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MOLECULAR METABOLISM

An ancestral role for the mitochondrial pyruvate carrier in glucose-stimulated insulin secretion

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ABSTRACT

Objective: Transport of pyruvate into the mitochondrial matrix by the Mitochondrial Pyruvate Carrier (MPC) is an important and rate-limiting step in its metabolism. In pancreatic β-cells, mitochondrial pyruvate metabolism is thought to be important for glucose sensing and glucose-stimulated insulin secretion.

Methods: To evaluate the role that the MPC plays in maintaining systemic glucose homeostasis, we used genetically-engineered *Drosophila* and mice with loss of MPC activity in insulin-producing cells.

Results: In both species, MPC deficiency results in elevated blood sugar concentrations and glucose intolerance accompanied by impaired glucose-stimulated insulin secretion. In mouse islets, β-cell MPC-deficiency resulted in decreased respiration with glucose, ATP-sensitive potassium (K_{ATP}) channel hyperactivity, and impaired insulin release. Moreover, treatment of pancreas-specific MPC knockout mice with glibenclamide, a sulfonylurea K_{ATP} channel inhibitor, improved defects in islet insulin secretion and abnormalities in glucose homeostasis *in vivo*. Finally, using a recently-developed biosensor for MPC activity, we show that the MPC is rapidly stimulated by glucose treatment in INS-1 insulinoma cells suggesting that glucose sensing is coupled to mitochondrial pyruvate carrier activity.

Conclusions: Altogether, these studies suggest that the MPC plays an important and ancestral role in insulin-secreting cells in mediating glucose sensing, regulating insulin secretion, and controlling systemic glycemia.

Keywords Stimulus-coupled secretion; Insulin; β-Cell; Diabetes; Pyruvate; Mitochondria; *Drosophila*

1. INTRODUCTION

Mitochondrial pyruvate metabolism requires transport across the impermeable inner mitochondrial membrane (IMM). Recent work has shown that mitochondrial pyruvate import is mediated by two proteins, the Mitochondrial Pyruvate Carrier 1 and 2 (MPC1 and MPC2), which form a hetero-oligomeric complex in the IMM [1,2]. Deletion of either MPC1 or MPC2 leads to destabilization and degradation of the complex, effectively resulting in a MPC double knockout and significantly reduced mitochondrial pyruvate uptake [1–3]. Mice with partial loss of MPC function [4], *Drosophila* with constitutive MPC1 deletion [2], and mammalian cells with MPC knockdown via RNAi [5] are viable and outwardly normal. However, global and constitutive loss of MPC1 [6] or MPC2 [4] in mice leads to lethality at early embryonic stages.

Mitochondrial pyruvate metabolism is thought to play an important role in the ability of pancreatic β-cells to respond appropriately to increased glucose concentrations by secreting insulin [7–9]. Oxidation of pyruvate by the pyruvate dehydrogenase complex results in increased ATP production, which inhibits ATP-sensitive potassium (K_{ATP}) channels, depolarizes the β-cell, and promotes calcium influx to drive insulin release. In addition, several studies have shown that the production of anaplerotic products by pyruvate carboxylation in the mitochondrial matrix promotes insulin granule exocytosis by Ca^{2+}-independent mechanisms [7,10–12]. Chemical inhibition or RNAi-mediated knockdown of the MPC in INS-1 cells and isolated rat islets reduced oxygen consumption rates, ATP content, and glucose-stimulated insulin secretion (GSIS) [13]. While relatively little is known about the role of the MPC in human islet insulin secretion, this same study showed that MPC inhibition in isolated human islets produced effects similar to those

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Abbreviations: IMM, inner mitochondrial membrane; MPC1 and MPC2, Mitochondrial Pyruvate Carrier 1 and 2; GSIS, glucose-stimulated insulin secretion; IPCs, Insulin-producing Cells; OCR, oxygen consumption rates; DILP2, *Drosophila* insulin-like peptide 2; Pdx1, pancreatic and duodenal homeobox 1; GTT, glucose tolerance test; ITT, insulin tolerance test; RESPYR, REporter Sensitive to PYRuvate

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seen in rat islets [13]. In addition, mutant mice that carry a partial loss-of-function mutation in *Mpc2* are also hypoinsulinemic and glucose intolerant [4]. These studies support the model that MPC function is required in the β-cell for GSIS and proper glucose homeostasis. Validation of this model *in vivo*, however, requires the characterization of the phenotypes associated with β-cell-specific MPC knockout. To complicate this issue further, recent work has shown that with marked depletion or even complete deletion of MPC proteins, adaptive mechanisms exist that can circumvent the block in pyruvate import [3,14–16]. For instance, conversion of pyruvate into amino acids, especially alanine, was shown to support hepatic gluconeogenesis in liver MPC knockout mice by bypassing the need for MPC activity [3,14]. Thus, we sought to evaluate the role of the MPC in GSIS in β-cells in a comprehensive manner by using both *Drosophila* and mouse models. Herein, we demonstrate that the MPC plays a central role in GSIS and systemic glucose homeostasis. MPC deficiency in *Drosophila* or the β-cells of mice led to elevated blood glucose concentrations, glucose intolerance, and reduced GSIS. Pancreas-specific MPC deficiency resulted in impaired islet glucose metabolism and K_{ATP} channel hyperactivity. Moreover, treatment with the K_{ATP} channel inhibitor glibenclamide rescued the defects in GSIS both *in vitro* and *in vivo*. Finally, glucose increased MPC activity in cultured INS-1 cells in a concentration-dependent manner, suggesting that glucose sensing is coupled to mitochondrial pyruvate transport and utilization to support efficient GSIS. Taken together with previous work in human islets [13], these data demonstrate an important and ancestral role for the MPC in glucose sensing and the regulation of insulin secretion that is conserved from *Drosophila* to humans.

2. MATERIALS AND METHODS

2.1. Animal studies

Drosophila dMPC1 mutants (*dMPC1*^{1}/*dMPC1*^{2} transheterozygotes) and genetically-matched precise-excision control strains have been described previously [2]. Unless otherwise noted, experiments were conducted with 6–12 week old mice of both sexes. All vertebrate animal experiments were approved by the Animal Studies Committee of Washington University School of Medicine.

2.2. *Drosophila* dietary treatments

Drosophila stocks were maintained on a standard cornmeal-molasses diet at 25 °C. To alter dietary sugar concentrations, media was prepared using either low (2% sucrose) or high (18%) sugar concentrations along with 10% yeast in water. For the lifespan studies, males were transferred to the indicated diet within one day of eclosion, then transferred to new vials every 2–5 days. To assay the effect of dietary sugar concentrations on metabolite levels, animals were raised on standard media and transferred to the indicated diet within 2–4 days of eclosion. Metabolites were measured within 8–12 days of transfer.

2.3. Fly metabolite measurements

Whole-animal glucose, trehalose, triacylglycerol, glycogen, and protein measurements were performed using standard colorimetric assays [17]. All other metabolites were measured by metabolomic profiling using gas chromatography/mass spectrometry as described [17]. To measure circulating glucose in *Drosophila*, ~30 adult females were punctured in the thorax with a tungsten needle and centrifuged at 13,000 g through DNA columns (Zymo- Spin IIIC #C1006-50) twice in a centrifuge at 4 °C. Hemolymph was then diluted 1/200 in PBS and heat-treated at 70 °C for 10 min to inactivate enzymes, after which the glucose concentration was measured as described [2].

2.4. Western blot analysis

To assay AKT phosphorylation, flies were aged for 8–12 days on high sugar media and then homogenized. Protein was resolved by SDS-PAGE and immunoblotted using standard methods with antibodies to phospho-AKT (Cell Signaling #4691, 1:1000 dilution), pan-AKT (Cell Signaling #4054, 1:1000 dilution), and beta-Tubulin (Chemicon MAB380, 1:100,000 dilution), followed by chemiluminescent detection.

2.5. Immunostaining of *Drosophila* insulin-producing cells (IPCs)

Brains were dissected in cold PBS and fixed for 20 min at room temperature in 4% paraformaldehyde in PBS. Following several washes in PAT (PBS + 0.5% Triton X-100) brains were blocked with 5% normal donkey serum overnight. Primary antibodies directed against DILP2 [18] and dMPC1 [5] were used at 1:500 concentration for 24 h at 4 °C. Rat Alexa 488-conjugated secondary antibodies (Jackson 212-545-168) and rabbit Cy3-conjugated secondary antibodies (Jackson 711-165-152) were used at 1:800 dilution at 4 °C. Brains were mounted in SlowFade Gold™ (Invitrogen) and imaged using an Olympus FV1000 confocal microscope. Z stack images were taken through the depth of fluorescence of the IPCs using identical settings.

2.6. DILP2 secretion assay

For measuring circulating DILP2 in *dMPC1* mutants, the HA-FLAG tagged DILP2 (DILP2-HF) transgene in a *dilp2*^{1} mutant background was recombined into a *dMPC1*^{1}/*dMPC1*^{2} transheterozygote mutant background [19]. To disrupt MPC function in the IPCs, *UAS-Dcr2; dilp2*^{1} *DILP2HF dilp2-GAL4/UAS-MPC1 RNAi; dilp2*^{1} *DILP2HF dilp2-GAL4* flies were used as described along with *dilp2*^{1} *DILP2HF* controls [19]. Briefly, adult male progeny were fasted for 16 h and then fed 2 M glucose for 30 min. The posterior end of the abdomen was dissected to collect circulating hemolymph in PBS and the remaining carcasses were homogenized in PBS containing 1% Triton X-100 to assay for total remaining DILP2-HF. HA-FLAG peptide standards from 0, 20, 40 80, 160, 320 and 640 pg/ml were generated for a linear standard curve. 96-well ELISA plate (Thermo Scientific MaxiSorp Immulon 4 HBX, Cat# 3855) coated with mouse anti-FLAG antibody (Sigma F1804, M2 monoclonal) and 1-Step Ultra TMB ELISA Substrate (Thermo Scientific 34029) were utilized for the ELISA assays. Circulating DILP2-HF (pg/fly) versus total remaining peptide was calculated to determine the percent secretion relative to controls ($n \geq 4$ biological replicates per condition).

2.7. Generation of β-cell specific Mpc2 deficient mice

Mice harboring a conditional floxed *Mpc2* allele have been previously described [3]. *Mpc2* floxed mice were crossed with mice expressing *Cre* recombinase driven by the rat insulin promoter [20] (RipCreMpc2$^{-/-}$ mice), the pancreatic and duodenal homeobox 1 (*Pdx1*) promoter [21] (PdxCreMpc2$^{-/-}$ mice), or a *Pdx1* promoter-driven tamoxifen-regulated Cre [22] (PdxCre^{ER}Mpc2$^{-/-}$ mice). To conditionally-delete *Mpc2*, PdxCre^{ER}Mpc2$^{-/-}$ mice were injected i.p. with tamoxifen (50 μg/g body weight) for 5 consecutive days commencing at 5–6 weeks of age. Littermate *Mpc2* floxed mice not expressing *Cre* (fl/fl) were used for controls in all experiments.

2.8. Glucose and insulin tolerance tests

For the *Drosophila* glucose tolerance test (GTT), adult male flies were aged 5–9 days on standard media, fasted overnight on fasting media (1% agar in water), fed 10% glucose/1% agar for 2 h, and subsequently transferred to fasting media for two or 4 h. Glucose

