Mitochondrial Metabolism of Pyruvate Is Essential for Regulating Glucose-stimulated Insulin Secretion*

Received for publication, September 27, 2013, and in revised form, March 21, 2014. Published, JBC Papers in Press, March 27, 2014, DOI 10.1074/jbc.M113.521666

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Background: Pyruvate metabolism plays an essential role in pancreatic β-cells.

Results: Pharmacological and siRNA-mediated inhibition of mitochondrial pyruvate carrier-1 and -2 inhibit β-cell metabolism and insulin secretion.

Conclusion: Pyruvate entry into β-cell mitochondria is critical for regulating insulin release.

Significance: Mitochondrial metabolism of pyruvate plays a key role in generating signals in response to nutrients that control insulin release.

It is well known that mitochondrial metabolism of pyruvate is critical for insulin secretion; however, we know little about how pyruvate is transported into mitochondria in β-cells. Part of the reason for this lack of knowledge is that the carrier gene was only discovered in 2012. In the current study, we assess the role of the recently identified carrier in the regulation of insulin secretion. Our studies show that β-cells express both mitochondrial pyruvate carriers (Mpc1 and Mpc2). Using both pharmacological inhibitors and siRNA-mediated knockdown of the MPCs we show that this carrier plays a key role in regulating insulin secretion in clonal 832/13 β-cells as well as rat and human islets. We also show that the MPC is an essential regulator of both the ATP-regulated potassium (K<sub>ATP</sub>) channel-dependent and -independent pathways of insulin secretion. Inhibition of the MPC blocks the glucose-stimulated increase in two key signaling molecules involved in regulating insulin secretion, the ATP/ADP ratio and NADPH/NADP<sup>+</sup> ratio. The MPC also plays a role in the in vivo glucose homeostasis as inhibition of MPC by the pharmacological inhibitor α-cyano-β-(1-phenylindol-3-yl)-acrylate (UK5099) resulted in impaired glucose tolerance. These studies clearly show that the newly identified mitochondrial pyruvate carrier sits at an important branching point in nutrient metabolism and that it is an essential regulator of insulin secretion.

Glucose is an important physiological stimulus for insulin secretion from pancreatic β-cells. A key pathway in glucose-regulated insulin secretion is mitochondrial glucose metabolism, which can lead to a rise in the ATP/ADP ratio. The increase in the cytosolic ATP/ADP ratio leads to an inhibition of ATP-regulated potassium channels (K<sub>ATP</sub> channels) that depolarizes the cell membrane and activates voltage-gated calcium channels, allowing calcium influx (1–3). Calcium leads to exocytosis of insulin secretory granules (4). However, it is apparent that the effects of glucose on insulin secretion extend beyond just closing K<sub>ATP</sub> channels. For example, when cytosolic calcium levels are clamped at an elevated level using diazoxide to open K<sub>ATP</sub> channels and KCl to depolarize the plasma membrane, glucose can still exert a dose-dependent effect on insulin secretion (5, 6). However, the details of this K<sub>ATP</sub> channel-independent pathway, often called the amplifying pathway, have yet to be fully characterized.

Mitochondrial pyruvate metabolism plays a significant role in the amplifying pathway. Most metabolic fuels (e.g. glucose and succinate) that are capable of stimulating insulin secretion in β-cells via a rise in the ATP/ADP ratio can also contribute to anaplerosis (7–9). Anaplerosis appears to be an essential component of the amplifying pathway and plays a key role in glucose-stimulated insulin secretion (GSIS) (7–9). Anaplerosis also forms the basis for a number of hypotheses of alternate signaling molecules involved in insulin secretion with posited anaplerosis-derived coupling factors including GTP (10–12), glutamate (13–15), malonyl-CoA/long chain acyl-CoA (16, 17), and NADPH (18–21).

Glucose metabolism in the glycolytic pathway leads to the generation of NADH and pyruvate. Pyruvate sits at a critical branching point in glucose metabolism in β-cells as it can be metabolized in the cytosol by lactate dehydrogenase or enter mitochondria to be metabolized by pyruvate dehydrogenase or pyruvate carboxylase (PC). Pyruvate metabolism by lactate dehydrogenase is not thought to play a role in GSIS (22, 23), whereas mitochondrial metabolism of pyruvate by pyruvate dehydrogenase and PC is critical for regulating insulin release.

*This work was supported in part by Canadian Institute of Health Research Grant MOP 86638 and Natural Sciences and Engineering Research Council of Canada Grant RGPIN 371723-2009. Human islet isolation was funded by the Alberta Diabetes Foundation and the University of Alberta (to P. E. M.).
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The abbreviations used are: K<sub>ATP</sub>, channel; ATP-regulated potassium channel; α-CCH; α-cyano-4-hydroxyxynamic acid; UK5099, α-cyano-β-(1-phenylindol-3-yl)-acrylate; DNP, 2,4-dinitrophenol; GSIS, glucose-stimulated insulin secretion; MPC, mitochondrial pyruvate carrier; PC, pyruvate carboxylase; KBB, Krebs-Ringer bicarbonate buffer; Ctrl, control; NT, no treatment; MCT, membrane monocarboxylate transporter.

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For pyruvate to be metabolized in the mitochondria, it must first be transported across the inner mitochondrial membrane. The protein(s) responsible for pyruvate transport into mitochondria was initially identified in yeast in 2003 (24) followed by their recent identification in mammalian cells in 2012 (25, 26). These mitochondrial carriers have not yet been fully characterized in β-cells or even in any other cell types. Most of the studies on the mitochondrial pyruvate carrier (MPC) have been done with the specific inhibitor of pyruvate transport, α-cyano-4-hydroxycinnamic acid (α-CHC), which was developed in the 1970s (27, 28). This inhibitor has facilitated experiments to determine the contribution of mitochondrial pyruvate transport to GSIS, yielding inconsistent results. Studies in rat islets (29), HIT cells (30), and MIN6 cells (31) showed that inhibition of pyruvate transport blocked GSIS, whereas a study with 832/13 cells showed no effect (32), and two other studies showed a mouse (33) and rat islets (34) that pyruvate transport inhibition leads to a stimulation of insulin secretion. A more potent mitochondrial pyruvate carrier inhibitor, α-cyano-β-(1-phenylindol-3-yl)-acrylate (UK5099), which is now commercially available, has not yet been tested in β-cells (28).

If mitochondrial pyruvate transport is critical for insulin secretion then its inhibition should show global effects on mitochondrial glucose metabolism and GSIS as both pyruvate dehydrogenase and PC act on pyruvate in the mitochondrial matrix. With the more potent MPC inhibitor UK5099 and the identification of two MPC genes (Mpc1 and Mpc2), we undertook studies to fully elucidate the contribution of mitochondrial pyruvate metabolism to nutrient-regulated insulin secretion.

MATERIALS AND METHODS

Reagents—All reagents were obtained from Sigma unless otherwise specified.

Cell Lines—The 832/13 cell line (35) derived from INS-1 rat insulinoma cells (36) was used for these experiments. The cells were a gift from J. B. Newgard and were cultured as described previously (18, 35, 37).

Cell Insulin Secretion Assay—Insulin secretion in response to glucose was measured as described previously (18, 37). The secretion medium consisted of Krebs-Ringer bicarbonate buffer (KRB) (4.38 mm KCl, 1.20 mm MgSO4, 1.50 mm KH2PO4, 129 mm NaCl, 10 mm HEPES, 5 mm NaHCO3, 3.11 mm CaCl2, pH 7.4, 0.1% (w/v) BSA). Briefly, cells were plated in 12-well plates at 0.5 × 10^6 cells/well (unless otherwise stated) and grown to 100% confluence. Cells were pretreated for 2 h in KRB with 2 mm glucose and then treated for 2 h in KRB containing glucose plus/minus drug at concentrations as indicated under “Results.” For the leucine plus glutamine studies, 832/13 cells were pretreated for 2 h in KRB with 2 mm glucose followed by the addition of either 1 mm leucine and 1 mm glutamine or 10 mm leucine and 10 mm glutamine for 1 h. For the KCl plus diazoxide studies, 832/13 cells were pretreated for 2 h in KRB with 2 mm glucose followed by the addition of either 2, 6, or 8 mm glucose plus/minus 30 mm KCl and 100 μM diazoxide for 1 h. The buffer was collected and assayed for insulin by the Coat-A-Count radioimmunoassay kit (Siemens, Los Angeles, CA).

Oxygen Consumption—Oxygen consumption was measured using an XF24 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) (38). Briefly, 832/13 cells were plated at 4.5 × 10^4 cells/well and grown to 100% confluence. On the day of the assay, cells were pretreated with 2 mm glucose for 2 h in the same manner as for the insulin secretion assay. Oxygen consumption was then measured during the following treatments for the given time frame: 2 mm glucose, 0–30 min; 8 mm glucose plus/minus UK5099 (at concentrations indicated in the figure legends), 30–65 min; 5 μM oligomycin, 65–85 min; 50 μM 2,4-dinitrophenol (DNP) with 20 mm pyruvate, 85–115 min; 5 μM rotenone and 5 μM myxothiazol, 115–145 min. Injection of these drugs was used to analyze cellular oxygen consumption. Oxygen consumption associated with ATP turnover was determined as the difference between the oxygen consumption in response to oligomycin and 8 mm glucose. Proton leak across the inner mitochondrial membrane was assessed as the difference between oxygen consumption in response to oligomycin and rotenone plus myxothiazol. DNP and pyruvate were used to determine the maximum respiratory capacity of the cells. Rotenone and myxothiazol were also used to assess oxygen consumption not associated with the electron transport chain.

Analysis of Mpc1 and Mpc2 mRNA Expression—To assess the expression of Mpc1 and Mpc2 in the 832/13 cell line, mRNA was isolated from 832/13 cells using the Aurum Total RNA kit (Bio-Rad), and cDNA was synthesized with the iScript cDNA Synthesis kit (Bio-Rad). The primers used to amplify the rat Mpc1 and Mpc2 mRNA (NCBI Reference Sequence accession numbers NM_133561 and NM_001077643, respectively) were as follows: Mpc1: forward, 5′-AGATGAGTAACGCGCCTCATGTCCT-3′; reverse, 5′-AGCCGAGATTGTTTGGGGAT-3′; Mpc2: forward, 5′-TGCTGCCAAGAAATGAGGCGCG-3′; reverse, 5′-GCACAGTGGATTGAGCTGTGTGTA-3′. Platinum Blue PCR Supermix (Invitrogen) was used to amplify the target sequences. Resulting products were separated on a 1.2% agarose gel. Quantitative PCR analysis of Mpc1 and Mpc2 cDNA was performed using SsoFast EvaGreen Supermix reagents (Bio-Rad) on a StepOnePlus PCR system (Applied Biosystems, Burlington, Ontario, Canada). Expression of rat cyclophilin B was used as a control gene (NCBI Reference Sequence accession number NM_022536.1).

siRNA Transfection and Generation of Adenoviruses Expressing siRNAs against Mpc1 and Mpc2—Expression of the MPC proteins was suppressed by the introduction of siRNA duplexes (Integrated DNA Technologies, Coralville, IA). Two 25-bp duplexes were tested for each gene. For Mpc1, the targeted sites were at positions 195 (siMPC1-1, GUU UAC GAA UUC UCC AGA GAU UAU CA) and 123 (siMPC1-2, CUA UCU CAU GAG UAC GCA CUU CU) relative to the start codon; for Mpc2, the first nucleotides of the target site were 63 (siMPC2-1, GUU GUU GCU GCC AAA GAA AAU GA) and 36 (siMPC2-2, CUA CCA CCG ACU CAU GGA UAA AG). A previously used scrambled siRNA sequence (GAG ACC CUA UCC GUG AUU A) with no known target was used as a control (siCtrl) (37). siRNA duplexes were introduced into 832/13 cells at 40–50% confluence using INTERFERin nucleofection (Polyplus Transfection, New York, NY). siRNA adenoviruses against Mpc1 and Mpc2 or a control siRNA adenovirus were generated by Welgen (Worcester, MA) using siRNA sequences for siControl,
MPC1-2, and MPC2-2 described above. Experiments were performed 72 h after transfection.

Viability—Cell viability was measured using CellTiter Blue (Promega, Madison, WI) according to the manufacturer’s protocol. The assay is based on cellular reduction of resazurin to resorufin. Appearance of resorufin was monitored by fluorescence emission at 585 nm using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA) with excitation at 555 nm. For UK5099-treated cells, cells were allowed to recover for 1 h before measuring cell viability. Data were expressed as fold relative to no treatment or siCtrl.

Islet Isolation and Secretion—Human islets were provided by the Alberta Diabetes Institute Islet Core at the University of Alberta in Edmonton with the assistance of the Human Organ Procurement and Exchange Program and the Trillium Gift of Life Network in the procurement of donor pancreata for research (islets from four donors with an average age of 70) (39, 40). Rat (from male Sprague-Dawley rats of 300–400 g) and mouse islets were isolated and cultured as described previously (18, 37). The buffer used for insulin secretion assays consisted of 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 5 mM NaHEPES, 24 mM NaHCO₃ (Fisher), and 0.1% (w/v) BSA at pH 7.4. The islet insulin secretion assay was performed as described previously (18, 37). Islet insulin content was assessed after the secretion assay as described previously (41).

Measurement of Nucleotides Using HPLC—832/13 cells were plated in 6-well plates and grown to 100% confluence. Cells were incubated in KRB with 2 mM glucose for 2 h followed by treatment in KRB containing either low (2 mM) or high (8 mM) glucose with or without 150 μM UK5099 for 1 h. Cells were harvested and snap frozen in a dry ice and ethanol bath. Nucleotides were extracted by adding 100 μl of 0.075 mM KOH to the frozen pellet and probe sonicated on ice. After a 1-min incubation, 100 μl of buffer A (60 mM KH₂PO₄, 4 mM tetrabutylammonium hydroxide sulfate, pH to 7.0 with KOH) was added. Samples were vortexed and filtered through a 50-kDa centrifugal filter (Millipore). Flow-through was collected and run on an Agilent 1100 HPLC with a Porshell 120 column (Agilent, Santa Clara, CA) were fasted for 16 h prior to glucose challenge. UK5099 (32 μmol/kg of body weight) or DMSO in PBS was injected into the intraperitoneal cavity 30 min before injecting glucose (1.5 mg of glucose/g of body weight). Blood glucose levels were measured at 0, 10, 20, 30, 60, and 120 min after glucose injection.

Statistical Analysis—All results are given as mean ± S.E. Statistical significance was assessed by Student’s t test or by one- or two-way analysis of variance followed by multiple comparisons with a Holm-Sidak correction.

RESULTS

Pharmacological Inhibition of the MPC Reduces GSIS in 832/13 Cells—To initially assess the role of the MPC in insulin secretion, the pharmacological MPC inhibitors α-CHC and the more potent UK5099 (28) were used. These inhibitors inhibit MPC by reversibly binding to an essential thiol group in the protein (43). Both drugs were found to dose-dependently inhibit insulin release in response to glucose (Fig. 1); however,
Figure A: Graph showing OCR (% basal) over time for different conditions.

Figure B: Bar graph showing area under the curve (relative units) for different conditions.

Figure C: Graph showing OCR (pmol O₂/min) over time for different conditions.
there were some unique differences. The commonly used MPC inhibitor α-CHC has been shown to inhibit insulin secretion in some studies (29–31), stimulate in others (33, 34), and do nothing in other studies (32). For most of these studies in β-cells, they have used a 1 mM α-CHC concentration, which is also known to inhibit PC (44). Because of these inconsistent results we performed a comprehensive analysis of the effects of α-CHC on insulin secretion in 832/13 cells. We show that α-CHC was without effect on basal insulin secretion; however, it consistently inhibited insulin secretion at 6 mM glucose (Fig. 1A). At a higher stimulatory concentration of glucose (8 mM glucose), α-CHC effects were consistent with only 100 and 150 μM showing any effect on insulin secretion. These results suggest that α-CHC shows inconsistent results that depend on the concentration of α-CHC and the concentration of glucose. A second generation, more potent commercially available MPC inhibitor, UK5099 (28), had no significant effect on basal insulin secretion and showed a more consistent inhibition of insulin secretion at both 6 and 8 mM glucose in 832/13 cells using a concentration range of 10–150 μM UK5099 (Fig. 1B). Because UK5099 was more effective at inhibiting insulin secretion it was chosen to use in further experiments.

The MPC Inhibitor UK5099 Reduces the Dynamic Glucose-stimulated Oxygen Consumption in 832/13 Cells—It has long been suggested that the MPC plays an essential role in mitochondrial metabolism and insulin secretion in β-cells, but this has not yet been confirmed in part due to inconsistent results with the MPC inhibitor α-CHC and because of the lack of identification of the carrier gene. To assess the role of the MPC in the glucose-stimulated increase in oxygen consumption, we measured dynamic oxygen consumption with the second generation MPC inhibitor UK5099 in 832/13 cells. UK5099 significantly inhibited the glucose-stimulated rise in oxygen consumption in a dose-dependent manner and at 150 μM reduced oxygen consumption below basal levels (Figs. 2, A and B). Oligomycin was used to assess ATP turnover at high glucose. ATP turnover was dose-dependently reduced by UK5099 from 196.8 ± 17.3 pmol of O₂/min (no treatment (NT) control) to 127.1 ± 22.4 (50 μM UK5099), 30.5 ± 17.9 (100 μM UK5099), and 50.8 ± 11.0 (150 μM UK5099) pmol of O₂/min. DNP (an uncoupler that accelerates fuel-driven oxygen consumption) and pyruvate (fuel) were used to assess maximal pyruvate-stimulated oxygen consumption. UK5099 dose-dependently reduced mitochondrial pyruvate-driven oxygen consumption (Fig. 2, A and B), suggesting that the drug reduces mitochondrial pyruvate transport. In addition, the effects of UK5099 on reducing mitochondrial pyruvate-driven oxygen consumption was slightly enhanced by changing the sequence of the addition of UK5099 (Fig. 2C). Interestingly, 100 and 150 μM UK5099 also inhibited oxygen consumption that was independent of the electron transport chain as measured by blocking the electron transport chain with rotenone (inhibits the transfer of electrons from iron-sulfur centers in complex I to ubiquinone) and myxothiazol (inhibitor of the mitochondrial cytochrome b₆(f complex (complex III)) (Fig. 2, A and B). This suggests that some of the non-electron transport chain oxygen consumption requires mitochondrial pyruvate transport. Proton leak was not affected by addition of UK5099 (data not shown).

UK5099 Reduces ATP Levels and Increases AMP and ADP Levels in 832/13 Cells—We next sought to determine whether or not the essential energy cofactors, adenine nucleotides, were affected by UK5099. For DMSO-treated controls (no treatment), the ATP/ADP ratio increased significantly in response to high glucose (8 mM) as we have seen previously (Fig. 3) (18, 20, 37). ATP levels at both low glucose (2 mM) and high glucose (8 mM) were significantly lower in UK5099-treated cells (150 μM) as compared with no treatment control cells (Fig. 3A). AMP and ADP levels were significantly elevated at both low and high glucose in UK5099-treated cells as compared with no treatment controls (Fig. 3A), suggesting that the loss of ATP was due to conversion to AMP and ADP. The net result of these changes was a significant reduction of the ATP/ADP ratio by UK5099 as compared with no treatment controls (Fig. 3B).

UK5099 Does Not Alter Cell Viability—One possible explanation for the decrease in oxygen consumption, ATP levels, and insulin secretion is a decrease in cell viability. To assess this, we used the cell viability reagent CellTiter Blue. Relative to no treatment controls (1.0 ± 0.1), 100 μM UK5099 had a cell viability of 0.94 ± 0.1, and 150 μM UK5099 had a cell viability of 0.97 ± 0.1, suggesting that, even at the higher UK5099 concentration of 150 μM, cell viability was only marginally affected.

UK5099 Reduces Both the K₅₀, Channel-dependent and -independent Pathways Regulating Insulin Secretion—Although ATP turnover and the ATP/ADP ratio were significantly reduced by UK5099, which suggests that the MPC is critical for the K₅₀, channel-dependent pathway of insulin secretion, we also wanted to know whether or not the K₅₀, channel-independent pathway was altered by MPC inhibition. The K₅₀, channel-independent pathway involves factors that regulate insulin secretion that work independently of K₅₀, channels. First, we confirmed the role of the K₅₀, channel-dependent pathway in 832/13 cells by incubating cells with diazoxide and KCl at low glucose (2 mM). Using this experimental setup, 150 μM UK5099 was found to inhibit insulin secretion (Fig. 4A). Increasing the concentration of glucose to 6 and 8 mM in the presence of diazoxide and KCl was used to assess the role of the
K<sub>ATP</sub> channel-independent pathway. Under these conditions, UK5099 also strongly inhibited insulin secretion, which suggests a role for the MPC in the K<sub>ATP</sub> channel-independent pathway (Fig. 4A). The studies in Fig. 4A suggest that both the K<sub>ATP</sub> channel-dependent and -independent pathways are altered by inhibiting the MPC. Interestingly, we also found that glutamine- and leucine-stimulated insulin secretion was significantly reduced in 832/13 cells by 150 μM UK5099 as compared with no treatment control cells (Fig. 4B), suggesting a role for mitochondrial pyruvate transport via the MPC in amino acid-stimulated insulin secretion.

Two of the metabolic coupling factors suggested to play a key role in the K<sub>ATP</sub> channel-independent pathway are the NADPH/NADP<sup>+</sup> ratio and the GTP/GDP ratio. 150 μM UK5099 significantly reduced both the NADPH/NADP<sup>+</sup> ratio (Fig. 5A) and the GTP/GDP ratio at both low and high glucose (Fig. 5B).

**UK5099 Does Not Affect Cortical F-actin Remodeling in 832/13 Cells or Dispersed Mouse β-Cells**—Cortical actin remodeling has been shown to play a key role in nutrient-stimulated insulin vesicle movement in β-cells (45). We next sought to understand whether or not this process involves mitochondrial metabolism of pyruvate. 10 mM glucose stimulated a significant increase in actin remodeling in both DMSO control-
and UK5099-treated dispersed mouse β-cells (150 μM) (Fig. 6A), suggesting that the ability of glucose to promote actin remodeling is independent of mitochondrial pyruvate metabolism and that the relevant nutrient-derived signal is not ATP or NADPH. Similar results were found in the 832/13 cells (data not shown).

**MPC1 and MPC2 Are Expressed in 832/13 Cells, and Both Play a Key Role in Regulating Insulin Release**—It has only recently been shown that there are two mammalian MPCs (25, 26); however, the role of these carriers in insulin secretion has not yet been investigated. We have found that both Mpc1 and Mpc2 are expressed in 832/13 cells. Relative to an internal control gene, cyclophilin B, the expression of Mpc1 was 1.2 ± 0.1, and that of Mpc2 was 1.6 ± 0.2. To investigate their roles in insulin secretion, we designed two siRNAs against both MPC1 (MPC1-1 and MPC1-2) and MPC2 (MPC2-1 and MPC2-2). MPC1 expression was significantly reduced by both siRNAs designed against MPC1 (MPC1-1 and MPC1-2) (Fig. 7A). We also found that the siRNAs against MPC1 had no effect on MPC2 expression (Fig. 7A). Likewise, the siRNAs against MPC2 significantly reduced the expression of MPC2 without an effect on MPC1 gene expression (Fig. 7A). All of the siRNAs against MPC1 and MPC2 significantly reduced insulin secretion in response to glucose (Fig. 7B).

**The siRNA MPC1-1 Reduces the Dynamic Glucose-stimulated Oxygen Consumption in 832/13 Cells**—Because MPC1 had the most dramatic effects on GSIS, we next sought to assess the role of MPC1 in the glucose-stimulated increase in oxygen consumption in 832/13 cells. The MPC1 siRNA significantly inhibited the glucose-stimulated rise in oxygen consumption in a manner similar to the pharmacological inhibitor UK5099 (Fig. 8). ATP turnover was also significantly reduced by the MPC1 siRNA from 135.6 ± 12.2 (control) and 173.3 ± 14.6 (siControl) to 111.2 ± 12.2 pmol of O2/min (siRNA MPC1-1) (p < 0.05). DNP- and pyruvate-stimulated oxygen consumption was significantly reduced by siMPC1-1 (Fig. 8), confirming that the siRNA against MPC1 reduced mitochondrial pyruvate transport. The siRNA against MPC1, however, did not affect oxygen consumption that was independent of the electron transport chain as measured by blocking the electron transport chain with rotenone and myxothiazol (Fig. 8). Proton leak was also not affected by the siRNA against MPC1 (data not shown). Finally, knocking down MPC1 or MPC2 had no effect on cell viability as assessed with CellTiter Blue (data not shown).

**UK5099, AdsiMPC1, and AdsiMPC2 Inhibited Insulin Secretion from Isolated Rat and Human Islets**—To confirm that the effects of MPC inhibition were not unique to the 832/13 cell line, we assessed insulin secretion in rat islets in response to UK5099. UK5099 dose-dependently inhibited insulin secretion

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**FIGURE 5.** The MPC inhibitor UK5099 reduces the glucose-stimulated increase in the NADPH/NADP⁺ and GTP/GDP ratios from 832/13 cells. A, NADPH/NADP⁺ levels in response to glucose. B, GTP/GDP levels in response to glucose (n = 6). ***, p < 0.001 for NT cells versus UK5099-treated cells at 2 mM glucose; ###, p < 0.001 for NT cells versus UK5099-treated cells at 8 mM glucose. Error bars represent S.E.

**FIGURE 6.** The MPC inhibitor does not block glucose-stimulated cortical actin remodeling in dispersed mouse β-cells. A, representative images are shown at the top with intensity line scans for F-actin staining intensity below. B, the average F-actin peak intensities are shown. Mouse β-cell identity was confirmed by insulin staining (n = 286–320 actin peaks from 143–160 β-cells from three mice). *, p < 0.05 for 1 mM glucose control cells versus 10 mM glucose control cells; #, p < 0.05 for 1 mM glucose UK5099-treated cells versus 10 mM glucose UK5099-treated cells. Error bars represent S.E. AU, arbitrary units.
in isolated rat islets (Fig. 9A). UK5099 also inhibited rat islet insulin secretion in response to 30 mM KCl plus 100 |M diazoxide in the presence or absence of glucose (Fig. 9B), suggesting that MPC plays a role in both the $K_{ATP}$ channel-dependent and -independent pathways. Glucose-stimulated insulin secretion was also inhibited in rat islets by an siRNA adenovirus targeting MPC1 (AdsiMPC1) and an siRNA adenovirus targeting MPC2 (AdsiMPC2) as compared with treating rat islets with either no adenovirus (NT) or a control siRNA adenovirus (AdsiControl) (Fig. 9C). AdsiMPC1 inhibited MPC1 gene expression by 62 ± 3%, and AdsiMPC2 reduced MPC2 gene expression by 58 ± 4% as compared with both NT and AdsiControl-treated islets. Infecting rat islets with both AdsiMPC1 and AdsiMPC2 at the same time led to a greater inhibition of glucose-stimulated insulin secretion as compared with rat islets treated with a control siRNA adenovirus (Fig. 9C). UK5099 also dose-dependently inhibited insulin secretion in isolated human islets (Fig. 9D). Insulin content was not affected by treating islets with either UK5099, AdsiMPC1, or AdsiMPC2 (data not shown).

**UK5099 Increased the Glucose Excursion during an Intraperitoneal Glucose Tolerance Test in C57BL/6 Mice**—We next sought to assess whether or not the MPC plays a role in vivo glucose tolerance. UK5099 (32 mg/kg) caused a significantly greater glucose excursion at 30, 60, and 120 min as compared with DMSO control mice (Fig. 10A). The area under the curve was also significantly increased by UK5099 as compared with DMSO control mice (Fig. 10B). These studies suggest that the MPC plays a significant role in regulating glucose tolerance in vivo.

**DISCUSSION**

Mitochondrial dysfunction plays an important role in the development of type 2 diabetes (46, 47). One critical aspect of mitochondrial metabolism is entry of pyruvate into mitochondria. Given the critical role of mitochondrial metabolism of...
Pyruvate in insulin secretion it is surprising that we know very little about how pyruvate is transported into mitochondria in $H_9252$-cells. Most of the studies on $H_9252$-cell mitochondrial pyruvate transport have involved high concentrations of a less potent inhibitor ($H_9251$-CHC) (29–34), and to date, there have been no studies on the carrier gene itself. Part of the reason for this lack of knowledge is that the carrier gene was only recently identified in mammalian cells (25, 26). Using newly available tools we have undertaken studies to gain more insight into the role of mitochondrial pyruvate transport via the MPC in regulating insulin release.

Because most of the studies on $H_9252$-cell mitochondrial pyruvate transport have given inconsistent results in terms of its effects on insulin secretion, we decided to reinvestigate this key area using a more potent commercially available MPC inhibitor, UK5099. UK5099 has been shown to be an effective inhibitor of pyruvate transport in non-$H_9252$-cells and has been shown to inhibit mitochondrial pyruvate metabolism (28). Most of the MPC inhibitors have also been shown to inhibit the plasma membrane monocarboxylate transporters (MCTs); however, the $K_i$ values for MCTs are 2–3 orders of magnitude higher than the $K_i$ values for the MPC (27, 48). Although UK5099 has been shown to inhibit MCT, we do not see this as a problem because primary $H_9252$-cells do not express significant amounts of MCT, and although the clonal 832/13 cell line does express MCT, the use of glucose as our stimulus bypasses this transporter. Studies with these inhibitors have also suggested that the MPC may transport ketones such as acetoacetate and $H_9252$-hydroxybutyrate (49). Interestingly, most of the studies on the MPC in $H_9252$-cells have used the inhibitor $H_9251$-CHC at a concentration of $H_11011$1m M. This concentration of $H_9251$-CHC can also inhibit PC (44). This makes interpreting the $H_9251$-CHC results difficult because PC plays an important role in $H_9252$-cell insulin secretion (9, 50). Our studies provide a clearer picture of the role of the MPC in the regulation of insulin secretion using both pharmacological and siRNA approaches.

Recently, two mammalian genes, $Mpc1$ and $Mpc2$, have been identified to play a role in mitochondrial pyruvate transport (25, 26). The role of these carriers in pyruvate transport was confirmed by measuring UK5099-inhibitable $[14C]$pyruvate uptake in isolated mitochondria. In addition, knockdown of these carriers in mouse embryonic fibroblasts inhibited pyruvate-dependent respiration. The authors also found that some children with lactic acidosis and hyperpyruvatemia had muta-

FIGURE 9. The MPC inhibitor UK5099, AdsiiMPC1, and AdsiiMPC2 inhibit glucose-stimulated insulin secretion from rat and human islets. A, glucose-stimulated insulin secretion in response to UK5099 from rat islets ($n = 10$). B, 30 mM KCl and 100 μM diazoxide-stimulated insulin secretion in the presence or absence of glucose from rat islets ($n = 6$). C, glucose-stimulated insulin secretion in response to treating rat islets with either no adenovirus (NT), control siRNA adenovirus (AdsiiControl), siRNA adenovirus targeting MPC1 (AdsiiMPC1), and/or an siRNA adenovirus targeting MPC2 (AdsiiMPC2) ($n = 6$). D, glucose-stimulated insulin secretion in response to UK5099 from human islets ($n = 6$). For A, C, and D, $\#$, p < 0.05; $\#$, p < 0.01; $\##$, p < 0.001 for NT islets versus UK5099-treated islets at 8 mM glucose and *, p < 0.05; **, p < 0.01; ***, p < 0.001 for NT islets versus UK5099-treated islets at 10 mM glucose. For B, $\#$, p < 0.01 for NT islets versus UK5099-treated islets at 2 mM glucose plus 30 mM KCl and 100 μM diazoxide; ***, p < 0.001 for NT islets versus UK5099-treated islets at 10 mM glucose plus 30 mM KCl and 100 μM diazoxide. Error bars represent S.E.
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A

![Graph showing the effect of the MPC inhibitor UK5099 on glucose excursion during an intraperitoneal glucose tolerance test in C57BLK mice.](image)

**FIGURE 10.** The MPC inhibitor UK5099 increases the glucose excursion seen during an intraperitoneal glucose tolerance test in C57BLK mice. A, C57BLK mice were preinjected with UK5099 (32 μmol/kg) 30 min prior to starting the intraperitoneal glucose tolerance test (1.5 mg of glucose/g of body weight) (n = 8). B, area under the curve of A. *p < 0.05; **p < 0.01; ***p < 0.001 for control mice versus UK5099-treated mice. Error bars represent S.E.

Consistent with this finding, we determined that oxygen consumption, the ATP/ADP ratio, and the NADPH/NADP⁺ ratio were all reduced by the inhibition of the MPC. These effects were seen without any detriment to cell survival. All of these effects can be explained by reduced pyruvate transport into mitochondria and flux through the tricarboxylic acid cycle. These studies provide the first detailed characterization of the role of mitochondrial pyruvate transport in β-cell insulin secretion.

Lower doses of UK5099 could inhibit insulin secretion while having little effect on respiration possibly because 1) UK5099 may have effects that are independent of MPC, or 2) small amounts of inhibition of MPC may affect pathways that do not depend on respiration, for example entry of pyruvate into the tricarboxylic acid cycle via PC. Selective inhibition of pyruvate entry into the tricarboxylic acid cycle via PC would reduce alternate signaling molecules thought to be involved in regulating insulin secretion without significantly altering respiration and ATP levels. Two possible alternate signaling molecules involve changes in the NADPH/NADP⁺ or GTP/GDP ratio, which have been shown to be reduced in these studies. Also, 150 μM UK5099 reduced oxygen consumption below that of basal respiration, whereas the ATP levels at 8 mM glucose were only reduced by about 20%. The lack of a more pronounced effect of ATP versus respiration can be explained by the time frame for the effects of UK5099. Unlike UK5099 effects on respiration, UK5099 does not directly reduce ATP content already present in the cells. We also noticed that we needed higher doses of UK5099 in islets to inhibit insulin secretion; this was likely due to penetration of the drug to the islet core.

Short-term inhibition of MPC with UK5099 had a more dramatic effect on respiration, ATP/ADP ratio, and insulin secretion as compared with long-term inhibition of the MPC using siRNAs against MPC. One reason for this difference could be that the knockdown of the carriers (between ~60 and 70%) may not have been sufficient to see similar effects to UK5099. Another explanation of the difference is that UK5099 inhibits both MPC1 and MPC2, whereas the siRNAs target only one carrier at a time. This last explanation is supported by the data showing that knockdown of both carriers with adenoviruses in rat islets led to a greater effect on the inhibition of insulin secretion.

Mitochondrial pyruvate metabolism by PC plays an essential role in β-cells (9, 50). For example, when 832/13 cells were stably transfected with a short hairpin RNA (shRNA) targeting PC it led to an inhibition of GSIS that was proportional to the inhibition of PC activity (51). Pharmacological inhibition of PC with phenylacetic acid was also shown to dose-dependently inhibit insulin secretion in INS-1 cells (52, 53) and rat islets (52, 54). Key PC-generated anaplerotic substrates such as citrate, isocitrate, and/or malate can exit the mitochondria and be converted back to pyruvate, concomitantly generating potential coupling factors such as NADPH in a process known as pyruvate cycling (for reviews, see Refs. 9 and 55). PC-driven anaplerosis has also been shown to correlate with glucose responsiveness in INS-1-derived cell lines (53). Although this study confirms the requirement of mitochondrial pyruvate in the regulation of insulin secretion further studies are required to elu-
citate the exact downstream pathway(s) involved in the ability of pyruvate to regulate insulin secretion.

It has been suggested that nutrient stimulation of β-cells leads to actin remodeling allowing insulin secretory vesicles to approach the plasma membrane (56). However, in our studies, nutrient stimulation of cortical actin remodeling was not affected even though we saw dramatic changes in the ATP/ADP, GTP/GDP, and NADPH/NADP⁺ ratios, suggesting that these factors are not required for this effect. This suggests that some other novel nutrient-derived factors other than these signaling molecules are playing a role in this process. We also found that amino acid (leucine and glutamine) stimulation of insulin secretion also requires a functional MPC. One possible explanation for this effect is that mitochondrial glutamate metabolism provides substrates for the generation of pyruvate, which could then feed into the pyruvate cycling pathway. The pyruvate cycling pathway plays a role in regulating insulin secretion and requires a functional MPC.

In the current study, we show for the first time that pyruvate transport through the MPC plays a critical role in regulating insulin secretion from pancreatic β-cells. The MPC regulates insulin secretion not only in response to glucose but also in response to amino acids, suggesting that it sits at an important point connecting cytosolic nutrient metabolism to mitochondrial metabolism. We show that mitochondrial pyruvate transport is important in regulating glucose oxidation and the ATP/ADP, GTP/GDP, and NADPH/NADP⁺ ratios in β-cells. In conclusion, our study provides the first detailed description of the link between glycolytic production of pyruvate and its eventual metabolism in the mitochondria in β-cells.

Acknowledgments—We thank the Human Organ Procurement and Exchange and Trillium Gift of Life Network programs for help in obtaining human pancreas for research and James Lyon for efforts in human islet isolation.

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