Cytosolic and Mitochondrial Malic Enzyme Isoforms Differentially Control Insulin Secretion*

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In islet β-cells and INS-1 cells both the high activity of malic enzyme and the correlation of insulin secretion rates with pyruvate carboxylase (PC) flux suggest that a pyruvate-malate cycle is functionally relevant to insulin secretion. Expression of the malic enzyme isoforms in INS-1 cells and rat islets was measured, and small interfering RNA was used to selectively reduce isoform mRNA expression in INS-1 cells to evaluate its impact on insulin secretion. The cytosolic NADP⁺-specific isoform (ME1) was the most abundant, with the mitochondrial isoforms NAD⁺-preferred (ME2) expressed at ~50%, and the NADP⁺-specific (ME3) at ~10% compared with ME1. Selective reduction (89 ± 2%) of cytosolic ME1 mRNA expression and enzyme activity significantly reduced glucose (15 mM: 41 ± 6%, p < 0.01) and amino acid (4 mM glutamine ± 10 mM leucine: 39 ± 6%, p < 0.01)-stimulated insulin secretion. Selective small interfering RNA reduction (51 ± 6%) of mitochondrial ME2 mRNA expression did not impact glucose-induced insulin secretion, but decreased amino acid-stimulated insulin secretion by 25 ± 4% (p < 0.01). Modeling of the metabolism of [U-¹³C]glucose by its isotopic distribution in glutamate indicates a second pool of pyruvate distinct from glycolytically derived pyruvate in INS-1 cells. ME1 knockdown decreased flux of both pools of pyruvate through PC. In contrast, ME2 knockdown affected only PC flux of the pyruvate derived from glutamate metabolism. These results suggest a physiological basis for two metabolically and functionally distinct pyruvate cycles. The cycling of pyruvate by ME1 generates cytosolic NADPH, whereas mitochondrial ME2 responds to elevated amino acids and serves to supply sufficient pyruvate for increased Krebs cycle flux when glucose is limiting.

It is now generally accepted that in addition to the key role of the ratio of ATP to ADP on closure of the ATP-dependent K⁺-channel, other metabolically derived second messengers are needed to promote insulin secretion from pancreatic islet β-cells. Many of these second messengers are believed to result from the export of the Krebs cycle intermediates from the mitochondria to the cytosol (1–5). By necessity the export of these second messengers from the mitochondria must be matched by an equivalent input of substrates (anaplerosis) to prevent the depletion of the Krebs cycle intermediates and detrimental effects on ATP generation. Approximately one-half of the glucose (or pyruvate) metabolized by the mitochondria of islet β-cells enters the Krebs cycle through pyruvate dehydrogenase (PDH), with the other half entering through the anaplerotic pathway of pyruvate carboxylase (PC) (5–10). In rat clonal INS-1 cells, rates of glucose-stimulated insulin secretion were shown to directly correlate with the rates of PC flux (10, 11).

In addition to replenishing Krebs cycle intermediates by PC, the input of glutamate into the Krebs cycle by glutamate dehydrogenase and/or transaminase represents another significant and physiologically important anaplerotic pathway (10, 12, 13). We recently presented evidence that whereas glutamate anaplerosis is increased in the presence of glutamine, it is the significant increase of PC flux from combined glutamine plus leucine metabolism that correlates best with enhanced insulin secretion (10). Thus, non-PDH metabolism of pyruvate is coupled either directly or indirectly with insulin secretion. Non-canonical (i.e. KₐTP independent)-stimulated insulin secretion via mitochondria-derived second messengers has received intensive effort with evidence in support of several candidate metabolites stimulating insulin exocytosis (1–5). Alternatively, a passive role for PC flux can be envisioned. The tight coupling of insulin secretion and plasma glucose concentration from the β-cell is dependant on the directly proportional increase in glycolytic flux (14, 15). However, because the β-cell lacks any appreciable capacity to either store the excess glucose as glycogen or eliminate it as lactate, the increased glycolytic flux would lead to feedback inhibition unless energy demand increases at the same pace as input (9, 16). Substrate cycling mediated through PC would provide an alternative mechanism to dissipate the increased glycolytic flux, thereby preventing feedback inhibition and allowing the β-cell to be metabolically responsive to changes in plasma glucose concentrations.

Malate represents one of the principle Krebs cycle intermediates exported from the mitochondria to the cytosol, where malic enzyme can then regenerate pyruvate from malate for cycling back to the mitochondria (5). β-Cells, in contrast to the liver, do not express phospho-enol-pyruvate carboxykinase and pyruvate cycling can only occur as the result of malic enzyme

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‡ The abbreviations used are: PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; ME1, malic enzyme 1; ME2, malic enzyme 2; ME3, malic enzyme 3; siRNA, small interfering RNA; KRB, Krebs-Ringer bicarbonate buffer; LC, liquid chromatography; MS, mass spectrometry.
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EXPERIMENTAL PROCEDURES

Cell Culture—Initial stocks of clonal INS-1 832/13 cells were provided by Dr. Christopher Newgard (Duke University) (26). Cells were determined to be mycoplasma-free (Cambrex, Mycoalert), and cultured as monolayers in RPMI 1640 complete medium with 11.1 mM D-glucose supplemented with 10% (v/v) fetal bovine serum, antibiotics (10,000 units/ml penicillin and 10 mg/ml streptomycin), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mecaptoethanol. All reagents were purchased from Sigma unless otherwise specified. Cells were cultured into 6-well plates at a density of 2.5 × 10^5 cells per well and incubated at 37 °C in the presence of humidified 5% CO_2, 95% air for 3 days to reach 50% confluence for transfection. The INS-1 cells were maintained for 2 additional days post-transfection to reach 80–100% confluence for mRNA knock-down analysis and insulin secretion assays.

Although INS-1 832/13 cells differ from pancreatic β-cells in some aspects of glucose metabolism (16), we, and others, have shown that many of the mechanisms coupling metabolism with insulin secretion are similar to islet β-cells. Significantly for this study, mitochondrial oxidation, anaplerosis, and as we show here, malic enzyme activity are similar in INS-1 cells and rat pancreatic islets (3, 4, 10, 11).

Construction of Small Interfering RNAs to Silence Malic Enzyme Expression—21-Nucleotide siRNA sequences targeting the specific malic enzyme genes for each of the three rat malic enzyme isoforms were constructed using the NCBI sequencing data base and synthesized using Qiagen’s custom siRNA facility. The NCBI accession numbers used to design the targets and primers are rat ME1, NM_012600; rat ME2, XM_001053085; and rat ME3, XM_341880. siRNAs for two different regions of each isoform were constructed for analysis of effective mRNA knockdown. BLAST analysis was used to verify that each sequence had no significant homology to any other known gene. The siRNA duplex consisted of the sense strand and its complementary antisense strand to provide a 2-nucleotide dithymidine overhang at each 3’ terminus.

siRNAs were synthesized by Qiagen with 19-base pair ribonucleic acid sequences with 5’ dideoxythymidine overhangs of the following sequences (“a” and “b” designate our siRNA constructs for each malic enzyme isoform): ME1a, AACCAGAGATCCAGGTCTCTT and ME1b, AAGCAAGAGGCTCTTTTATC; ME2a, AACGGCTTGTAGTAAAGGCC and ME2b, AAAGCAATGGCCGCTATCAAC. Duplexes were resuspended and annealed as per the manufacturer’s recommendation. Similar results were obtained with either siRNA for each isoform.

siRNA Knockdown of Malic Enzyme Isoforms—siRNA transfection constructs were made using the RNAiFect Transfection Reagent kit (Qiagen). Once the INS-1 cells reached 50% confluence in a 6-well plate, media was replaced with 1 ml of OptiMEM with Glutamax (Invitrogen) transfection media. Each well was then treated with the transfection complex consisting of 6 μl of 20 μM stock siRNA, 9 μl of Qiagen RNAiFect reagent, and 100 μl of RNAiFect buffer, and then incubated at 37 °C for ~6 h. The transfected 6-well plates were then incubated in RPMI as described above. Insulin secretion in response to glu-

activity (17, 18). The relatively high activity of both PC and cytosolic malic enzyme can support a robust rate of pyruvate cycling, and this cycle has been hypothesized to be a necessary component of glucose-stimulated insulin secretion (5, 11, 19). A pyruvate cycle mediated by PC and cytosolic malic enzyme occurs at the expense of one ATP, but leads to the exchange of reducing equivalents from mitochondrial NADH to cytosolic NADPH. The shift in redox state toward increased concentration of NADPH with increased pyruvate cycling may couple increased mitochondrial activity with downstream events in the cytosol leading to insulin secretion (16, 20, 21).

There also exists the potential for an alternative pyruvate cycle within the mitochondrial matrix via a mitochondrial localized isoform of malic enzyme. In mammalian cells, three isoforms of malic enzyme are known. In addition to the cytosolic NADP⁺-dependent isoform, malic enzyme 1 (ME1), two mitochondrial isoforms exist: malic enzyme 2 (ME2) with a preference for NAD⁺, and an NADP⁺-dependent malic enzyme 3 (ME3). Less well established is the potential for the mitochondrial isoforms, ME2 and ME3, to play a role in fuel-stimulated insulin secretion because mitochondrial malic enzyme activity will not provide an avenue for any exchange of reducing equivalents or other substrates from the mitochondria to the cytosol. Mandella and Sauer (22) characterized the enzymatic properties of the mitochondrial isoforms, and suggested that the high K_m value of ME2 for malate and NAD⁺ will minimize the conversion of malate to pyruvate under conditions of ample pyruvate supply, but will provide an alternative source of pyruvate from fumarate precursors such as glutamine when glycolytic flux is low (22). In brain and tumor cells, mitochondrial malic enzyme utilizes glutamine as a respiratory fuel by shunting glutamate-derived malate toward the formation of pyruvate and acetyl-CoA (23, 24). The combination of glutamine and leucine is equipotent to glucose for stimulating insulin secretion in vitro (12, 13), and mitochondrial malic enzyme may be utilized by the β-cell to couple amino acid metabolism with insulin secretion.

To provide further insight into the apparent requirement of PC flux and pyruvate cycling in fuel-stimulated insulin secretion, we have measured the relative expression levels of the cytosolic and mitochondrial isoforms of malic enzyme in 832/13 INS-1 cells and rat islets. Prior experimental evidence implicating pyruvate cycling as a key regulator of insulin secretion has been deduced from modeling studies, activation of one arm of the cycle, or from small molecule inhibitors of malate transport (11, 25). However, these methods do not clearly differentiate the roles of PC flux from pyruvate cycling, nor can they distinguish cytosolic from mitochondrial pyruvate cycling. In this article, we report the results of selective reduction of cytosolic ME1 and mitochondrial ME2 isoforms, and hence pyruvate cycling in INS-1 cells. These experiments allow direct determination of the impact on both insulin secretion and rates of anaplerotic flux from the 1) reduction in pyruvate cycling between cytosol and mitochondria catalyzed by ME1, distinct from 2) reduced pyruvate cycling confined to the mitochondria catalyzed by ME2.
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cose or amino acid stimulation was performed 48 h after transfection.

A non-silencing fluorescent siRNA (Qiagen, Alexa Fluor) had no effect on glucose-stimulated insulin secretion, or mRNA expression of ME1, ME2, or ME3. Thus, the observed effects of ME1 and ME2 knockdown using siRNA were not due to non-specific effects of siRNA transfection. Transfection efficiency was estimated from fluorescent-labeled siRNA and was apparent from 24 to 72 h post-transfection with optimal transfection efficiency at 48 h. Transfection efficiency was estimated to be over 90% (data not shown).

Quantitative Real-time PCR Analysis of Malic Enzyme Isoforms—Cells were extracted using the RNeasy Mini Kit (Qiagen) with RNase-free DNase (Qiagen) from 6-well plates that had been set aside for RNA and were not used for the substrate stimulation. RNA was reverse transcribed (Stratagene, Strato- cycler (Bio-Rad)). Forward and reverse sequence primers (KECK facility, Yale University) and fluorescent-labeled internal probes (Applied Biosystems, TaqMan) were designed specifically for each malic enzyme isoform and normalized to actin. Sequences for primers and probes used were: ME1, 5'-ATGGAAGAAGGATTTATCAAG-3' and 5'-GGCTTCTAGGCCTTTATTCACTT-3', internal probe, VIC-AGCCACCATGACCTGTTC-3', and 5'-GGCTTCTAGGTCTGTCAGGGTCC-3', internal probe, VIC-AGCCACCATGACCTGTTC-3'.

Quantitative real-time PCR (Bio-Rad) using Quantitect PCR reagent (Qiagen) was evaluated using the Opticon 2 Monitor analysis software version 2.02 provided by MI Research. Reaction efficiencies were determined to be greater than 98.7% for actin, ME1, ME2, and ME3.

Cytosolic NADH-dependent Malic Enzyme 1 Activity—Fresh INS-1 cell extracts from the malic enzyme isoform knock-down studies were analyzed for malic enzyme activity. NADH-dependent malic enzyme was measured spectropho
tometrically (340 nm) at 32 °C in a 96-well plate (250 μl/well). The assay was performed under the following reaction conditions: pH 7.4, 100 mM Tris/HCl, 1 mM MnCl2, 1 mM NH4Cl, 100 mM KCl, 1.25 mM NADP (freshly prepared), and 10 μM l-malate. All cell samples were run in duplicate with or without malate as substrate for test and control conditions, respectively. Cell extracts were added to all of the wells last, immediately shaken, and the absorbance read every 1 min for 40 min. Enzyme activity was determined by subtracting the activity of the control wells for each sample from the test wells run with l-malate. The resulting slopes of absorbance versus time were averaged and normalized to protein content.

Mitochondrial NADH Preferred Malic Enzyme 2 Activity—Mitochondria were isolated by a modification of methods previously described (27). Briefly, INS-1 cells were grown to 80–100% confluence on 10 150-cm2 plates in RPMI. The cells were immediately placed on ice, washed with ice-cold phosphate-buffered saline and quickly extracted by the cell scraping technique in ice-cold isotonic mitochondria extraction buffer containing 65 mM sucrose, 215 mM d-mannitol, 5 mM HEPES, 3 mM MgCl2, 5 mM KH2PO4, and 5 mM KHCO3 (made fresh daily). The remainder of the protocol was carried out at 4 °C. Cell suspensions were centrifuged at low speed (~100 × g) to gently pellet the cells. The supernatant was aspirated and the cell pellet was re-suspended in 1.0 ml of mitochondria buffer. The sample was transferred to a pre-cooled B-pestle Dounce and was passed 50 times, centrifuged for 3.5 min at 1.8 × 104 × g to remove heavy membranes, and re-extracted in isotonic buffer. The supernatants from both low speed spins were then centrifuged for 5 min at 8.5 × 105 × g to pellet out the mitochondria. The high-speed pellet was washed by re-suspension in mitochondria buffer and centrifuged a second time. The final pellet of isolated mitochondria was re-suspended in 1.0 ml of mitochondria buffer. Aliquots were taken for protein analysis (Bio-Rad, Lowry method) and the cytochrome c oxidase assay, the remainder of the sample was used for the 13C labeling studies. The cytochrome c oxidase assay was performed on intact and sonicated mitochondria to determine mitochondrial integrity. Mitochondrial integrity was determined to be greater than 79 ± 6%. Cytosolic contamination of less than 3% was determined by glycerol-3-P dehydrogenase activity in 300 mM triethanolamine-HCI buffer (pH 7.4, 0.13 mM NAD+).

The freshly isolated mitochondria (1 mg protein) were then incubated in mitochondrial buffer with ADP (1.25 mM) in the presence of either [U-13C]fumarate (1 mM), or [U-13C]glutamate (1 mM) plus leucine (10 mM). After a 20-min incubation, the reaction was quenched with ice-cold perchloric acid (30%) and sonicated to disrupt the mitochondria. The solution was neutralized with potassium hydroxide, centrifuged, and the supernatant was lyophilized and resuspended in D2O for NMR analysis followed by LC/MS/MS determination of pyruvate isotopic enrichment.

LC/MS/MS Analysis of [13C]Pyruvate—Quantitative analysis of the isotopomer distribution of [13C]pyruvate was performed with Applied Biosystems 4000 Q Trap LC/MS/MS system. Multiple reaction monitoring (negative-mode) of negatively charged ions from the TurboIonSpray probe was used to determine abundance of the unlabeled (86.9/43.3), single-labeled (87.9/43.3 and 87.9/44.3), double-labeled (88.9/45.3 and 88.9/44.3), and triple-labeled (89.9/45.3) pyruvate mass isotopomers. A 5-μl sample of the mitochondrial extract was loaded onto a LUNA 5u C8 (2) 100A 250 × 4.6-mm column (Phe
nomenex, Torrance, CA) and eluted using a linear gradient starting with 75% buffer A, 25% B changing to 5% buffer A, 95% buffer B over 10 min. Buffer A was 95% water with 5% acetonitrile (2 mM ammonium acetate) and buffer B was 5% water with 95% acetonitrile (2 mM ammonium acetate). These conditions provided for the necessary baseline resolution of pyruvate from oxaloacetate because they ionize to produce similar ion fragment spectra. Pyruvate peak identification was based on the retention time of 13C-labeled and unlabeled standards.

Glucose- and Amino Acid-stimulated Insulin Secretion—Transfected and non-treated INS-1 cells were equilibrated in Krebs-Ringer buffer (KRB) ±99% fatty acid-free bovine serum
albumin with 3 mM glucose for 2 h and then incubated in 2 ml of the appropriate secretagogue KRB mixture (G3 (3 mM Glc), G15 (15 mM Glc), G3 + 4 mM Gln + 10 mM Leu, or G15 + 4 mM Gln + 10 mM Leu) for an additional 2 h. Media aliquots were then taken and stored in the −20 °C freezer for analysis. Cells in the remaining media were placed on ice, washed with ice-cold phosphate-buffered saline, and treated with 0.1% Triton prior to protein analysis.

Isotopomer Analysis of Anaplerotic Pathways—The quantitative analysis of anaerobic pathways was determined by incubating the transfected and non-transfected INS-1 cells using identical protocols as described above to determine the effects of malic enzyme knockdown on insulin secretion, except that [U-13C]glucose was substituted for natural abundance glucose. At the end of the incubation period, the amino acids were extracted and the isotopomer distributions of glutamate, aspartate, and alanine were determined using either 13C NMR or gas chromatography-mass spectrometry (10). The relative fluxes of labeled and unlabeled substrates into the Krebs cycle were calculated from the isotopomer distribution of glutamate, aspartate, and alanine using the program “tcacalc” (10, 28).

Insulin Secretion Analysis—Media extracts were measured for insulin concentration using the High Range Insulin ELISA kit (ALPCO) and analyzed on a spectrophotometric plate reader at 450 nm. All media extracts were normalized to cell protein concentration using a Micro-BCA Protein Assay Kit (Pierce).

RESULTS

Relative Expression Levels of Malic Enzyme Isoforms—mRNA levels of each of the three malic enzyme isoforms, cytosolic ME1, mitochondrial ME2, and mitochondrial ME3, were determined for both INS-1 832/13 cells and rat islets. All cell and islet mRNA expression levels for each of the malic enzyme isoforms were normalized to actin expression and further compared with the non-treated sample. Reaction efficiencies for all primer/probe mixtures were greater than 98.7%. mRNA expression from all three isoforms of malic enzyme were present in the INS-1 832/13 cells as was determined using quantitative real-time PCR with the two predominant forms ME1 and ME2 accounting for over 97% of malic enzyme expression. Expression levels of mitochondrial isoforms ME2 and ME3 are shown relative to cytosolic ME1 (Fig. 1A). Similar expression patterns were observed in rat islets when compared with the INS-1 cells, where ME1 and ME2 accounted for 93% of malic enzyme expression (Fig. 1B). Because in both models, the mitochondrial isoform ME3 was a minor species, our studies focused on the roles of cytosolic ME1 and mitochondrial ME2 in glucose- and amino acid-stimulated insulin secretion.

Cytosolic NADP+-dependent Malic Enzyme 1 Activity—Enzyme assays were performed on the INS-1 whole cell lysates in the presence of NADP+ and L-malate in the reaction buffer to assess ME1 activity. There was no change in malic enzyme activity in cells treated with the nonspecific siRNA control compared with the non-treated cells (p = 0.87, n = 3 experiments done in duplicate). However, we observed a similar reduction in ME1 activity in cells treated with either siRNA construct (ME1a, 58 ± 2%, p < 0.001, n = 6 experiments done in triplicate; and ME1b, 54 ± 2%, p < 0.001, n = 4 experiments done in triplicate) compared with the non-treated control cells.

Mitochondrial NADP+-preferred Malic Enzyme 2 Activity—Assays to determine the activity of ME2 in isolated mitochondria that are based on the spectrophotometric measured rates of NAD+ reduction cannot distinguish between the activities of malic enzyme and malate dehydrogenase. We therefore used an isotopic tracer approach to demonstrate mitochondrial malic enzyme activity. Intact mitochondria were isolated from INS-1 cells and incubated in the presence of ADP and either [U-13C]fumarate (1 mM) or [U-13C]glutamate (4 mM) plus leucine (10 mM). Under these conditions, pyruvate becomes labeled only if malic enzyme is present. NMR analysis indicated the uptake and metabolism of both the [U-13C]fumarate and the [U-13C]glutamate to the level of [U-13C]malate. However, the pyruvate concentration was below reliable NMR calculations of isotopomer populations. To determine the isotopic incorporation into mitochondrial pyruvate, we used LC/MS/MS to measure the mass isotopomer distribution of pyruvate (Table 1). The distribution of mass isotomers included [U-13C]pyruvate (m+3), which could only be derived from the conversion of [U-13C]malate. The other single- (m+1) and double (m+2)-labeled isotomers of pyruvate are derived following cycling through the Krebs cycle. The relative mass isotopomer distribution of the pyruvate that was measured experimentally corresponded closely to the predicted distribution calculated by the modeling program “tcasim” (28). These

![Figure 1](http://www.jbc.org/)
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FIGURE 2. Malic enzyme 1 (cytosolic, NAD(P)+-specific) knockdown and the effect on insulin secretion. A, expression levels of malic enzyme isoforms in INS-1 cells after transfection with ME1-targeted siRNA (*, p = 0.02 compared with non-treated cells). B, cumulative rates of insulin secretion from INS-1 cells after transfection with ME1-targeted siRNA compared with non-treated cells (**, p < 0.05 compared with non-treated cells under the same incubation conditions; ‡, p < 0.05 compared with basal glucose secretion). Cells were equilibrated for 2 h at 3 mM glucose and incubated in KRB under the conditions specified. Insulin was analyzed by enzyme-linked immunosorbent assay. No significant differences were observed for either ME1 siRNA construct; therefore the data presented here are the pooled results for both ME1 siRNA constructs (n = 9). Data are mean ± S.E.

Enzyme Knockdown—All of the data for mRNA expression and functional assays were determined using two siRNA sequences for each malic enzyme isoform targeting two distinct locations on the gene to rule out any off pathway effects of RNA interference. We observed no significant differences between each siRNA construct (ME1a versus ME1b, and ME2a versus ME2b) in the efficacy for reducing mRNA expression or their effects on insulin secretion, therefore we report here the pooled results for ME1 (a/b) and ME2 (a/b). ME1 siRNA transfection decreased ME1 mRNA expression 89.9 ± 2% (p = 0.02, n = 6), with no effect on either ME2 or ME3 (Fig. 2A). Insulin secretion was determined in KRB at 3 mM glucose (G3) and 15 mM glucose (G15) alone or supplemented with 4 mM glutamine (Q) and 10 mM leucine (L). At G15 a 4-fold increase (p = 0.001, n = 6) in the non-treated cells was observed when compared with G3 (Fig. 2B). Knockdown of ME1 in the INS-1 cells blunted insulin secretion by 41 ± 6% (p = 0.005, n = 6) (Fig. 2B). In a similar fashion, ME1 knockdown reduced amino acid-stimulated insulin secretion (Gln + Leu) by 39 ± 6% (p = 0.008, n = 6) (Fig. 2B) (amino acid secretagogue stimulation was evaluated at basal glucose (G3) to maintain normal β-cell physiology in the INS-1 rat insulinoma cell line). Insulin secretion stimulated by the combination of high glucose and amino acids (G15 + Gln + Leu) was also blunted to a similar degree 38 ± 2% (p = 0.0006, n = 6) (Fig. 2B) as the result of ME1 knockdown.

To determine whether ME2 had any functional role in insulin secretion, the expression of ME2 was reduced using a siRNA specific to the ME2 isoform. mRNA expression of ME2 was reduced by 51.4 ± 4% (p = 0.0003, n = 9) without affecting ME1 or ME3 (Fig. 3A). In contrast to the global reduction in fuel-stimulated insulin secretion observed with siRNA knockdown of the cytosolic isoform ME1, the knockdown in ME2 had no affect on insulin secretion stimulated by 15 mM glucose (p = ns, n = 9, Fig. 3B). However, we found that this reduction in ME2 expression led to a 25 ± 4% (p = 0.005, n = 9) reduction in amino acid-stimulated insulin secretion (G3 + Gln + Leu) as shown in Fig. 3B.

Effect of KCl-induced Insulin Secretion on siRNA Malic Enzyme Knockdown—As shown in Fig. 4, insulin secretion in response to membrane depolarization using potassium chloride was unaffected by siRNA knockdown of malic enzyme or by the nonspecific siRNA control. These data support our hypothesis that the decreased rates of insulin secretion resulting from the cytosolic and mitochondrial malic enzyme knockdown are a result of reduced pyruvate cycling.

FIGURE 3. Malic enzyme 2 (mitochondrial, NAD(P)+-preferred) knockdown and the effect on insulin secretion. A, expression levels of malic enzyme isoforms in INS-1 cells after transfection with ME2-targeted siRNA (*, p = 0.0003 compared with non-treated cells). B, cumulative rates of insulin secretion from INS-1 cells after transfection with ME2-targeted siRNA compared with non-treated cells (**, p < 0.05 compared with non-treated cells under same incubation conditions; ‡, p < 0.05 compared with basal glucose secretion). Cells were equilibrated for 2 h at 3 mM glucose and incubated in KRB under the conditions specified. Insulin was analyzed by enzyme-linked immunosorbent assay. No significant differences were observed for either ME2 siRNA construct, therefore the data presented here are the pooled results for both ME2 siRNA constructs (n = 12). Data are mean ± S.E.
other from an unlabeled pool. siRNA knockdown of either ME1 or ME2 had no effect on the anaplerotic pathways of PC or glutamate anaplerosis under basal conditions. However, at 15 mM glucose, ME1 knockdown decreased PC flux by ~80%, with equivalent reductions in flux of the labeled and unlabeled pools. A lesser (~47%), but still significant decrease in PC flux was observed when the INS-1 cells were challenged with G3 plus glutamine and leucine. In contrast, reduced activity of ME2 abolished flux of the pyruvate from the unlabeled pyruvate pool when challenged either by G15 (~59% reduction), or by G3 plus glutamine and leucine (~68% reduction), but had no significant effect on PC flux of the 13C-labeled pyruvate pool. The marked reduction in the unlabeled pyruvate pool with decreased expression of the mitochondrial isofrom of malic enzyme provides a physiological basis for two separate pyruvate pools, a mixed cytosolic pool and a non-equilibrating mitochondrial pool. Although, additional studies are needed, these results are also suggestive of substrate channeling of glutamine through several Krebs cycle enzymes to malic enzyme.

DISCUSSION

Role of Malic Enzyme in Pyruvate Cycling and Fuel-stimulated Insulin Secretion—Insulin secretion rates are tightly coupled with increases in mitochondrial metabolism (1, 2, 14, 15). In addition to the requirement for increased mitochondrial ATP production rates, it is also recognized that other products of mitochondrial metabolism are required for fuel-stimulated insulin secretion. The generation of several of these putative second messengers is the result of the pyruvate carboxylase flux leading to an increase in the concentration of Krebs cycle intermediates and the export of citrate, isocitrate, and malate into the cytosol (1–5). In fact, mitochondria isolated from pancreatic islets will export malate at rates comparable with the carboxylation of pyruvate by PC, which is comparable with the rate of pyruvate decarboxylation by PDH (5). The extramitochon-
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drial fate of these compounds to stimulate the release of insulin is unclear and is currently an area of intensive research (1–5, 9).

One well documented fate of extramitochondrial malate is the oxidative decarboxylation to pyruvate catalyzed by cytosolic malic enzyme (5, 17). The high activity of pyruvate carboxylase and malic enzyme in the β-cell has led to the hypothesis this “pyruvate cycle” plays a key role in the sensing of the increased β-cell metabolic rate in response to increased plasma glucose levels and leads to a proportional increase in insulin secretion (1, 5, 11).

In vivo, the severalfold higher rates of insulin secretion in Zucker fatty rats compared with Zucker lean rats was associated with 2–3-fold increases in the activities of pyruvate carboxylase and malic enzyme in the islets (19). Mouse islets, in contrast, had no detectable malic enzyme activity, and the absence of this malate-pyruvate cycle was proposed to explain the weak second-phase of insulin secretion from mouse islets compared with rat islets (29). Finally, in several clonal rat INS-1 cell lines, the responsiveness of glucose-stimulated insulin secretion correlated to increases in pyruvate cycling as calculated from isotopomer analysis (11).

However, direct evidence for the role of malic enzyme in insulin secretion is limited to small molecule inhibitor studies that targeted the malate shuttle, preventing malate export from the mitochondria to the cytosol (25). As an alternative to small molecule inhibition, we have focused our efforts on specific inhibition using siRNA methodology. Three malic enzyme isoforms are known to be expressed in mammalian tissues. Because, these isoforms differ in substrate specificity, NADP+ and NAD+, and cellular compartmentation, one cytosolic and two mitochondrial, we hypothesized that they could have different roles in the generation of metabolic second messengers of insulin secretion. In contrast to previous studies-reporting only cytosolic NADP+-dependent malic enzyme could be measured in rat islets, we found significant mRNA expression of the cytosolic malic enzyme isoforms in both a stable rat insulinoma cell line (INS-1 832/13), and in isolated rat islets.

The Role of Cytosolic Malic Enzyme 1 in Fuel-stimulated Insulin Secretion (Fig. 6)—We found that the cytosolic NADP+-dependent isoform is required for both glucose and amino acid-stimulated insulin secretion and supports the concept that NADPH acts as a second messenger to enhance insulin secretion. At stimulatory glucose concentrations, glycolytic flux is sufficient to drive PDH and PC flux at rates sufficient to increase ATP and NADPH synthesis rates necessary to facilitate insulin secretion. As we have shown previously, stimulating insulin secretion by the addition of glutamine and leucine, 15 mM glucose, or the combination correlates tightly with rates of increased PC flux (10). Because the rate of pyruvate carboxylation is the sum of pyruvate that is cycled and that which is irreversibly lost to other products, knockdown of ME1 will only affect the rate of NADPH synthesis from pyruvate cycling. The synthesis and export of other mitochondrial metabolic second messengers, the net rate of Krebs cycle flux, and mitochondrial ATP synthesis rates should remain unchanged.

Whether NADPH is acting directly, or indirectly, to facilitate insulin exocytosis cannot be determined from these studies. The potential of NADP(H) to directly affect insulin secretion rates by its binding to the nucleotide inhibitory site of the KATP channel has been reported by Dabrowski et al. (30). Mechanistically, rather than the NADP(H) redox state acting passively as a modulator of ion currents, the high rate of pyruvate cycling suggests that a continuous regeneration of NADPH is needed for the sustained exocytosis of insulin during the second phase of insulin release. Recently, Ivarsson et al. (21) have shown that increased activity of the glutaredoxin system lead to increased exocytosis by patch-clamp experiments of purified β-cells (21). Electrons transferred from NADPH to glutaredoxin can be used to reduce disulfides in target proteins or mediate the reversible glutathionylation of proteins. The reversible modification of proteins by these NADPH-glutaredoxin-mediated reactions would be responsive to changes in β-cell metabolic rates and would provide acute regulation of fuel-stimulated insulin secretion rates.

The Role of Mitochondrial Malic Enzyme 2 in Fuel-stimulated Insulin Secretion (Fig. 6)—In contrast to the role of cytosolic malic enzyme to generate NADPH, it is likely that the mitochondrial NAD+-dependent isoform functions as a means to increase metabolic flux through PC and PDH in response to increased amino acid concentrations and independent of glycolytic flux. Earlier studies in the rat adrenal cortex illustrated the affect of various substrates on ME2 activity and found high activity of the enzyme with the addition of substrates such as glutamine and other fumarate precursors (22). Based on these findings, it was suggested that ME2 may preserve flux through the Krebs cycle by forming pyruvate when glucose is not the primary substrate (22).

At low glucose concentration, the flux of pyruvate into the mitochondria may be sufficient only to drive PDH flux for ATP generation, and pyruvate cycling needed to maintain a threshold redox state of NADPH. When glutamine and leucine are provided, the increased flux through mitochondrial malic enzyme and PC provides sufficient mitochondrial pyruvate to increase both ATP synthesis rates and the generation of cytosolic NADPH from pyruvate cycling. The increased rates of ATP and NADPH synthesis lead to increased rates of insulin secretion. To our knowledge this is the first report to support a role for the mitochondrial isoform of malic enzyme in amino acid-stimulated insulin secretion.

Recent studies of the functional correlation between anaplerosis and insulin secretion using a 13C-isotopomer approach suggest the possibility of two metabolically distinct pools of pyruvate. It was hypothesized that one pool was derived from glycolysis and was used primarily to maintain PDH flux, whereas a second pool provided substrate for pyruvate cycling (11). The results presented here provide a physiological basis for this interpretation of the modeling results. Substrate channeling of Krebs cycle intermediates from substrate entering at the level of succinyl-CoA has been previously shown to occur (31), and would provide a mechanism to feed substrate into mitochondrial pyruvate independent of glycolytically derived pyruvate. We propose that the channeling of glutamine to mitochondrial malic enzyme would create a pool of mitochondrial pyruvate that is initially distinct from pyruvate originating from either glycolysis or cycling through cytosolic malic enzyme...
enzyme, and that this could account for two slowly equilibrating pools of pyruvate.

In summary, the compartmentation of malic enzyme leads to a cytosolic pyruvate cycle and a mitochondrial pyruvate cycle, both cycles being functionally important for the coupling of metabolic rate to insulin secretion (Fig. 6). The cytosolic pyruvate cycle catalyzed by ME1 is necessary for the redox control of insulin secretion by NADPH. Whereas, glutamine/leucine-stimulated insulin secretion requires mitochondrial malic enzyme to provide sufficient pyruvate for increased mitochondrial ATP production and the export of malate to the cytosol to generate NADPH. In conclusion, differences in compartmentation and substrate specificity lead to functionally distinct roles for the cytosolic and mitochondrial isoforms of malic enzyme and can account for metabolically separate pools of pyruvate in insulin secreting cells.

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REFERENCES
