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Mitochondrial pyruvate carrier 2 hypomorphism in mice leads to defects in glucose-stimulated insulin secretion

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Mitochondrial Pyruvate Carrier 2 Hypomorphism in Mice Leads to Defects in Glucose-Stimulated Insulin Secretion


INTRODUCTION

Pyruvate is an important three-carbon intermediate in energy metabolism and is a central substrate in carbohydrate, fat, and amino acid catabolic and anabolic pathways. Pyruvate is generated in the cytoplasm through glycolysis and subsequently is transported into the mitochondrion for oxidation or carboxylation, which is required for several critical metabolic processes (Figure 1A). For example, mitochondrial pyruvate carboxylation in the mitochondrial matrix results in the formation of oxaloacetate (an anaplerotic reaction), which is required for the biosynthesis of glucose via gluconeogenesis. Pyruvate entry into the mitochondrial matrix is also required for pyruvate oxidation, which is a prerequisite for the production of reducing equivalents in the tricarboxylic acid (TCA) cycle and production of citrate for de novo lipogenesis. Both pyruvate oxidation and carboxylation are critical for glucose-stimulated insulin secretion (GSIS) in pancreatic β cells (Jensen et al., 2008; Prentki et al., 2013; Sugden and Holness, 2011). Elevated blood glucose causes an increase in the intracellular ratio of ATP/ADP via increased mitochondrial pyruvate oxidation (Prentki et al., 2013; Sugden and Holness, 2011). ATP inhibits KATP channels, leading to depolarization, Ca²⁺ influx, and the release of insulin into the circulation (Huopio et al., 2002). Anaplerotic mitochondrial pyruvate metabolism activates pyruvate cycling pathways that alter NADPH, and regulates insulin secretion by inhibiting KATP channel activity as well (Jensen et al., 2008).

The existence of carrier-assisted transport of pyruvate across the inner mitochondrial membrane (IMM) was demonstrated in the 1970s (Halestrap, 1975; Halestrap and Denton, 1975; Papa et al., 1971). However, the proteins that facilitate pyruvate import into the mitochondrial matrix have only recently been identified (Bricker et al., 2012; Herzig et al., 2012). New evidence has emerged that the mitochondrial pyruvate carrier (MPC) is composed of two proteins, MPC1 and MPC2, which form a hetero-oligomeric complex in the IMM, and that both proteins are required for complex activity and stability (Bricker et al., 2012; Herzig et al., 2012). The MPC protein complex has been determined to be essential for mitochondrial pyruvate transport in Drosophila and yeast (Bricker et al., 2012; Herzig et al., 2012). In humans, mutations in MPC1 have been identified and associated with defects in mitochondrial pyruvate metabolism, lactic acidosis, hyperpyruvataemia, severe illness, and failure to thrive (Bricker et al., 2012; Brivet et al., 2003). Since its discovery, interest in the MPC complex as a drug target for cancer, neurological disorders, and metabolic diseases has been extremely
Recent work suggests that insulin-sensitizing thiazolidinedione compounds bind the MPC complex (Colca et al., 2013) and modulate mitochondrial pyruvate oxidation (Divakaruni et al., 2013; Colca et al., 2013). Thus, a better understanding of MPC function has the potential to advance our knowledge about intermediary metabolism and impact drug discovery for current public health problems.

Herein, we report the generation and characterization of two MPC2-deficient mouse strains. Although complete MPC2 deficiency resulted in embryonic lethality, mice expressing a truncated, partially functional MPC2 protein were viable. Diminished pyruvate metabolism led to elevated blood lactate concentrations, particularly when mice were challenged with stimuli that increased glucose metabolism. The mutant mice also exhibited elevated blood glucose during glucose tolerance tests (GTTs) and pyruvate tolerance tests (PTTs) that was likely due to reduced GSIS. Additionally, glucose intolerance could be corrected by administration of KATP channel-inhibiting sulfonylurea drugs. These studies demonstrate the importance of mitochondrial pyruvate transport to glucose homeostasis in a mammalian system and support the continued exploration of mitochondrial function in complex metabolic diseases.

RESULTS

Tissue Expression of MPC Proteins

We first isolated various tissues from C57Bl/6 mice to determine relative Mpc mRNA and MPC protein expression. We detected large variations in MPC protein (Figures 1B and 1C) and mRNA expression (Figure 1D), with the highest levels observed in tissues with greater mitochondrial abundance. Isolation and equal loading of mitochondrial lysate from these tissues somewhat normalized MPC1 and MPC2 protein expression (Figures S1A and S1B). However, MPC protein was still most abundant in highly oxidative tissues even when corrected for mitochondrial protein content.

Generation of Mpc2-Deficient Mice

We targeted the Mpc2 allele for deletion by using zinc-finger nuclease (ZFN) technology. The first line of mice harbored a deletion of 2 nt just downstream of the start codon (Figure S2A), resulting in a frameshift mutation and likely complete loss of protein product (Mpc2/C0/C0 mice). The mutation results in embryonic lethality in homozygous embryos. However, the number of heterozygous mice present at weaning was as predicted by Mendelian ratios (p = 0.717; Table S1), and these mice were overtly indistinguishable from their wild-type (WT) littermates. Lethality in homozygous embryos likely occurs between embryonic day 11 (E11) and E13, a period when robust mitochondrial biogenesis takes place (Câmara et al., 2011; Larsson et al., 2011; Nilsen et al., 2011; Larsson et al., 2013).
Timed mating studies yielded expected Mendelian ratios at embryonic days 10 and 11 (p = 0.496; Table S2), but no viable homozygote embryos at E13 (Table S3). Western blots of the tissues of heterozygous (Mpc2+/-) mice demonstrated only a modest reduction in MPC2 and MPC1 protein compared with WT controls (Figure S2B). Pyruvate-driven oxygen consumption by isolated mitochondria from Mpc2+/- mice was not diminished compared with WT mitochondria (Figure S2C).

A second attempted knockout strategy resulted in the deletion of 20 nt including the original start codon for MPC2 translation. Despite the deletion, Mpc2 mRNA was expressed at normal levels (data not shown) and MPC2 protein translation proceeded from a second downstream start codon (Figure S2A). This resulted in a truncated protein that lacked the first 16 amino acids of the N terminus (MPC2Δ16). Pups homozygous for the truncated allele were born at the expected Mendelian ratios (p = 0.995; Table S1) and were outwardly normal. The truncated protein, detected by western blotting with an antibody against the C terminus of MPC2, was less abundant than the WT protein in all tissues examined, except for brown adipose tissue (BAT; Figure 2A). The MPC2Δ16 mutation led to a marked reduction in the abundance of MPC1 protein, likely reflecting the maintenance of a stoichiometric ratio of MPC1 and MPC2 protein in the complex. Immunoprecipitation studies also indicated that the Mpc2Δ16 mutant protein commounprecipitated less MPC1 protein, suggesting that the interaction between the two proteins is significantly weakened by the loss of these 16 amino acids of MPC2 (Figure 2B). The abundance of the pyruvate dehydrogenase (PDH) complex and pyruvate carboxylase (PC) proteins was not altered in various tissues of the Mpc2Δ16 mice, suggesting a lack of significant downstream alterations in pyruvate metabolic enzymes (Figure 2A). Western blotting of subfractionated kidney and heart demonstrated that the truncated Mpc2Δ16 protein properly localized to mitochondria (Figure 2C) and specifically to the IMM in liver (Figure 2D).

Isolated Mpc2Δ16 Mitochondria Have Reduced Oxygen Consumption

The Mpc2Δ16 mice were healthy and appeared outwardly normal, suggesting that the MPC complex was at least partially functional. Plasma triglyceride was decreased and plasma ketone concentrations were increased in Mpc2Δ16 mice compared with WT control mice (Table 1), suggesting an increased reliance on fat oxidation. Possibly consistent with a reduced capacity for mitochondrial pyruvate import, blood lactate concentrations were also significantly elevated (Table 1).

Therefore, we evaluated the effects of the MPC2 truncation on oxygen consumption in isolated mitochondria. We used heart and kidney tissue due to their high mitochondrial density and high MPC expression. Mpc2Δ16 mitochondria from heart or kidney exhibited 25%–30% lower rates of pyruvate/malate-stimulated respiration in 2,4-dinitrophenol (DNP)-uncoupled conditions compared with WT controls (Figure 3A). Addition of methyl-pyruvate, a membrane-permeable pyruvate analog, rescued oxygen consumption to WT levels in Mpc2Δ16 mitochondria. Additionally, maximal oxygen consumption rates in Mpc2Δ16 mitochondria with glutamate/malate or glucose support were less than those in WT control mitochondria (Figure 3B).
succinate/rotenone were unaltered compared with WT controls. Together, these data suggest a specific effect of the Mpc2<sup>D16</sup> mutation on mitochondrial pyruvate oxidation.

**Mitochondrial Membrane Potential Is Normal in Fibroblasts from Mpc2<sup>D16</sup> Mice**

Given the observed defect in pyruvate oxidation in Mpc2<sup>D16</sup> tissues, we investigated whether these mitochondria displayed any sign of overt dysfunction by assessing the mitochondrial membrane potential (ΔΨ<sub>M</sub>) in isolated adult fibroblasts of WT and Mpc2<sup>D16</sup> mice. Tetramethylrhodamine, ethyl ester, perchlorate (TMRE) staining revealed no difference in ΔΨ<sub>M</sub>, a comprehensive measure of overall mitochondrial function, in Mpc2<sup>D16</sup> fibroblasts compared with WT controls (Figure 3B). The lack of effect on mitochondrial membrane potential was likely due to the small decrease in pyruvate-stimulated respiration and the maintained ability to metabolize other substrates.

**Exercise Endurance Is Normal in MPC2<sup>Δ16</sup> Mice**

During exercise, significant quantities of pyruvate, which is interconverted to lactate, are generated via anaerobic glycolysis in skeletal muscle. Lactate, transported by the blood to the liver, is converted back to glucose that can again be used anaerobically as an energy source in muscle. This process, known as the Cori cycle (Katz and Tayek, 1999; Pilkis et al., 1988), depends on pyruvate import in liver mitochondria. Therefore, we challenged Mpc2<sup>Δ16</sup> homozygote and Mpc2/<sup>±</sup> mice using an acute endurance exercise bout of gradually increasing intensity until exhaustion (Figure 3C). The average blood glucose concentration of the Mpc2<sup>Δ16</sup> mice was not different from that of WT controls in the 60 min after exercise (Figure 3C). However, the average blood lactate of the Mpc2<sup>Δ16</sup> was approximately 2-fold higher compared with WT controls immediately after exercise and remained significantly elevated for 30 min postexercise (Figure 3C). Postexercise blood lactate and glucose concentrations were not different between Mpc2/<sup>±</sup> and WT mice (Figure 3C). Despite the robust differences in blood lactate concentrations postexercise, we observed no significant differences in acute endurance exercise capacity among the genotypes (Figure 3C).

**Elevated Blood Glucose and Lactate in Mpc2<sup>Δ16</sup> Mice following Pyruvate Injection**

To test the effects of the Mpc2 mutations on pyruvate disposal in vivo, we subjected Mpc2<sup>Δ16</sup> homozygotes and

### Table 1. Baseline Plasma Parameters

<table>
<thead>
<tr>
<th>Glucose (mg/dl)</th>
<th>125.1 ± 5.7</th>
<th>124.3 ± 5.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate (mmol/l)</td>
<td>1.6 ± 0.09</td>
<td>2.3 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.51 ± 0.09</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>61.8 ± 2.2</td>
<td>54.8 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td>0.58 ± 0.05</td>
<td>0.67 ± 0.05</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>65.3 ± 2.6</td>
<td>58.3 ± 3.7</td>
</tr>
<tr>
<td>Total ketones (mmol/l)</td>
<td>0.15 ± 0.01</td>
<td>0.25 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>p < 0.05 versus WT. Data are expressed as mean ± SEM.

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Figure 3. Mpc2<sup>Δ16</sup> Mice Have a Diminished Capacity for Pyruvate Utilization, but Exercise Capacity Is Unimpaired

(A) DNP-stimulated rates of oxygen consumption by mitochondria isolated from heart or kidney of WT or Mpc2<sup>Δ16</sup> mice in the presence of the indicated substrates (n = 8).

(B) TMRE staining of tail fibroblasts isolated from WT or Mpc2<sup>Δ16</sup> mice (n = 8, separate experiments in duplicate).

(C) Schematic of the exercise protocol. Shown are blood glucose and blood lactate curves for WT, Mpc2/<sup>±</sup>, and Mpc2<sup>Δ16</sup> mice following graduated-intensity exercise on a motor-driven treadmill (n = 6), and elapsed time until exhaustion for WT, Mpc2/<sup>±</sup>, and Mpc2<sup>Δ16</sup> mice during graduated-intensity exercise (n = 6). Data are presented as mean ± SEM. *p < 0.05, Mpc2<sup>Δ16</sup> versus WT.
Mpc2<sup>+/−</sup> mice to an intraperitoneal (i.p.) PTT. Following pyruvate injection, blood lactate increased 5-fold in all groups. However, the decline in blood lactate concentration was significantly slower in Mpc2<sup>Δ16</sup> mice compared with WT controls (Figure 4A). Blood glucose and lactate levels were not different between Mpc2<sup>+/−</sup> and WT mice. Compared with WT mice, Mpc2<sup>Δ16</sup> mice also exhibited a significantly greater blood glucose excursion (Figure 4A). These data are consistent with reduced pyruvate utilization resulting in elevated blood lactate concentrations.

To specifically evaluate the effect of the Mpc2<sup>Δ16</sup> mutation on pyruvate gluconeogenesis and other intracellular pathways of pyruvate metabolism, we repeated the pyruvate challenge in Mpc2<sup>Δ16</sup> mice with uniformly labeled [U-<sup>13</sup>C]pyruvate. The mice were sacrificed, plasma and tissue were collected 60 min postinjection, and fractional <sup>13</sup>C enrichments were evaluated by mass spectrometry. There were no differences in hepatic oxaloacetate, malate, or fumarate concentrations or enrichments (Figure S3), indicating that liver mitochondrial pyruvate anaplerosis is intact in Mpc2<sup>Δ16</sup> mice. Although the fractional enrichment of plasma glucose was unaffected by the Mpc2<sup>Δ16</sup> mutation (Figure 4B), the plasma glucose concentration and therefore the total amount of <sup>13</sup>C-labeled plasma glucose were significantly elevated in Mpc2<sup>Δ16</sup> mice versus WT mice (Figure 4B). These data suggest that the capacity for pyruvate-derived gluconeogenesis is intact in Mpc2<sup>Δ16</sup> mice. Incorporation of <sup>13</sup>C-label into plasma lactate was not different between Mpc2<sup>Δ16</sup> and WT control groups (Figure 4C). However, due to the increased lactate concentration in the blood, <sup>13</sup>C-labeled plasma lactate was significantly elevated in Mpc2<sup>Δ16</sup> mice compared with WT controls (Figure 4C), consistent with decreased mitochondrial pyruvate metabolism in extrahepatic tissues. Additionally, expression of liver gluconeogenic enzymes was unaltered in Mpc2<sup>Δ16</sup> mice, except...
that Pck2, which encodes the mitochondrial form of phosphoenolpyruvate carboxykinase, was significantly decreased (Figure 4D), suggesting that compensatory activation of these genes does not explain the intact gluconeogenic flux. Altogether, these data suggest that hepatic gluconeogenesis is not impaired in Mpc2<sup>D16</sup> mice.

**Mpc2<sup>D16</sup> Mice Are Sensitive to Insulin, but Have Reduced Plasma Insulin and Elevated Blood Glucose**

One possible explanation for the elevated blood glucose concentration in the PTT studies is that Mpc2<sup>D16</sup> mice are insulin resistant. We conducted insulin tolerance tests (ITTs) to examine insulin effectiveness. Although blood lactate during the ITT was significantly elevated in Mpc2<sup>D16</sup> mice compared with WT mice, blood glucose concentrations at all time points and the area under the curve (AUC) were not different, suggesting that insulin-stimulated glucose disposal is normal in Mpc2<sup>D16</sup> mice (Figure 5A). Insulin-stimulated phosphorylation of Akt in liver was significantly enhanced (Figure 5B) and was unaltered in gastrocnemius muscle (Figure 5C) of Mpc2<sup>D16</sup> mice compared with WT control mice. These data suggest that the Mpc2<sup>D16</sup> mice have normal sensitivity to the effects of insulin.

In contrast, Mpc2<sup>D16</sup> mice exhibited elevated blood lactate and trended toward a higher glucose AUC (p = 0.078) during an i.p. GTT (Figure 5D). Plasma insulin concentration was significantly lower in Mpc2<sup>D16</sup> mice compared with WT controls at baseline and 30 min after glucose injection (Figure 5E), suggesting that glucose intolerance in Mpc2<sup>D16</sup> mice was due to insufficient insulin secretion. Similarly, blood insulin concentration in Mpc2<sup>D16</sup> mice compared with WT controls was also significantly lower 15 min after a bolus pyruvate injection (Figure 4E).

**Impaired GSIS in Mpc2<sup>D16</sup> Mice Is Corrected by Glibenclamide**

The data obtained in GTT studies suggested a defect in GSIS by pancreatic islet β cells of Mpc2<sup>D16</sup> mice. Both MPC1 and MPC2 are expressed in β cells, as demonstrated by colocalization with insulin in immunohistochemical staining of pancreatic sections (Figure 6A). Western blotting analyses confirmed that MPC1 and MPC2 proteins are expressed in isolated pancreatic islets and demonstrated that the expression of the MPC2<sup>D16</sup> protein was markedly reduced compared with WT MPC2 (Figure 6B). Islet insulin content in Mpc2<sup>D16</sup> mice was not different from that in WT controls (Figure 6C).
Figure 6. Elevated Blood Glucose and Defective GSIS in Mpc2^{Δ16} Mice Are Corrected by Glibenclamide Treatment

(A) Images from immunofluorescent staining of pancreatic islets from C57Bl/6 mice with antibodies against MPC1 (green), MPC2 (green), or insulin (red) are shown.

(B) Western blot depicting the abundance of MPC2 and MPC1 protein using protein lysates from isolated islets from Mpc2^{Δ16} mice and WT controls.

(C) Insulin content of islets isolated from WT and Mpc2^{Δ16} mice (n = 6).

(D) Insulin secretion by isolated pancreatic islets from WT and Mpc2^{Δ16} mice in response to the indicated stimuli (n = 6).

(E) Blood glucose and blood lactate curves during an i.p. GTT for WT and Mpc2^{Δ16} mice treated with glibenclamide or saline vehicle (n = 11–13).

(F) Plasma insulin from blood collected during the experiment described in (E) (n = 11–13). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01 Mpc2^{Δ16} versus WT.
We examined GSIS in isolated pancreatic islets from WT and Mpc2
mutant mice. Compared with WT islets, Mpc2 mutant islets exhibited reduced insulin secretion in response to 23 mM glucose (Figure 6D). β cell glucose sensing requires mitochondrial pyruvate metabolism, resulting in closure of the KATP channels (Huopo et al., 2002). We therefore examined the effects of glibenclamide, a sulfonylurea drug that inhibits KATP channel activity, on GSIS in Mpc2 mutant islets. As we hypothesized, the GSIS defect was rescued by the addition of glibenclamide to high-glucose media (Figure 6D). Additionally, Mpc2 mutant islets exhibited normal insulin secretion in response to KCl (Figure 6D). Supplementing high-glucose medium with glutamine, a metabolic substrate that does not require the MPC complex to enter the mitochondrion to be oxidized, also corrected the defect in GSIS (Figure 6D). These findings collectively suggest that the Mpc2 insulin secretion defect is specific to stimulation by mitochondrial pyruvate metabolism rather than to a general deficiency in insulin production or defects in the insulin secretory machinery.

To confirm the relevance of these findings in vivo, we administrated glibenclamide to Mpc2 mice with a bolus of glucose in GTT studies. Vehicle-treated Mpc2 mice exhibited elevated blood glucose concentrations compared with WT mice at 30 and 60 min postinjection, but blood glucose concentrations in Mpc2 mice treated with glibenclamide were not different from those in vehicle-treated WT mice (Figure 6E). Administration of glibenclamide did not affect plasma lactate concentrations in mice of either genotype. Glibenclamide administration to Mpc2 mice resulted in increased plasma insulin concentrations, which were reduced in Mpc2 mice versus WT mice, at 15 and 30 min after injection (Figure 6F). These data further support the model in which whole-body Mpc2 deficiency leads to a defect in GSIS that is improved by KATP channel inhibition.

DISCUSSION

Carrier-mediated transport of pyruvate across the IMM is a critical step in intermediary metabolism, which is required for both anaerobic and capillary mitochondrial pyruvate metabolism. We found that complete loss of MPC2 led to embryonic lethality, but expression of a truncated MPC2 protein that exhibited hypomorphic activity was sufficient to support life. The most striking phenotypes of the Mpc2 mutant mice were elevated plasma lactate concentrations, particularly in the context of increased glucose concentrations, and a defect in β cell GSIS leading to relative glucose intolerance. These findings are consistent with a function for MPC2 in regulating mitochondrial pyruvate utilization and reveal important roles for MPC2 in regulating whole-body glucose homeostasis.

As mitochondrial pyruvate import is required in a number of critical metabolic pathways, it was not unexpected that complete ablation of Mpc2 resulted in embryonic lethality. Human mutations in MPC1 are associated with neonatal and juvenile mortality (Briot et al., 2003). We were unable to recover viable Mpc2 mutant embryos after E11, which is when mitochondrial function begins to play a larger role in embryonic metabolism (Camara et al., 2011; Larsson et al., 1998; Metodiev et al., 2009; Park et al., 2007). Mpc2 mutant mice exhibited only a modest reduction in MPC2 and MPC1 protein abundance, and were phenotypically indistinguishable from WT littermates in every parameter we examined. Our inability to detect a phenotype from the Mpc2 mutant mice, even under conditions of metabolic challenge, likely highlights an excess transport capacity of the MPC complex.

We also serendipitously created a truncated mutant MPC2 protein as a consequence of the unpredictable results obtained by ZFN-mediated deletion. Although the Mpc2 mutant has a nucleotide deletion larger than that of the Mpc2 mouse (20 bp versus 2 bp, respectively), it utilizes a downstream methionine as an alternative start site for translation of a truncated MPC2 protein. Because the original start codon is ablated by the 20 bp deletion but is intact in the 2 bp deletion, this allows an alternative ribosomal entry sequence to be enforced. This start codon may be less efficient or the resulting protein less stable, given that MPC2 protein is less abundant than full-length MPC2. Most tissues in the Mpc2 mutant mice also had a significant reduction in abundance of the MPC1 protein. Previous work has shown that MPC1 and MPC2 must be coexpressed for proper assembly of the multimeric MPC complex, and that depletion of either protein leads to reduced abundance of the other (Bricker et al., 2012; Colca et al., 2013). It is likely that the reduced abundance of MPC1 in the Mpc2 mutant is due to the lower quantity of Mpc2 compared with WT protein. A diminished or weakened protein-protein interaction between the two proteins is also a potential factor in the reduced complex stability, since less MPC1 was communoprecipitated with Mpc2 compared with WT protein. However, the expression of MPC1 was only modestly affected by MPC2 truncation in BAT and islets, suggesting a tissue-specific regulation of MPC complex stability or the possible existence of homodimeric MPC protein complexes.

The truncated MPC2 protein is properly localized to the IMM and presumably, based on the phenotypes of the mutant mice, forms a transporter complex that is functionally hypomorphic. A number of mitochondrial carrier proteins of the IMM do not utilize canonical N-terminal mitochondrial localization sequences (Ferramosca and Zara, 2013; Pfanner and Geissler, 2001; Harbauer et al., 2014). Instead, these carriers utilize internal sequences that have yet to be defined. Indeed, analysis by mitochondrial localization prediction programs, which predict mitochondrial targeting based on these canonical N-terminal localization sequences, did not produce a high-scoring prediction of mitochondrial localization for full-length or Δ16 MPC2 protein. However, the focus of this paper was not to define the targeting sequence, because that has been elusive for most mitochondrial proteins that lack the N-terminal canonical sequence.

Given that conversion of pyruvate into glucose by liver and kidney requires pyruvate to enter mitochondria for conversion to oxaloacetate, we expected that the Mpc2 mutant mice might be hypoglycemic in conditions requiring gluconeogenesis. Previous work using isolated liver preparations and a chemical inhibitor of MPC activity showed an acute inhibition of hepatic glucose output (Martin-Requero et al., 1986; Rognstad, 1983; Thomas and Halestrap, 1981). However, whether carrier-mediated pyruvate transport by MPC plays a significant role in controlling gluconeogenesis has been debated (Groen et al., 1983, 1986; Halestrap and Armstrong, 1984), and the hypomorphic function...
of the MPC transport complex was not a limiting factor in the glu-
coneogenic capacity of Mpc2<sup>Δ16</sup> mice. Plasma <sup>13</sup>C-labeled
glucose levels were actually increased in [L<sup>13</sup>Cl<sup>3</sup>]-PTT studies and Mpc2<sup>Δ16</sup> mice were not hypoglycemic following exhaustive
exercise, suggesting that the Cori cycle was intact. The observa-
tion that gluconeogenesis was not impaired is not entirely
unprecedented, given that previous studies have shown that
an up to 85% reduction in phosphoenolpyruvate carboxykinase
expression does not impact gluconeogenic flux (Burgess et al.,
2007). Further work with other, more complete, liver-
specific loss-of-function models will be needed to define the
role that MPC complex proteins play in regulating hepatic
 gluconeogenesis.

Another interesting phenotype of the Mpc2<sup>Δ16</sup> mice was the
hyperglycemia detected during PTT and GTT studies. Given
that the insulin-stimulated decrease in blood glucose concentra-
tion was normal in ITT experiments and pyruvate metabolism is
important for β cell glucose sensing (Lu et al., 2002; Prentki
et al., 2013; Sugden and Holness, 2011), we suspected that insu-
lin secretion might be defective. Indeed, plasma insulin concen-
tration was diminished in Mpc2<sup>Δ16</sup> mice compared with WT mice
during the GTT and PTT studies, and isolated islets from Mpc2<sup>Δ16</sup>
mice exhibited an attenuated insulin secretion response following
the administration of 23 mM glucose. Glucose sensing and GSIS require mitochondrial pyruvate
oxidation and carboxylation. β cells express very little lactate
dehydrogenase (LDH), and most of the pyruvate generated
from glycolysis enters the mitochondrion for further metabolism
(Malmgren et al., 2009; Schult et al., 1997). Pyruvate carboxyla-
tion and oxidation inhibit ATP-sensitive potassium channels
(K<sub>ATP</sub>), depolarizing the β cell membrane, opening voltage-
dependent calcium channels, and triggering the release of
insulin into the circulation (Huopio et al., 2002). We found that
the defects in insulin secretion by isolated islets and hypoinsuli-
nemia/hyperglycemia in Mpc2<sup>Δ16</sup> mice were corrected by the
sulfonylurea glibencamide, which functions by binding and
inhibiting the regulatory subunit of K<sub>ATP</sub> channels. It is likely
that glibencamide is able to circumvent the reduced capacity
of Mpc2<sup>Δ16</sup> mutants for mitochondrial pyruvate metabolism.

The observation that glucose, which relies upon its own IMM
carrier (Indiveri et al., 1998), is able to stimulate insulin secretion
in Mpc2<sup>Δ16</sup> islets also supports the notion of a specific defect in
mitochondrial pyruvate metabolism. Consistent with these data,
while this paper was in review, Patterson et al. (2014) used chem-
ical inhibitors and small interfering RNA (siRNA) methods to sug-
gest that MPC inhibition suppressed pyruvate metabolism and
GSIS in isolated islets and β cell lines. Taken together, these
studies demonstrate that the hypoinsulinemia of Mpc2<sup>Δ16</sup>
mice is a specific defect in GSIS and not a broader indication of
β cell dysfunction.

The molecular identification of MPC proteins has stimulated
interest in targeting this complex for the treatment of many
chronic disease states. Pharmacologic approaches to modulate
the activity of the MPC complex could yield treatments for broad
categories of obesity-related metabolic diseases, neurodegen-
erative conditions, and neoplastic diseases associated with
impaired pyruvate metabolism (Constantin-Teodosiu, 2013;
Schell and Rutter, 2013; Stacpoole, 2012). For example, the
MPC complex has recently been identified as a binding site for
the thiazolidinedione class of insulin sensitizers (Colca et al.,
2013), which acutely modulate mitochondrial pyruvate oxidation
(Divaarun et al., 2013; Colca et al., 2013). The extent to which
regulation of mitochondrial pyruvate import by thiazolidine-
diones affects insulin sensitization remains unclear, but could
be addressed by MPC loss-of-function mouse models. Screens
to identify small molecules that enhance, inhibit, or selectively
modulate MPC activity may identify novel therapeutics with
applicability to myriad chronic diseases.
Previously described (Huss et al., 2004). Fibroblasts from WT and Mpc2

Isolation of primary adult murine fibroblasts from tail clip was performed as previously described (Huss et al., 2004). Fibroblasts from WT and Mpc2

Acute Exercise Protocol

WT, Mpc2+/−, and Mpc2−/− mice performed a single bout of treadmill exercise to exhaustion. The mice were fasted for 4 hr prior to the exercise session. Following a 3 min familiarization period on the treadmill, the exercise protocol consisted of graduated intensity running at 5 m/min for 5 min, 10 m/min for 5 min, 15 m/min for 10 min, 25 m/min for 15 min, and 30 m/min until the mouse fatigued. The mice were encouraged to run with a mild electric stimulus (20 V) positioned at the end of the treadmill and were determined to be exhausted by their refusal to remain on the treadmill belt for 5 s. Postexercise blood glucose and lactate levels were determined at 0, 5, 10, 20, 30, 60, and 120 min after exhaustion.

Measurement of Baseline Plasma Parameters

Mice were fasted for 4 hr and tail blood glucose and lactate concentrations were determined using a One-Touch Ultra glucometer (LifeScan), respectively. Blood samples for plasma analyses were collected by mandibular bleeding. Plasma insulin was measured using an ultrasensitive ELISA (Crystal Chem). Plasma triglyceride and cholesterol concentrations were measured by Infinity assay kits (Thermo Fisher Scientific). Nonesterified fatty acids (NEFAs) and total ketone concentrations were measured using enzymatic assays (Wako Diagnostics).

GTT, PTT, and ITT

For the GTT and PTT, mice were fasted overnight for 16 hr and housed on aspen chip bedding. The mice were injected i.p. with 1 g/kg body weight glucose or with 2 g/kg body weight Na-pyruvate dissolved in sterile physiological saline. For the ITT, mice were fasted for 6 hr and injected i.p. with a 0.75 U/kg body weight insulin. Tail blood glucose and lactate concentrations were determined at 0, 30, 60, and 120 min after challenge as described above. The total AUC was calculated using the trapezoidal rule. Blood samples for plasma insulin quantification were collected by mandibular bleeding as described above. A subset of mice were injected i.p. with 2 g/kg body weight Na-pyruvate and sacrificed at 15 min to determine plasma insulin concentrations during pyruvate challenge. To examine insulin-stimulated protein phosphorylation, a subset of mice were injected i.p. with 10 mU/g body weight human insulin 15 min before sacrifice to collect liver and muscle tissue. The glibenclamide rescue studies were conducted in the context of a standard GTT, with sample time points adjusted to 0, 15, 30, and 60 min after challenge. Glibenclamide was administered at 0.1 mg/kg body weight in a glucose solution that contained 0.1% DMSO. Blood was collected at each time point by means of mandibular bleeding as described above.

13C-Labeled Pyruvate Studies

Pyruvate labeling studies were conducted as previously described (Mendez-Lucas et al., 2013). Briefly, mice were fasted for 16 hr and injected i.p. with 2 g pyruvate per kg body weight with a 10% enrichment of uniformly labeled [U-13C3]Na-pyruvate (Cambridge Isotope Laboratories). The mice were sacri-

fieded at 60 min postinjection and plasma was analyzed by gas chromatography-mass spectrometry as previously described (Sunny and Bequet, 2010).

Pancreas Immunohistochemistry

Protein expression of MPCs in pancreatic islets was determined in paraffin-embedded pancreatic sections from WT mice cut on glass slides. The slides were rehydrated, permeabilized with trypsin, blocked in 1% BSA, and probed with guinea pig anti-insulin (Abcam) and either rabbit anti-MPC1 or rabbit anti-MPC2 (gifts from Michael Wolfgang). All antibodies were diluted 1:200 in buffer containing 1% BSA and incubations were conducted overnight. The sections were then washed three times for 5 min in PBS and probed with 1:1,000 donkey anti-rabbit green or 1:1,000 donkey anti-guinea pig red Alexa Fluor secondary antibodies (Invitrogen) for 2 hr. The slides were again washed three times for 5 min in PBS, mounted with Vectashield + DAPI, and imaged on an epifluorescence microscope (Nikon Instruments).

Islet Isolation and Insulin Release Experiments

Islet isolation and insulin release studies were performed as previously described (Nemedi et al., 2004). Briefly, after the mice were euthanized, the pancreases were removed and injected with HBSS (Sigma) containing collagenase (0.5 mg/ml, pH 7.4). They were digested for 4 min at 37°C, and the islets were isolated by hand. Following overnight incubation in 3 mM glucose, CMRL-1066 medium, islets (10 per well in 12-well plates) were incubated for 60 min at 37°C in CMRL-1066 + 1 mM glucose, 23 mM glucose, 23 mM glucose + 1 μM glibenclamide, 1 mM + 30 mM KCl, or 23 mM glucose + 10 mM glutamine, as indicated. After the incubation period, the medium was removed and assayed for insulin release. Experiments were repeated in triplicate. To estimate the islet insulin content, groups of ten islets were disrupted by ethanol-HCl extraction and sonicated on ice. Insulin secretion and content were measured using a rat insulin radiomunoassay (Millipore) according to the manufacturer’s instructions.

Statistical Analyses

Statistical comparisons were made using ANOVA, chi-square test, or t test. All data are presented as means ± SEM, with a statistically significant difference defined as p < 0.05.

Supplemental Information

Supplemental Information includes three figures and four tables and can be found with this article online at [http://dx.doi.org/10.1016/j.cellrep.2014.05.017].

Author Contributions

P.A.V. and K.S.M. performed experiments, analyzed data, generated figures, and wrote the manuscript. G.G.S. and M.S.R. performed experiments, analyzed data, generated figures, and wrote portions of the manuscript. K.T.C. and G.W.M. performed experiments and edited the manuscript. W.G.M. performed experiments, analyzed data, generated figures, and wrote portions of the manuscript. S.L.C. performed experiments and edited the manuscript. J.R.C. and R.F.K. analyzed data and edited the manuscript. S.C.B. and B.N.F. analyzed data and wrote portions of the manuscript.

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