirmation stabilizes the hyper-reactive cysteine, prevents nucleotide-binding and represents a catalytically incompetent state of the enzyme [107]. Further, pH-rate studies propose that the protonation of this



cysteine residue is required for optimal interaction of PEPCK-M with its substrates [126]. Catalytic studies revealed that  $\text{Zn}^{2+}$  inhibits PEPCK-M activity in the direction of OAA formation because it favors sulfhydryl ligands and stabilizes the Cys307-coordinated tetrahedral geometry. Further, in vitro PEPCK-M activity studies require  $\beta$ -mercaptoethanol ( $\beta$ -ME), suggesting that the inactive conformation is stabilized without, and diminished with  $\beta$ -mercaptoethanol [111]. Transcriptional control tightly mediates PEPCK-C expression, and since the PEPCK-C protein has a half-life of 6 h [136], this mechanism of conformational regulation could also provide a mechanism of immediate regulation of PEPCK-C. Indeed, the requirement of reducing agents in enzymatic assays, such as dithiothreitol (DTT) or  $\beta$ -ME was also noted for PEPCK-C [119]. Importantly, the optimal pH for both PEPCK reactions depends on the divalent metal ion utilized because of subtle different secondary and tertiary enzyme structures at different proton concentrations [107,119,125].

In summary, since PEPCK activity relies on the availability of divalent metals as well as OAA and GTP, PEPCK seems to be prone to substrate regulation. Moreover, energy metabolism, redox state, ATP/ADP ratio as well as TCA cycle flux are other factors regulating PEPCK activity [137]. Given the lack of known regulators of PEPCK-M expression, its enzymatic flux is consequently much more susceptible to vary based on product and substrate concentrations. Hormones, such as insulin, glucagon, catecholamines and cortisol may all indirectly regulate PEPCK-M via controlling gluconeogenic substrate availability.

## 6. Is there a role of PEPCK-M in gluconeogenesis?

Presently, PEPCK-C is generally believed to account for virtually all the gluconeogenesis from mitochondrial-derived precursors, at least in rodents [138]. PEPCK-C is often considered as the only isoform and it is even frequently abbreviated simply as PEPCK. However, as noted above, it is less appreciated that there are actually two distinct isoforms that reside in either the cytosol or the mitochondria [8]. The cytosolic isoform has attracted the majority of attention despite PEPCK-M being the first isoform discovered and 40% of the cellular PEP can be found in the mitochondrial fraction [139–142].

PEPCK-C is well-known for its role in gluconeogenesis and its function has been extensively studied and reviewed [76,86,138,143,144]. Apart from gluconeogenesis the following functions have been suggested: glyceroneogenesis, cataplerosis/anaplerosis, amino acid synthesis, PEP/pyruvate cycling. Similar functions of both PEPCK enzymes are suggested since they both catalyze the same reaction [76]. The common denominator of both PEPCK enzymes is PEP as a product that may be utilized for biochemical processes. PEP is needed for glucose production but also serves for triglyceride, amino acids and nucleic acid synthesis. The source of PEP for these synthetic processes may be dependent on the tissue, metabolic condition, energy status and precursor availability [100]. While PEPCK-C function is remarkably well studied, the biology of PEPCK-M is poorly investigated. PEPCK-M is highly conserved in all eukaryotes studied, suggesting PEPCK-M an important biological role [138].

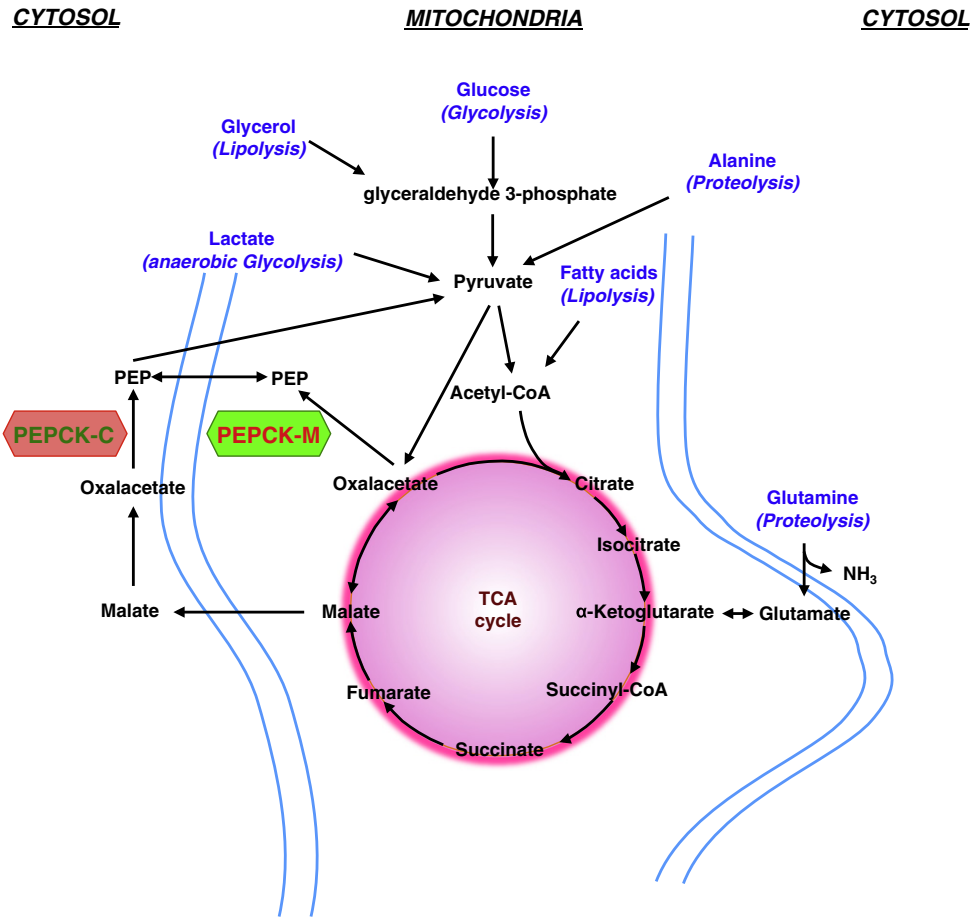
An inferior role for PEPCK-M in gluconeogenesis has primarily been supported by in vitro studies [145–148]. Deletion and overexpression studies of PEPCK-C in the liver confirmed a role of PEPCK-C in gluconeogenesis and glyceroneogenesis [137,145–147,149,150]. Surprisingly, though, several genetic models of PEPCK-C deletion maintain normal fasting glucose and glucose turnover rates. This suggests that other mechanism(s) must exist that can support glucose production [146,147]. Notably, there is no correlation between PEPCK-C mRNA, protein, or activity in humans with diabetes [151]. The current dogma of PEPCK-C as sole regulator of cataplerotic PEP production may need to be recalibrated. Here, we will consider the possibility that PEPCK-M has been overlooked and might have a potential role in gluconeogenesis.

### 6.1. A hint from PEPCK-C knockout animals

In support for an exclusive role of PEPCK-C in glucose production, homozygous whole-body PEPCK-C knockout mice die within 2–3 days of birth that is associated with hypoglycemia [146,147]. However, these pups could not be rescued by exogenous glucose and had normal plasma lactate. Interestingly, deletion of glucose-6-phosphatase in mice did not cause death until the age of 10 days. These mice were profoundly hypoglycemic due to diminished gluconeogenesis, but, in contrast, the mice could be rescued by glucose [152]. Noteworthy, given that PEPCK-C is dramatically induced at time of birth [89], it may be possible that these PEPCK-C null mice could survive once they made it through the neonatal phase (for example as a cre/lox mice with 80% reduction at birth and a 90–100% reduction later on). Neonatal death of homozygous whole-body PEPCK-C knockout animals may indicate other important functions of PEPCK-C.

Based on current models of gluconeogenesis, one would predict that hepatic PEPCK-C deficiency would cause hypoglycemia, in particular during states of negative energy balance such as fasting. Surprisingly, heterozygous mice lacking hepatic PEPCK-C ( $\text{PCK1}^{\text{lox/lox}} \text{ Alb-Cre}$ ) remain euglycemic under fed and fasted conditions, although these mice show evidence of hepatic steatosis, insulin resistance and exercise intolerance [145–147]. Even with less than 10% of whole body PEPCK-C activity (a level comparable to endogenous PEPCK-M) mice have normal concentrations and turnover rates of glucose, lactate, and glycerol in vivo [146,147]. In this setting, the absolute rates of PEP production necessary for gluconeogenesis are not significantly reduced [148]. Only, when PEPCK-C is completely eliminated does a significant reduction in PEP synthesis occur in perfused livers [148]. This degree of PEPCK-C suppression does not occur in vivo and suggests other physiologic mechanisms normally regulate PEPCK flux besides just PEPCK-C expression. In order to explain the observed euglycemia in PEPCK-C knockout animals, extra-hepatic gluconeogenesis was suggested to compensate, whereas the alternative hepatic pathway to glucose production was ruled out due to the assumed lack of PEPCK-M activity in rodent liver [146]. This is supported by the observation that renal gluconeogenesis has an enormous capacity and can completely compensate during the extrahepatic phase of liver transplants [13,153–155]. Intriguingly, liver-specific PEPCK-C deficient mice develop fatty livers, have reduced TCA cycle flux, and accumulate TCA cycle intermediates indicating that they are indeed PEPCK-C deficient [137,146,147,156].

Both PEPCK isoforms depend on normal mitochondrial function to generate energy and gluconeogenic precursors. However, PEPCK-C knockout animals have varying severity of mitochondrial dysfunction [145,148,156]. Notably, the degree of PEPCK-C deficiency in liver-specific knockout mice correlates with the extent of the TCA cycle defect. Complete PEPCK-C loss impairs mitochondrial function with a 20% reduction in  $\text{O}_2$  consumption and 85% reduction in the TCA cycle flux [148]. Consequently, liver-specific PEPCK-C knockout mice accumulate mitochondrial intermediates (including citrate, succinate, fumarate, malate and aspartate), have a high mitochondrial NADH/NAD<sup>+</sup> ratio and severe fatty livers (but normal malonyl CoA). In addition, these mice show changes in expression of metabolic and mitochondrial genes. Recently, these mice have been used to confirm a role of PEPCK-M in gluconeogenesis [145]. However, a 100-fold overexpression of PEPCK-M in the livers of these mice did not have any detectable consequence in vivo and just a marginal improvement (~8%) of glucose production from PEP in perfused livers. The interpretation was that PEPCK-M lacks a primary role and only supports PEPCK-C as primary determinant of gluconeogenesis. Another interpretation is that rodent livers do not actually lack PEPCK-M (Table 1) but rather the normal levels of PEPCK-M cannot work in the setting of severely impaired mitochondrial function. Of note,  $\beta$ -cells have equally high levels of anaplerotic and cataplerotic metabolism compared to the liver. However,  $\beta$ -cells do not express PEPCK-C [7], and still have properly functioning mitochondria. Thus, there must



**Fig. 3.** Gluconeogenic substrates entering the gluconeogenic pathway. Graph shows entry point of gluconeogenic substrates, such as lactate, glycerol or amino acids. Both PEPCKs synthesize PEP from OAA that can feed the TCA cycle (anaplerosis) or serve for various biosynthetic processes (cataplerosis), such as gluconeogenesis. Alanine and glutamine are the main amino acids in the blood and arise during starvation from muscular protein breakdown (*proteolysis*). Likewise, fatty acids and glycerol are released from triglyceride breakdown (*lipolysis*) during fasting. Unlike glycerol, acetyl-CoA (fatty acid breakdown) does not contribute to cataplerotic OAA or PEP production or other gluconeogenic intermediates. Glycerol and glucose enter via glyceraldehyde 3-phosphate. Lactate forms in the muscle during *anaerobic glycolysis* and enters the gluconeogenic pathway via pyruvate and is the main gluconeogenic precursor in the kidney and liver.

be something unique to hepatic PEPCK-C null mice causing mitochondrial dysfunction. Accordingly, it is difficult to conclude if changes in gluconeogenesis are caused by the loss of PEPCK-C enzymatic activity *per se* or because of other metabolic deficiencies. However, in contrast to the perfused livers from these mice, *in vivo* basal gluconeogenesis is not reduced in different PEPCK-C knockout animals. Regardless, given the dependence of both isoforms on normal mitochondrial function, results from PEPCK-C knockouts do not preclude a significant role for PEPCK-M.

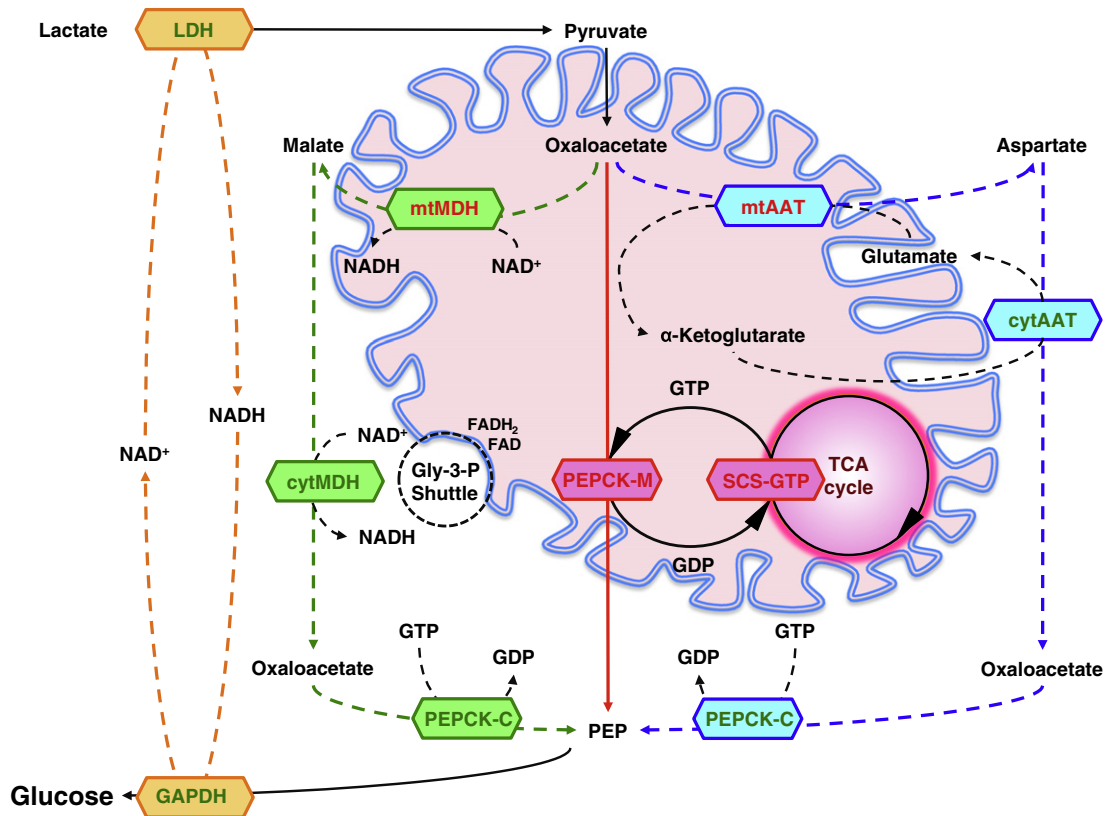
Besides the general view that PEPCK-M is not sufficiently active in rodent liver, a few publications (Table 1) [82–85,135] suggest contrary and would challenge the above mentioned studies [137,146,147]. Surprisingly, in the livers of chronically glucose-infused rats, a model of Type-2 diabetes with inappropriate increases in glucagon and insulin, PEPCK-C was suppressed but PEPCK-M increased in accordance with endogenous glucose production rates [135]. These results point towards an undiscovered role for PEPCK-M in gluconeogenesis [146,147]. Finally, PEPCK-C and PEPCK-M may operate in parallel, thus PEPCK-M may compensate for PEPCK-C knockout animals as long as mitochondrial function remains intact. Taken together, there does appear to be a potential role for PEPCK-M in gluconeogenesis, but inactivation or inhibition of the mitochondrial isoform would need to be performed to solidify its role.

#### 6.2. Could PEPCK-M participate in gluconeogenesis?

Given the possibility that PEPCK-M's activity in the liver may have been underestimated, is there a possibility that it has a role in

gluconeogenesis? Unlike PEPCK-C, PEPCK-M is constitutively expressed, hence could provide a continuous supply of PEP. PEPCK-M is not inducible by glucagon [157] but this also does not rule out a gluconeogenic role. Both PEPCK isoforms convert OAA to PEP [158]. However, a potential limitation of the PEPCK-M pathway is its dependency on mtGTP. One TCA cycle turn makes only one mtGTP. Unless there is an alternative source of mtGTP during states of high demand, SCS-GTP flux would set an upper limit to mtPEP production. Presently the capacity of PEPCK-M to accommodate mtGTP from the TCA cycle in rodent liver is unknown. In contrast, PEPCK-C is not dependent on mtGTP and in principle would have the capacity to produce extra PEP albeit at higher energetic cost (Fig. 5). So while PEPCK-M may support basal needs, during times of high glucose demand, collaboration between the isoforms may likely be required. Thus, a system with two PEPCKs would provide an increased degree of metabolic flexibility.

In addition to changes in protein expression induced by hormonal signaling, gluconeogenic flux is potentially regulated turning on and off a futile PEP cycle (pyruvate → OAA → PEP → pyruvate) [4,5,7,159]. Here, this anaplerotic/cataplerotic cycling could operate in three ways: 1) redirecting anaplerotic substrates to pyruvate (including substrates that enter the TCA cycle as 4- and 5-carbons such as aspartate, glutamate and glutamine) and 2) consuming ATP that could otherwise be used for gluconeogenesis to buffer substrate flux and 3) providing additional amounts of metabolic cycling to fine tune metabolic flux while ensuring adequate TCA cycle intermediates [4].



**Fig. 4.** Gluconeogenesis from lactate. Lactate as substrate generates NADH via lactate dehydrogenase (LDH) in the cytosol necessary for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction of gluconeogenesis (orange). Mitochondrial oxaloacetate (OAA) has four pathways to cytosolic phosphoenolpyruvate (PEP), whereas three are energetically preferable for gluconeogenesis: The *PEPCK-M* pathway (red) is the most direct pathway and uses mtGTP produced by succinyl-CoA synthetase (SCS-GTP) in the TCA cycle. The *PEPCK-C/aspartate* pathway (blue) uses mitochondrial (mtAAT) and cytosolic (cytAAT) transamination reactions via aspartate aminotransferase (AAT) and needs shuttling of glutamate and  $\alpha$ -ketoglutarate to generate OAA in the cytosol. OAA is converted by PEPCK-C that hydrolyses cytosolic GTP for the production of cytosolic PEP. The *PEPCK-C/malate* pathway (green) uses mitochondrial (mtMDH) and cytosolic (cytMDH) malate dehydrogenase (MDH). Mitochondrial MDH converts OAA in malate and malate is transferred to the cytosol, where cytosolic MDH forms OAA. This creates an excess NADH oxidized by the glycerol-3-phosphate shuttle.

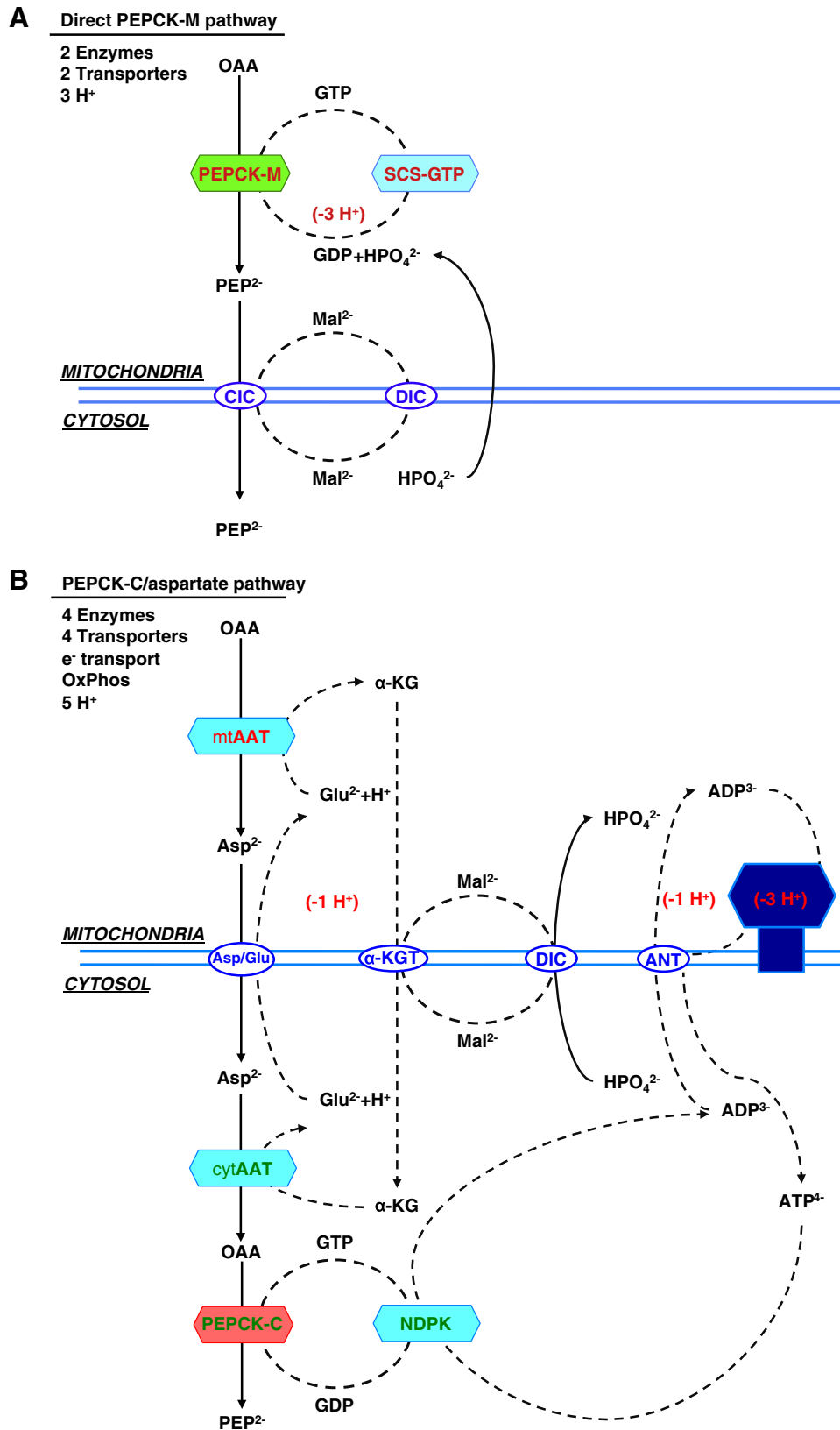
### 6.3. Gluconeogenic substrates

Generally, any metabolite that enters the TCA cycle to build OAA can contribute to glucose production once converted to PEP (Fig. 3). Glucose can be synthesized *de novo* from mitochondrial-dependent substrates (e.g., lactate, pyruvate, glutamine, alanine, and propionate) or from cytosolic substrates (e.g., glycerol and fructose). All of the mitochondrial gluconeogenic precursors have an absolute requirement for the PEPCK reaction. During each turn of the TCA cycle, citrate synthase consumes as much OAA as is produced by malate dehydrogenase (MDH). Balanced carbon outflow into synthetic pathways (cataplerosis) requires compensation by carbon inflow (anaplerosis) into the TCA cycle pool. Thus, the carbons of acetyl-CoA generated by lipolysis or PDH cannot directly contribute to gluconeogenesis without an additional source of OAA. Unlike fatty acids from triglyceride breakdown, glycerol can contribute to glucose production and enters gluconeogenesis (or glycolysis) via glyceraldehyde-3-phosphate. Pyruvate, lactate, and alanine become anaplerotic substrates when they are carboxylated into OAA by PC. The amino acids alanine and glutamine must first be deaminated in order for them to enter the TCA cycle. Since aminotransferase reactions require a net acceptor of the amide nitrogen, amino acids must be either oxidatively deaminated by GDH to release ammonia or processed in the ornithine cycle to generate urea. Methionine, threonine and the branched chain amino acids are added to the TCA cycle through the metabolism to propionyl-CoA. Similarly the final  $\gamma$  oxidation of the occasional odd-chain fatty acids also generates propionyl-CoA. Propionyl-CoA then must be carboxylated by the biotin-dependent enzyme propionyl-CoA carboxylase before it can enter the TCA cycle as succinyl-CoA [20].

Lactate forms in the muscle during anaerobic glycolysis and enters the gluconeogenic pathway via pyruvate and is the main gluconeogenic precursor in the kidney and liver. Noteworthy, the kidney has been suggested to prefer glutamine [160] while the liver may favor alanine as gluconeogenic precursors [13].

Each mitochondrial substrate has its own advantages and disadvantages. Based on turnover rates, lactate produced by the Cori cycle is quantitatively the most important gluconeogenic substrate. Given that glucose is the predominant source of lactate, once glycogen stores are depleted, lactate cannot make a net contribution to the glucose pool in the setting of ongoing glucose oxidation. Pyruvate, too, is derived primarily from glucose, but because of rapid equilibration across lactate dehydrogenase (LDH) is about one tenth the concentration of circulating lactate. Alanine is a significant gluconeogenic contributor, but about half of the carbon in the glucose-alanine cycle also comes from glucose itself with the remaining coming from amino acids from protein breakdown [12,15]. Glutamine is the most concentrated circulating amino acid and only a small fraction contains carbons from glucose making it a net contributor to the glucose pool. Some propionate comes from the breakdown of certain amino acids and odd-chain fatty acids, but its significant contribution to gluconeogenesis comes from the gut flora following meals and is an especially important source of glucose for ruminants.

Each mitochondrial substrate may favor one or the other PEPCK isoform in order to provide PEP while maintaining a favorable cytosolic redox. Ultimately, all mitochondrial substrates entering gluconeogenesis oxidize NADH at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) step. If glucose is made from lactate, NADH for GAPDH is produced in the cytosol by LDH [161]. Therefore, when the substrate for



**Fig. 5.** (A) direct PEPCK-M pathway, (B) aspartate/PEPCK-C pathway, (C) malate/PEPCK-C pathway and (D) citrate/PEPCK-C pathway. Enzymes, transporters and metabolites involved in the 4 metabolic pathways from mitochondrial oxaloacetate to cytosolic PEP. The charge valences of the metabolites that are used by the different transporters are listed as superscripts. The accounting is based on the number of protons equivalents generated (in green) or consumed (in red) by each of the metabolic steps. Synthesis of both ATP and GTP within the mitochondria was assumed to be equivalent to 3 protons, the transport of one ATP out of the mitochondria and glutamate into the mitochondria each consume one proton. Oxidation of mitochondrial NADH pumps 10 protons, while cytosolic translocates 6 via the glycerol-3-phosphate step since the malate aspartate shuttle is not available. Abbreviations: OAA, oxaloacetate; PEP, Phosphoenolpyruvate; CIC, citrate isocitrate transporter; DIC, dicarboxylate transporter; Asp, aspartate;  $\alpha$ KG,  $\alpha$ -ketoglutarate; Asp/Glu, aspartate glutamate transporter;  $\alpha$ KGT,  $\alpha$ -ketoglutarate transporter; ANT, adenine nucleotide transporter; Mal, malate; Glu, Glutamate; mtAAT, mitochondrial aspartate aminotransferase, cytAAT, cytosolic aspartate aminotransferase; mtMDH, mitochondrial malate dehydrogenase; cytMDH, cytosolic malate dehydrogenase; mtGlyc-3-PDH, mitochondrial glycerol 3 phosphate dehydrogenase; cytGlyc-3-PDH, cytosolic glycerol-3-phosphate dehydrogenase; NDPK, nucleotide diphosphokinase.



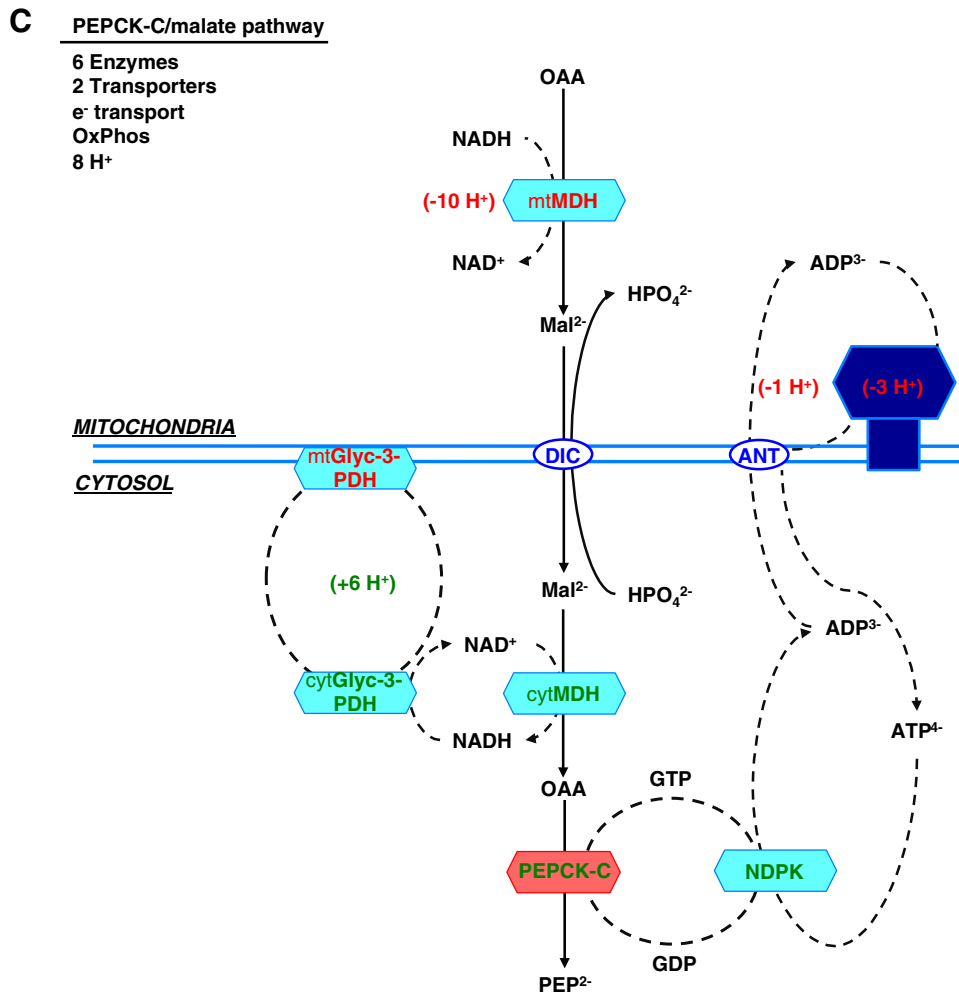


Fig. 5 (continued).

gluconeogenesis is lactate, PEPCK-M is favored since a shift of reducing equivalents from the mitochondria to the cytosol is not required (Fig. 4) [78]. The study from Gstraunthler et al. [162] identifies lactate as a likely substrate for PEPCK-M. Cells from pig kidney cortex (LLC-PK<sub>1</sub>F<sup>+</sup>) expressing both PEPCK isoforms grow in a medium containing pyruvate and lactate, whereas cells from opossum kidney (OK<sub>GNG</sub>+) that express only PEPCK-M could be only maintained in a medium with lactate. This may be explained by the requirement of pyruvate, propionate or alanine to generate cytosolic NADH. In this case, mitochondrial-derived OAA was proposed to be converted to malate in the matrix and shuttled to the cytosol where cytMDH would make both NADH and OAA [76]. Subsequently, PEPCK-C metabolizes cytosolic OAA to PEP, leaving NADH for GAPDH step of gluconeogenesis [76]. Special consideration for alanine depends on whether its amide nitrogen is eventually disposed as ammonia or as urea. While ammonia release (via transamination of  $\alpha$ -ketoglutarate into glutamate followed by GDH metabolism) generates mitochondrial NAD(P)H, urea metabolism could increase NADH in the cytosol or mitochondria depending on where the MDH reaction needed for the ornithine cycle occurs. Glutamine catabolism may be advantageous during a prolonged fast as it provides the energy, carbons, as well as mtGTP for efficient mtPEP formation. Again, the mechanism of ammonia disposal may determine whether PEPCK-C or PEPCK-M is favored.

#### 6.4. PEPCK-M is the more direct pathway for PEP production

As noted, lactate is quantitatively the most important mitochondrial gluconeogenic substrate. Lactate is continuously produced (even in times

of hyper- or hypoinsulinemia) and lactate turnover increases during euglycemic hyperinsulinemic clamps. Since PEPCK-C is suppressed by insulin but PEPCK-M is constitutively expressed, then the mitochondrial isoform is well poised to continuously produce PEP from lactate. In contrast, the release of amino acids into the blood from skeletal muscle is variable and therefore requires the hormone-regulateable adaptation of PEPCK-C gluconeogenesis [78]. Thus, it has been suggested that PEPCK-M is the enzyme for gluconeogenesis from lactate while PEPCK-C is preferred for glucose production from pyruvate and amino acids depending on supply and demand [163]. Taken together, in terms of energetics, enzymatics, and transport, PEPCK-M metabolism of lactate is a more efficient pathway from mitochondrial OAA to cytosolic PEP (Fig. 4). Indeed, a mitochondrial PEPCK location makes sense since the initial step of OAA formation also occurs in the mitochondria. In principle, there are four possible pathways to generate PEP in the cytosol [76] (Figs. 4 and 5):

- 1) *PEPCK-M pathway* (conversion to PEP via PEPCK-M and SCS-GTP)
- 2) *PEPCK-C/aspartate pathway* (transamination to aspartate by AAT)
- 3) *PEPCK-C/malate pathway* (reduction to malate via MDH)
- 4) *PEPCK-C/citrate pathway* (conversion to citrate by citrate synthase).

In theory, a *PEPCK-M pathway* (Fig. 5A) would need just two enzymes (SCS-GTP and PEPCK-M) in order to generate mtPEP and is independent of the need for oxidative phosphorylation. The transport of mtPEP into the cytosol via the CIC then occurs with the lowest cumulative energetic cost [7]. In contrast, to deliver OAA in the cytosol for PEPCK-C, the OAA first must be transferred out of the mitochondria.

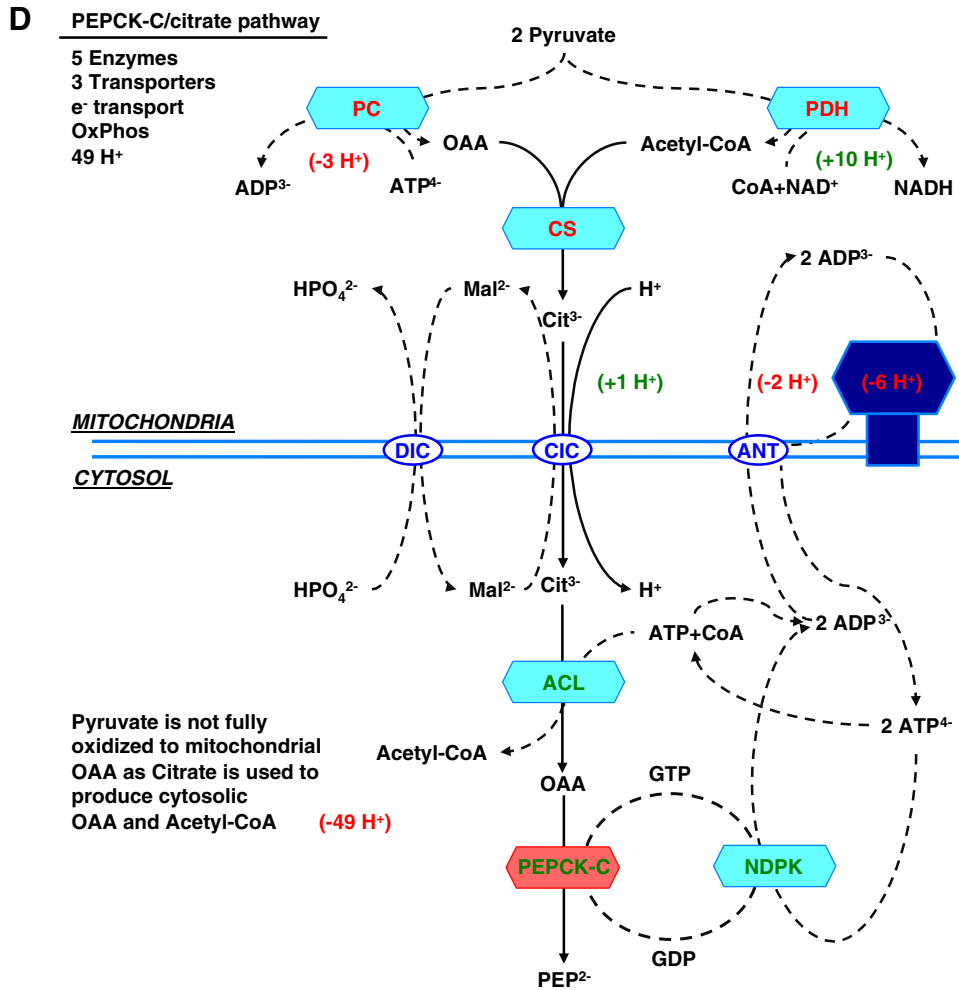


Fig. 5 (continued).

Since OAA lacks a transporter then it is either transaminated to aspartate or reduced to malate.

The *PEPCK-C/aspartate pathway* (Fig. 5B) is more complex and involves four enzymes, electron transport and oxidative phosphorylation. OAA is first transaminated to aspartate in the mitochondria and subsequently transported into the cytosol where it is transaminated back into OAA via cytosolic aspartate aminotransferase. Continuous transport of glutamate into, and  $\alpha$ -ketoglutarate out of the mitochondrion is required for the transamination reaction and therefore the process is restricted by the availability of these other substrates [20]. Four transporters are involved to obtain cytosolic PEP from mitochondrial OAA. This raises the energetic cost to 40% because two additional protons must be delivered to the matrix coupled to the transport of glutamate and ATP.

The *PEPCK-C/malate pathway* (Fig. 5C) utilizes mtMDH to reduce OAA to malate and is favored by a highly reduced matrix. Malate shuttles to the cytosol where cytMDH oxidizes malate and uses NAD<sup>+</sup> to generate OAA and an excess NADH. The PEPCK-C/malate pathway then requires the glycerol-3-phosphate dehydrogenase shuttle in order to balance cytosolic redox. Altogether, it needs four enzymes, energetic expensive shuttles as well as the electron transport and oxidative phosphorylation for a net cost of >60% more energy.

The *PEPCK-C/citrate pathway* (Fig. 5D), if solely used to generate PEP, is energetically the most expensive pathway to generate PEP in the cytosol. It requires four enzyme reactions (citrate synthase, ATP-citrate lyase, NDPK, PEPCK-C), two transporters (DIC, ANT), electron transport and oxidative phosphorylation. Further, it would cost two pyruvates

and two ATPs (for PC and ATP-citrate lyase reaction). Also, pyruvate is not fully oxidized in the TCA cycle and carbons are instead transferred to the cytosol to form OAA and acetyl-CoA. Thus, considering the loss of acetyl-CoA oxidation, then the energetic cost for this pathway is very expensive (~49 protons). Citrate export favors lipid synthesis rather than gluconeogenesis as it generates acetyl-CoA and OAA in the cytosol. Formation of NADPH by malic enzyme could then energize lipid synthesis, but would deprive the cytosol of PEP that could be used for gluconeogenesis.

In summary, the PEPCK-M pathway is in theory the most direct pathway for PEP production in the cytosol. It offers significant metabolic advantages over any described PEPCK-C pathways. These include greater energy efficiency and less dependence upon oxygen consumption—qualities especially favorable for fasting and exercise. This efficiency may explain why PEPCK-M is the only isoform found in the livers of birds of flight that are highly dependent on Cori cycling [163,164]. At this time, PEPCK-M has not been directly evaluated experimentally as a potential contributor to gluconeogenesis. Here we raise the interesting possibility that it may be more significant than previously considered.

## 7. PEPCK-M deficiency

Alterations in PEPCK-C gene expression and its metabolic effect have been intensively studied in animal models [24,137,146,147,165–168]. To date there has been no animal model studied with altered PEPCK-M expression. Human cases of PEPCK-M deficiency were initially reported in some children that died prematurely due to liver failure. Analysis of

fibroblasts (which only have PEPCK-M) detected the defect and the residual PEPCK activities were 18% and 16% respectively [169–171]. The deficiency was observed with failure to thrive, fasting hypoglycaemia, glucagon insensitivity, lactic acidemia, hypotonia, hepatomegaly and liver function impairment. Autopsy revealed massive fat deposition in the liver and kidneys. However, a later study suggested that the lowered PEPCK-M activity was not the primary defect and that mitochondrial DNA depletion was to blame [172]. The reports of Vidnes and Sovik in 1976 [173–175] describe the phenotype of a boy whose liver lacked PEPCK-C and suffered from persistent hypoglycemia and died in his early childhood. PEPCK-C activity was virtually zero [175]. Interestingly, total hepatic PEPCK activity was normal, and closer investigation revealed that the boy did not have primary PEPCK-C deficiency but rather suppressed PEPCK-C expression due to hyperinsulinemia. Predominant  $\alpha$ -cells over  $\beta$ -cells and hyperplasia of the islets of the endocrine pancreas suggest the possibility of a hormonal defect rather than PEPCK-C deficiency. Noteworthy, it is uncertain whether a primary gene defect exists or any other defect regulating PEPCK activity (e.g. hormones, another PEPCK regulating protein). In addition, the performed PEPCK activity assays did not distinguish between the two isoforms. However, total PEPCK deficiency (low PEPCK-C plus PEPCK-M activity) is likely to cause severe hypoglycemia and early death [169,176,177].

## 8. Summary and concluding remarks

Since the discovery of PEPCK, the knowledge of its characteristics and biological role has increased tremendously. The enzyme is not exclusively involved in glucose production but may have broader metabolic functions in “balancing” the TCA cycle. Indeed, it plays an important role in cataplerosis of TCA cycle intermediates and is required for gluconeogenesis and glyceroneogenesis. In the past, PEPCK (mainly PEPCK-C) was shown to play a pivotal role as a regulator of both carbohydrate and lipid metabolism. Prior to the cloning of the genes of each isoform, it was not appreciated that each PEPCK isoform comes from two distinct nuclear genes, from different chromosomes with different sequences and catalytic properties. The cytosolic isoform has had considerable attention in the livers of most studied animals – the rat and the mouse – because of reports of insignificant PEPCK-M [8,76,89,137,146,147,178–181]. This conclusion, as well as strong hormonal transcriptional regulation led to considerable early enthusiasm primarily for PEPCK-C.

Nevertheless, mammalian livers express two PEPCK isoforms (PEPCK-C and PEPCK-M). Both catalyze the metabolism of OAA into PEP using GTP as a phospho-donor [76]. As the two isoforms are highly homologous and depend upon GTP hydrolysis, the function, as well as regulation, of PEPCK-M has been assumed to be similar to PEPCK-C [100,138]. At the present time, the metabolic contribution of PEPCK-M to gluconeogenesis and glyceroneogenesis remains unknown. Upon reconsideration, PEPCK-C may be favored to convert pyruvate and amino acids to glucose during fasting while PEPCK-M may favor lactate from the Cori cycle [76]. Another way to interpret the lack of hormonal regulation of PEPCK-M is that it is constitutively active, unlike PEPCK-C that disappears when insulin is elevated. It is tempting to speculate that PEPCK-M provides the foundation of gluconeogenesis while PEPCK-C contributes additional capacity to augment glucose production in times of need.

The regulation of PEPCK-M may be explained not by expression but rather by its dependency upon mtGTP. It is well disposed to regulate cataplerosis when there is adequate TCA flux. In pancreatic  $\beta$ -cells this mitochondrial metabolic flux pathway couples TCA cycle flux with anaplerotic flux to trigger insulin secretion. In the gluconeogenic tissues we raise the hypothesis that a similar pathway exists. Thus the mtGTP/PEPCK-M pathway may be a significant determinant of endogenous glucose production that is not turned off by hormonal regulation. Such “unresponsiveness” may ensure a continuous level of PEP synthesis. In the background of “basal” PEPCK-M activity the hormonally regulated PEPCK-C pathway can be turned on and off as needed without a

risk of hypoglycemia. This is supported by the observation that even a 90% reduction of whole-body or 100% hepatic PEPCK-C activity did not result in changes in glycemia until the mice were stressed [146]. A continuous supply of PEP by PEPCK-M, even in the presence of high insulin, may also be important for other biosynthetic pathways, such as glycogen or triglycerides. Noteworthy, half of the postprandial glycogen synthesis still comes from gluconeogenesis when insulin levels are increased (and PEPCK-C suppressed) [18,182,183]. Further, healthy humans derive 50% of endogenous glucose production from gluconeogenesis even in the immediate post-absorptive phase when glycogen stores are not depleted [184]. Theoretically, having a background pathway for continuous PEP production by PEPCK-M protects against hypoglycemia, which could be caused by complete cessation of gluconeogenesis. The PEPCK-C pathways expand the overall capacity for gluconeogenesis as needed in response to hormonal and metabolic cues. However, PEPCK-C expression may only have minimal control over basal rates of gluconeogenesis. Given that humans have a much higher relative PEPCK-M activity [185], the specter is raised that the mitochondrial isoform could play an even more significant role in normal or pathologic, insulin-independent, fasting and fed glucose production.

In summary, both PEPCK isoforms convert TCA cycle OAA into PEP as they consume GTP. PEP can re-enter the TCA cycle (anaplerosis, “PEP cycle”) [7], or exit the TCA cycle (cataplerosis) to ultimately feed carbons for a number of downstream process, such as gluconeogenesis and glyceroneogenesis [158]. Overall metabolic milieu and type of carbons entering the TCA cycle may ultimately determine the relative activities of each isoform. While PEPCK-M ensures continuous cataplerotic PEP production, PEPCK-C may augment gluconeogenic capacity when needed. As this mitochondrial mtGTP/PEPCK-M pathway may regulate the two faces of glucose homeostasis – glucose clearance and glucose production – we envision PEPCK-M and SCS-GTP as a “metabolic tachometer” that uses mtGTP to “sense” TCA cycle flux. The role of PEPCK-M in gluconeogenesis needs to be confirmed.

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## References

- [1] B.R. Landau, et al., Contributions of gluconeogenesis to glucose production in the fasted state, *J. Clin. Invest.* 98 (2) (1996) 378–385.
- [2] E. Cersosimo, P. Garlick, J. Ferretti, Insulin regulation of renal glucose metabolism in humans, *Am. J. Physiol.* 276 (1 Pt 1) (1999) E78–E84.
- [3] C. Meyer, et al., Effects of physiological hyperinsulinemia on systemic, renal, and hepatic substrate metabolism, *Am. J. Physiol.* 275 (6 Pt 2) (1998) F915–F921.
- [4] J.G. Jones, et al., Hepatic anaplerotic outflow fluxes are redirected from gluconeogenesis to lactate synthesis in patients with Type 1a glycogen storage disease, *Metab. Eng.* 11 (3) (2009) 155–162.
- [5] I. Magnusson, et al., Noninvasive tracing of Krebs cycle metabolism in liver, *J. Biol. Chem.* 266 (11) (1991) 6975–6984.
- [6] O.E. Owen, S.C. Kalhan, R.W. Hanson, The key role of anaplerosis and cataplerosis for citric acid cycle function, *J. Biol. Chem.* 277 (34) (2002) 30409–30412.
- [7] R. Stark, et al., Phosphoenolpyruvate cycling via mitochondrial phosphoenolpyruvate carboxykinase links anaplerosis and mitochondrial GTP with insulin secretion, *J. Biol. Chem.* 284 (39) (2009) 26578–26590.
- [8] R.C. Nordlie, H.A. Lardy, Mammalian liver phosphoenolpyruvate carboxykinase activities, *J. Biol. Chem.* 238 (1963) 2259–2263.
- [9] R.G. Kibbey, et al., Mitochondrial GTP regulates glucose-stimulated insulin secretion, *Cell Metab.* 5 (4) (2007) 253–264.
- [10] A. Consoli, et al., Determination of Krebs cycle metabolic carbon exchange in vivo and its use to estimate the individual contributions of gluconeogenesis and glycogenolysis to overall glucose output in man, *J. Clin. Invest.* 80 (5) (1987) 1303–1310.
- [11] J. Wahren, P. Felig, L. Hagenfeldt, Physical exercise and fuel homeostasis in diabetes mellitus, *Diabetologia* 14 (4) (1978) 213–222.
- [12] J.E. Gerich, Physiology of glucose homeostasis, *Diabetes Obes. Metab.* 2 (6) (2000) 345–350.
- [13] M. Stumvoll, et al., Human kidney and liver gluconeogenesis: evidence for organ substrate selectivity, *Am. J. Physiol.* 274 (5 Pt 1) (1998) E817–E826.
- [14] M. Stumvoll, et al., Renal glucose production and utilization: new aspects in humans, *Diabetologia* 40 (7) (1997) 749–757.

- [15] J.E. Gerich, Role of the kidney in normal glucose homeostasis and in the hyperglycaemia of diabetes mellitus: therapeutic implications, *Diabet. Med.* 27 (2) (2010) 136–142.
- [16] A. Gastaldelli, et al., Separate contribution of diabetes, total fat mass, and fat topography to glucose production, gluconeogenesis, and glycogenolysis, *J. Clin. Endocrinol. Metab.* 89 (8) (2004) 3914–3921.
- [17] D.G. Maggs, et al., Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial, *Ann. Intern. Med.* 128 (3) (1998) 176–185.
- [18] M. Roden, et al., The roles of insulin and glucagon in the regulation of hepatic glycogen synthesis and turnover in humans, *J. Clin. Invest.* 97 (3) (1996) 642–648.
- [19] M. Roden, K.F. Petersen, G.I. Shulman, Nuclear magnetic resonance studies of hepatic glucose metabolism in humans, *Recent Prog. Horm. Res.* 56 (2001) 219–237.
- [20] J.M. Berg, J.L. Tymoczko, L. Stryer, *Biochemistry*, 6th ed., W. H. Freeman, New York, 2006.
- [21] M.J. Gibala, et al., Tricarboxylic acid cycle intermediate pool size and estimated cycle flux in human muscle during exercise, *Am. J. Physiol.* 275 (2 Pt 1) (1998) E235–E242.
- [22] A. Kelly, C.A. Stanley, Disorders of glutamate metabolism, *Ment. Retard. Dev. Disabil. Res. Rev.* 7 (4) (2001) 287–295.
- [23] C. MacMullen, et al., Hyperinsulinism/hyperammonemia syndrome in children with regulatory mutations in the inhibitory guanosine triphosphate-binding domain of glutamate dehydrogenase, *J. Clin. Endocrinol. Metab.* 86 (4) (2001) 1782–1787.
- [24] D.O. Lambeth, et al., Expression of two succinyl-CoA synthetases with different nucleotide specificities in mammalian tissues, *J. Biol. Chem.* 279 (35) (2004) 36621–36624.
- [25] J.D. Wikstrom, et al., A novel high-throughput assay for islet respiration reveals uncoupling of rodent and human islets, *PLoS One* 7 (5) (2012) e33023.
- [26] C. Li, et al., Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets, *J. Biol. Chem.* 278 (5) (2003) 2853–2858.
- [27] E.E. McKee, et al., Origin of guanine nucleotides in isolated heart mitochondria, *Biochem. Biophys. Res. Commun.* 257 (2) (1999) 466–472.
- [28] E.E. McKee, et al., Guanine nucleotide transport by atractyloside-sensitive and -insensitive carriers in isolated heart mitochondria, *Am. J. Physiol. Cell Physiol.* 279 (6) (2000) C1870–C1879.
- [29] A. Voza, et al., Identification of the mitochondrial GTP/GDP transporter in *Saccharomyces cerevisiae*, *J. Biol. Chem.* 279 (20) (2004) 20850–20857.
- [30] J.D. Johnson, et al., Genetic evidence for the expression of ATP- and GTP-specific succinyl-CoA synthetases in multicellular eucaryotes, *J. Biol. Chem.* 273 (42) (1998) 27580–27586.
- [31] A. Kowluru, Adenine and guanine nucleotide-specific succinyl-CoA synthetases in the clonal beta-cell mitochondria: implications in the beta-cell high-energy phosphate metabolism in relation to physiological insulin secretion, *Diabetologia* 44 (1) (2001) 89–94.
- [32] E.F. Kadrmas, P.D. Ray, D.O. Lambeth, Apparent ATP-linked succinate thiokinase activity and its relation to nucleoside diphosphate kinase in mitochondrial matrix preparations from rabbit, *Biochim. Biophys. Acta* 1074 (3) (1991) 339–346.
- [33] A. Kowluru, M. Tannous, H.Q. Chen, Localization and characterization of the mitochondrial isoform of the nucleoside diphosphate kinase in the pancreatic beta cell: evidence for its complexation with mitochondrial succinyl-CoA synthetase, *Arch. Biochem. Biophys.* 398 (2) (2002) 160–169.
- [34] M. Tokarska-Schlattner, et al., The nucleoside diphosphate kinase D (NM23-H4) binds the inner mitochondrial membrane with high affinity to cardiolipin and couples nucleotide transfer with respiration, *J. Biol. Chem.* 283 (38) (2008) 26198–26207.
- [35] U. Schlattner, et al., Mitochondrial kinases and their molecular interaction with cardiolipin, *Biochim. Biophys. Acta* 1788 (10) (2009) 2032–2047.
- [36] U. Schlattner, et al., Dual function of mitochondrial Nm23-H4 protein in phosphotransfer and intermembrane lipid transfer: a cardiolipin-dependent switch, *J. Biol. Chem.* 288 (1) (2013) 111–121.
- [37] P. Hahn, M. Novak, Development of brown and white adipose tissue, *J. Lipid Res.* 16 (2) (1975) 79–91.
- [38] B. Amutha, et al., GTP is required for iron–sulfur cluster biogenesis in mitochondria, *J. Biol. Chem.* 283 (3) (2008) 1362–1371.
- [39] D.J. Pagliarini, et al., A mitochondrial protein compendium elucidates complex I disease biology, *Cell* 134 (1) (2008) 112–123.
- [40] Z. Drahota, et al., Phosphoenolpyruvate shuttle—transport of energy from mitochondria to cytosol, *FEBS Lett.* 157 (2) (1983) 347–349.
- [41] A. Szollosi, et al., Glucose stimulates  $\text{Ca}^{2+}$  influx and insulin secretion in 2-week-old beta-cells lacking ATP-sensitive  $\text{K}^{+}$  channels, *J. Biol. Chem.* 282 (3) (2007) 1747–1756.
- [42] A. Szollosi, M. Nenquin, J.C. Henquin, Overnight culture unmasks glucose-induced insulin secretion in mouse islets lacking ATP-sensitive  $\text{K}^{+}$  channels by improving the triggering  $\text{Ca}^{2+}$  signal, *J. Biol. Chem.* 282 (20) (2007) 14768–14776.
- [43] S.J. Ashcroft, P.J. Randle, Enzymes of glucose metabolism in normal mouse pancreatic islets, *Biochem. J.* 119 (1) (1970) 5–15.
- [44] M.J. MacDonald, Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets. Further implication of cytosolic NADPH in insulin secretion, *J. Biol. Chem.* 270 (34) (1995) 20051–20058.
- [45] M.J. MacDonald, Influence of glucose on pyruvate carboxylase expression in pancreatic islets, *Arch. Biochem. Biophys.* 319 (1) (1995) 128–132.
- [46] M.J. MacDonald, Estimates of glycolysis, pyruvate (de)carboxylation, pentose phosphate pathway, and methyl succinate metabolism in incapacitated pancreatic islets, *Arch. Biochem. Biophys.* 305 (2) (1993) 205–214.
- [47] G.W. Cline, et al., Rates of insulin secretion in INS-1 cells are enhanced by coupling to anaplerosis and Krebs cycle flux independent of ATP synthesis, *Biochem. Biophys. Res. Commun.* 415 (1) (2011) 30–35.
- [48] G.W. Cline, et al.,  $^{13}\text{C}$  NMR isotopomer analysis of anaplerotic pathways in INS-1 cells, *J. Biol. Chem.* 279 (43) (2004) 44370–44375.
- [49] D. Lu, et al.,  $^{13}\text{C}$  NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS), *Proc. Natl. Acad. Sci. U.S.A.* 99 (5) (2002) 2708–2713.
- [50] M.J. MacDonald, C. Chang, Do pancreatic islets contain significant amounts of phosphoenolpyruvate carboxykinase or ferroactivator activity? *Diabetes* 34 (1985) 246–250.
- [51] C.B. Newgard, et al., Stimulus/secretion coupling factors in glucose-stimulated insulin secretion: insights gained from a multidisciplinary approach, *Diabetes* 51 (Suppl. 3) (2002) S389–S393.
- [52] R.L. Pongratz, et al., Cytosolic and mitochondrial malic enzyme isoforms differentially control insulin secretion, *J. Biol. Chem.* 282 (1) (2007) 200–207.
- [53] C. Guay, et al., A role for ATP-citrate lyase, malic enzyme, and pyruvate/citrate cycling in glucose-induced insulin secretion, *J. Biol. Chem.* 282 (49) (2007) 35657–35665.
- [54] E. Heart, et al., Role for malic enzyme, pyruvate carboxylation, and mitochondrial malate import in glucose-stimulated insulin secretion, *Am. J. Physiol. Endocrinol. Metab.* 296 (6) (2009) E1354–E1362.
- [55] J.W. Joseph, et al., The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion, *J. Biol. Chem.* 281 (47) (2006) 35624–35632.
- [56] R.L. Pongratz, R.G. Kibbey, G.W. Cline, Investigating the roles of mitochondrial and cytosolic malic enzyme in insulin secretion, *Methods Enzymol.* 457 (2009) 425–450.
- [57] S.M. Ronnebaum, et al., A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion, *J. Biol. Chem.* 281 (41) (2006) 30593–30602.
- [58] S.M. Ronnebaum, et al., Silencing of cytosolic or mitochondrial isoforms of malic enzyme has no effect on glucose-stimulated insulin secretion from rodent islets, *J. Biol. Chem.* 283 (43) (2008) 28909–28917.
- [59] M.V. Jensen, et al., Metabolic cycling in control of glucose-stimulated insulin secretion, *Am. J. Physiol. Endocrinol. Metab.* 295 (6) (2008) E1287–E1297.
- [60] M.L. Odegaard, et al., The mitochondrial 2-oxoglutarate carrier is part of a metabolic pathway that mediates glucose- and glutamine-stimulated insulin secretion, *J. Biol. Chem.* 285 (22) (2010) 16530–16537.
- [61] F. Palmieri, The mitochondrial transporter family (SLC25): physiological and pathological implications, *Physiol. Rev.* 447 (5) (2004) 689–709.
- [62] A.J. Garber, F.J. Ballard, Phosphoenolpyruvate synthesis and release by mitochondria from guinea pig liver, *J. Biol. Chem.* 244 (17) (1969) 4696–4703.
- [63] S. Passarella, et al., The role of mitochondrial transport in energy metabolism, *Mitochondrion* 2 (5) (2003) 319–343.
- [64] J. Satrustegui, B. Pardo, A. Del Arco, Mitochondrial transporters as novel targets for intracellular calcium signaling, *Physiol. Rev.* 87 (1) (2007) 29–67.
- [65] R.M. Denton, et al., Regulation of mammalian pyruvate dehydrogenase, *Mol. Cell. Biochem.* 9 (1) (1975) 27–53.
- [66] C.F. Peng, et al., Factors that influence phosphoenolpyruvate-induced calcium efflux from rat liver mitochondria, *Biochem. Biophys. Res. Commun.* 56 (1974) 134–141.
- [67] L. Boquist, NADP-linked dismutation and concentrations of citrate, cytosolic free  $\text{Ca}^{2+}$  and phosphoenolpyruvate in islet B-cells stimulated with glucose, *Biochem. Int.* 14 (3) (1987) 531–538.
- [68] I.V. Deaciuc, N.B. D'Souza, H.I. Miller, A novel mechanism for  $\text{Ca}^{2+}$ -dependent regulation of hepatic gluconeogenesis: stimulation of mitochondrial phosphoenolpyruvate synthesis by  $\text{Ca}^{2+}$ , *Int. J. Biochem.* 24 (1) (1992) 129–132.
- [69] A.L. Shug, E. Shrago, Inhibition of phosphoenolpyruvate transport via the tricarboxylate and adenine nucleotide carrier systems of rat liver mitochondria, *Biochem. Biophys. Res. Commun.* 53 (2) (1973) 659–665.
- [70] P. Chudapongse, Further studies on the effect of phosphoenolpyruvate on respiration-dependent calcium transport by rat heart mitochondria, *Biochim. Biophys. Acta* 423 (2) (1976) 196–202.
- [71] H.S. Sul, E. Shrago, A.L. Shug, Relationship of phosphoenolpyruvate transport, acyl coenzyme A inhibition of adenine nucleotide translocase and calcium ion efflux in guinea pig heart mitochondria, *Arch. Biochem. Biophys.* 172 (1) (1976) 230–237.
- [72] I. Roos, M. Crompton, E. Carafoli, The effect of phosphoenolpyruvate on the retention of calcium by liver mitochondria, *FEBS Lett.* 94 (1978) 418–421.
- [73] G. Szabadkai, M.R. Duchon, Mitochondria: the hub of cellular  $\text{Ca}^{2+}$  signaling, *Physiology (Bethesda)* 23 (2008) 84–94.
- [74] H.G. Wood, C.H. Werkman, The utilization of  $\text{CO}_2$  by the propionic acid bacteria, *Biochem. J.* 32 (7) (1938) 1262–1271.
- [75] M.F. Utter, K. Kurahashi, Mechanism of action of oxalacetate carboxylase from liver, *J. Am. Chem. Soc.* 75 (1953) 785–787.
- [76] R.W. Hanson, Y.M. Patel, Phosphoenolpyruvate carboxykinase (GTP): the gene and the enzyme, *Adv. Enzymol. Relat. Areas Mol. Biol.* 69 (1994) 203–281.
- [77] C.A. Hebda, T. Nowak, The purification, characterization, and activation of phosphoenolpyruvate carboxykinase from chicken liver mitochondria, *J. Biol. Chem.* 257 (10) (1982) 5503–5514.
- [78] S. Modaresi, et al., Human mitochondrial phosphoenolpyruvate carboxykinase 2 gene. Structure, chromosomal localization and tissue-specific expression, *Biochem. J.* 333 (Pt 2) (1998) 359–366.
- [79] C.J. Hedeskov, K. Capito, Pancreatic islet metabolism of pyruvate and other potential regulators of insulin release. Effects of starvation, *Horm. Metab. Res.* 10 (1980) 8–13.
- [80] C.J. Hedeskov, K. Capito, P. Thams, Phosphoenolpyruvate carboxykinase in mouse pancreatic islets ATP-induced changes in sensitivity to  $\text{Mn}^{2+}$  activation, *Biochim. Biophys. Acta* 791 (1984) 37–44.
- [81] M.J. MacDonald, et al., Lack of gluconeogenesis in pancreatic islets: expression of gluconeogenic enzyme genes in islets, *Horm. Metab. Res.* 24 (4) (1992) 158–160.



- [82] D.B. Horn, et al., Alterations in key gluconeogenic regulators with age and endurance training, *Metabolism* 46 (4) (1997) 414–419.
- [83] D. Saggerson, C.J. Evans, The activities and intracellular distribution of nicotinamide-adenine dinucleotide phosphate-malate dehydrogenase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase in rat, guinea-pig and rabbit tissues, *Biochem. J.* 146 (2) (1975) 329–332.
- [84] M.S. Usatenko, Hormonal regulation of phosphoenolpyruvate carboxykinase activity in liver and kidney of adult animals and formation of this enzyme in developing rabbit liver, *Biochem. Med.* 3 (4) (1970) 298–310.
- [85] R.J. Heitzman, I.D. Herriman, C.B. Mallinson, Some effects of glucocorticoids on the subcellular distribution of the activities of citrate synthase and phosphoenolpyruvate carboxykinase in livers of rats and cows, *FEBS Lett.* 20 (1) (1972) 19–21.
- [86] R.W. Hanson, L. Reshef, Glyceroconeogenesis revisited, *Biochimie* 85 (12) (2003) 1199–1205.
- [87] L. Reshef, R.W. Hanson, F.J. Ballard, A possible physiological role for glyceroconeogenesis in rat adipose tissue, *J. Biol. Chem.* 245 (22) (1970) 5979–5984.
- [88] F.J. Ballard, I.T. Oliver, Carbohydrate metabolism in liver from foetal and neonatal sheep, *Biochem. J.* 95 (1965) 191–200.
- [89] F.J. Ballard, R.W. Hanson, Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver, *Biochem. J.* 104 (3) (1967) 866–871.
- [90] N. Benvenisty, L. Reshef, Developmental expression and modification of genes, *Biol. Neonate* 52 (2) (1987) 61–69.
- [91] J.R. Girard, et al., Fuels, hormones, and liver metabolism at term and during the early postnatal period in the rat, *J. Clin. Invest.* 52 (12) (1973) 3190–3200.
- [92] I.J. Arinze, On the development of phosphoenolpyruvate carboxykinase and gluconeogenesis in guinea pig liver, *Biochem. Biophys. Res. Commun.* 65 (1) (1975) 184–189.
- [93] M.M. McGrane, et al., Metabolic effects of developmental, tissue-, and cell-specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice, *J. Biol. Chem.* 265 (36) (1990) 22371–22379.
- [94] M.A. van Roon, et al., Accumulation of carbamoylphosphate-synthetase and phosphoenolpyruvate-carboxykinase mRNA in embryonic rat hepatocytes. Evidence for translational control during the initial phases of hepatocyte-specific gene expression in vitro, *Eur. J. Biochem.* 178 (1) (1988) 191–196.
- [95] N. Benvenisty, L. Reshef, Developmental acquisition of DNase I sensitivity of the phosphoenolpyruvate carboxykinase (GTP) gene in rat liver, *Proc. Natl. Acad. Sci. U.S.A.* 84 (5) (1987) 1132–1136.
- [96] C.L. Eisenberger, et al., Differential regulation of the rat phosphoenolpyruvate carboxykinase gene expression in several tissues of transgenic mice, *Mol. Cell. Biol.* 12 (3) (1992) 1396–1403.
- [97] D. Mencher, L. Reshef, Effect of triamcinolone on renal and hepatic phosphoenolpyruvate carboxykinase in the newborn rat. Changes in the rate of synthesis of the enzyme and in the activity of its translatable messenger RNA, *Eur. J. Biochem.* 94 (2) (1979) 581–589.
- [98] A. Zorzoli, I.J. Turkenkopf, V.L. Mueller, Gluconeogenesis in developing rat kidney cortex, *Biochem. J.* 111 (2) (1969) 181–185.
- [99] G.A. Alleyne, G.H. Scullard, Renal metabolic response to acid base changes. I. Enzymatic control of ammoniogenesis in the rat, *J. Clin. Invest.* 48 (2) (1969) 364–370.
- [100] E.G. Beale, B.J. Harvey, C. Forest, PCK1 and PCK2 as candidate diabetes and obesity genes, *Cell Biochem. Biophys.* 48 (2–3) (2007) 89–95.
- [101] E.G. Beale, et al., Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP). Structures of the protein, messenger RNA, and gene, *J. Biol. Chem.* 260 (19) (1985) 10748–10760.
- [102] G.M. Carlson, T. Holyoak, Structural insights into the mechanism of phosphoenolpyruvate carboxykinase catalysis, *J. Biol. Chem.* 284 (40) (2009) 27037–27041.
- [103] P. Dunten, et al., Crystal structure of human cytosolic phosphoenolpyruvate carboxykinase reveals a new GTP-binding site, *J. Mol. Biol.* 316 (2) (2002) 257–264.
- [104] T.A. Johnson, T. Holyoak, Increasing the conformational entropy of the Omega-loop lid domain in phosphoenolpyruvate carboxykinase impairs catalysis and decreases catalytic fidelity, *Biochemistry* 49 (25) (2010) 5176–5187.
- [105] S.M. Sullivan, T. Holyoak, Enzymes with lid-gated active sites must operate by an induced fit mechanism instead of conformational selection, *Proc. Natl. Acad. Sci. U.S.A.* 105 (37) (2008) 13829–13834.
- [106] R.M. Stiffin, et al., Differential inhibition of cytosolic PEPCK by substrate analogues. Kinetic and structural characterization of inhibitor recognition, *Biochemistry* 47 (7) (2008) 2099–2109.
- [107] T. Holyoak, S.M. Sullivan, T. Nowak, Structural insights into the mechanism of PEPCK catalysis, *Biochemistry* 45 (27) (2006) 8254–8263.
- [108] S.M. Sullivan, T. Holyoak, Structures of rat cytosolic PEPCK: insight into the mechanism of phosphorylation and decarboxylation of oxaloacetic acid, *Biochemistry* 46 (35) (2007) 10078–10088.
- [109] C.Y. Chen, Y. Sato, V.L. Schramm, Isotope trapping and positional isotope exchange with rat and chicken liver phosphoenolpyruvate carboxykinases, *Biochemistry* 30 (17) (1991) 4143–4151.
- [110] C.T. Lewis, B.E. Haley, G.M. Carlson, Formation of an intramolecular cystine disulfide during the reaction of 8-azidoguanosine 5'-triphosphate with cytosolic phosphoenolpyruvate carboxykinase (GTP) causes inactivation without photolabeling, *Biochemistry* 28 (24) (1989) 9248–9255.
- [111] A.L. Makinen, T. Nowak, A reactive cysteine in avian liver phosphoenolpyruvate carboxykinase, *J. Biol. Chem.* 264 (21) (1989) 12148–12157.
- [112] L.A. Bentle, H.A. Lardy, P-enolpyruvate carboxykinase ferroactivator. Purification and some properties, *J. Biol. Chem.* 252 (4) (1977) 1431–1440.
- [113] L.A. Bentle, R.E. Snoke, H.A. Lardy, A protein factor required for activation of phosphoenolpyruvate carboxykinase by ferrous ions, *J. Biol. Chem.* 251 (10) (1976) 2922–2928.
- [114] M.L. Merryfield, D.C. Kramp, H.A. Lardy, Purification and characterization of a rat liver ferroactivator with catalase activity, *J. Biol. Chem.* 257 (8) (1982) 4646–4654.
- [115] N.S. Puneekar, H.A. Lardy, Phosphoenolpyruvate carboxykinase ferroactivator 1. Mechanism of action and identity with glutathione peroxidase, *J. Biol. Chem.* 262 (14) (1987) 6714–6719.
- [116] P.P. Chee, H.A. Lardy, Isolation from erythrocytes of a green hemoprotein with ferroactivator activity, *J. Biol. Chem.* 256 (8) (1981) 3865–3870.
- [117] R.I. Brinkworth, et al., Mn<sup>2+</sup>-sensitive and -insensitive forms of phosphoenolpyruvate carboxykinase (GTP), *J. Biol. Chem.* 256 (21) (1981) 10795–10802.
- [118] W. Hoppner, et al., Is the p29 protein involved in the rapid regulation of phosphoenolpyruvate carboxykinase (GTP)? *J. Biol. Chem.* 266 (26) (1991) 17257–17260.
- [119] A. Sato, T. Suzuki, H. Kochi, Purification and characterization of cytosol-specific phosphoenolpyruvate carboxykinase from chicken liver, *J. Biochem.* 100 (3) (1986) 671–678.
- [120] D.D. Holten, R.C. Nordlie, Comparative studies of catalytic properties of guinea pig liver intra- and extramitochondrial phosphoenolpyruvate carboxykinases, *Biochemistry* 4 (1965) 723–731.
- [121] O. Barzu, et al., Nucleotide specificity of pyruvate kinase and phosphoenolpyruvate carboxykinase, *Biochim. Biophys. Acta* 452 (2) (1976) 406–412.
- [122] J.J. Hlavaty, T. Nowak, Characterization of the second metal site on avian phosphoenolpyruvate carboxykinase, *Biochemistry* 39 (6) (2000) 1373–1388.
- [123] Y. Goto, et al., Purification and characterization of cytosol phosphoenolpyruvate carboxykinase from bullfrog (*Rana catesbeiana*) liver, *J. Biochem.* 86 (1) (1979) 71–78.
- [124] D.E. Ash, et al., Mammalian and avian liver phosphoenolpyruvate carboxykinase. Alternate substrates and inhibition by analogues of oxaloacetate, *J. Biol. Chem.* 265 (13) (1990) 7377–7384.
- [125] M.H. Lee, C.A. Hebda, T. Nowak, The role of cations in avian liver phosphoenolpyruvate carboxykinase catalysis. Activation and regulation, *J. Biol. Chem.* 256 (24) (1981) 12793–12801.
- [126] T. Holyoak, T. Nowak, pH dependence of the reaction catalyzed by avian mitochondrial phosphoenolpyruvate carboxykinase, *Biochemistry* 43 (22) (2004) 7054–7065.
- [127] N. Ishihara, G. Kikuchi, Studies on the functional relationship between the phosphopyruvate synthesis and the substrate level phosphorylation in guinea-pig liver mitochondria, *Biochim. Biophys. Acta* 153 (4) (1968) 733–748.
- [128] M. Suzuki, et al., Cloning and reporter analysis of human mitochondrial phosphoenolpyruvate carboxykinase gene promoter, *Gene* 338 (2) (2004) 157–162.
- [129] S. Savon, P. Hakimi, R.W. Hanson, Expression of the genes for the mitochondrial and cytosolic forms of phosphoenolpyruvate carboxykinase in avian liver during development, *Biol. Neonate* 64 (1) (1993) 62–68.
- [130] Y.Y. Lin, et al., Protein acetylation microarray reveals that NuA4 controls key metabolic target regulating gluconeogenesis, *Cell* 136 (6) (2009) 1073–1084.
- [131] W. Jiang, et al., Acetylation regulates gluconeogenesis by promoting PEPCK1 degradation via recruiting the UBR5 ubiquitin ligase, *Mol. Cell* 43 (1) (2011) 33–44.
- [132] Y. Xiong, et al., Regulation of glycolysis and gluconeogenesis by acetylation of PKM and PEPCK, *Cold Spring Harb. Symp. Quant. Biol.* 76 (2011) 285–289.
- [133] D.H. Jones, D.M. Raymer, S.L. Schoelen, The activity of phosphoenolpyruvate carboxykinase throughout the lactation cycle of the guinea pig mammary gland, *Proc. Soc. Exp. Biol. Med.* 192 (1) (1989) 16–22.
- [134] C. Agca, et al., Cloning and characterization of bovine cytosolic and mitochondrial PEPCK during transition to lactation, *Physiol. Genomics* 11 (2) (2002) 53–63.
- [135] R.A. Jamison, et al., Hyperglucagonemia precedes a decline in insulin secretion and causes hyperglycemia in chronically glucose-infused rats, *Am. J. Physiol. Endocrinol. Metab.* 301 (6) (2011) E1174–E1183.
- [136] M.F. Hopgood, F.J. Ballard, Synthesis and degradation of phosphoenolpyruvate carboxylase in rat liver and adipose tissue. Changes during a starvation-re-feeding cycle, *Biochem. J.* 134 (2) (1973) 445–453.
- [137] S.C. Burgess, et al., Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase, *J. Biol. Chem.* 279 (47) (2004) 48941–48949.
- [138] R.W. Hanson, Thematic minireview series: a perspective on the biology of phosphoenolpyruvate carboxykinase 55 years after its discovery, *J. Biol. Chem.* 284 (40) (2009) 27021–27023.
- [139] M.F. Utter, K. Kurahashi, Mechanism of action of oxalacetic carboxylase, *J. Biol. Chem.* 207 (2) (1954) 821–841.
- [140] M.F. Utter, K. Kurahashi, Purification of oxalacetic carboxylase from chicken liver, *J. Biol. Chem.* 207 (2) (1954) 787–802.
- [141] M.F. Utter, K. Kurahashi, I.A. Rose, Some properties of oxalacetic carboxylase, *J. Biol. Chem.* 207 (2) (1954) 803–819.
- [142] E.A. Siess, et al., Effect of glucagon on metabolite compartmentation in isolated rat liver cells during gluconeogenesis from lactate, *Biochem. J.* 166 (2) (1977) 225–235.
- [143] R.W. Hanson, A.J. Garber, Phosphoenolpyruvate carboxykinase. I. Its role in gluconeogenesis, *Am. J. Clin. Nutr.* 25 (10) (1972) 1010–1021.
- [144] R.W. Hanson, L. Reshef, Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression, *Annu. Rev. Biochem.* 66 (1997) 581–611.
- [145] A. Mendez-Lucas, et al., PEPCK-M expression in mouse liver potentiates, not replaces, PEPCK-C mediated gluconeogenesis, *J. Hepatol.* 59 (1) (2013) 105–113.
- [146] P. She, et al., Mechanisms by which liver-specific PEPCK knockout mice preserve euglycemia during starvation, *Diabetes* 52 (7) (2003) 1649–1654.
- [147] P. She, et al., Phosphoenolpyruvate carboxykinase is necessary for the integration of hepatic energy metabolism, *Mol. Cell. Biol.* 20 (17) (2000) 6508–6517.
- [148] S.C. Burgess, et al., Cytosolic phosphoenolpyruvate carboxykinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver, *Cell Metab.* 5 (4) (2007) 313–320.
- [149] Y. Sun, et al., Phosphoenolpyruvate carboxykinase overexpression selectively attenuates insulin signaling and hepatic insulin sensitivity in transgenic mice, *J. Biol. Chem.* 277 (26) (2002) 23301–23307.

- [150] A. Valera, et al., Transgenic mice overexpressing phosphoenolpyruvate carboxykinase develop non-insulin-dependent diabetes mellitus, *Proc. Natl. Acad. Sci. U.S.A.* 91 (19) (1994) 9151–9154.
- [151] V.T. Samuel, et al., Fasting hyperglycemia is not associated with increased expression of PEPCK or G6Pc in patients with type 2 diabetes, *Proc. Natl. Acad. Sci. U.S.A.* 106 (29) (2009) 12121–12126.
- [152] K.J. Lei, et al., Glucose-6-phosphatase dependent substrate transport in the glycogen storage disease type-1a mouse, *Nat. Genet.* 13 (2) (1996) 203–209.
- [153] E. Cersosimo, P. Garlick, J. Ferretti, Renal substrate metabolism and gluconeogenesis during hypoglycemia in humans, *Diabetes* 49 (7) (2000) 1186–1193.
- [154] M. Stumvoll, et al., Effects of glucagon on renal and hepatic glutamine gluconeogenesis in normal postabsorptive humans, *Metabolism* 47 (10) (1998) 1227–1232.
- [155] M. Stumvoll, et al., Role of glutamine in human carbohydrate metabolism in kidney and other tissues, *Kidney Int.* 55 (3) (1999) 778–792.
- [156] P. Hakimi, et al., Phosphoenolpyruvate carboxykinase and the critical role of cataplerosis in the control of hepatic metabolism, *Nutr. Metab. (Lond.)* 2 (2005) 33.
- [157] C.M. Croniger, et al., Mini-series: modern metabolic concepts, phosphoenolpyruvate carboxykinase revisited, II. Control of PEPCK-C gene expression, *Biochem. Mol. Biol. Educ.* 30 (6) (2002) 353–362.
- [158] J. Yang, S.C. Kalhan, R.W. Hanson, What is the metabolic role of phosphoenolpyruvate carboxykinase? *J. Biol. Chem.* 284 (40) (2009) 27025–27029.
- [159] J.G. Jones, et al., Measurement of gluconeogenesis and pyruvate recycling in the rat liver: a simple analysis of glucose and glutamate isotopomers during metabolism of [1,2,3-(13)C3]propionate, *FEBS Lett.* 412 (1) (1997) 131–137.
- [160] C. Meyer, et al., Renal substrate exchange and gluconeogenesis in normal postabsorptive humans, *Am. J. Physiol. Endocrinol. Metab.* 282 (2) (2002) E428–E434.
- [161] H.D. Söling, J. Kleineke, Gluconeogenesis: Its Regulation in Mammalian Species, John Wiley & Sons, Inc., New York, 1976, 369–462.
- [162] G. Gstraunthaler, H. Holcomb, N.P. Curthoys, Subcellular localization of PEPCK and metabolism of gluconeogenic substrates of renal cell lines, *J. Am. Soc. Nephrol.* 4 (1993) 887.
- [163] M. Watford, et al., The unique role of the kidney in gluconeogenesis in the chicken. The significance of a cytosolic form of phosphoenolpyruvate carboxykinase, *J. Biol. Chem.* 256 (19) (1981) 10023–10027.
- [164] H.D. Soling, et al., Relationship between intracellular distribution of phosphoenolpyruvate carboxykinase, regulation of gluconeogenesis, and energy cost of glucose formation, *Eur. J. Biochem.* 37 (2) (1973) 233–243.
- [165] Y. Olswang, et al., A mutation in the peroxisome proliferator-activated receptor gamma-binding site in the gene for the cytosolic form of phosphoenolpyruvate carboxykinase reduces adipose tissue size and fat content in mice, *Proc. Natl. Acad. Sci. U.S.A.* 99 (2) (2002) 625–630.
- [166] S. Franckhauser, et al., Increased fatty acid re-esterification by PEPCK overexpression in adipose tissue leads to obesity without insulin resistance, *Diabetes* 51 (3) (2002) 624–630.
- [167] P. Hakimi, et al., Overexpression of the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) in skeletal muscle repatterns energy metabolism in the mouse, *J. Biol. Chem.* 282 (45) (2007) 32844–32855.
- [168] R.W. Hanson, P. Hakimi, Born to run; the story of the PEPCK-Cmus mouse, *Biochimie* 90 (6) (2008) 838–842.
- [169] F.A. Hommes, et al., Two cases of phosphoenolpyruvate carboxykinase deficiency, *Acta Paediatr. Scand.* 65 (1976) 233–240.
- [170] P.T. Clayton, et al., Mitochondrial phosphoenolpyruvate carboxykinase deficiency, *Eur. J. Pediatr.* 145 (1–2) (1986) 46–50.
- [171] B.H. Robinson, J. Taylor, S. Kahler, Mitochondrial phosphoenolpyruvate carboxykinase deficiency in a child with lacticacidemia, hypotonia and failure to thrive, *Am. J. Hum. Genet.* 31 (1979) 60A.
- [172] J.V. Leonard, et al., Mitochondrial phosphoenolpyruvate carboxykinase deficiency, *Eur. J. Pediatr.* 150 (1991) 198–199.
- [173] J. Vidnes, Gluconeogenesis in infancy and childhood. I. A method for the study of the in vivo gluconeogenesis from alanine and glycerol, *Scand. J. Clin. Lab. Invest.* 36 (4) (1976) 347–356.
- [174] J. Vidnes, O. Sovik, Gluconeogenesis in infancy and childhood. II. Studies on the glucose production from alanine in three cases of persistent neonatal hypoglycaemia, *Acta Paediatr. Scand.* 65 (3) (1976) 297–305.
- [175] J. Vidnes, O. Sovik, Gluconeogenesis in infancy and childhood. III. Deficiency of the extramitochondrial form of hepatic phosphoenolpyruvate carboxykinase in a case of persistent neonatal hypoglycaemia, *Acta Paediatr. Scand.* 65 (3) (1976) 307–312.
- [176] M. Matsuo, et al., Hepatic phosphoenolpyruvate carboxykinase deficiency: a neonatal case with reduced activity of pyruvate carboxylase, *J. Inher. Metab. Dis.* 12 (3) (1989) 336–337.
- [177] R.H. Fiser Jr., H.L. Melsner, D.A. Fisher, Hepatic phosphoenolpyruvate carboxykinase (PEPCK) deficiency: a new cause of hypoglycaemia in childhood, *Pediatr. Res.* 8 (1974) 432.
- [178] N.W. Cornell, et al., Subcellular location of phosphoenolpyruvate carboxykinase in hepatocytes from fed and starved rats, *J. Nutr.* 116 (6) (1986) 1101–1108.
- [179] M.J. MacDonald, C.M. Chang, Pancreatic islets contain the M2 isoenzyme of pyruvate kinase. Its phosphorylation has no effect on enzyme activity, *Mol. Cell. Biochem.* 68 (2) (1985) 115–120.
- [180] H.D. Soling, et al., Regulation of gluconeogenesis in the guinea pig liver, *Eur. J. Biochem.* 16 (2) (1970) 289–302.
- [181] T.J. Wiese, D.O. Lambeth, P.D. Ray, The intracellular distribution and activities of phosphoenolpyruvate carboxykinase isozymes in various tissues of several mammals and birds, *Comp. Biochem. Physiol. B* 100 (2) (1991) 297–302.
- [182] M.C. Moore, et al., Sources of carbon for hepatic glycogen synthesis in the conscious dog, *J. Clin. Invest.* 88 (2) (1991) 578–587.
- [183] R. Taylor, et al., Direct assessment of liver glycogen storage by <sup>13</sup>C nuclear magnetic resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects, *J. Clin. Invest.* 97 (1) (1996) 126–132.
- [184] K.F. Petersen, et al., Contribution of net hepatic glycogenolysis to glucose production during the early postprandial period, *Am. J. Physiol.* 270 (1 Pt 1) (1996) E186–E191.
- [185] M. Diesterhaft, E. Shrago, H.J. Sallach, Human liver phosphoenolpyruvate carboxykinase: evidence for a separate mitochondrial and cytosol enzyme, *Biochem. Med.* 5 (1971) 297–303.