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Mitochondrial pyruvate transport: a historical perspective and future research directions

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Abstract

Pyruvate is the end-product of glycolysis, a major substrate for oxidative metabolism, and a branching point for glucose, lactate, fatty acid and amino acid synthesis. The mitochondrial enzymes that metabolize pyruvate are physically separated from cytosolic pyruvate pools and rely on a membrane transport system to shuttle pyruvate across the impermeable inner mitochondrial membrane (IMM). Despite long-standing acceptance that transport of pyruvate into the mitochondrial matrix by a carrier-mediated process is required for the bulk of its metabolism, it has taken almost 40 years to determine the molecular identity of an IMM pyruvate carrier. Our current understanding is that two proteins, mitochondrial pyruvate carriers MPC1 and MPC2, form a hetero-oligomeric complex in the IMM to facilitate pyruvate transport. This step is required for mitochondrial pyruvate oxidation and carboxylation – critical reactions in intermediary metabolism that are dysregulated in several common diseases. The identification of these transporter constituents opens the door to the identification of novel compounds that modulate MPC activity, with potential utility for treating diabetes, cardiovascular disease, cancer, neurodegenerative diseases, and other common causes of morbidity and mortality. The purpose of the present review is to detail the historical, current and future research investigations concerning mitochondrial pyruvate transport, and discuss the possible consequences of altered pyruvate transport in various metabolic tissues.

Keywords

pyruvate; mitochondria; MPC; membrane transport

INTRODUCTION

Pyruvate is a critical intermediate that can be used in a variety of anabolic and catabolic pathways, including oxidative metabolism, re-synthesis of glucose (gluconeogenesis), synthesis of new lipids (de novo lipogenesis) and cholesterol synthesis, and maintenance of the tricarboxylic acid (TCA) cycle flux. Pyruvate metabolism for these processes requires mitochondrial import, which is a carrier-mediated and regulated process. The purpose of the present review is to discuss the historical and recent discoveries about mitochondrial

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pyruvate transport. Cytosolic and mitochondrial pyruvate metabolism are only briefly described because it has already been reviewed in depth [1].

FORMATION OF CYTOSOLIC PYRUVATE

Pyruvate can be created from several sources in the cytosol. A predominant source is from the breakdown of glucose through anaerobic glycolysis into two molecules of pyruvate, ultimately produced by the enzyme pyruvate kinase (Figure 1). A significant proportion of pyruvate is produced via oxidation of lactate by lactate dehydrogenase. Pyruvate can also be re-formed from malate by cytosolic malic enzyme, which plays an important role in shuttling mitochondrial TCA cycle metabolites such as citrate, malate and oxaloacetate between the cytosol and mitochondria. One last significant source of pyruvate is from the catabolism of three-carbon amino acids. Alanine transaminase (ALT) catalyses the reversible reaction of alanine and 2-oxglutarate into glutamate and pyruvate. There is also a component of ALT activity in the mitochondrial matrix, and alanine can be transported into mitochondria and then converted to pyruvate [2,3]. Serine, threonine, glycine, cysteine and tryptophan can all be converted to pyruvate as well. Altogether, these comprise the primary sources of cytosolic pyruvate.

In most tissues, much of the cytosolic pyruvate generated by these pathways is imported into the mitochondria. However, pyruvate can also be reduced to lactate by the bi-directional cytosolic enzyme lactate dehydrogenase (LDH), of which there are two distinct isoforms: LDH-A which favours the production of lactate from pyruvate, and LDH-B which favours the production of pyruvate from lactate [4]. LDH-A is also known as the M isoform (for muscle), whereas LDH-B is alternatively referred to as the H or heart isoform [5]. Appropriately, glycolytic skeletal muscle is a robust lactate-producing tissue, whereas the heart is a pyruvate-oxidizing organ.

EVIDENCE FOR A MITOCHONDRIAL PYRUVATE TRANSPORTER

Pyruvate dehydrogenase and carboxylase enzymes are localized to the mitochondrial matrix and, therefore, pyruvate must be transported from the cytosol through both the outer and the inner mitochondrial membranes. Similar to most other ions and metabolites, pyruvate probably traverses the outer mitochondrial membrane through the large, relatively non-specific, voltage-dependent anion channel (VDAC), or porin [6]. Consistent with this, humans deficient in VDAC1 show impaired pyruvate oxidation and ATP production [7]. As the proton gradient across the inner mitochondrial membrane (IMM) must be maintained for ATP production to occur, and diffusion of pyruvic acid across this membrane would collapse this gradient, logically there should be a transport mechanism with a proton symport. How pyruvate is transported through the IMM has, however, been a long-standing mystery undergoing intense investigation and debate.

It was first suggested that pyruvate in its undissociated (acidic) form could cross the IMM freely [8]. This seemed to contradict the consensus that the IMM was a selective barrier for other metabolites and, shortly after this, strong evidence was presented that pyruvate crossed the membrane via a specific transporter [9]. Purified rat liver mitochondria were pre-incubated with inhibitors of pyruvate metabolism and the electron transport chain, mixed

with radiolabelled pyruvate, and then centrifuged into perchloric acid ($HClO_4$) to quickly terminate transport and the metabolic reactions. Pyruvate transport displayed saturation kinetics that argued for the existence of a specific transporter. Importantly, that paper also suggested for the first time that pyruvate transport was coupled to a proton symport. These findings proved controversial because the report was quickly followed by another that argued against the 'substrate accumulation method' for determining the rate and regulation of membrane transport, and noted that both normal and denatured mitochondria accumulated pyruvate in a similar manner [10]. This new report suggested that absorption of pyruvate into the membrane, rather than transport through it, accounted for mitochondrial pyruvate binding. This theory of pyruvate absorption by the membrane was later rejected [11,12]. Regardless of these findings, without specific inhibitors of pyruvate transport, this cast doubt on the existence of carrier-mediated mitochondrial pyruvate transport.

Inhibitors of pyruvate transport

The major breakthrough that solidified the evidence for the existence of a pyruvate transporter was the identification of a specific inhibitor [13]. This inhibitor, α-cyano-4-hydroxycinnamate (CHC), is an analogue of the *enol* form of pyruvate with an attached aromatic ring. The inhibitor reduced pyruvate oxidation by rat liver mitochondria, but had no effect on pyruvate's metabolic enzyme activities [pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC) and LDH] in permeabilized mitochondrial extracts. This effect was fairly specific for pyruvate, because there was no inhibition of mitochondrial acetate, lactate or butyrate accumulation. The inhibitor also reduced accumulation of pyruvate and lactate, but not acetate or butyrate, in erythrocytes, which have no mitochondria, suggesting that a monocarboxylate carrier affected by the inhibitor was also expressed on the plasma membrane.

Several other cinnimate analogues were later found to be more potent than CHC for inhibiting pyruvate transport [14]. α-Cyanocinnimate, α-cyano-5-phenyl-2,4-pentadienoate, 4-hydroxycinnamate and α-cyano-β-(1-phenylindol-3-yl)-acrylate (also known as UK-5099) were all shown to inhibit pyruvate-stimulated respiration with IC_{50} values in the nanomolar range [14]. It appeared that the important features of the inhibitors were the α-cyanopropenoate group and the hydrophobic aromatic side chain. Of these, UK-5099 was the most potent, causing inhibition at only 50 nM, and it remains the current gold standard mitochondrial pyruvate carrier (MPC) inhibitor.

A variety of other diverse compounds has been found to inhibit pyruvate import with varying levels of potency. The anthracycline chemotherapeutic drug doxorubicin can decrease the mitochondrial binding of CHC and inhibit pyruvate transport in rat heart mitochondria, with an IC_{50} of 125 μM [15]. This inhibition was proposed to be indirect via interaction with the abundant IMM phospholipid cardiolipin, similar to the doxorubicin-mediated inhibition of the mitochondrial phosphate carrier [16]. The antibiotic gatifloxacin also inhibits mitochondrial pyruvate uptake by renal and hepatic mitochondria [17], and silibinin inhibits the MPC in liver mitochondria [18]. Last, it has been reported that zaprinast, a phosphodiesterase inhibitor, blocks mitochondrial pyruvate transport and decreases pyruvate-stimulated oxygen consumption, with no effect on PDH activity [19].

Several compounds containing thiazolidine rings have been found to bind and inhibit the MPC. Experimental thiazolidine compounds designed as ATP-sensitive potassium (K^+_{ATP}) channel agonists for use as anti-diabetic drugs exerted metabolic effects [20] similar to those previously observed in response to CHC [21]. These compounds have a double bond that could be activated to allow Michael addition of a thiol group from the MPC, which had been shown to occur for both CHC and UK-5099 [22]. These thiazolidine compounds inhibited pyruvate-stimulated respiration, and suppressed pyruvate uptake into rat liver and yeast mitochondria, with an even higher potency than UK-5099 [20]. Insulin-sensitizing thiazolidinediones (troglitazone, rosiglitazone, pioglitazone and MSDC-0160) also decreased pyruvate-stimulated respiration in skeletal muscle cells [23]. Furthermore, armed with new information about the identity of the proteins comprising the MPC, this work showed that knockdown of the MPC subunits by shRNA further sensitized this inhibition by thiazolidinediones [23]. Colca et al. [24] also described two thiazolidinedione compounds (MSDC-0602 and MSDC-0160) which bound to the proteins that make up the MPC (described below). It is interesting that none of the thiazolidinedione compounds used by Colca et al. [24] or Divakaruni et al. [23] has the double bond previously believed to be critical for binding to the MPC [22]. Whether the interaction with the MPC is required for the insulin-sensitizing effects of these drugs, and the chemical structures and properties that allow these drugs to interact with the MPC proteins, remain to be firmly established.

Properties and kinetics of the MPC

The discovery of specific inhibitors and establishment of the pyruvate transport assay sparked a number of studies into the transport properties of the MPC and helped us understand the basic principles of pyruvate transport. The kinetics of MPC activity were dissected and the K_m, activation energy and V_{max} for the MPC were determined [14]. It was postulated that a proton symport was required for mitochondrial pyruvate import. Indeed, early work suggested that symport with a proton, or exchange with a hydroxyl ion, occurred with pyruvate import and this proton flux was also inhibited by UK-5099 [14]. Later, it was determined that transport occurs primarily with symport of a proton, and not exchange with an hydroxyl ion [25]. Some work suggested that DL-palmitoylcarnitine and L-carnitine caused a decrease in mitochondrial pyruvate content [26,27]. However, this was discounted in later papers [25,28]. Others postulated that oxoacid export might provide substrate for the pyruvate transporter, and evidence was provided that pyruvate and 3-hydroxybutyrate may be exchanged across the IMM [26]. When mitochondria were preloaded with β-hydroxybutyrate, pyruvate import could be enhanced 3-fold [25]. Acetoacetate was also shown to be transported through the same carrier as pyruvate, and this transport is accompanied by proton exchange [12,29]. Altogether, these studies conducted by several groups in the 1970s have helped us understand the basic tenets of mitochondrial pyruvate transport. However, the purification and identification of the proteins that constitute the MPC would prove problematic and elusive in the subsequent decades.

Trials and tribulations in identification of the MPC

Protein complexes with pyruvate transport activity were purified by two separate groups in 1986 [30,31]. Isolated beef heart mitochondria were sonicated in a buffer with Triton X-114 and cardiolipin to collect submitochondrial particles, and then subjected to hydroxyapatite

chromatography. The eluate was then reconstituted into liposomes, and pyruvate transport sensitive to CHC was observed. A number of proteins ranging in size from 12 kDa to 94 kDa were observed on silver-stained SDS/PAGE gels. Similar procedures using Triton X-100 also purified several proteins of approximately 30 kDa with CHC-sensitive pyruvate/acetoacetate exchange activity from rat liver mitochondria [32]. Although the authors suggested that at least one of these proteins was the MPC, absolute purification of the carrier was not achieved.

Evidence that inhibitors stably bound to the MPC [33,34] suggested that MPC could be purified by using an immobilized inhibitor as bait. Hydroxyapatite eluate of beef heart mitochondria was passed through immobilized 2-cyano-4-hydroxycinnamate on Sepharose, and proteins of 31.5 and 34 kDa were found to be bound to the column [35]. The same procedure with yeast mitochondria isolated proteins of 26 and 50 kDa [36]. The proteins were not specifically identified from these studies, and it should be noted that the sizes (approximately 30 kDa) are similar to that of the plasma membrane monocarboxylate carrier, which has been reported by one group to be expressed in mitochondria from muscle cells [37–39]. Another study took advantage of the observation that *α*-cyanocinnamate inhibited the irreversible binding of the thiol-blocking reagent *N*-phenylmaleimide to the MPC [40]. Using *N*-[^{3}H]phenylmaleimide, they then showed that *α*-cyanocinnamate blocked the radiolabelling of a 15-kDa protein in both rat liver and heart mitochondria. This protein was later isolated and sequenced as the 15-kDa subunit of cytochrome *c* oxidase IV (COXIV) [41].

These failures to identify the MPC in non-biased assays led investigators to take a candidate gene approach. The vast majority of IMM metabolite carriers belong to the mitochondrial carrier family, known as the solute carrier 25 (SLC25) family in humans. These proteins are typically 30–35 kDa in size, contain six transmembrane domains (Figure 2) and have, in the last couple of years, been expertly reviewed [42,43]. Using mitochondria from 18 mutant yeast strains with deletion of these carriers revealed that knockout of a 41.9-kDa protein encoded by the *YIL006w* gene resulted in low pyruvate uptake and diminished sensitivity to UK-5099 [41]. Although it was believed that this was a pyruvate transporter, this protein was later definitively shown to be the mitochondrial NAD^{+} transporter in liposomal reconstitution experiments [44]. The decreased activity of the NAD^{+}-requiring PDH enzyme in the *YIL006w* mutants probably accounts for the observed phenotype. Palmieri et al. [45] subsequently attributed functions to all but eight of the yeast mitochondrial carrier family proteins without identifying a pyruvate transporter.

Molecular identification of the MPC

Two to three years ago, two independent research groups used genetic and bioinformatic approaches to identify a hetero-oligomeric complex of proteins now named MPC1 and MPC2 (formerly known as Brp44L and Brp44, respectively) as being both necessary and sufficient for pyruvate transport [46,47]. Bricker et al. [46] screened for proteins with unknown functions that were highly conserved from yeasts to humans, with the assumption that conservation was an indicator of essential function. This group determined that MPC1 and MPC2 were 12-and 15-kDa proteins, respectively, which formed an oligomeric complex

of approximately 150 kDa in the IMM, and that both proteins were needed for complex stability. Although smaller than the SLC25 family proteins, the molecular mass of the MPC proteins is consistent with several of the proteins observed in early isolation attempts [30,31]. The stoichiometry of the proteins in the high-molecular-mass complex and the membrane topology of the complex are unknown. MPC1 and MPC2 are predicted to each contain two or three transmembrane helices, depending on which prediction tools are used; this is dissimilar to the SLC25 family proteins which canonically contain six transmembrane domains (Figure 2) (MPC1 UniProt# Q9Y5U8; MPC2 UniProt# O95563; aligned with Clustal W2). The topology of the MPC proteins needs to be experimentally determined, although modelling [48] would suggest that the two proteins prefer an opposite orientation with regard to localization of the N- and C-terminal tails (Figure 2). Recent work has also suggested a structural similarity, including three transmembrane domains, to the semiSWEET family of bacterial sugar transporters and the PQ-loop family of amino acid transporters [49]. Another recent review has used this information to generate a model of the complex's structure [50]. This similarity and the link to a family of sugar and amino acid transporters could also support the role for MPC proteins in intermediate carbohydrate transport.

Genetic and pharmacological evidence is mounting that the MPC proteins constitute the MPC complex. Genetic deletion of MPC1 resulted in almost complete loss of pyruvate transport in yeast mitochondria, and human cells with siRNA-mediated knockdown of either MPC1 or MPC2 exhibited significantly reduced pyruvate-stimulated mitochondrial respiration [46]. Co-expression of murine MPC1 and MPC2 in *Lactococcus lactis* was sufficient to facilitate pyruvate import into the bacterium, and this transport was sensitive to UK-5099 [47]. Herzig et al. [47] noted that MPC1- or MPC2-deficient yeast strains grew slowly in amino-acid-free medium, suggesting an inability to utilize glucose oxidation. Deletion of MPC1 in both yeasts and *Drosophila* spp. also resulted in build-up of glycolytic intermediates and depletion of TCA cycle intermediates. Later studies conducted in mammalian cells confirmed that MPC1 or MPC2 RNAi suppressed acetyl-CoA formation [51] and incorporation of [^{13}C]glucose into TCA cycle intermediates, and suggested that the ability of cells with MPC inhibition to survive was due to compensatory increases in glutamate oxidation in the mitochondria [52,53]. In addition, mice with constitutive global deletion of MPC2 were found to die at approximately embryonic day 11 [54], when a burst of mitochondrial biogenesis occurs [55]. Constitutive deletion of MPC1 in mice is also reported to be embryonically lethal [50]. It is of interest that mice harbouring a hypomorphic allele of MPC2, which expressed a truncated, partially functional protein, were viable, but exhibited a metabolic phenotype consistent with diminished mitochondrial pyruvate transport [54]. A 30% reduction in rates of pyruvate-mediated mitochondrial oxygen consumption was detected in mitochondria isolated from the heart or kidney, whereas oxygen consumption in the context of other metabolic substrates (glutamate or succinate) was normal. The mice also exhibited an elevated blood lactate concentration, especially when challenged with physiological stimuli associated with increased lactate/pyruvate metabolism. Finally, a human mutation in MPC1 resulted in a phenotype consistent with a defect in pyruvate transport including lactic acidosis and diminished pyruvate utilization [46,56].

Pharmacological evidence for the identity of the MPC proteins is also accumulating. An unbiased approach using thiazolidinediones that inhibit pyruvate-stimulated respiration [23] showed that these compounds could be specifically cross-linked to a 15-kDa IMM protein in mouse liver lysates [24]. Proteomic analyses showed this protein to be MPC2, and knockdown of MPC2 or MPC1 in *Drosophila* spp. abolished specific binding of the thiazolidinediones to the IMM. Furthermore, UK-5099 competed with a radiolabelled thiazolidinedione probe for binding to the 15-kDa protein. In addition, knockdown of MPC1 or MPC2 sensitized cells to the thiazolidinedione-mediated inhibition of pyruvate respiration [23]. Altogether, these studies conducted in yeasts, *Drosophila* spp., mice and humans are all consistent with MPC1 and MPC2 proteins comprising the long-sought-after MPC.

Many aspects of our basic biochemical understanding of the MPC complex, such as the stoichiometry of the hetero-oligomeric complex and whether MPC1 and MPC2 are the only proteins required for complex activity, are still incompletely understood. Available evidence suggests that the MPC transports pyruvate by acting as a facilitative carrier, rather than forming a channel, but the mechanism by which MPC proteins carry pyruvate across the membrane is unclear. This has caused some debate about whether these proteins comprise the totality of the MPC and whether MPC1 and MPC2 are instead regulators of the PDH complex [57]. Although it is possible that MPC1 and MPC2 are only accessory or stabilizing subunits of the MPC, and that other proteins are integrated into the complex to catalyse pyruvate transport, the experiments on MPC1/MPC2 constitution into *Lactococcus lactis*, performed by Herzig et al. [47], dictate against this. Also, recent work has indicated that genetic inhibition of the MPC proteins does not affect PDH complex activity in cultured cells [53]. In our opinion, the evidence that MPC1 and MPC2 are at least components of the MPC and integral to MPC complex stability is widely accepted [57,58]. Obviously, additional work is needed to fully dissect the molecular and biochemical nature of the MPC.

MITOCHONDRIAL PYRUVATE METABOLISM

Once in the matrix, pyruvate can be metabolized by two different routes. Most of the pyruvate in oxidative tissues is converted to acetyl-CoA (and NADH from NAD^+) by the PDH complex (see Figure 1). This acetyl-CoA then enters the TCA cycle and these carbons are predominantly converted to CO_2. The energy created from turning pyruvate into CO_2 produces the reducing equivalents NADH and $FADH_2$, which are important for generating the proton gradient required for oxidative phosphorylation and ATP production. In heart and oxidative skeletal muscle, oxidation by the PDH is the predominant fate for mitochondrial pyruvate.

The acetyl-CoA formed by PDH can also be used for the anabolic production of fatty acids, cholesterol and acetylcholine (see Figure 1). Acetyl-CoA is combined with oxaloacetate by citrate synthase to form citrate. This citrate can exit the mitochondria and is cleaved back to acetyl-CoA and oxaloacetate by citrate lyase in the cytosol. Acetyl-CoA carboxylase irreversibly carboxylates the acetyl-CoA into malonyl-CoA, which is then used by fatty acid synthase to produce palmitoyl-CoA. Conversely, the acetyl-CoA can be converted to acetoacetyl-CoA and ultimately mevalonate by two reactions catalysed by

hydroxymethylglutarate (HMG)-CoA synthase and HMG-CoA reductase. This mevalonate is then converted by several steps into cholesterol.

Carboxylation by PC is the other fate for mitochondrial pyruvate. PC produces oxaloacetate [59] and is highly expressed in liver, kidney, brown adipose and pancreatic β cells. Although the PDH reaction is used predominantly for ATP production, the PC reaction is used for the production and replenishment of TCA cycle intermediates, which is known as anaplerosis [60]. TCA cycle intermediates such as oxaloacetate, citrate, 2-oxoglutarate and succinyl-CoA are used in the biosynthetic pathways of gluconeogenesis, lipogenesis, biogenesis of amino acids and haem generation, respectively, and anaplerosis ensures that the mitochondrial concentrations of these intermediates are adequate so that the TCA cycle flux remains unperturbed. In addition to channelling metabolites towards these biosynthetic pathways, oxaloacetate generated by PC serves as an acceptor for the acetyl-CoA formed by PDH to initiate the TCA cycle.

PHYSIOLOGY AND PATHOPHYSIOLOGY OF MITOCHONDRIAL PYRUVATE TRANSPORT

In hepatic metabolism

Mitochondrial pyruvate import and metabolism are important in the liver for the production of new glucose and lipids [61,62]. Due to the abundance and ease of mitochondrial isolation from liver lysates, much of what we know about mitochondrial pyruvate transport comes from the liver. The liver is normally a net consumer of lactate and pyruvate. Under fed conditions, pyruvate oxidation may be a significant fate of pyruvate because the PDH complex is active during these conditions [63]. Under fasted conditions, the PDH is inhibited and pyruvate is primarily carboxylated by PC [63,64]. This switch in metabolic fates is crucial to enhancing the flux of the carbon in pyruvate into the gluconeogenic pathway via synthesis of oxaloacetate, conversion to malate and export to the cytosol, for eventual conversion back to new glucose (see Figure 1). As PC is a mitochondrial matrix protein, and the dephosphorylation of phospho*enol*pyruvate to form pyruvate is an irreversible reaction, the transport of pyruvate across the IMM would seem to be a prerequisite step in gluconeogenesis. Moreover, hepatic mitochondrial ketone export, which is another important component of the fasting response, may also be an important function of the MPC [25,65]. Ketone body export/pyruvate import across the IMM could be a regulatory step to enhance hepatic gluconeogenesis and maintain the mitochondrial membrane potential [25,66,67]. Hepatic glucose production from gluconeogenesis is important physiologically during prolonged food deprivation or exercise, but unabated gluconeogenesis also contributes to chronic hyperglycaemia in diabetes. As a result of the implications of this process in physiology and pathophysiology, several studies have examined the role of pyruvate import on gluconeogenesis.

It has been suggested that conditions of increased gluconeogenic flux result in enhanced activity of the MPC complex. Glucagon, adrenaline, insulin deficiency and cortisol increase pyruvate carboxylation, probably by increasing mitochondrial pyruvate import [68–71], e.g. using Triton X-114 isolation and reconstitution of pyruvate transport activity into liposomes,

it was shown that livers from rats with streptozotocin-induced Type 1 diabetes displayed a 193% increase in activity [72], which was not observed in diabetic rats treated by daily insulin injection [73]. In contrast, other work has suggested that hormonal stimulation does not directly increase mitochondrial pyruvate import, but rather stimulates mitochondrial respiration, increasing the proton motive force and ATP:ADP ratio, thus activating PC [74–77]. With the molecular identity of the MPC now known, it will be possible to investigate the transcriptional and post-translational regulation of the proteins to examine how these changes alter function during physiological/pathophysiological scenarios. Liver MPC2 expression is enhanced in mice after 24 h of fasting [78] and in diabetic *db*/*db* mice [79]. These increases in expression are modest compared with other gluconeogenic enzymes, probably as a result of the importance of the MPC to pyruvate oxidative metabolism under fed, non-gluconeogenic conditions. Post-translational modifications may also affect MPC activity, but how MPC proteins are modified and how these post-translational modifications affect MPC activity require further investigation.

It has been calculated that the V_{max} for pyruvate transport (42 nmol/min per mg of protein) [14] could be limiting for gluconeogenesis because perfused rat livers stimulated with glucagon or fatty acids could produce glucose from lactate at 100–200 μmol/h per g, which would require mitochondrial pyruvate transport rates of 65–125 nmol/min per mg [80,81]. After accounting for inhibitor-insensitive pyruvate transport, other reports calculated even lower rates for the MPC [29], increasing the likelihood that MPC flux was a regulatory step for gluconeogenesis. Indeed, treatment with MPC inhibitors reduced glucose output by isolated perfused liver preparations [82–85]. CHC was able to limit pyruvate carboxylation in liver mitochondria, and decrease gluconeogenesis in kidney cortex slices [21] and from pyruvate, lactate and serine in rat liver cells [3,82,83,86]. The antibiotic gatifloxacin was also shown to inhibit mitochondrial pyruvate import and decrease glucose production in both the kidney and the liver, probably contributing to the hypoglycaemia associated with gatifloxacin therapy [17]. Last, it has also been suggested that silibinin decreases hepatic gluconeogenesis by inhibiting the MPC [18].

However, other studies using MPC inhibitors have cast doubt on whether MPC flux is a limiting step in hepatic gluconeogenesis [83]. Our recent study using mice expressing a truncated, partially functional, MPC2 protein with modest reductions in pyruvate transport found no defect in gluconeogenesis after exhaustive exercise, fasting or a bolus intraperitoneal pyruvate injection [54]. Furthermore, flux of [^{13}C]pyruvate into glucose or TCA cycle intermediates in the liver was also unaffected. The probable explanation for the outcomes of these studies is that, under conditions of high lactate concentrations, the capacity for pyruvate transport into mitochondria is far in excess of that needed for gluconeogenesis [83]. Alternatively, pyruvate interconversion into gluconeogenic substrates (e.g. alanine) that can enter mitochondria independently of the MPC could compensate for loss of the MPC. Models of more severe MPC deficiency in liver, possibly with other experimental interventions, are needed to determine the overall importance of pyruvate transport in hepatic gluconeogenesis and to rule out alternative mechanisms.

In pancreatic β-cells

Impairments in glucose-stimulated insulin secretion (GSIS) are an important step in the development of hyperglycaemia and the progression of diabetes. Pancreatic islet β-cells have the capacity to sense glucose concentrations, and higher plasma glucose concentrations stimulate insulin secretion via increased β-cell metabolism of glucose and pyruvate [87]. Glucose that enters the β-cell is predominantly metabolized by glycolysis, and the resulting pyruvate primarily enters the mitochondrion [88] due to the extremely low expression of LDH-A in β-cells [89,90]. Once pyruvate has entered the β-cell mitochondrion, it can be either carboxylated by PC or oxidized by PDH [88,91–95]. Important roles for both these mitochondrial pyruvate metabolic pathways have been demonstrated in controlling insulin secretion. It is believed that oxidation of pyruvate stimulates production of ATP and increases the intracellular ATP concentration. This regulates insulin secretion by suppressing the activity of K^+_{ATP} efflux channels (see Figure 1). ATP inhibits K^+_{ATP} channel activity, leading to K^+ accumulation, membrane depolarization, Ca^{2+} influx and release of insulin into the circulation [96]. Indeed, mice with β-cell-specific knockout of a subunit of the PDH complex (Pdha) exhibited hypoinsulinaemia, glucose intolerance and impaired GSIS in isolated islets and *in vivo* during hyperglycaemic clamp [97,98]. Other evidence supports an important role for pyruvate carboxylation and anaplerosis in GSIS [93–95,99]. Anaplerotic mitochondrial pyruvate metabolism activates pyruvate cycling pathways that alter NADPH and regulates insulin secretion by inhibiting K^+_{ATP} channel activity as well [100]. It is likely that both the pyruvate-mediated production of mitochondrial ATP and anaplerotic products play important roles in insulin secretion via regulation of K^+_{ATP} channel activity [87].

As altering mitochondrial pyruvate entry via the MPC would affect pyruvate metabolism by both PC and PDH, this could be an important role for regulating GSIS. In support of this, ectopic over-expression of LDH-A in β-cells, thus artificially enhancing conversion of pyruvate to lactate, impairs GSIS [101–103]. Inhibitors of mitochondrial pyruvate transport have also been shown to decrease GSIS in INS1 cells [104,105], *ob*/*ob* obese mouse islets [106], and isolated rat and human islets [105]. UK-5099 was also shown to increase blood glucose excursion during a glucose tolerance test, although it should be noted that plasma insulin was not measured and it is not clear which tissues were affected by this inhibitor [105]. On the other hand, two early studies observed either no effect [107] or enhanced GSIS [108] with CHC treatment of isolated islets. Importantly, both of these studies utilized islet stimulation buffers containing BSA, which may have inhibited CHC entry into the cells [14]. The MSDC-0160 thiazolidinedione compound shown to bind MPC2 [24] improved human islet insulin content, but also did not affect GSIS [109].

Now that the MPC constituents have been identified, genetic approaches can be taken to dissect the role of this complex in GSIS. Indeed, we recently demonstrated that expression of a truncated, hypomorphic MPC2 protein in mice resulted in basal hypoinsulinaemia and hyperglycaemia during a glucose tolerance test [54]. Isolated islets from these animals displayed defective GSIS, which could be circumvented by stimulation with a different respiratory substrate such as glutamate, or bypass of the mitochondria by K^+_{ATP} blockade with either the sulfonylurea glibenclamide or KCl [54]. Glibenclamide treatment also

decreased blood glucose and increased plasma insulin *in vivo* during a glucose tolerance test [54]. This is consistent with other work showing that knockdown of MPC1 or MPC2 by siRNA decreased GSIS in INS1 cells and isolated rat and human islets [105]. Altogether, these findings constitute strong evidence that the MPC complex plays an important role in mediating glucose sensing in pancreatic β-cells and controlling GSIS.

In myocardial metabolism

The myocardium has the ability to metabolize fatty acids, glucose, lactate, amino acids and ketone bodies, depending on the substrate milieu and the neurohormonal inputs provided [110]. Up to 95% of the heart's ATP generation comes from mitochondrial oxidation, and typically approximately 60–90% of this mitochondrial ATP production comes from fatty acids, whereas 10–40% is from pyruvate oxidation [111]. The pyruvate is typically derived from both glycolysis and lactate oxidation in almost equal amounts [111], because the heart expresses high levels of the LDH-B isoenzyme which favours the conversion of lactate to pyruvate. The heart is a net consumer of lactate even at almost maximal cardiac workload [112]. Importantly, acute metabolic stress, such as ischaemia, and chronic stresses, such as hypertrophy and heart failure, are known to drastically alter myocardial substrate availability and metabolism. These effects on pyruvate oxidation, which occur during ischaemia [113] or diabetes [114], are discussed further below.

A fair amount is known about the transport properties of pyruvate in the heart [14,21]. Shearman and Halestrap [115] used the inhibitor α-cyano-β-(1-phenylindol-3-yl)acrylate to determine the MPC content in heart mitochondria, and calculated that the MPC's abundance in the heart was almost identical to its expression in the liver, and that pyruvate transport was rate-limiting for pyruvate oxidation by heart mitochondria during ADP-stimulated respiration. In agreement, we observed similarly high levels of MPC1/MPC2 expression in both mouse heart and mouse liver, and also observed a 30% decrease in pyruvate-stimulated respiration in heart mitochondria from mice with a truncated MPC2 protein [54]. In addition, Paradies and Ruggiero [116,117] suggested that hyperthyroidism increased rat heart MPC activity [116], whereas hypothyroid rats displayed decreased heart MPC activity [117]. These authors also observed age-related decreases in heart MPC activity in rats [118,119]. Last, in working guinea-pig hearts, it was suggested that MPC flux did not play acontrolling role in the rate of pyruvate oxidation because PDH activity was determined to be slower than the rate of pyruvate import [120]. The researchers did note that CHC drastically reduced pyruvate oxidation at normal workloads, and almost completely abolished the increases in pyruvate oxidation that occur after treatment with α- or β-adrenergic agonists [120].

During ischaemia, oxygen supply is insufficient to support mitochondrial ATP synthesis, and thus flux through the electron transport chain is reduced. In this scenario, pyruvate oxidation is decreased and dependence on anaerobic glycolysis is increased [121]. This results in increased lactate production, which can be exacerbated if cardiac work increases [122]. Pyruvate oxidation is reduced during ischaemia, and activation of PDH activity with dichloroacetate improved cardiac function [123]. It is of interest that administration of pyruvate during reperfusion has been shown to increase PDH activity and improve

myocardial function [124]. It is possible that reversing the effects of ischaemia on MPC flux and therapeutically enhancing mitochondrial pyruvate import could improve cardiac outcomes.

An overall decline in mitochondrial metabolism has been observed in chronic, decompensated, heart failure [125,126], with specific decreases in pyruvate oxidation being observed [127,128]. This is thought to be due to decreased PDH activity caused by increased PDH kinase expression, which inactivates PDH [129–132]. However, decreased activity of the MPC could also be involved, and warrants investigation in heart failure.

Last, the observation that doxorubicin inhibits the MPC [15] suggests that this decreased mitochondrial pyruvate import could contribute to the development of doxorubicin-induced cardiomyopathy which occurs in up to 10% of cancer patients treated with doxorubicin [133]. Most of doxorubicin's deleterious effects on the heart are attributed to reactive oxygen species generation, yet treatment with antioxidants appears to have little effect on the cardiotoxicity [134]. Increased anaerobic glycolysis and lactate formation have been observed in hearts from doxorubicin-treated rats [135], suggesting that there may indeed be a defect in mitochondrial pyruvate import. Doxorubicin has also been shown to decrease carnitine transport across the IMM [136], causing reduced fatty acid oxidation [137], and thus creates a 'starved heart', also observed in other types of heart failure. Clearly, further investigation is needed to confirm the role of mitochondrial pyruvate import in various forms of heart failure and to examine whether targeting the MPC to enhance mitochondrial pyruvate import would have therapeutic effects on heart failure or ischaemic heart disease.

In adipose tissue

Glucose is taken up by adipocytes and converted into new fatty acids in the process of de novo lipogenesis. The initial steps of de novo lipogenesis require mitochondrial pyruvate entry and metabolism to form citrate. This citrate is then exported back to the cytosol for fatty acid synthesis (see Figure 1). In an early study, Halestrap and Denton [21] determined that CHC inhibited fatty acid synthesis from glucose or fructose, but not from acetate, in epididymal fat pads. In brown adipose tissue (BAT) mitochondria, fatty acids make up the predominant energy source for uncoupled respiration and thermogenesis. However, complete oxidation of the acetyl-CoA formed by fatty acid β-oxidation requires condensation of the acetyl-CoA with oxaloacetate in the TCA cycle. The anaplerotic products produced by pyruvate carboxylation enhance fatty acid oxidation in BAT mitochondria, probably by replenishment of TCA cycle intermediates as discussed above [138]. The MPC complex could also play an important role in BAT de novo lipogenesis, although this has not yet been examined in detail. In agreement with the importance of mitochondrial pyruvate import in brown adipocytes, the MPC1/MPC2 proteins are highly expressed in BAT compared with other mouse tissues, even when corrected for mitochondrial protein content [54]. The MSDC-0160 and MSDC-0602 thiazolidinedione compounds, which bind MPC2, but do not activate peroxisome-proliferator-activated receptor γ [139], induce browning of BAT progenitor cells [24]. High concentrations of UK-5099 also enhanced the expression of the BAT marker UCP1 (uncoupling protein 1) [24]. This potentially indicates that mitochondrial pyruvate metabolism is important in the

browning process. Consistent with this, lactate administration caused ‘browning’ of adipocytes in culture via mechanisms that are not entirely clear, but may involve effects on the redox status [140]. Given the potential importance of mitochondrial pyruvate import in both fatty acid formation and oxidation in adipose tissue, as well as the possible role that pyruvate/lactate metabolism may play in BAT cell differentiation, it is likely that the MPC has multifaceted effects on fat cell metabolism and biology.

In the nervous system

The central nervous system relies almost exclusively on glucose and pyruvate metabolism to generate ATP. Neurons readily import lactate, which is then converted to pyruvate and oxidized in the mitochondria. Defects in mitochondrial function are associated with several progressive neurodegenerative diseases [141]. There may also be a link between specific defects in mitochondrial pyruvate metabolism, because reduced PDH activity has been detected in neurons from mouse and human brains in the context of several neurological diseases [142–144]. Consistent with this, increased pyruvate and lactate levels have been observed in the cerebrospinal fluid of patients with Alzheimer’s disease [145] and in the blood of patients with Parkinson’s disease [146]. Reduced PDH flux has also been observed in the brains of patients with Alzheimer’s disease despite there being no reduction in PDH protein levels [143]. One possible explanation for this observation is that MPC’s activity is diminished, leading to reduced flux into PDH. Information about the roles played by the MPC in brain mitochondrial metabolism is extremely limited compared with other tissues. It is known that phenylpyruvate, 2-oxoisocaproate [147] and CHC [21] inhibit pyruvate import and oxidation in brain mitochondria. In addition to the need for pyruvate oxidation for ATP synthesis, brain mitochondrial pyruvate metabolism is also required for the formation of acetyl-CoA and the subsequent synthesis of the neurotransmitter acetylcholine [148], which is decreased in several neurodegenerative diseases [149]. Thus, although evidence is currently limited, there is a strong rationale for a prominent role of mitochondrial pyruvate transport in maintaining neuronal function, and evidence that perturbations in MPC activity could contribute to the progression of neurodegenerative diseases. Future studies using mice with targeted MPC gene disruption in neurons, as well as use of compounds that target MPC activity, should help in our understanding of these concepts.

In cancer

Most tumour cells display a metabolic phenotype defined by enhanced glycolysis and decreased oxidative phosphorylation, which was discovered by Otto Warburg in the 1920s [150] and has since been termed the ‘Warburg effect’. The consequence of these alterations is elevated lactate production from anaerobic glycolysis. Several molecular adaptations in glucose and mitochondrial metabolism have been identified in tumour cell lines and neoplastic cells *in vivo* which allow the cancerous cell to produce this metabolic phenotype, e.g. cancer cells directly alter pyruvate metabolism by phosphorylation of PDH kinase 1 to inactivate PDH and mitochondrial pyruvate oxidation [151]. In addition, dimerization and inactivation of pyruvate kinase M2, which result in decreased pyruvate formation, have also been observed in tumour cells [152].

Given its position at the interface between glycolysis and mitochondrial pyruvate metabolism, it seems likely that alterations in MPC activity mightbe observed in tumour cells. One early study of mitochondrial pyruvate transport showed that MPC activity was significantly decreased in Ehrlich's ascites tumour cells [153]. Reduced mitochondrial pyruvate import, compared with normal hepatocytes, was later observed in several other liver tumour cell lines [154]. This report also defined MPC's increased K_m value for pyruvate in these tumours, suggesting that the MPC may be physically different in these cells, potentially due to post-translational modifications. More recently, it was determined that MPC proteins were under-expressed in a variety of tumour cell lines and solid tumours [155]. Likewise, chemical inhibition of tumour xenografts with CHC slightly enhanced tumour growth [52]. Enhancing MPC activity by forced over-expression in the tumour cells was found to reduce tumour growth in soft agar assays and in mice *in vivo*. The data published thus far suggest that the inhibition of the MPC may have its most dramatic effects on the stem cells that mediate solid tumour expansion. Additional studies into the mechanisms by which cancer cells could limit MPC activity to enhance tumour growth are an area of active investigation [156]. The idea that tumour growth or metastasis could be inhibited by enhancing the activity of the MPC is a tempting target for new anti-cancer agents.

CONCLUDING REMARKS

Mitochondrial pyruvate metabolism is an important process in normal organ function and pathology. The recent identification of the proteins comprising the MPC opens up vast possibilities for investigation of the roles of altered mitochondrial pyruvate transport in various common acquired diseases. In our opinion the four most accessible and important areas now available for study are: (i) a need for proof-of-concept studies in higher organisms, with conditional loss of function for MPC proteins to define their roles in mitochondrial metabolism and identify pathways that might compensate for loss of MPC function; (ii) the determination of the topology of the MPC proteins in the membrane, the structure and stoichiometry of the MPC proteins in the complex, and whether other proteins are a component of the MPC complex will be important for understanding how the complex functions as a transporter and for rational drug design; (iii) the determination of how the MPC's activity and capacity are regulated at the transcriptional level and by post-translational modifications in physiological and pathophysiological contexts; and (iv) defining whether genetic variations or mutations in the genes encoding the MPC proteins can be linked to various chronic diseases is another area that has not yet been explored. The end goal of these new areas will be to gain a better understanding of mitochondrial pyruvate transport at a mechanistic level, and so enable the design of novel MPC-targeted therapies for several chronic diseases that constitute current public health problems.

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Abbreviations

ALT	alanine transaminase
BAT	brown adipose tissue
CHC	α-cyano-4-hydroxycinnamate
GSIS	glucose-stimulated insulin secretion
HMG	hydroxymethylglutarate
IMM	inner mitochondrial membrane
LDH	lactate dehydrogenase
MPC	mitochondrial pyruvate carrier
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
SLC25	solute carrier 25
TCA	tricarboxylic acid
VDAC	voltage-dependent anion channel

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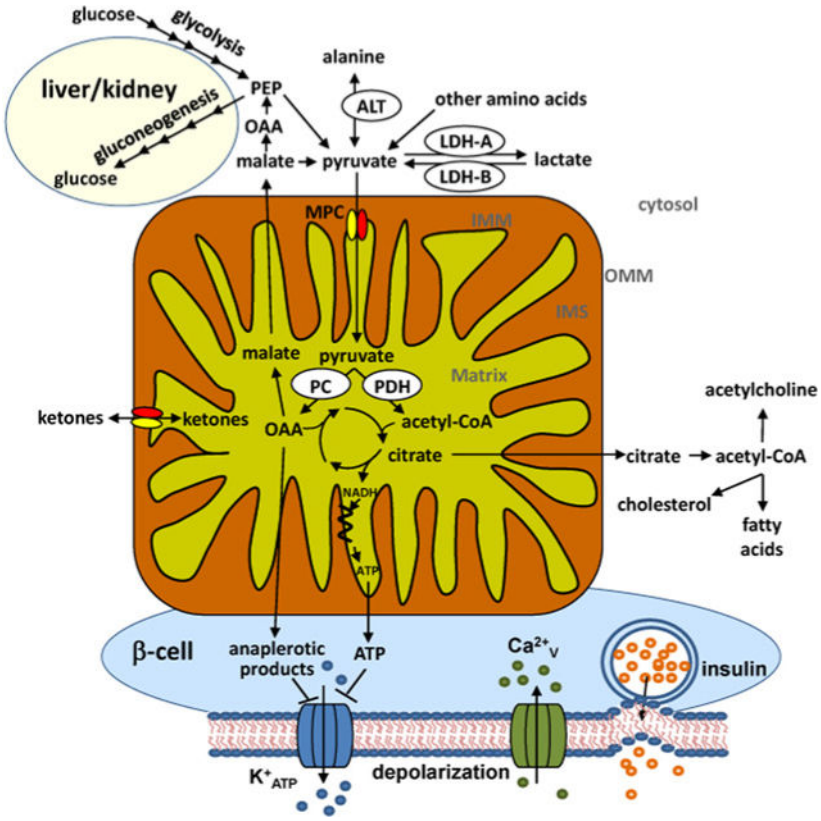

Figure 1. Pyruvate metabolic pathways

Pyruvate can be formed in the cytosol by glycolysis, or conversion from alanine by ALT, from lactate by LDH-B or from malate by malic enzyme (ME). Pyruvate crosses the outer mitochondrial membrane (OMM) probably via the VDAC into the intermembrane space (IMS). Pyruvate is then transported across the IMM by the MPC. It has also been suggested that the MPC transports ketone bodies across the IMM. In the mitochondrial matrix, pyruvate can be either oxidized into acetyl-CoA by PDH or carboxylated to oxaloacetate (OAA) by PC. Although pyruvate oxidation is important for the production of reducing equivalents for ATP synthesis, citrate formed in the TCA cycle can also be exported to the cytosol, converted to acetyl-CoA, and used to produce new fatty acids, cholesterol or acetylcholine. OAA produced by PC can be exported to the cytosol and converted to phospho*enol*pyruvate (PEP), which can then be used to form glucose in gluconeogenic tissues such as the liver, kidney and intestine. Last, both mitochondrial energy produced from pyruvate oxidation and anaplerotic intermediates produced by pyruvate carboxylation play a role in the stimulation of insulin secretion in pancreatic β-cells by inhibiting K^+_{ATP} channels, causing depolarization of the plasma membrane, and Ca^{2+} influx through Ca^{2+}_V channels, and allowing insulin secretory vesicle fusion and insulin release.

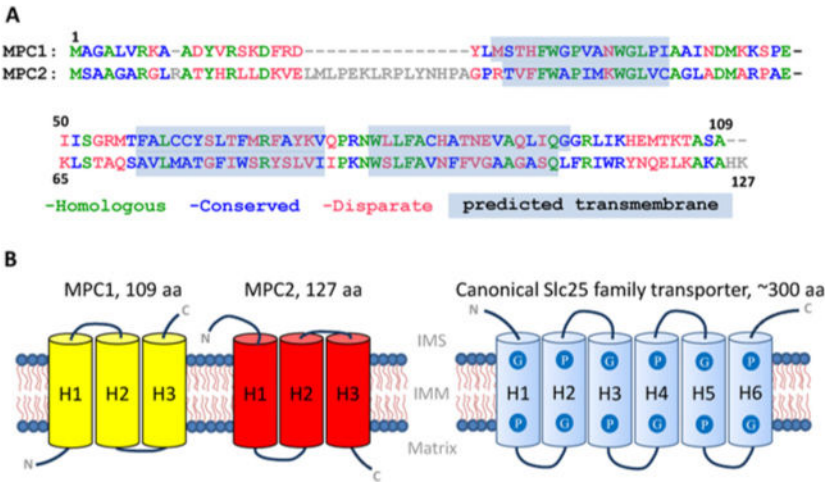

Figure 2. Predicted molecular structure of MPC proteins

(**A**) An alignment of human MPC1 and MPC2 proteins is shown. Sequence conservation is colour-coded and predicted transmembrane domains are in shaded boxes. (**B**) A schematic representation of the predicted transmembrane topology of monomeric MPC proteins is shown and compared with the canonical SLC25 family proteins. MPC proteins, which are predicted to contain two or three transmembrane helices compared with six in SLC25 family proteins, are predicted to adopt opposing topological orientations and are also known to form hetero-oligomeric complexes. However, the stoichiometry and structure of these higher-order aggregates are not known.