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Investigating the Roles of Mitochondrial and Cytosolic Malic Enzyme in Insulin Secretion

Rebecca L. Pongratz, Richard G. Kibbey, and Gary W. Cline

Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA

Abstract

Glucose homeostasis depends upon the appropriate release of insulin from pancreatic islet β -cells. Postprandial changes in circulating nutrient concentrations are coupled with graded release of stored insulin pools by the proportional changes in mitochondrial metabolism. The corresponding increased synthesis rates of both ATP and of anaplerotic metabolites have been shown to be mediators for nutrient-stimulated insulin secretion. Anaplerosis leads to the export of malate or citrate from the mitochondria, both of which can be recycled through metabolic pathways to reenter the Krebs cycle. These metabolic cycles have the net effect of either transferring mitochondrial reducing equivalents to the cytosol, or of efficiently providing pyruvate to facilitate responsive changes in the Krebs cycle flux in proportion to increased availability of glutamate and anaplerotic flux through glutamate dehydrogenase. Here, we describe siRNA knock-down and isotopic labeling strategies to evaluate the role of cytosolic and mitochondrial isoforms of malic enzyme in facilitating malate–pyruvate cycling in the context of fuel-stimulated insulin secretion.

1. Introduction

Secretion of insulin from pancreatic β -cells plays a central role in regulating whole-body metabolism after a meal. The ability of the β -cell to respond to the postprandial increases in plasma glucose and amino acids with graded rates of insulin release rests in the coupling of cellular metabolism with exocytosis of the insulin stores. Overarching the mechanistic explanations for nutrient-stimulated insulin secretion is the influx of Ca^{2+} that follows increases in glycolytic and mitochondrial ATP production and the depolarization of the cellular membrane after closure of ATP-sensitive potassium channels (K_{ATP}). Delving further into the coupling mechanisms though, many researchers have shown that stimulated insulin secretion is enhanced by a K_{ATP} -independent, yet metabolism-dependent, mechanism to generate other “second messengers.” Despite intensive research by many groups, the identification of these second messengers is still uncertain and controversial. Several plausible pathways and metabolites have been proposed to fill the role of second messenger, and it is generally accepted that mitochondrial anaplerotic pathways contribute to their production (for a review: MacDonald *et al.*, 2005).

Anaplerosis increases the concentrations of several Krebs cycle intermediates generating a large surplus of mitochondrial malate and citrate that is exported to the cytosol. The relatively high activity of both pyruvate carboxylase (PC) and cytosolic malic enzyme in the β -cell supports the concept that the mechanisms linking metabolism with insulin secretion may include a β -cell pyruvate–malate cycle (Fig. 24.1) (Liu *et al.*, 2002; Lu *et al.*, 2002; MacDonald, 1995; Pongratz *et al.*, 2007). Malate that is exported from the mitochondria to the cytosol is regenerated to pyruvate by cytosolic malic enzyme for cycling back to the mitochondria. Cytosolic malic enzyme, together with ATP citrate lyase and malate dehydrogenase, is also central to recycling of citrate back to pyruvate. Farfari *et al.* (2000) suggested that this citrate–malate–pyruvate cycle serves to regenerate NAD^+ and maintain glycolytic flux. Cytosolic citrate can also be cycled independent of malic enzyme via isocitrate for input to the Krebs cycle at the level of α -ketoglutarate. These pyruvate cycles all lead to the exchange of reducing equivalents from mitochondrial NADH to cytosolic NADPH. The shift in redox state towards increased concentration of NADPH with increased pyruvate cycling may couple increased mitochondrial activity with downstream events in the cytosol leading to insulin secretion (Ashcroft and Christie, 1979; Ishihara *et al.*, 1999; Ivarsson *et al.*, 2005).

There also exists the potential for an alternative malate–pyruvate cycle within the mitochondrial matrix via mitochondrial-localized isoforms of malic enzyme. In addition to the cytosolic NADP^+ -dependent isoform, malic enzyme 1 (ME1), two mammalian mitochondrial isoforms exist; malic enzyme 2 (ME2) with a preference for NAD^+ , and an NADP^+ -dependent malic enzyme 3 (ME3). Mandella and Sauer (1975) 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. In other detailed studies of the kinetic regulation of malic enzyme by Teller *et al.* (1992), the results indicate that heteroenzyme interactions of ME2 and the PDH complex make ME2-generated pyruvate a better substrate for PDH than the pool of free pyruvate. Thus, mitochondrial malic enzyme may enhance the flux of glutamine as a respiratory fuel by shunting glutamate-derived malate towards the formation of pyruvate, and provide a mechanism to couple the metabolism of glutamine, in the presence of leucine, with enhanced Krebs cycle flux.

This chapter describes strategies used in our laboratory to investigate the hypothesis that malic enzyme is integral to the coupling of metabolism with insulin secretion. Herein are described the methods used to confirm the expression and activity of the cytosolic and mitochondrial isoforms of malic enzyme in insulin secreting cells, to determine whether nutrient-stimulated insulin secretion is affected by selective changes in the activities of each isoform, and to identify metabolic pathways that may be regulated by the activity of malic enzyme isoforms.

2. Malic Enzyme mRNA Expression in Rat Insulinoma INS-1 832/13 Cells, Rat Islets, and Mouse Islets

Three malic enzyme isoforms are known to be expressed in mammalian tissue, differing in substrate specificity, NADP⁺ or NAD⁺, and cytosolic (ME1) or mitochondrial (ME2 and ME3) compartmentation. We used quantitative real time PCR (RTqPCR) to determine the relative mRNA expression levels of the malic enzyme isoforms in INS-1 cells, mouse islets, and rat islets.

2.1. RNA isolation

RNA can be obtained from cells and isolated islets using RNA extraction kits such as the RNeasy Mini Kit (Qiagen, Germantown, Maryland). Cells (+/- siRNA transfection) are grown in 6-well plates until they are 80–90% confluent. The cells are treated with 350 μ l of lysis buffer containing fresh β -mercaptoethanol (BME) and homogenized using a 1 ml syringe with a 20 G needle and passed 8–10 times as per the manufacturer's protocol. The remainder of the steps is followed as suggested by the manufacturer's protocol either on bench top or using the automated Qiacube system (Qiagen). Both manual and automated extractions yield the same quality of RNA. The DNase step which is suggested in the manual is highly recommended. For cells, RNA is eluted off the column with 50 μ l of RNase free water and placed on ice for spectrophotometer analysis.

If extracting RNA from isolated rat or mouse islets, 100–150 islets with no exocrine tissue contamination are hand-picked into 1.7 ml tubes on ice using a glass pipette with a flamed tip to smooth the edges so that the islets do not shear. Islets are then centrifuged at a low speed of 100 rcf for 5 min at 4°C and any extra medium is removed carefully by aspiration. The samples are then placed on bench top at room temperature and 350 μ l of lysis buffer containing BME is added to each islet sample tube. Islets are left at room temperature for 10–15 min then vortexed briefly to ensure that all membranes are broken. The vortex step should not exceed 5 s. The islet lysates are then homogenized as described above for the cells and the remainder of the protocol steps are the same except islet RNA is eluted off the column using 30 μ l of water in order to yield RNA at a sufficient concentration for the reverse transcription step.

2.2. Reverse transcription of RNA to cDNA

A fraction of the RNA sample is diluted 1:10 and read on the spectrophotometer to determine RNA concentration and purity. Five microliters of cell and islet RNA are brought to 50 μ l using RNase/DNase free water and placed in a 50- μ l quartz cuvette. The spectrophotometer (Biochrom Ultra-spec 2100 *pro*, General Electric, Waukesha, Wisconsin) should be turned on for at least 15 min prior to the spectrophotometer reading to ensure that the tungsten (or deuterium for some spectrometers) bulb is warmed up. The background absorbance at 260 and 280 nm of RNase/DNase free water is measured as a blank. Absorbance of the samples is then measured and the concentration (μ g/ μ l) of RNA is calculated from the absorbance at 260 nm where 1 Optical Density unit at 260 nm for RNA molecules is equal to 40 ng/ μ l of RNA. RNA purity is determined from the 260/280 nm ratio of absorbances. A ratio of 1.9 is optimal (range from 1.8 to 2.0) for successful RTqPCR.

Islet RNA concentrations range from ~ 0.04 to $0.08 \mu\text{g}/\mu\text{l}$ for ~ 125 mouse islets, ~ 0.08 to $0.2 \mu\text{g}/\mu\text{l}$ for ~ 125 rat islets, and 0.2 to $0.6 \mu\text{g}/\mu\text{l}$ for confluent cells in 1 well (35 mm diameter) of a 6-well plate. At these concentrations, there is enough total RNA to proceed with the reverse transcription procedure. The reverse transcription reaction works well with a total of $2 \mu\text{g}$ RNA in the final RT reaction volume. We use reverse transcriptase with a volume of $50 \mu\text{l}$ per sample reaction mixture ($44 \mu\text{l}$ from Step 1 plus $6 \mu\text{l}$ from Step 2) as follows.

For Step 1, combine the following in a PCR tube, $2 \mu\text{l}$ of $100 \mu\text{M}$ 9-mer random primers (Agilent Technologies, Santa Clara, California) yielding a final reaction concentration of $4 \mu\text{M}$, $5 \mu\text{l}$ of 40 mM dNTPs (10 mM per nucleotide) (Denville Scientific, Metuchen, New Jersey) yielding a final reaction concentration of $4 \mu\text{M}$, and the purified RNA to yield a total of $2 \mu\text{g}$ RNA. However, the volume of purified RNA should not exceed $37 \mu\text{l}$. For example, if the RNA concentration is $0.1 \mu\text{g}/\mu\text{l}$, then $20 \mu\text{l}$ of the RNA solution would be needed (i.e., $2 \mu\text{g}$ total RNA $\div 0.1 \mu\text{g RNA}/\mu\text{l} = 20 \mu\text{l}$). Adjust to a final volume of $44 \mu\text{l}$ with RNase free/DNase free water (in this example, $17 \mu\text{l}$ would be needed: $44 \mu\text{l} - 2 \mu\text{l} - 5 \mu\text{l} - 20 \mu\text{l} = 17 \mu\text{l}$). Mix and spin briefly. This initial mixture is heated at 70°C for 5 min so that the primers can anneal to the RNA sequence.

For Step 2, the remainder of the mix, consisting of $5 \mu\text{l}$ of $10 \times$ RT enzyme buffer (NEB, Ipswich, Massachusetts) and $1 \mu\text{l}$ of $200 \text{ U}/\mu\text{l}$ M-MuLV reverse transcriptase (NEB), is added to the sample PCR tube and the RNA is reverse transcribed to complementary DNA (cDNA) using the following reverse transcriptase (RT-PCR) protocol: incubate for 1 h at 42°C , inactivate the enzyme for 10 min at 90°C , and then cool to 4°C . The cDNA from the reverse transcriptase reaction can be stored at 4°C or immediately analyzed using RTqPCR.

2.3. RTqPCR for ME1 and ME2 mRNA expression in cells and islets

The cDNA prepared in Section 2.2 from the cells or the islets is diluted 1:10 with RNase free/DNase free water for RTqPCR analysis. Standard curves for RTqPCR amplification efficiencies are obtained from four sequential 1:5 serial dilutions (1:5 to 1:625) of the cDNA from either the control cells or the islets. Efficiency of the primers is calculated from the slope of the standard curve where the efficiency is equal to $-1 + 10^{(-1/\text{slope})}$. Efficiencies between 1.9 and 2.1 are generally accepted for a doubling with each amplification cycle. If the efficiency is beyond this range, then new primer sequences should be designed.

The sensitivity of the RTqPCR instrument will determine the reaction size. For a $10\text{-}\mu\text{l}$ SYBR green reaction, components include; $0.5 \mu\text{l}$ of $10 \mu\text{M}$ forward primer for the target gene, $0.5 \mu\text{l}$ of $10 \mu\text{M}$ reverse primer for the target gene, $5 \mu\text{l}$ of $2 \times$ Fast SYBR green mix containing Taq polymerase (Applied Biosystems, Foster City, California) and $4 \mu\text{l}$ of 1:10 diluted cDNA. For $10 \mu\text{l}$ TaqMan internal fluorescent probe reaction, the components include; 1 ml custom TaqMan (Applied Biosystems) $10 \times$ primer/probe mix ($10 \times$ primer/probe master mix stock is prepared as follows; $50 \mu\text{l}$ of $10 \mu\text{M}$ forward primer, $50 \mu\text{l}$ of $10 \mu\text{M}$ reverse primer and $25 \mu\text{l}$ of $10 \mu\text{M}$ probe and can be stored at -20°C), $5 \mu\text{l}$ of $2 \times$ internal probe enzyme mix (Applied Biosystems (TaqMan Fast RT PCR Universal Master mix)) containing Taq polymerase and $4 \mu\text{l}$ of 1:10 diluted cDNA. Reactions are run in optically clear plates on a real-time PCR instrument (Applied Biosystems 7500 software

version 4.1). The RTqPCR protocol will be specific to the primer mix. The protocol that we used is as follows. Step 1 is 95 °C for 20 s with one repeat. Step 2 is 95 °C for 3 s, then increased to 60 °C for 30 s, with 40 repetitions. For SYBR Green, there is an additional step for the dissociation curve, as follows. Step 3 is 95 °C for 15 s, increased to 60 °C for 60 s, increased to 95 °C for 15 s, then cooled to 60 °C for 15 s, with one repeat.

Primers for either TaqMan or SYBR green RTqPCR analysis for ME1 and ME2 are as follows:

ME1 forward 5'-ATGGAGAAGGAAGGTTTATCAAAG-3'
 ME1 reverse 5'-GGCTTCTAGGTTCTTCATTCTTC-3'
 ME2 forward 5'-GGCTTTAGCTGTTATTCTCTGTGA-3'
 ME2 reverse 5'-TGAATATTAGCAAGTGATGGGTAAA-3'
 ME3 forward 5'-AGATAAGTTCGGAATAAATTGCCT-3'
 ME3 reverse 5'-CATCATTGAACATGCAGTATTTGT-3'

Internal probes for both ME1 and ME2 for TaqMan analysis are as follows:

ME1 probe FAM-GGGCGTGCTTCTCTCACAGAAGA-TAMRA
 ME2 probe FAM-CCCGACACATCAGTGACACCGTTT-TAMRA
 ME3 probe FAM-GACTTTGCCAATGCCAATGCCTTC-TAMRA

The advantage of using internal probe technology is that the probe sequence, which anneals to the template downstream of the sequence primers, has a reporter dye (i.e., FAM) on the 5' end and the quencher dye (i.e., TAMRA) on the 3' end. As the primer extends, laying down complimentary base pairs on the template, it will reach the probe which the Taq DNA polymerase in the enzyme mix cleaves and the reporter dye is released and then quenched. Each amplification cycle can then be quantified upon the release of the reporter dye. When using SYBR green, since the dsDNA fluorophores are not specific to the gene target sequence, dissociation curves must be checked for each primer set to ensure that the amplification copies are quantified for the target sequence only, and not other nonspecific PCR products such as primer dimers. The dissociation curve should have a single peak at the appropriate melting temperature (T_m). Primer dimers result in two peaks in the dissociation curve. When determining small interfering ribonucleic acid (siRNA) knock-down, all mRNA transcript copies (CTs) are analyzed using $\Delta\Delta CT$ analysis where message is normalized to an endogenous housekeeping gene such as beta actin and then also to the non-treated control. When determining overall relative expression of a gene product in a particular tissue type, mRNA expression is calculated using $\Delta\Delta CT$ analysis where message is normalized to an endogenous housekeeping gene and then relative fold expression is compared to a second gene of interest. Figure 24.2 shows the relative mRNA expression of ME1, ME2, and ME3 in INS-1 832/13 cells and rat islets.

3. siRNA Knock-Down of ME1 and ME2 in INS-1 832/13 β -Cells

In order to assess the relevance of ME1 and ME2 for nutrient-stimulated insulin secretion from pancreatic islet β -cells, we used the strategy of effectively blocking or “knocking-down” gene expression with double stranded siRNA sequences. Because of the low mRNA

expression levels of ME3, we focused only on ME1 and ME2 in our studies; however, a similar strategy could be followed to assess the role of ME3. The key to RNA interference is the double stranded RNA configuration where nonendogenous dsRNA is recognized by the cell initiating a cascade of events which ultimately leads to silencing of endogenous RNA that is homologous to the dsRNA. siRNA 21-mer duplexes, consisting of a sense strand and its complementary antisense strand, are characterized by a 2-nucleotide dithymidine overhang at each 3' terminus and 5' phosphate groups. siRNAs are assembled into ribonuclease induced silencing complexes (RISC) which then unwind the siRNA to form single stranded RNA (ssRNA). The unwinding of the double stranded siRNA requires ATP and it is this configuration that activates the RISC complex which then seeks out homologous mRNA targets. Once this occurs, the RISC complex cleaves the mRNA, exonucleases degrade the sequence and the message is silenced (Bernstein *et al.*, 2001; Dalmay *et al.*, 2000; Hammond *et al.*, 2001). We chose 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.

3.1. ME1 and ME2 siRNA design

The National Center for Biotechnology Information (NCBI) provides a database where specific sequences for a particular gene in an organism can be searched in order to serve as DNA target sequences for siRNA construction. The selected nucleotide sequence can then be analyzed using the Basic Local Alignment Search Tool (BLAST) program in order to verify that it is not homologous to any other gene in the genome except for the target gene under investigation. Commercially available custom 21-mer siRNA sequences can be purchased through various vendors and designed using the specific requirements for each vendor's design tools. We found that the siRNAs from Qiagen were very successful for transfection and decreased mRNA expression and function of ME1 and ME2. The siRNA duplex consists of the sense strand and its complementary antisense strand to provide a 2-nucleotide dithymidine overhang at each 3' terminus. The DNA target sequences for ME1 (a and b) and ME2 (a and b) are as follows:

ME1a: AACCAGGAGATCCAGGTCCTT and ME1b: AAGCCAAGAGGCCTCTTATC,
ME2a: AACGGCTTGCTAGTTAAGGGC and ME2b: AAAGCCATGGCCGCTATCAAC.

The 19-base pair siRNA consisting of dsRNA for each target sequence are as follows:

ME1a: (CCAGGAGAUCCAGGUCCUU)d(TT)(AAGGACCUGGAUCUCCUGG)d(TT)
ME1b: (GCCAAGAGGCCUCUUUAUC)d(TT)(GAUAAAGAGGCCUCUUGGC)d(TT)
ME2a: (CGGCUUGCUAGUUAAGGGC)d(TT)(GCCCCUUAACUAGCAAGCCG)d(TT)
ME2b: (AGCCAUGGCCGCUAUAAC)d(TT)(GUUGAUAGCGCCAUGGCU)d(TT)

The duplexes are resuspended and annealed as per manufacturer's recommendation and require a simple preparation of heating to 90 °C in a water bath for 1 min to disrupt any hairpins that may have formed during synthesis and then cooling to 37 °C for 1 h to allow the RNA to reanneal into an uninterrupted double stranded complementary sequence configuration.

3.2. siRNA transfection optimization in INS-1 832/13 β -cells

Success in transfection of mammalian cells either in stable cell cultures or primary cell lines requires consideration of multiple interacting factors. Although stable cell cultures tend to transfect at a much higher efficiency than primary cells, optimization of the conditions for effective siRNA gene silencing requires an iterative approach. In addition to the cell type, other factors such as passage number, confluence, transfection reagent, and siRNA concentration can influence the effectiveness of siRNA transfection and function in the stable cell line. Positive and negative siRNA controls are useful for optimizing transfection efficiency for each particular cell model. Fluorescent labeled nonspecific siRNAs are commercially available and function as both a positive control and negative control (Qiagen). The fluorescent labeled siRNA functions as a positive control for transfection efficiency since siRNA incorporation into a cell can be visualized using fluorescence microscopy or quantified using fluorescence spectroscopy. The nonspecific siRNA sequence functions as a negative control to ensure the absence of nonspecific knock-down of the target gene, since the siRNA duplex has been created and tested to have no sequence homology to a functional mammalian gene. Once transfection efficiency is optimized for siRNA concentration and incorporation into the cell using control siRNAs, it is then necessary to transfect multiple siRNA sequences corresponding to various locations of the selected gene to test for mRNA down-regulation or “knock-down” and the effect, if any, on protein expression. It is also important to note that even with successful knock-down of mRNA expression, the effects upon the translation and function of the protein should be determined. It is also essential to monitor the duration of transfection with regard to protein turnover since rates of protein turnover vary for different proteins (McManus and Sharp, 2002). INS-1 832/13 cells, like many other mammalian cell lines, can incorporate siRNAs through lipid-mediated transfection (Qiagen RNAifect). This method requires specially designed lipophilic reagents which carry the siRNAs across the lipid bilayer of the plasma membrane and into the cell.

3.3. Protocol for experimental siRNA KD of ME1 and ME2 in INS-1 832/13 β -cells

INS-1 cells are grown in 6-well plates until they reach 50% for transfection. Plates are set up so that independent experiments evaluating ME1 and ME2 functional assays such as glucose and amino acid stimulated insulin secretion or enzyme activity assays, as well as, RNA extraction for mRNA expression can be performed simultaneously on the same passage of cells to ensure that siRNA knock down of message translates to functional down regulation of the ME1 and ME2 proteins. The transfection mixture is prepared based on optimization of the manufacturer's suggested protocol in 1 ml of serum-free OptiMEM with Glutamax (Gibco (Invitrogen), Carlsbad, California) transfection media and then placed on the cells. This will vary from cell type to cell type. In our hands, a 1:5 ratio of siRNA to RNAifect reagent (Qiagen) where 6 μ l (1.8 μ g) of 20 μ M siRNA is brought up to 100 μ l with EC transfection buffer provided in the kit and 9 μ l of transfection reagent is applied to each well of a 6-well plate in 1 ml OptiMEM. The cells are incubated in the mixture at 37 °C for 6–15 h and then replenished with nutrient-rich RPMI media. The transfected cells are incubated for an additional 48 h and glucose/amino acid stimulation and RNA extraction for mRNA expression is performed.

4. Enzymatic Assays to Determine Activity of Cytosolic and Mitochondrial Malic Enzymes

In addition to assessing the effectiveness of siRNA to reduce mRNA expression levels, relative enzyme activity levels were used to confirm changes in protein levels. Cytosolic malic enzyme is NADP⁺-dependent and can be determined in the whole cell lysate without interference from malate dehydrogenase which uses NAD⁺ for oxidation of malate to oxaloacetate. In contrast, a similar approach to measure the activity of mitochondrial malic enzyme by following the rate of reduction of NAD⁺ in the presence of malate is confounded by the presence and high activity of MDH in both the cytosol and mitochondria. Previously, this problem has been resolved by purification of mitochondrial malic enzyme on an affinity column. Our concern that differences in recovery may mask differences in activity, prompted us to develop an alternate isotopic-labeling approach that would allow us to measure mitochondrial malic enzyme activity in intact isolated mitochondria. The rationale for these experiments is that in the absence of PEPCK activity, the only mechanism for the appearance of uniformly ¹³C-labeled pyruvate ([¹³C₃]pyruvate) when incubating mitochondria with either uniformly ¹³C-labeled fumarate ([¹³C₄]fumarate or glutamate ([¹³C₅]glutamate) is by the activity of the mitochondrial isoforms of malic enzyme. To evaluate any contribution of PEPCK activity, we include phenylalanine (1 mM final concentration) to inhibit pyruvate kinase (PK) activity and conversion of PEP to pyruvate.

4.1. Cytosolic malic enzyme activity assay

The assay to determine cytosolic enzyme activity consisted of extraction of whole cell lysate, incubation of the cell lysate in the presence of malate and NADP⁺, monitoring the reduction of NADP⁺ spectrophotometrically, and normalizing to total cellular protein. We describe the protocol for the assay in INS-1 cells; however, the methodology can be readily used for determining malic enzyme activity in the extracts of isolated islets. The method as described requires ~1–2 mg total cell protein in a volume of 6 ml.

4.1.1. Extraction of whole cell lysate—INS-1 cells are cultured in 6-well plates until they reach 80–90% confluence, typically, $\sim 5.0 \times 10^5$ cells per well of a 6-well plate are extracted. Protein concentrations for each well range between 200 and 400 μ g. On the day of extraction, cells are placed on ice, washed with ice-cold PBS then lysed using 1 ml of ice-cold 0.1% triton per well. The cells remain on the ice for roughly 15–30 min and wells are then scraped using a plastic cell lifter (Corning Incorporated, Corning, New York), mixed by pipetting and transferred into 1.7 ml plastic tubes on ice.

4.1.2. Malic Enzyme 1 assay reaction mixture stock components—All stocks are prepared prior to the experiment and stored as separate solutions at room temperature unless otherwise indicated. The assay components include; 250 mM Tris/HCl pH 7.4, 50 mM MnCl₂, 40 mM NH₄Cl, 1 M KCl, and 20 mM NADP⁺ which must be prepared fresh and immediately placed on ice. Since NADP⁺ powder is hygroscopic, the chemical was brought to room temperature prior to weighing in order to ensure an accurate concentration. One hundred millimolar of the enzyme reaction substrate, L-malate, is prepared, brought to pH

7.4 and stored at 4 °C. On the day of the experiment, the enzyme assay reagent mixture is prepared as described in Table 24.1.

A 150- μ l aliquot of this reaction mixture is placed into each well of a 96-well clear flat bottom plate compatible for spectrophotometric analysis on a 96-well plate reader. (We used NUNC (Thermo Fisher Scientific), Rockford, Illinois, Immuno MicroWell 96-well plates and a Dynex, Chantilly, Virginia, MRX Revaluation Plate Reader.) Test and control wells are set up in duplicate for each cell sample where 50 μ l of H₂O is placed in test wells; 75 μ l of H₂O is placed in control wells. Twenty-five microliters of the enzyme substrate L-malate is added to the test wells only. The final concentrations of the assay components in each well are as follows: ~100 mM (93 mM) Tris/HCl, ~1 mM (0.93) MnCl₂, ~1 mM (0.93) NH₄Cl, ~100 mM KCl, ~1 mM (0.93) NADP⁺, and 10 mM L-malate. The reaction plate is then brought to room temperature on bench-top. Twenty-five microliters of the cell lysate samples are then added to each of the test and control wells using a multidispensing pipette (Rainin, Oakland, California, EPD-3 PLUS) where the control wells are seeded first to avoid cross contamination of the substrate. The plate is mixed by shaking briefly and immediately read at 340 nm for 40 min with data collected every minute beginning immediately after an initial 5 s shaking step. The optical densities (OD) for each minute of data collection can then be plotted and slopes determined for cytosolic malic enzyme 1 activity in both test and control wells where control wells are subtracted from the test wells and data are then normalized to whole cell protein concentration. Activity = OD/min/mg protein. If desired, this can be expressed in terms of reduction rates of NAD⁺ or NADP⁺ with a suitable standard curve. For the purposes of these experiments, the relative changes in ME activity in control and siRNA-treated cells, as assessed by DOD, are sufficient to evaluate the relation between changes in mRNA expression and enzyme activity.

4.1.2.1. Protein assay: Protein concentrations in the whole cell lysates are assayed using the Micro BCA assay (Pierce, Thermo Fisher Scientific, Rockford, Illinois) following the manufacturer's protocol and measured at an absorbance of 595 nm. The standard curves are made from 1:2 serial dilutions of the 2 mg/ml BSA standard provided in the kit with final concentrations ranging from 20 to 0.625 μ g/ml.

4.2. Mitochondrial malic enzyme activity assay

The assay to determine mitochondrial enzyme activity consisted of extraction and purification of intact viable mitochondria, incubation of the mitochondria in the presence of [U-¹³C]-labeled substrates, fumarate or glutamate, followed by extraction of the mitochondrial metabolites and analysis by LC/MS/MS to determine the ¹³C-enrichment of pyruvate and other Kreb's cycle intermediates. We describe the protocol for the assay in INS-1 cells; however, the methodology can be readily used for determining malic enzyme activity in the extracts of isolated islets. The method as described requires ~1.5 mg mitochondrial protein to perform the kinetic analysis using LC/MS/MS to determine the time course of ¹³C incorporation into pyruvate.

4.2.1. Isolation of mitochondria—About 48–72 h prior to isolation of the mitochondria, INS-1 cells are seeded at a density of $\sim 1.0 \times 10^7$ cells/plate into 150 cm² cell culture

dishes. Each dish yields ~0.5 mg mitochondrial protein. Two dishes are needed for the LC/MS/MS kinetic studies. The cells should be cultured in RPMI-1640 complete media with 11.1 mM D-glucose supplemented with 10% (v/v) fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M BME, 10,000 U/ml penicillin, and 10 mg/ml streptomycin. After 2-days culture in 5% CO₂ at 37 °C, the cell density should be ~90% confluent, and ready for isolation of the mitochondria.

Mitochondria are isolated under conditions to preserve function but to minimize enzymatic activity. All media, isolation buffers, centrifuge tubes, and centrifuges should be pre-cooled to between 0 and 4 °C. Fifteen milliliters of mitochondrial extraction buffer is needed for extraction of mitochondria from three 150 cm² plates (5 ml/plate). The plates are placed on ice and the growth medium is removed by aspiration. The cells in each plate are then quickly washed with 5 ml of ice-cold Phosphate Buffered Saline, PBS. The PBS is immediately removed by aspiration and replaced with 5 ml of ice-cold isotonic mitochondrial extraction buffer containing 65 mM sucrose, 215 mM D-mannitol, 5 mM HEPES, 3 mM MgCl₂, 5 mM KH₂PO₄, and 5 mM KHCO₃. The mitochondrial extraction buffer should be prepared fresh on the day of the extraction, as detailed in Table 24.2. The adherent cells are quickly scraped from the plates using a sturdy cell lifter (Corning), and the cell suspensions are transferred into one 15-ml conical centrifuge tubes for three plates. The cell suspensions are centrifuged at a low speed of ~100 rcf for 5 min at 4 °C. The supernatant is then removed by aspiration, and the cell pellet is gently resuspended in ice-cold mitochondrial buffer (1 ml/culture plate) until a homogeneous mixture is obtained. Transfer 3 ml of the cell suspension to a pre-cooled (4 °C) 5-ml glass-teflon Potter Elvehjem homogenizer, and homogenize with the vertical passing of 50 strokes between teflon pestle and glass vial/mortar. We found that to obtain a cleaner mitochondrial preparation with minimal cytosolic contamination, it is best to resuspend the cell pellet in 1 ml/culture plate, and do multiple rounds of douncing rather than to concentrate the cell pellet with less volume per culture plate. The homogenate is then split into 1.7-ml microcentrifuge tubes (1 ml/tube), and the sheered cells are centrifuged for 3.5 min at 1800 rcf at 4 °C to pellet the nucleus, sheered plasma membrane, and other cellular fragments. The supernatant, containing the mitochondria, is transferred to new 1.7-ml tube and placed on ice while additional mitochondria trapped in the pellet are harvested two more times to yield the maximum amount of mitochondria as follows. The pellet is resuspended in 0.5 ml of mitochondrial extraction buffer and centrifuged for 3.5 min at 1800 rcf at 4 °C. The supernatant, with the mitochondria, is again transferred to a new tube and kept on ice. The process is repeated once more. The supernatants from the second and third spins are combined with the first mitochondrial supernatant in a 1.7-ml tube. (The pellet containing the membrane fragments can now be discarded.) The mitochondria are then pelleted by centrifugation of all the supernatant tubes for 5 min at a higher speed of 10,800 rcf at 4 °C. Carefully aspirate the supernatant from the yellowish mitochondrial pellet. At higher speeds, you may see a white fluffy band above the pellet. If this occurs, remove the white band with the rest of the supernatant by aspiration. The mitochondrial pellet from one tube is resuspended in 1.0 ml of extraction buffer and transferred to the next tube containing the second mitochondria pellet, and then resuspended to obtain one homogeneous suspension of mitochondria. The mitochondria are pelleted with a final high-speed centrifugation for 5 min at 4 °C. Aspirate the supernatant and resuspend the pellet in 300 μ l

of the mitochondrial extraction buffer for the kinetic labeling experiments for assessment of mitochondrial malic enzyme activity. A 30- μ l aliquot of the mitochondrial suspension is taken for protein analysis by the Bradford method (Bio-Rad Laboratories, Hercules, California, reagent).

4.2.1.1. Protein Assay: Protein concentrations of the mitochondrial suspension are assayed at this point to determine the dilution needed to achieve a concentration of 1 mg protein/ml. The Bradford method (Bio-Rad protein reagent) must be used as sucrose interferes with the micro BCA assay. Protein assays are performed following manufacturers protocol measuring absorbance at 595 nm with a standard curve from 40 to 1.2 μ g/ml.

4.2.1.2. Cytochrome c assay: Preliminary experiments should be performed to assess the integrity of the mitochondria isolated from the cell lysate as described above. The cytochrome *c* oxidase assay kit (Sigma Aldrich, St. Louis, Missouri) based on cytochrome *c* oxidase enzyme (EC 1.9.3.1) assay allows for the detection of intact mitochondria by assessing outer membrane integrity via cytochrome *c*. Pure fractions of intact mitochondria are assayed following the manufacturer's protocol at 550 nm where changes in absorption of cytochrome *c* when reduced and then reoxidized determines cytochrome *c* activity. Pure mitochondrial fractions containing intact mitochondria will yield a positive change in absorption. A positive control is included in the assay kit. Negative controls include pure cytosolic fractions from the isolation and lysed mitochondria subjected to brief sonication.

4.2.1.3. Glycerol-3-P dehydrogenase assay: In order to be sure that the mitochondria fraction from the isolation is not contaminated with cytosolic components, both the cytosolic and mitochondrial fractions from the isolation were saved and assayed for α -glycerophosphate dehydrogenase (EC 1.1.1.8) activity, following the manufacturer's (Sigma Aldrich) recommendation monitoring the change in absorption at 340 nm to determine glycerol-3-P dehydrogenase activity. Pure mitochondrial fractions, free of cytosolic contamination, should have no activity as evidenced by no change in absorption. Glycerol-3-P dehydrogenase activity should only be evident in the cytosolic fraction from the supernatants saved from the high-speed spins.

4.2.2. Kinetic analysis of mitochondrial malic enzyme activity—To validate the activity of mitochondrial malic enzyme, we use electrospray tandem mass spectroscopy (LC/MS/MS) to measure the appearance of [$^{13}\text{C}_3$]pyruvate when freshly isolated mitochondria are incubated with NAD^+ , ADP^+ , and either [$^{13}\text{C}_4$]fumarate or [$^{13}\text{C}_5$]glutamate with leucine. A 96-well format with eight time points and four mitochondrial dilutions is used to obtain the time course of [$^{13}\text{C}_3$]pyruvate synthesis. In order to collect aliquots at the timed intervals, we make the following preparations.

First prepare eight identical 1.7-ml tubes prepared as listed in Table 24.3.

Next prepare two sequential 1:5 dilutions of the undiluted mitochondrial suspension (~ 1.5 mg mitochondrial protein in 300 μ l prepared from three confluent 150 cm^2 dishes) that was prepared as described above in Section 4.2.1. For the 1:5 mitochondrial dilution, 150 μ l of undiluted mitochondria plus 600 μ l mitochondrial buffer is prepared. For the 1:25

mitochondrial dilution, 150 μ l of 1:5 diluted mitochondria plus 600 ml mitochondrial buffer is prepared.

Place the eight previously prepared 1.7 ml tubes with the enzyme assay reagent mixtures (Table 24.3) in a 37 °C water bath for 3–5 min with the lids open (be sure that no water splashes in). Have a 96-well plate on ice ready and labeled so that each column corresponds to a time point and each row corresponds to the incubation tube. Add 150 μ l of 1 mM acetic acid to all 96 wells to immediately quench the reaction once an incubated aliquot is taken. Prior to adding the mitochondria, take 50 μ l from each 1.7 ml tube and place in the corresponding 0 min column of the 96-well plate. Add 50 μ l of the mitochondria to each tube, using the undiluted and each dilution sample for two replicate tubes (e.g., undiluted mitochondria in tubes 1 and 2, 1:5 diluted mitochondria in tubes 3 and 4, 1:25 diluted mitochondria in tubes 5 and 6). Immediately mix by pipetting and collect 50 μ l from each tube in the same order as mitochondria samples were added. This will be time 0.5 min and spike into appropriate well in the 96-well plate. Follow this procedure for all of the sample tubes for each of the remaining time-points: 2.5, 5, 10, 15, 30, and 60 min.

4.2.2.1. Electrospray tandem mass spectrometry for determining ^{13}C -enrichment of Kreb's cycle intermediates and pyruvate: The samples are then thawed and transferred to 96-well filter plates, the supernatant is collected by centrifugation through the filter, and analyzed by electrospray tandem mass spectrometry (LC/MS/MS) for ^{13}C -enrichment of pyruvate, malate, citrate, and glutamate. In our laboratory, LC/MS/MS analyses of metabolites are performed on an Applied Biosystems API4000 QTrap interfaced to a Shimadzu, Kyoto, Japan, HPLC (LC-20AD, SIL-20AC, CTO-20A). Metabolites were eluted from a Dionex, Sunnyvale, California, Aclaim Polar Advantage (C16, 5 μ m, 120 Å, 4.5 $\times$ 250 mm) column (40 °C) with acetonitrile:water buffered with 2 mM ammonium acetate using a linear gradient from 5% to 95% acetonitrile at a flow rate of 600 μ l/min. Metabolite concentrations and enrichments are determined by electrospray ionization monitoring of the positive product ion transition pairs of pyruvate (87.0/32.0), citrate (191.0/87.0), succinate (117.0/73.0), glutamate (146.0/128.0), and malate (133.0/71.0). Isotopic labeling is monitored with incremental increases in the masses of the parent and daughter ions to account for the ensemble of potential isotopic isomers. To monitor the synthesis rate of [U- ^{13}C]pyruvate from [U- ^{13}C]fumarate, the transition pair for m + 3 pyruvate of 90.0/32.0 is shown in Fig. 24.3.

5. Calculating Relative Rates of Anaplerotic Pathways from ^{13}C -Glutamate Isotopomer Distribution

The NMR ^{13}C -isotopomer and modeling approach that we used has the advantage that a comprehensive picture of the entry into the Kreb's cycle of multiple substrates through multiple pathways can be determined in a single experiment. The metabolism of [U- ^{13}C]glucose results in labeling of Kreb's cycle intermediates that provides an experimentally convenient means of simultaneously quantifying relative flux rates through multiple pathways from a single isotopic tracer input. From the NMR analysis of the molecular distribution of ^{13}C -label within glutamate, the contributions of glucose (through

PDH) and fatty acids (by β -oxidation) into acetyl CoA are calculated. The analysis also provides the relative flux rates of substrates entering through several anaplerotic entry points including glucose through PC, and glutamate through glutamate dehydrogenase (GDH) when glutamine plus leucine are supplied as secretagogues (Fig. 24.4).

Metabolism of [U- ^{13}C]glucose results in labeling of Kreb's cycle intermediates that are determined by relative rates of PC and PDH, as well as the entry of other metabolites into the cycle. PDH flux initially labels both Glu 4 and 5 (Glu4 + 5). Further turns of the Kreb's cycle randomize the doubly labeled isotopomer to either Glu4 + 5 or Glu1 + 2. When PDH is the only entry point for Kreb's cycle flux, the mixture of glutamate iso-topomers quickly reaches a steady-state distribution of glutamate labeled in all five carbons. In contrast, PC flux of [U- ^{13}C]pyruvate (with unlabeled acetyl-CoA), initially labels Glu2 + 3 and Glu1 + 2 + 3. At steady-state, ~70% of the isotopomers consist of Glu1 + 2, Glu2 + 3, and Glu1 + 2 + 3. Because of these differences in steady-state isotopomer distributions, the proportion of PC to PDH flux can be readily determined from the glutamate isotopomer distribution. Furthermore, the isotopomer distribution also provides a measure of the pathways and relative flux rates of unlabeled substrates (fat, other three carbon precursors, or GDH) contributing to Kreb's cycle flux. The relative rates of labeled and unlabeled substrates are calculated as the product of the precursor enrichment and the relative rates for each step as determined by the program "tcacalc" (Malloy *et al.*, 1990).

5.1. ^{13}C -labeling of cells for isotopomer studies of anaplerotic pathways

INS-1 cells (+/- siRNA KD of malic enzyme), at 80% confluence, are pre-incubated for 2 h at low glucose (2 mM) in KRB to establish basal conditions. The cells are then washed to remove unlabeled glucose, and then incubated for 2 h in DMEM, or KRB, with [U- ^{13}C]glucose (Cambridge Isotope Laboratories, Miamisburg, Ohio, 99% ^{13}C , 2.5 mM or 15 mM), alone or supplemented with 4 mM glutamine and 10 mM leucine (singly or in combination). At the end of the 2 h isotopic labeling period, the media is removed and frozen for later analysis. The monolayer of cells is quenched with ice-cold water, quickly scraped with a cell lifter, and transferred to a 50 ml conical tube with a 200 ml aliquot saved for protein and insulin assays. The remainder of the supernatant (and media) is frozen on dry ice, lyophilized, and redissolved in 400 μl D $_2$ O for NMR analysis.

5.2. ^{13}C NMR analysis of the ^{13}C isotopic isomers of glutamate

The ^{13}C positional isotopomer distribution of glutamate is determined by ^{13}C NMR spectroscopy. In our laboratory, we used an AVANCE 500-MHz spectrometer (Bruker Instruments, Inc. Billerica, Massachusetts). Spectra were acquired with TR = 0.5 s, NS = 10,000, 16 K data, and Waltz-16 broadband proton decoupling. Correction factors for differences in T_1 relaxation times should be determined from fully relaxed spectra of standard solutions (Cline *et al.*, 2004).

An example of the ^{13}C NMR spectrum of glutamate isotopomers that are generated by INS-1 cells after incubation with 15 mM [U- $^{13}\text{C}_6$]glucose in KRB is shown in Fig. 24.5. The isotopic isomers are best identified from the ^{13}C - ^{13}C J -coupling constants, and quantified

by integration of line fitting (NUTS, Acorn, Inc) to the glutamate resonances at C2 (~55 ppm), C3 (~28 ppm), and C4 (~34 ppm).

5.2.1. Analysis of glutamate 3—The ^{13}C isotopic isomers determined from analysis of C3 are glutamate triply labeled at C2–C3–C4 (triplet), glutamate doubly labeled at C2–C3 or C3–C4 (doublet). The center resonance of C3 is the sum of the C2–C3–C4 isotopomer, and glutamate labeled at C3, but not C2 or C3. The triplet at C3 is due to the similarity of the coupling constants of 35 Hz for both C2–C3 and C3–C4.

5.2.2. Analysis of glutamate 4—In contrast to the triplet seen at glutamate C3 (for C2–C3–C4), when glutamate is triply labeled at C3–C4–C5, the differences in the coupling constants of 35 Hz for C3–C4, and 51 Hz for C4–C5, results in a quartet. From the coupling constant of 51 Hz, it can be determined that only the doubly labeled isotopomer, glutamate C4–C5, is present. The doubly labeled isotopomer, glutamate C3–C4 ($J = 35$ Hz) is undetectable in this spectrum.

5.2.3. Analysis of glutamate 2—Glutamate 2 is interpreted in the same way as glutamate 4. In contrast to the spectrum of glutamate 4, both of the doubly labeled isotopomers C2–C3 ($J = 35$ Hz), and C1–C2 ($J = 50$ Hz) can be observed.

5.3. Calculation of metabolic fluxes with tcacalc

Steady-state flux rates, relative to Krebs's cycle flux, are calculated from the isotopomers of glutamate observed at carbon positions 2, 3, and 4 in the ^{13}C NMR spectra, and modeled using the program tcacalc (Malloy *et al.*, 1990), and is available for download from the UTSW Medical Center Rogers NMR Center (<http://www4.utsouthwestern.edu/rogersnmr/software.htm>).

Using the nomenclature adopted for tcacalc (Fig. 24.4), the model provides the best fit to our NMR data by calculating the relative fluxes of acetyl-CoA synthetase (ACS), lactate dehydrogenase (LDH), pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC), pyruvate kinase (PK), anaplerosis leading to succinyl-CoA (YS), and enrichments of “lactate” and “fat.” Fat includes all metabolite inputs into acetyl-CoA other than glucose (e.g., leucine). All flux rates are referenced to a citrate synthase (CS) flux of 1, and is equivalent to Krebs's cycle flux. CS flux is modeled as the sum of PDH flux of labeled and unlabeled pyruvate, and of ACS flux, as presented in Fig. 24.4. YS represents unidirectional anaplerotic flux of substrates that lead to succinyl-CoA. As originally designed, YS represents flux of propionate into the Krebs's cycle. In our experiments with glutamine plus leucine as the secretagogues, YS represents the rate of synthesis of α -ketoglutarate from glutamate (Figs. 24.1 and 24.4: GDH).

An example input file with initial parameters and sequential addition of the pathways of pdh, pk, and ys, used to calculate fluxes is as follows:

```
fat12      0.5  0.1
LAC123    0.5  0.1
YPC       0.7  0.2
```


Add pdh 0.3 0.2
 Add pk 0.3 0.2
 Add ys 0.5 0.1

An example of a data file with fractional isotopomer distribution determined from the ^{13}C NMR spectrum of INS-1 cells incubated for 2 h in KRB with 15 mM glucose is

CO Created using tcasim and altered to have 3% error

CO FAT2 0.30 FAT12 0.40 YS 0.05 AS3 0.50 CYCLES 35.00

GLU2S 0.039
 GLU2D23 0.333
 GLU2D12 0.096
 GLU2Q 0.531
 GLU3S 0.037
 GLU3D 0.232
 GLU3T 0.730
 GLU4S 0.000
 GLU4D34 0.000
 GLU4D45 0.319
 GLU4Q 0.680

Define C3C4 GLU3F/GLU4F 0.804

Define C2C4 GLU2F/GLU4F 0.753

The above input file represents the fractional area for the multiplets at each of carbon resonances.

Using these inputs, tcacalc calculated the following fluxes:

ACS 0.857
 Fat0 0.123
 Fat12 0.877
 LDH 0.593
 Lac0 0.008
 Lac123 0.992
 PDH 0.143
 YPC 1.230
 YS 0.379
 ASO 1.000
 PK 0.780

From which we calculated the following Kreb's cycle parameters:

Relative flux rates: Acetyl-CoA entry: ACS + PDH = 1.00

Relative flux rates: Anaplerotic entry: YPC + YS = 1.61

Acetyl-CoA from [U-¹³C]glucose: $ACS \times Fat12 + PDH \times Lac123 = 0.89$

Acetyl-CoA from other sources: $ACS \times Fat0 + PDH \times Lac0 = 0.11$

The relative rates of labeled and unlabeled substrates can be calculated as the product of the precursor enrichment and the relative rates.

Anaplerotic flux from [U-¹³C]glc: $YPC \times Lac123 = 1.22$

Anaplerotic flux from unlabeled pyr: $YPC \times Lac0 = 0.01$

Anaplerotic flux from glutamate: $YS \times ASO = 0.38$

The percent anaplerosis to total Kreb's cycle flux can be calculated as:

$$\%Anaplerosis: 100 \times (PC+YS) / (PC+YS+PDH+ACS) = 62\%$$

Although open to interpretation, Lu *et al.* (2002) proposed that an index of pyruvate cycling can be calculated as the average of the flux rates of PC and of PK.

Pyruvate cycling: $(PC + PK)/2 = 1.01$.

6. Discussion

The pancreatic islet β -cell is uniquely specialized to tightly couple its metabolism and rates of insulin secretion with the levels of circulating nutrient fuels. Unlike other metabolically responsive cells, such as myocytes and hepatocytes, β -cells cannot accommodate increased glycolytic flux by storing the excess glucose as glycogen or eliminate it as lactate. Without an increase in energy demand that matches the increased glycolytic flux, feedback inhibition would limit the ability of the metabolic responsiveness of the cell. Substrate cycling, mediated by malic enzyme, provides a mechanism to allow glycolytic and mitochondrial fluxes to increase in direct proportion to circulating concentrations of glucose and other fuel secretagogues. We have used tcacalc to evaluate changes in the flux through glycolytic and Kreb's cycle pathways that are believed to play a role in the coupling of metabolism with insulin exocytosis. It should be noted though, that tcacalc represents a simplified model of metabolism within the β -cell, and has limited adaptability to incorporate the numerous intertwined pathways and substrate cycles that serve to modulate nutrient-stimulated insulin secretion. A pressing need exists to develop modeling programs that have the flexibility to include new pathways as they are revealed, and to be able to incorporate the wealth of data available from both NMR isotopomer and mass spectrometric isotopolog analysis. For example, substrate cycling of the anaplerotic metabolites malate and citrate is modeled by tcacalc based on the assumption that the average of unidirectional fluxes through PK and PC into the Kreb's cycle is a valid approach for calculating a cycling flux mediated by malic enzyme. Our use of siRNA to alter the activity of malic enzyme was undertaken to directly test the validity of this assumption, and the inference that the rate of insulin secretion was influenced by malate/citrate/pyruvate cycling, as opposed to PC flux *per se*. Our approach rests on the premise that malic enzyme exerts measurable control over the rate of these

substrate cycles, and that this cycle is tied to the regulation of insulin secretion. Our results, as well as others, indicate that both the cytosolic and mitochondrial isoforms of malic enzyme exert metabolic control over the rate of pyruvate cycling in the rat insulinoma INS-1 832/13 cell line. However, recent results suggest that this may not be true in the context of the rat or mouse islets. These seemingly contradictory results highlight the need for a rigorous study of pyruvate cycling using metabolic control analysis. A low control coefficient for malic enzyme would result in little observable effect unless the activity of malic enzyme was virtually eliminated. In most cases, siRNA KD of malic enzyme is not absolute and a significant level of enzyme activity remains. Thus, one would observe little effect upon glucose-stimulated insulin secretion, despite the necessary involvement of malic enzyme and malate/pyruvate cycling in the coupling of metabolism with secretion. In conclusion, the redundancy of several complementary substrate cycles, the variance in their degree of metabolic control, and the species-dependency of expression and activity of malic enzyme in islet β -cells, makes it imperative to evaluate the role of malic enzyme in fuel-stimulated insulin secretion in human islet β -cells.

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Figure 24.1.

Anaplerotic substrate cycling pathways of insulin secreting cells: insulinoma INS-1 cells and pancreatic islet β -cells. Pyruvate cycling can occur via several redundant and complementary pathways. Cytosolic malic enzyme cycles malate derived from the export of mitochondrial malate, or derived indirectly from the exported citrate that is converted to oxaloacetate (OAA) by ATP:citrate lyase (ACL), and then malate by malate dehydrogenase. Pyruvate cycling can also occur entirely within the mitochondria by the conversion of mitochondrial malate into mitochondrial pyruvate by malic enzyme 2 (ME2). An alternative cycling pathway exists for return of citrate to α -ketoglutarate (α -KG) via the conversion of exported citrate to isocitrate and then α -KG. Each of these pathways can be considered a mechanism for the transfer of mitochondrial reducing equivalents to the cytosol in the form of NADPH.

Figure 24.2.

Malic enzyme isoform mRNA expression in INS-1 832/13 cells, and in isolated rat islets. INS-1 832/13 cells were cultured until they reached 80–90% confluence. 100–150 islets were picked after a 24 hour recovery in culture media. RNA was extracted in RNA extraction lysis buffer and isolated from the cells and islets. Purified RNA was reverse transcribed to cDNA and expression of the malic enzyme was determined using RTqPCR technology. mRNA expression was determined using $\Delta\Delta CT$ analysis where expression of each malic enzyme isoform was normalized to β -actin then compared of the expression of cytosolic ME1 where expression levels of the mitochondrial isoforms ME2 and ME3 are shown relative to ME1. Data are mean $\pm$ S.E.

Figure 24.3.

Time course of synthesis of $[\text{U}-^{13}\text{C}]$ pyruvate by mitochondria isolated from INS-1 cells overexpressing a dominant-negative HNF-1 α mutation (filled squares), and control INS-1 cells (filled diamonds). $[\text{U}-^{13}\text{C}]$ pyruvate are determined by LC/MS/MS analysis; integrated intensity are normalized to mitochondrial protein. Mitochondria were incubated with 2.5 mM $[\text{C}_4]$ fumarate and 0.5 mM ADP and GDP in iso-osmotic buffer as described in the text and Table 24.3.

Figure 24.4.

Model of Krebs' cycle used to determine relative flux rates through acetyl-CoA synthetase (ACS), pyruvate dehydrogenase complex (PDH), pyruvate carboxylase (PC), and glutamate dehydrogenase (GDH). Labeled substrate originates from exogenous $[U-^{13}C_6]$ glucose. Unlabeled substrates originate from exogenous glutamine, leucine, and from endogenous sources feeding the substrate pools designated as "fat", and "other pyruvate". Malic enzyme (ME) provides one possible pathway for cycling of anaplerotic flux of pyruvate via PC. Anaplerotic entry of glutamate (from glutamine) is at the level of α -ketoglutarate (α -KG) by the action of GDH, calculated as YS by "tcacalc". Acetyl-CoA is derived from either fat via ACS, or pyruvate via the PDH. Pyruvate can be derived from $[U-^{13}C]$ glucose (labeled pyr: filled circles), or from endogenous or cycling via Malic enzyme (unlabeled pyr: open circles). All fluxes are normalized by "tcacalc" to citrate synthase (CS) flux.

Figure 24.5.

Proton-decoupled ^{13}C -NMR spectrum of glutamate carbons 2, 3, and 4. Extracts of $\sim 30 \times 10^6$ cells were prepared after a 2 hour pre-incubation in KRB buffer containing 2 mM glucose, followed by 2 hour incubation at 37 °C in KRB with 15 mM $[\text{U-}^{13}\text{C}_6]$ glucose. Isotopic isomers are identified from the ^{13}C - ^{13}C $^1J_{\text{CC}}$ coupling constants as indicated above each carbon resonance, and the integrated areas for each isotopomer were determined by peak fitting for metabolic pathway modeling.

Table 24.1
Enzyme assay reagent mixture for malic enzyme 1

Substance	Stockconc. (mM)	Volume of stock (ml)	Final conc. (mM) in mix
Tris/HCl	250	10	155
MnCl ₂	50	0.5	1.55
NH ₄ Cl	40	0.625	1.55
KCl	1000	2.5	155
NADP ⁺	20	1.25	1.55

Table 24.2
Mitochondrial Iso-osmotic buffer (2×)

Concentration	Substance	Molecular weight	200 mL of 2× stock
65 mM	Sucrose	342.3	8.90 g
215 mM	D-Mannitol	182.18	15.66 g
5 mM	HEPES	238.31	0.238 g
3 mM	MgCl ₂	203.30	0.122 g
5 mM	KH ₂ PO ₄	136.09	0.136 g

The 2× buffer can be stored up to one month at 4 °C.

For 100 ml of 1X Mitochondria buffer made fresh on the day of isolation:
 Add 0.05 g KHCO₃ to 50 ml of 2× solution

1. Adjust the pH to 7.4 with 30% KOH (very little needed)
2. Add water to total final volume of 100 ml
3. Filter sterilize through 0.22 micron filter

Table 24.3
Enzyme assay reagent mixture for malic enzyme 2

Solutions	Quantity(μ l) Total volume: 500 μ l	Final concentration
Mitochondrial Iso-osmotic buffer (2 $\times$): from Table 24.2	250	1X
ADP	2.5 of 100 mM	0.5 mM
GDP	2.5 of 100 mM	0.5 mM
[$^{13}\text{C}_4$]Fumarate $\star$	12.5 of 100 mM	2.5 mM
MnCl ₂	50 of 10 mM	1 mM
H ₂ O	182.5	
[$^{13}\text{C}_5$]Glutamate plus leucine	12.5 of mixture of 100 mM ^{13}C -gln and 200 mM leu	2.5 mM ^{13}C -gln, 5 mM leu
Prepare 8–1.7 ml reaction tubes with the above reagents for the kinetic analysis of formation of ^{13}C -pyruvate by mitochondrial malic enzyme 2; determined by LC/MS/MS.		

$\star$ Alternatively to [$^{13}\text{C}_4$]Fumarate, use a mixture of [$^{13}\text{C}_5$]Glutamate and leucine, as below.