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Review

Anaplerotic roles of pyruvate carboxylase in mammalian tissues

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Abstract. Pyruvate carboxylase (PC) catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate. PC serves an anaplerotic role for the tricarboxylic acid cycle, when intermediates are removed for different biosynthetic purposes. In liver and kidney, PC provides oxaloacetate for gluconeogenesis. In adipocytes PC is involved in *de novo* fatty acid synthesis and glyceroneogenesis, and is regulated by the peroxisome proliferator-activated receptor-γ, suggesting that PC is involved in the metabolic switch controlling fuel partitioning toward lipogenesis. In islets, PC is necessary for glucose-induced insulin secretion by providing oxaloacetate to form malate that participates in the 'pyruvate/malate cycle' to shuttle 3C or 4C between mitochondria and cytoplasm. Hyperglycemia and hyperlipidemia impair this cycle and affect glucose-stimulated insulin release. In astrocytes, PC is important for *de novo* synthesis of glutamate, an important excitatory neurotransmitter supplied to neurons. Transcriptional studies of the PC gene pinpoint some transcription factors that determine tissue-specific expression.

Keywords. Pyruvate carboxylase, biotin carboxylase, glucose-induced insulin secretion, pyruvate/malate shuttle, pyruvate/citrate shuttle, type 2 diabetes, lipogenesis, glyceroneogenesis, astrocytes, glutamate synthesis.

Historical background

When pyruvate carboxylase [PC, EC 6.4.1.1] was first discovered in 1959 by M. F. Utter and D. B. Keech [1], it was identified as one of the four gluconeogenic enzymes which include phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (F1,6BPase) and glucose-6-phosphatase (G6Pase). PC was first isolated from chicken liver, where it was shown to be able to fix CO_2 to pyruvate and form oxaloacetate in the mitochondria [1]. The overall reaction catalyzed by PC occurs in two spatially distinct reactions (eq. i and ii), as follows:

$$\text{ATP} + \text{HCO}_3^- + \text{Enz-biotin} \xleftrightarrow{\text{Mg}^{2+},\ \text{acetyl-CoA}} \text{Enz-biotin-CO}_2 + \text{ADP} + \text{Pi} \quad \textbf{i}$$

$$\text{Pyruvate} + \text{Enz-biotin-CO}_2 \longleftrightarrow \text{Enz-biotin} + \text{oxaloacetate} \quad \textbf{ii}$$

Therefore, PC serves two biosynthetic purposes: it provides oxaloacetate for PEPCK to convert to phosphoenolpyruvate so that it can bypass the reaction catalyzed by pyruvate kinase, and it sustains the level of oxaloacetate in the Krebs cycle when its intermediates are withdrawn for several biosynthetic purposes [2, 3].

In recent years complementary DNA (cDNA) and gene cloning/sequencing, as well as structural studies undertaken by chemical modification, X-ray crystallography and site-directed mutagenesis have been performed to relate the structure to the function of this enzyme. PC has also been detected in non-gluconeogenic tissues, suggesting that PC may well be involved in other metabolic path-

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ways [4–6]. Here we review the diverse biochemical roles of PC in different mammalian tissues.

The gluconeogenic role of PC

Mammalian tissues, especially red blood cells and brain, rely solely on glucose as a primary fuel. Homeostatic mechanisms are in place to maintain blood glucose levels within a very narrow range, protecting the body from becoming hypoglycemic during prolonged fasting or developing hyperglycemia after ingesting a high carbohydrate diet. These adaptive mechanisms are achieved by the hormonal modulation of gluconeogenesis in the liver and kidney, and of the peripheral uptake of glucose by skeletal muscle, adipocytes and other tissues. Dietary glucose absorbed after a meal triggers the release of insulin, which exerts its effects by promoting glycogen synthesis and glycolysis either through post-translational or transcriptional control of some of regulatory enzymes in those pathways while inhibiting the gluconeogenic pathway. Hypoglycemia in response to fasting triggers the release of glucagon and glucocorticoids to promote glycogenolysis in liver to release glucose for essential needs and prevent the body from becoming even more hypoglycemic. Due to the limited amount of glycogen stored in liver, additional essential requirements for glucose must be synthesized by gluconeogenesis. In rats and mice, fasting promotes hepatic glucose production sustained by an increased pyruvate flux, and increases in PC activity and protein [7, 8].

Similar to other gluconeogenic enzymes, PC is positively regulated by glucagon and glucocorticoids while negatively regulated by insulin. Glucagon acutely controls PC activity by an increased rate of pyruvate carboxylation without an apparent change in PC protein. A study using a pyruvate inhibitor [alpha-cyano-beta-(1-phenylindol-3-yl)-acrylate] [9] demonstrated that glucagon controls PC activity by stimulating the respiratory chain, leading to an activation of pyruvate carboxylation [9]. The increase in respiratory chain activity in turn stimulates gluconeogenesis by generating ATP and by providing reducing equivalents to the mitochondria [10]. Glucocorticoids have long been known to stimulate gluconeogenesis [3], but relatively little is known about how these hormones regulate PC activity. Treating rat hepatocytes with dexamethasone has been shown to affect substrate supply to PC in rat hepatocytes [11]. A recent study in mice indicated that fasting alters PC gene transcription through a cyclic AMP (cAMP)-dependent mechanism. Mice carrying a mutation of the cAMP-response element binding protein (CREB) showed a global reduction of messenger RNAs (mRNAs) for gluconeogenic enzymes, including PC, PEPCK and G6Pase [12], indicating that CREB is an important transcriptional factor that regulates PC transcription during fasting. Identification of CREB-regulated genes by bioinformatics [13, 14] and by the recently described method of serial analysis of chromatin occupancy [15] has revealed the presence of a functional cAMP-responsive element (CRE) in the promoter of the rat PC gene that mediates the cAMP response.

Insulin has long been known to negatively regulate PC expression and other gluconeogenic enzymes. In the liver of streptozotocin-induced diabetic rats, pyruvate flux through the PC reaction and PC activity are increased twofold relative to control rats [7, 16]. This effect is mediated through an alteration of the plasma glucagon to insulin ratio. Normalization of plasma glucose with insulin reduced pyruvate flux through the PC reaction and brought PC activity back to normal levels [7, 16]. A similar result was obtained in genetically obese and diabetes-prone mice, which are characterized by increased hepatic glucose production as the result of increased activities of F1,6BPase [17].

Promoter studies of the PC gene in rats revealed the presence of an insulin-responsive element, which is similar to that of glyceraldehyde-3-phosphate dehydrogenase and that has been shown to mediate the insulin response [13]. Furthermore, pharmacological inhibition of PC by an inhibitor, phenylacetic acid (PAA), reduced hepatic gluconeogenesis, suggesting that PC could be a useful drug target for diabetes treatment [18].

The role of PC in pancreatic β-cells

The level of plasma glucose is tightly regulated by insulin secretion from pancreatic β-cells in response to the fluctuating level of glucose. The signaling cascade leading to insulin secretion involves an increase in cytosolic ATP concentration, which in turn triggers the closure of the ATP-sensitive potassium channel, resulting in depolarization of the plasma membrane [19–21]. This leads to an influx of Ca^{2+} into the β-cell followed by exocytosis of insulin granules [20, 22]. Insulin released from β-cells is then delivered to the target tissues, including liver, skeletal muscle and adipose tissue. Binding of insulin to its receptor on these target tissues results in diverse metabolic effects, i.e. it promotes glycogen synthesis in liver and muscle, and it promotes lipid storage, while inhibiting lipolysis in adipose tissues [23]. One of the remaining unanswered questions is what is the metabolic signal that enables β-cells to sense when and how much insulin to release.

After meals, the elevated glucose level in plasma is transported across the plasma membrane of β-cells by the glucose transporter GLUT2. Glucose is then phosphorylated by a β-cell-specific glucokinase which has a high Km for glucose [24, 25] to form glucose-6-phosphate, which subsequently enters the glycolytic pathway to produce pyruvate. In most tissues, pyruvate would be decarboxylated by pyruvate dehydrogenase (PDH) to produce

acetyl-coenzyme A (CoA), which subsequently enters the Krebs cycle for complete oxidation. In pancreatic islets, however, it has been found that the level and activity of PC is as high as in the gluconeogenic tissues, liver and kidney [6, 26–28]. Yet β-cells have no detectable activity of PEPCK [29, 30] to carry out gluconeogenesis, although they contain a significant level of G6Pase activity [31–35]. It appears that both PC and PDH are highly expressed in pancreatic islets, and their levels of expression and protein increase in response to high concentrations of glucose. These observations suggest that these two alternate routes of pyruvate utilization are important for glucose-induced insulin secretion [26, 36]. In rats, the PC gene possesses two tissue-specific promoters, the proximal and distal promoters [13], and it is the distal promoter which is active and inducible by glucose in β-cells [37]. Recent characterization of the distal promoter of the rat PC gene has shown that the Sp1/Sp3 transcription factor may be important in regulating transcriptional activity of the PC gene during induction by glucose [38].

Pyruvate could enter mitochondria either to undergo decarboxylation by PDH or carboxylation by PC. The question that remains unclear is, which of these two pathways is relevant to glucose-induced insulin release? It has been estimated that pyruvate derived from glucose is carboxylated by PC or decarboxylated by PDH to similar extents varying from 40–60% [39–41]. Recent studies using 13C nuclear magnetic resonance (NMR) to study pyruvate transport into the mitochondria of insulinoma cells demonstrated that mitochondrial pyruvate exists as two compartmentalized pools [42, 43]. One pool is carboxylated and varies in proportion to the glucose responsiveness of the insulinoma cells. In contrast, the other pool of pyruvate feeds acetyl-CoA into the Krebs cycle through PDH and therefore provides a sustained flux through the Krebs cycle and oxidative phosphorylation.

As the pyruvate carboxylation rate is very active due to the high abundance of PC protein in β-cells [6, 27], this raises an intriguing question as to what its metabolic consequences are. Obviously the high rate of pyruvate carboxylase activity could supply more oxaloacetate to the Krebs cycle, which could result in a proportional increase in Krebs cycle activity. Therefore, it would be expected that all Krebs cycle intermediates may be affected to a certain degree by the increased flux of oxaloacetate. However, some of these intermediates are shuttled from the mitochondria to the cytoplasm. Specifically, malate is exported from the mitochondria to the cytoplasm under conditions of high glucose concentration. MacDonald [6] was the first to describe the link between insulin secretion and the 'pyruvate/malate' shuttle, which operates across the mitochondrial membranes. As shown in Figure 1, this shuttle is initiated by the rapid carboxylation of pyruvate to oxaloacetate by PC. Oxaloacetate is then converted to malate in the mitochondria by malate dehydrogenase (MDH) [EC 1.1.1.37] and exported to the cytosol, where it is decarboxylated by the malic enzyme [EC 1.1.1.40] to pyruvate, which can re-enter the mitochondria for carboxylation again. More important, decarboxylation of malate to pyruvate also produces NADPH, which is thought to be a 'metabolic coupling factor' required for insulin secretion [6]. As this pathway occurs as a shuttle, a higher level of NADPH would be produced than that obtained from the pentose phosphate shunt. The increased level of oxaloacetate provided by PC activity would be utilized by the combined mitochondrial aspartate aminotransferase-α-ketoglutarate dehydrogenase reactions for the production of succinate. This intermediate is important for the synthesis of mevalonate, one of the potent insulin secretagogues [44].

In contrast, Farfari et al. [45] proposed an alternative cycle in which pyruvate is carboxylated to oxaloacetate, which is then converted to citrate by citrate synthase [EC 2.3.3.1]. Citrate formed in the mitochondria then enters the cytoplasm, where it undergoes cleavage catalyzed by ATP-dependent citrate lyase [EC 2.3.3.8] to oxaloacetate and acetyl-CoA. Oxaloacetate is subsequently reduced to malate by MDH in the cytoplasm. To complete the cycle, malate is converted back to pyruvate by the malic enzyme, thereby producing NADPH. As this 'citrate/malate' pathway also occurs in a cyclic fashion similar to that of the pyruvate/malate shuttle, substantial amounts of NADPH would also be produced [45]. Inhibition of PC activity by its specific inhibitor, PAA, reduced glucose-induced insulin secretion concomitant with the marked reduction of citrate and malate exported by mitochondria [36]. The involvement of NADPH in other biochemical pathways that may be related to insulin secretion in β-cells has recently been reviewed [46]. The role of NADPH in triggering glucose-induced insulin release has been shown to be regulated by two redox proteins, glutaredoxin and thioredoxin. Glutaredoxin potentiates the effects of NADPH on exocytosis, whereas thioredoxin antagonizes the NADPH effect [47].

Schuit et al. [41] have demonstrated that the rapid carboxylation of pyruvate derived from glycolysis occurs only in pancreatic β-cells and not in the non-β cells. This is largely due to the high abundance of PC protein and the lower ratio of lactate dehydrogenase to FAD-linked glycerol phosphate dehydrogenase in β-cells. This supports the view that anaplerosis is an essential pathway implicated in β-cell activation by glucose.

PC and β-cell adaptation to insulin resistance

Given the link between PC activity and glucose metabolism in the process of insulin secretion, it is likely that deterioration of the above pathway could lead to the development of type 2 diabetes. Insulin released from β-cells has major metabolic actions in three insulin-sensitive tis-

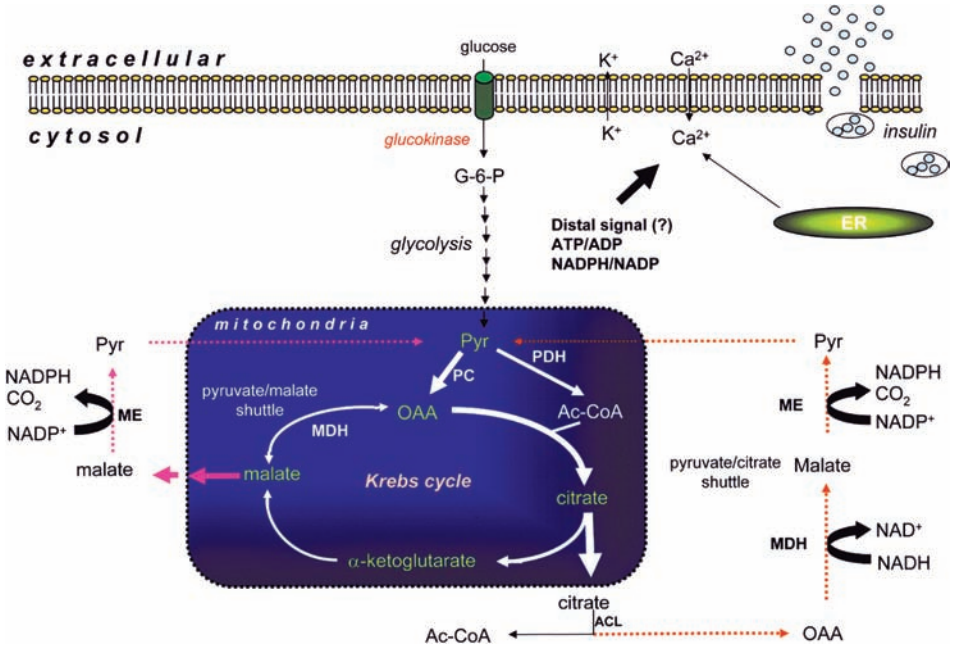

Figure 1. Anaplerotic role of PC in pancreatic islets. Glucose enters these cells via GLUT2 followed by its rapid phosphorylation by glucokinase. Subsequently glycolysis produces pyruvate that enters mitochondria either for carboxylation by PC to oxaloacetate or oxidation by PDH to acetyl-CoA. For the pyruvate/citrate cycle, oxaloacetate formed by PC is condensed with acetyl-CoA to form citrate, which exits the mitochondria and is cleaved by ATP-citrate lyase to produce acetyl-CoA and oxaloacetate. Oxaloacetate formed in the cytoplasm is converted via malate to pyruvate by cytosolic malate dehydrogenase and malic enzyme along with the production of NAD and NADPH, respectively. Pyruvate re-enters the mitochondria and repeats the above cycle. For the pyruvate/malate cycle, oxaloacetate formed by PC is converted to malate by mitochondrial malate dehydrogenase before being exported to the cytoplasm. Malate is then oxidatively decarboxylated to pyruvate by malic enzyme before re-entering the mitochondria. This shuttle produces NADPH, increases the level of mitochondrial derived ATP [21], and these, combined with a high NADPH/NADP ratio generated from pyruvate/malate or pyruvate/citrate cycles, serve as the metabolic signal triggering the closure of the ATP-sensitive potassium channels. This is followed by membrane depolarization and accumulation of intracellular Ca^{2+} which in turn triggers exocytosis of insulin granules. Pharmacological inhibition of PC activity impairs these cycles and lowers glucose-induced insulin secretion. AcCoA, acetyl-CoA; ACL, ATP-citrate lyase; G-6-P, glucose-6-phosphate; ME, malic enzyme; MDH, malic dehydrogenase; OAA, oxaloacetate; Pyr, pyruvate; PC pyruvate carboxylase; PDH, pyruvate dehydrogenase; ER, endoplasmic reticulum.

sues, viz. liver, skeletal muscle and adipose tissue. In liver, insulin promotes glycolysis [48] and glycogen synthesis [49], while it decreases hepatic glucose production by inhibiting gluconeogenesis [48] thus preventing hyperglycemia. In muscle, insulin promotes glucose uptake and stimulates glycogen synthesis. Insulin facilitates fat deposition in the adipose tissue by stimulating *de novo* lipogenesis and triglyceride synthesis while inhibiting lipolysis [23, 50]. Failure of insulin action in these tissues, due to either insulin resistance and/or inadequate insulin secretion, results in hyperglycemia, hyperlipidemia and an excess of plasma free fatty acids due secondarily to increased lipolysis. Under these conditions secretion of insulin progressively deteriorates through toxic reactions due to processes known as 'glucotoxicity' and 'lipotoxicity', which will be discussed below.

Glucotoxicity

Under normal physiological conditions, the concentration of blood glucose is tightly controlled within a narrow range, between 4–7 mM. Exposure of rat pancreatic islets to high concentrations of glucose results in an increase in the amount of PC and its activity [36] that accelerates the flux of pyruvate through pyruvate carboxylation. This is supported by the increased concentration of malate being released from isolated mitochondria in islets following a 48-h incubation with 16.7 mM glucose [51]. Liu et al. [51] found that islets exposed to the same high concentration of glucose lowered their PDH activity by 65% compared with the activity observed at the low concentration of glucose (5.5 mM). The reduction of pyruvate flux through PDH activity is also associated with a marked increase in the activity of the pyruvate/malate shuttle and enhanced glucose-induced insulin release. Interestingly, the reduction of PDH activity induced by glucose is reminiscent of the PDH deactivation induced by excess free fatty acids [51]. Roche et al. [52] performed a similar experiment and showed that rat insulinoma INS-1 cells, when exposed to high concentrations of glucose, increased their levels of citrate, malate and malonyl-CoA concomitant with a rise in the levels of acetyl-CoA car-

boxylase and fatty acid synthase, despite levels of PDH and PC proteins and their mRNAs being unchanged after 48 and 72 h. The authors suggested that the transcriptional control of PDH and PC genes may be operating differently in islets and cultured cell lines. These authors also suggested that under high concentrations of glucose, malonyl-CoA formed from acetyl-CoA inhibits fatty acid oxidation through inhibition of carnitine palmitoyl transferase I, thus promoting the accumulation of fatty acyl CoA, which is further converted to esterified lipids. Therefore, this should be considered as an early adaptive process to hyperglycemia and glucose sensitization of β-cells.

In partially pancreatectomized rats, the removal of 60% of the islets did not cause the rats to develop hyperglycemia. Indeed, the pyruvate/malate cycle activity was increased and accompanied by enhanced β-cell proliferation. The authors suggest that enhanced pyruvate carboxylation in pancreatectomized islets may have an important role in islet cell proliferation, preventing rats becoming hyperglycemic [53]. In rats with 90% of their islets removed by pancreatectomy, the situation was much worse. In this model, pancreatectomized rats developed hyperglycemia, and their remaining islets showed a global alteration in the expression of genes involved in β-cell function. Specifically, these islets show the down-regulation of mRNAs encoding islet-specific transcription factors, and all the important glucose-induced insulin sensing enzymes including GLUT2, glucokinase, mitochondrial glycerol phosphate dehydrogenase and PC [54]. These authors suggested that this is the key mechanism whereby the β-cells undergo dedifferentiaton as an outcome of the decompensatory mechanism caused by hyperglycemia. Normalization of the plasma glucose level with phlorizin treatment of partially pancreactectomized rats only partially restored the PC mRNA level.

Lipotoxicity

Chronic exposure to free fatty acids appears to exert a toxic effect on the glucose-induced insulin secretion of β-cells by affecting pyruvate metabolism or the pyruvate/malate shuttle system in similar way to that seen under 'glucotoxicity' conditions. For pancreatic islets of normoglycemic Zucker fatty rats exhibiting hyperlipidemia due to insulin resistance [55], or in cultured islets of normal rats chronically exposed to oleate [56], it was found that glycolysis-derived pyruvate entering decarboxylation through PDH was reduced by 35%. Conversely, pyruvate flux through pyruvate carboxylation was enhanced in parallel with increases of other mitochondrial enzymes that also participate in the pyruvate/malate shuttle including malate dehydrogenase, and malic enzyme [55]. Inhibition of PC activity by PAA also lowered glucose-induced insulin release [55]. These data suggest that PC and the pyruvate/malate shuttle activity are increased in the islets of Zucker fatty rats, and that this accounts for glucose mitochondrial metabolism being increased when PDH activity is reduced. This increase in the anaplerotic activity of PC, causing an increase in insulin secretion, appears to be a β-cell adaptation for maintaining a normal blood glucose level by compensating for insulin resistance [55].

As is the case with hyperglycemia, long-term exposure to high concentrations of fatty acids decreases the insulin secretory capacity of β-cells leading to diabetes. Islets of normal mouse cultured in the presence of oleate and/or palmitate appear to promote triglyceride synthesis and its deposition in β-cells [57]. The accumulation of triglycerides and other reactive lipid-derived intermediates in β-cells promotes a lipotoxic effect that results in the development of impaired glucose-induced insulin secretion [58, 59]. For example, increased ceramide synthesis in response to lipid load has been linked to β-cell apoptosis [60, 61]. Similarly, chronic exposure of rat insulinoma INS-1 (subline 823/13) or mouse insulinoma MIN6 cells to oleate/palmitate or palmitate markedly desensitized glucose-induced insulin secretion, resulting in the complete reduction of activity of the pyruvate/malate cycle [62]. Furthermore, significantly improved glucose-induced insulin secretion in fatty-acid treated 823/13 INS-1 cells or similarly treated islets could be achieved by the addition of dimethyl malate, a membrane-permeable ester of malate that enters into the pyruvate/malate shuttle after intracellular de-esterification [62]. Microarray and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of palmitate- and oleate-treated MIN6 cells demonstrated that the PC mRNA level was decreased [63, 64] in parallel with the decrease of PC protein [64]. These data suggest a key role of PC in lipid-induced impairment of glucose-induced insulin secretion in β-cells. The impairment of glucose-induced insulin release by free fatty acids could also be partially or completely restored by removal of fatty acids from the culture media [62, 64].

A similar phenomenon was also found in the islets of two models of genetically inherited type 2 diabetic rats, i.e. Zucker diabetic fatty rats (ZDF/Gm-fa/fa) and Goto-Kakizaki (GK) rats. These rodent models develop hyperglycemia and impaired glucose-induced insulin secretion. The islets of these two rat models show a 50% reduction in the levels and activity of PC, a key component of the pyruvate/malate shuttle [65, 66]. Of particular interest, normalization of the high blood glucose of the GK rat by insulin treatment brought the activity and the level of PC back to the normal level [65].

In summary, anaplerosis of Krebs cycle intermediates by pyruvate carboxylation is an important adaptive mechanism for glucose-induced insulin secretion in β-cells. This β-cell specialized-pyruvate/malate or -pyruvate/cit-

rate cycle through the activity of PC provides a metabolic coupling factor, NADPH, required for insulin secretion. Disruption of PC activity in β-cells by the PC inhibitor PAA impairs glucose-induced insulin secretion. Furthermore, physiological imbalance caused by hyperglycemia or hyperlipidemia appears to alter the pyruvate/malate or pyruvate/citrate cycle that generates NADPH, which at least in part impairs glucose-induced insulin secretion. Therefore, pharmacological strategies aiming to maintain PC activity in the context of glucose/lipotoxicity may be a suitable strategy to maintain insulin secretion and prevent the development of diabetes.

The role of PC in adipocytes

The murine 3T3-L1 adipocyte cell line provides a useful model to study adipocyte differentiation. Upon hormonal stimulation, 3T3-L1 preadipocytes undergo differentiation to mature adipocytes, which accumulate cytosolic triglyceride in a manner similar to mature adipocytes found in adipose tissue. The biochemical changes during differentiation of preadipocytes to mature adipocytes include induction of pro-adipogenic transcription factors such as C/EBPβ and C/EBPδ followed by expression of adipogenic transcription factors, i.e. C/EBPα and PPARγ [67]. C/EBPα and PPARγ subsequently switch on expression of adipocyte-specific genes whose products are involved in *de novo* fatty acid synthesis, fatty acid transport, fatty acid resterification and lipid deposition. As the rate of lipogenesis is exceptionally high, it is not surprising to see increased activities of acetyl-CoA carboxylase (ACC), and fatty acid synthase, both of which are required for long-chain fatty acid synthesis [4, 68, 69]. The activities of other enzymes which supply precursors for fatty acid synthesis, i.e. PC [4, 70, 71], citrate synthase, ATP-citrate lyase and malic enzyme [4, 68, 72, 73], are also increased during adipocyte differentiation. For example, the activities of ATP-citrate lyase, PC and malic enzyme are >20-fold higher in adipocytes than preadipocytes. As in pancreatic islets, PC does not serve a gluconeogenic role in adipocytes, as no activities of G6Pase and F1,6BPase can be detected. It is known that citrate is exported from mitochondria to cytoplasm, where it undergoes cleavage to acetyl-CoA and oxaloacetate by ATP-citrate lyase (see Fig. 2). Acetyl-CoA is a precursor of malonyl-CoA, the building block of long-chain fatty acids. The oxaloacetate formed in the cytoplasm is converted by malate dehydrogenase to malate, which is subsequently converted to pyruvate by malic enzyme. Pyruvate re-enters the mitochondria, where it is carboxylated to oxaloacetate and subsequently condensed with acetyl-CoA to citrate. This pathway was first proposed to be necessary for *de novo* fatty acid synthesis by Ballard and Hanson [74]. High activities of PC, ATP-citrate lyase and malic enzyme in differentiating adipocytes support the concept of this citrate/pyruvate cycle. Inhibition of the citrate efflux from mitochondria using specific inhibitors impairs lipid deposition in differentiating adipocytes, strengthening the idea that this citrate/pyruvate cycle is essential for *de novo* fatty acid synthesis [75]. In adipose tissue of genetically obese Zucker fatty rats, the activities of three enzymes, ATP-citrate lyase, malic dehydrogenase and malic enzyme, are abnormally high, providing further support for this idea [76]. A role for PC in lipogenesis is evidenced by the extremely low levels of PC mRNA and protein in preadipocytes, whereas these rapidly increase at an early stage of adipocyte differentiation concomitant with the expression of PPARγ, a master transcriptional regulator of adipogenesis. Forced expression of PPARγ2, an adipocyte-specific PPARγ isoform in 3T3-L1 preadipocytes, results in up-regulation of many adipocyte-specific genes, including a 40-fold increase in PC mRNA [77]. Transcriptional studies of the promoter of the murine PC reveals a *bona fide* PPARγ response element that functionally interacts with PPARγ *in vitro* and *in vivo* [78]. Stimulation of adipocytes of *ob/ob* mice with a PPARγ agonist (thiazolidinedione) strongly induced expression of PC mRNA [79]. The finding that PC is regulated by PPARγ further supports a lipogenic role of PC in adipocytes.

However, the role of PC is not restricted only to *de novo* fatty acid synthesis, as it is also involved in glyceroneogenesis, the process of synthesizing glycerol for esterification with free fatty acids to form triglycerides in differentiating adipocytes, as shown in Figure 2. The high rate of glyceroneogenesis is due to the presence of a high activity of the cytosolic PEPCK, which converts oxaloacetate to phosphoenolpyruvate (PEP). PEP is subsequently converted to glycerol via dihydroxyacetone phosphate for esterification with fatty acids to form triglycerides [80]. As the cytosolic oxaloacetate is provided by PC through the citrate/pyruvate shuttle, PC should be considered to be an important enzyme involved in glyceroneogenesis in differentiating adipocytes. A recent study in adipocytes of *ob/ob* mice treated with rosiglitazone, a thiazolidinedione agent that improves insulin sensitivity, showed an increase in the size of mitochondria and an increased rate of oxygen consumption that paralleled the increased expression of mitochondrial proteins and enzymes involved in fatty acid transport, fatty acid oxidation and energy expenditure. The level of PC protein and mRNA increased 3–4-fold concomitant with the increased re-esterification rate of free fatty acids in adipocytes treated with rosiglitazone [81]. These data suggest that PC and PEPCK may act in concert and determine the rate of fatty acid re-esterification in this cell type.

In addition to white adipocytes, PC is also expressed in brown adipocytes. A recent study demonstrated that PC expression is induced during brown adipocyte differenti-

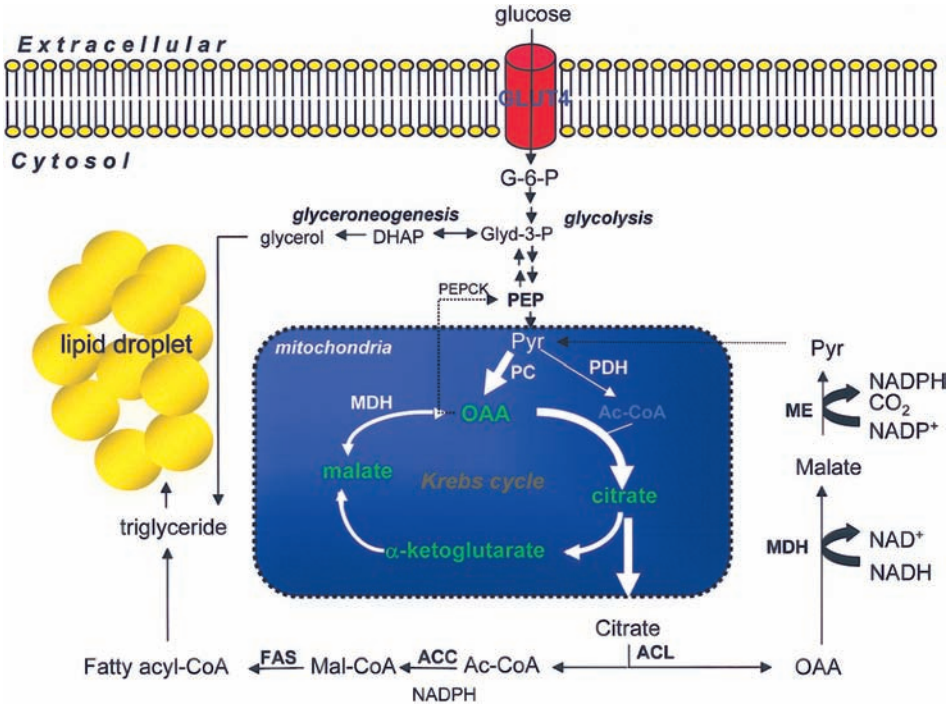

Figure 2. Anaplerotic role PC in adipocytes. In adipocytes, oxaloacetate formed by mitochondrial PC is condensed with acetyl-CoA via citrate synthase to form citrate. Citrate is then exported to the cytoplasm, where it is converted to oxaloacetate and acetyl-CoA by ATP-citrate lyase. Oxaloacetate is subsequently converted back to pyruvate by cytosolic MDH and ME before re-entering the mitochondria. The acetyl-CoA produced by ATP-citrate lyase is carboxylated by ACC to malonyl-CoA. Condensation of acetyl units from malonyl-CoA by FAS produces long-chain fatty acyl-CoA in the cytoplasm. This pathway is known as *de novo* fatty acid synthesis. Cytosolic PEPCK can convert oxaloacetate to phosphoenolpyruvate, which is subsequently converted via dihydroxyacetone phosphate to glycerol. This pathway is known as glyceroneogenesis. Glycerol is esterified with fatty acyl-CoA to form triglycerides that are stored in lipid droplets in adipocytes. CS, citrate synthase; AcCoA, acetyl-CoA; ME, malic enzyme; MDH, malic dehydrogenase; OAA, oxaloacetate; Pyr, pyruvate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; FAS, fatty acid synthetase; PEPCK, phosphoenolpyruvate carboxykinase; G-6-P, glucose-6-phosphate; Glyd, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate.

ation in a manner similar to that in white adipocytes [78]. The high rate of fatty acid degradation through the β-oxidation pathway in brown adipocytes provides a large amount of heat, known as thermogenesis, through mitochondrial uncoupling proteins [82]. Two non-exclusive potential roles may be fulfilled by PC. First, PC could be required to ensure complete fatty acid oxidation, as the β-oxidation of fatty acids yields large amounts of acetyl-CoA that must condense with oxaloacetate to enter the TCA cycle for complete oxidation. PC must therefore provide a continuous supply of oxaloacetate to condense with the acetyl-CoA produced. A study in hamsters demonstrated that pyruvate carboxylation was necessary for maximal oxygen consumption in norepinephrine-stimulated respiration, even when drainage of citric acid cycle intermediates for amino acid synthesis is eliminated. This shows the role of PC in the provision of oxaloacetate to promote thermogenesis by the oxidation of acetyl-CoA derived from fatty acid degradation [83, 84]. A second role of PC in brown adipocytes is its indirect promotion of thermogenesis by participating in *de novo* longchain-fatty acid synthesis for subsequent oxidation [78]. It is well known that cold-induced thermogenesis increases the rate of *de novo* fatty acid synthesis concomitant with expression of ATP citrate lyase and ACC1, enzymes required for citrate export and the production of malonyl-CoA, respectively. A recent study in mice showed that cold-induced thermogenesis caused >10-fold induction of PC mRNA and to a lesser extent of PC protein [78].

The role of PC in the nervous system

Synapse junctions of neurons in both the central and peripheral nervous systems require neurotransmitters to transmit an electrical impulse. Glutamate is of particular interest as it is one of the excitatory neurotransmitters and is also a precursor of GABA, another neurotransmitter. Glutamate is synthesized in the nervous system by astrocytes before being converted by glutamine synthetase [EC 2.7.7.42] to glutamine, which is secreted to the neuronal synapses. Glutamine is taken up by neurons and subsequently converted to glutamate by glutaminase [EC

3.5.1.2], and glutamate released from neurons is recycled to glutamine in astrocytes. This cycle is known as the 'glutamine-glutamate cycle' (for review see [85]). However, *de novo* synthesis of glutamate is only available from the Krebs cycle intermediate, α-ketoglutarate, via aspartate aminotransferase [EC 2.6.1.1], which is highly active in astrocytes. The operation of the glutamine-glutamate cycle therefore requires a continuous supply of oxaloacetate to be made available through the reaction catalyzed by PC, in order to provide α-ketoglutarate. This is supported by the high activity of PC in astrocytes, where there are no activities of the other gluconeogenic enzymes present to perform gluconeogenesis [86–89]. Furthermore, there is no PC activity detectable in neurons, indicating that the *de novo* synthesis of glutamate does not occur in neurons. Localization of PC by immunohistochemistry clearly shows that PC is present in astrocytes in the central nervous system [90] and in satellite and Schwann cells in the peripheral nervous system [91]. Pyruvate carboxylation appears to be a critical step in the pathway for synthesizing glutamate from TCA cycle intermediates. NMR analysis of glucose entering astrocytes indicated that carbon flux through the glutamate-glutamine cycle via pyruvate carboxylation constitutes an important metabolic pathway in brain [92, 93]. Inhibition of pyruvate carboxylation by using an inhibitor of carbonic anhydrase, an enzyme that supplies HCO_3^- for carboxylation of pyruvate to oxaloacetate reduced TCA cycle intermediate levels and glutamate production [94]. Increased levels of substrates for pyruvate carboxylation also enhanced production of glutamate in astrocytes [89]. The importance of PC in the nervous system can be clearly seen in mental retardation in PC-deficient patients who carry one or more forms of mutations of the PC gene and by the brain abnormalities that severely compromise their psychomotor development [95, 96].

Tissue-specific control of PC

In mammals, PC is regulated by two distinct promoters, the proximal and the distal, located upstream of the first coding exon [13, 97, 98]. In rat and mouse, alternative transcription of these two promoters produces mRNAs that share the same coding sequence but differ in their 5′-untranslated regions [13, 98]. The proximal promoter generates mRNA transcripts that are expressed in gluconeogenic tissues (liver and kidney) and adipose tissue, while the distal promoter produces the transcript which is ubiquitously expressed but is particularly highly abundant in pancreatic islets, where it is induced by glucose [30]. The different activities of these two promoters in various tissues suggest that both promoters are controlled in a tissue-specific manner [13]. For example, the proximal promoter, which is active in both gluconeogenic tissues and lipogenic tissues, appears to be primarily regulated by different specific transcription factors in these tissues. In adipocytes, the proximal promoter is regulated by PPARγ, which produces an ~40–60% reduction of PC mRNA and protein in mice with a disrupted gene for PPARγ2, an adipocyte-specific isoform. However, no such effect was seen in the liver of these knockout mice, indicating the independent regulation of PC in both tissues by PPARγ [78]. In liver the proximal promoter is either directly or indirectly regulated by CREB, a transcription factor that is activated via an elevated level of cAMP during fasting. Ectopic expression of a dominant-negative CREB mutant in transgenic mice markedly reduced PC mRNA in liver by 75%, suggesting that CREB is a positive regulator of the proximal promoter in liver. Unfortunately, there was no data for PC mRNA in adipose tissues of these transgenic mice [12].

For the distal promoter, specific proteins (Sp1and Sp3) and the nuclear factor Y (NF-Y) are the major transcription factors that positively and negatively regulate PC transcription under basal conditions, respectively [38]. The liver X receptors (LXRs), α and β, members of the nuclear hormone receptor family, have recently been implicated in transcriptional activation of the PC gene in mouse pancreatic islets and insulin secreting cells. Treatment of these cells with LXR agonist causes increased PC mRNA [99]. However, an LXR-responsive element in the promoter of the mouse PC gene has not yet been identified. A recent study has shown the involvement of the pancreatic duodenal homeobox 1 transcription factor (PDX1) in the transcription of PC gene [100]. Dominant-negative expression of a PDX1 mutant in rat islets resulted in a 3-fold increase of PC mRNA, indicating that PDX1 is an important tissue-specific factor that regulates PC transcription from the distal promoter in this tissue [100]. It will be interesting to see whether the carbohydrate-responsive elements identified in other glucose-responsive genes [101] are also involved in the regulation of PC transcription. Figure 3 shows various candidate proteins controlling transcription of the proximal and distal promoters in different tissues in response to different stimuli.

Conclusion

Additional anaplerotic roles for PC in mammalian tissues have been documented in the past decade. The observation of a high level expression of PC in pancreatic islets has led to the discovery of pyruvate/malate and/or pyruvate/citrate cycle(s), which are necessary for glucose-induced insulin release. Chronic exposure of β-cells to elevated levels of glucose and fatty acids impairs glucose-induced insulin release, and this is accompanied by reduction of PC activity and protein. These data suggest a critical role of PC in maintaining glucose-stimulated insulin secretion in

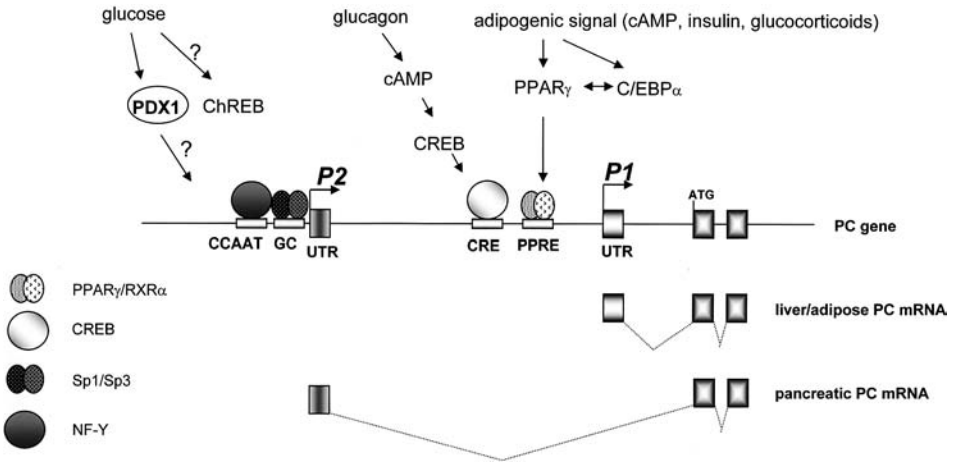

Figure 3. Tissue-specific control of PC by transcription factors. The mammalian PC gene has two alternative promoters, the proximal (P1) and the distal (P2) promoter. Transcription from the P1 promoter produces PC mRNA, which is found exclusively in gluconeogenic and adipose tissues, while transcription from the P2 promoter produces PC mRNA, which is ubiquitously expressed but is highly abundant in pancreatic islets [13, 37]. These two types of PC mRNA share the same coding exons but differ in their 5′-UTRs. Hormonal induction of adipocyte differentiation stimulates expression of adipogenic transcription factors, C/EBPα and PPARγ. PPARγ is then heterodimerized with RXRα, binds PPRE and activates transcription from the P1 promoter [78]. In liver, CREB activates P1 transcription, possibly via a CRE located upstream of PPRE [12, 13]. For the P2 promoter, Sp1/Sp3 bind a GC box and positively regulate P2 transcription, while NF-Y binds a CCAAT box located upstream of the GC box and acts as a negative regulator of Sp1/Sp3 [38]. P1, proximal promoter; P2, distal promoter; shaded boxes represent exons; rectangular boxes, binding sites for transcription factors; PDX1, pancreatic duodenal homeobox1; ChREB, carbohydrate response element binding protein; CREB, cAMP-response element binding protein; NF-Y, nuclear factor Y.

this cell type. An enhanced expression of PC in pancreatic islets may provide a useful strategy for the improvement of glucose-induced insulin secretion in type 2 diabetic patients. In differentiating adipocytes, the induction of PC expression suggests its role in *de novo* fatty acid synthesis and glyceroneogenesis, while it may also be indirectly involved in cold-induced thermogenesis in brown adipose tissue. The finding that PC is regulated by PPARγ in adipocytes indicates that thiazolidinediones would increase expression of PC in adipocytes and hence the capacity for glyceroneogenesis. This would reduce circulating fatty acid levels due to enhanced fatty acid esterification. Identification of transcription factors directing PC transcription will increase our understanding of the tissue-specific control of PC. If the three-dimensional structure of PC or its active site could be obtained by X-ray crystallography, it would greatly increase the probability of designing an inhibitor or agonist that could pharmacologically modulate PC activity.

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