Role for malic enzyme, pyruvate carboxylation and mitochondrial malate import in glucose-stimulated insulin secretion.

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Running head: Mitochondrial malate import in pancreatic beta cells

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Pyruvate cycling has been implicated in glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells. The operation of some pyruvate cycling pathways is proposed to necessitate malate export from the mitochondria and NADP+-dependent decarboxylation of malate to pyruvate by cytosolic malic enzyme (ME1). Evidence in favor of and against role of ME1 in GSIS has been presented by others using si-RNA-mediated suppression of ME1. ME1 was also proposed to account for methyl succinate (MS)-stimulated insulin secretion (MSSIS), hypothesized to take place via succinate entry to the mitochondria in exchange for malate, and subsequent malate conversion to pyruvate.

In contrast to rat, mouse β-cells are lacking ME1 activity, which was suggested to explain their lack of MSSIS. However this hypothesis was not tested. In this report, we demonstrate that while adenoviral-mediated over-expression of ME1 greatly augments GSIS in INS-1 832/13 cells, it does not restore MSSIS or significantly affect GSIS in pancreatic islets.

ME1 over-expression augments anaplerosis and GSIS in INS-1 832/13 cells, it is not likely involved in MSSIS and GSIS in pancreatic islets.

Insulin secretion from pancreatic β-cells is dependent upon the metabolism of secretory fuels. Apart from oxidative pathways leading to the rise in the ATP/ADP ratio and resulting in the K_ATP channel-dependent sequence of events (18; 34), it has been proposed that efflux of TCA cycle intermediates out of the mitochondria (9; 26; 28) leads to the synthesis of other coupling factors which couple β-cell metabolism with insulin secretion. Candidates for these coupling factors include NADPH, short-chain acyl-CoAs (SC-CoA), glutamate, long-chain acyl-CoAs (LC-CoA), and malonyl-CoA, however, evidence against malonyl-CoA and glutamate has been presented (1; 29; 36).

The efflux of TCA cycle intermediates is contingent upon the anaplerotic influx of pyruvate to the TCA cycle. Conversion of these TCA cycle intermediates back to pyruvate in the cytosol occurs via pyruvate cycling pathways. Pyruvate cycling has been implicated as an important component of glucose-mediated insulin secretion. According to the proposed model, pyruvate cycling can occur via the action of NADP+-dependent cytosolic malic enzyme (ME1), which enables the pyruvate/malate (26) and pyruvate/citrate (9) pathways. It can also take place via the action of cytosolic isocitrate.
dehydrogenase (ICDc), which enables the pyruvate/isocitrate pathway (41). In the malate/pyruvate pathway, malate is exported from the mitochondria to the cytosol and converted directly to pyruvate by ME1. In the pyruvate/citrate pathway, citrate is exported from the mitochondria to the cytosol, where malate is produced via aconitase, citrate lyase and malate dehydrogenase reactions, and then converted by ME1 to pyruvate. In the pyruvate/isocitrate pathway, isocitrate is exported from the mitochondria into the cytosol, and can be oxidized to \( \alpha \)-ketoglutarate (\( \alpha \)-KG) by ICDc, which then can enter mitochondria and be converted to malate via TCA cycle reactions.

A series of reports support the role of pyruvate cycling in glucose-stimulated insulin secretion (GSIS) by demonstrating that the level of GSIS correlates with an increase in pyruvate cycling (24), while inhibition of the pyruvate/isocitrate shuttle, via inhibition of either the ICDc or the citrate/isocitrate carrier, impairs GSIS (21; 41). Inhibition of the pyruvate/malate and pyruvate/citrate shuttles, via inhibition of ME1, also impairs GSIS (13; 39), while potentiation of GSIS by the membrane-permeable malate analog dimethyl-malate (DMM), has been proposed to take place via activation of pyruvate cycling (5). However, the role of ME1 in GSIS has been disputed in a recent report which showed that siRNA-mediated inhibition of ME1 does not affect GSIS in rat islets (44).

While all three pathways are operative in rat \( \beta \)-cells, only the isocitrate pathway operates in mouse \( \beta \)-cells, likely due to the absence or relatively low level of NADP⁺-dependent malic enzyme activity (2; 17; 27). Absence of the malate/pyruvate cycle in mouse islets was suggested to account for their weak second phase of GSIS (39) and their inability to secrete insulin in response to methyl-succinate (MS, a membrane permeable succinate analog) (27). It has been suggested that MS-stimulated insulin secretion (MSSIS) is contingent upon the presence of ME1 activity (27), in such a way that succinate import to the mitochondria is balanced by export of malate, and subsequent conversion of malate to pyruvate via ME1 in the cytosol. However, this hypothesis has not been tested.

This study was undertaken to determine the mechanism by which ME1 activity enhances insulin secretion and to test the hypothesis that the response to MS is contingent upon the presence of ME1 (27). Our data demonstrate that over-expression of ME1 potentiates GSIS in rat insulinoma INS-1 832/13 cells, and this effect is accompanied by an increase in mitochondrial malate and citrate levels, while the ATP/ADP ratio was not affected. However, introduction of ME1 activity to mouse pancreatic \( \beta \)-cells did not significantly augment GSIS, neither did it restore the secretory response to MS as previously hypothesized (27). These data suggest that while ME1 regulates metabolism and GSIS in INS1-832/13 cells, it is not directly involved in MSSIS or GSIS in islets.

**MATERIALS AND METHODS**

**Cell preparation and culture.** Clonal INS-1 832/13 cells and MIN-6 cells were provided by Drs. Christopher Newgard (Duke University) and Jun-ichi Miyazaki (Osaka University, Japan), and were maintained and cultured as described previously (19; 35). Male CD-1 mice or Sprague-Dawley rats (Charles River) were euthanized by halothane. All procedures were performed in accordance with the Institutional Guidelines for Animal Care in compliance with United States Public Health Service regulations. Pancreatic islets were isolated by collagenase (Roche, Indianapolis, IN) digestion (16). Islets were either used immediately after isolation for viral transduction, or after an overnight culture in RPMI supplemented with 10% fetal calf serum (Hyclone), penicillin/streptomycin and 5 mM glucose, and were dispersed by incubation in Ca²⁺/Mg²⁺ free PBS, 3 mM EGTA and 0.002% trypsin as described (16). Islet cells were plated on poly-D-lysine coated coverslips (MatTek, Ashland, MA) in 35 mm Petri dishes (Ca²⁺ studies, immunocytochemistry). INS-1 832/13 cells (at 60-70% confluency), single mouse islet cells and whole mouse islets were transduced with Ad-ME1-GFP or Ad-CV-GFP at 50 MOI (multiplicity of infection) for 12 hrs, after which viral media was replaced with appropriate growth media. Insulin secretion and Ca²⁺ responses were determined 48 hours after transduction. Transduction efficiency in single cells, determined from GFP fluorescence, reached more than 90% under these conditions. Islets and single cells were used 48 hours post-transduction. ME1 expression was monitored by GFP fluorescence and
measurement of ME1 mRNA and protein level, as well as ME1 enzymatic activity.

**Purification of ME1.** INS-1 832/13 cells, at 80% confluency, were fractionated using a cytosol fractionation kit (Calbiochem) according to the manufacturer’s protocol. Cytosolic fractions were further purified by NADPH affinity chromatography using the substrate activation method (47; 49). Briefly, 2',5'-ADP-agarose was added (0.2 ml volume of settled resin per volume of cytosol) and the suspension was rocked for 1 h at 4°C and then centrifuged at 2000 x g for 5 min. The supernatant was discarded, and the resin was washed five times with phosphate-buffered saline (PBS). ME1 was eluted by washing the resin with PBS containing 0.1% NP-40 and 5 mM NADPH (12). To remove NADPH from the eluate, eluate was first concentrated using an Amicon Ultra centrifugal filter 50 (Millipore, Billerica, MA). Resulting retentate was reconstituted to the original volume with PBS, and this process was repeated 6 times. Under these conditions, the concentration of NADPH in the resulting sample was less than 10 nM, as determined spectrophotometrically.

**Malic enzyme activity.** To determine the rate of reductive decarboxylation of malate, protein extracts (20 µg) or NADPH standard (1-100 nmol) were added to the reaction buffer (50 mM HEPES, pH 7.4, 5 mM MnCl₂, 50 µM NADP⁺, 10 mM malate) in a cuvette positioned inside a temperature-controlled chamber maintained at 37°C. The cuvette content was stirred to assure proper mixing. Fluorescence was measured at 340 nm excitation and 460 nm emission on a FluoroMax-3 fluorescence spectrophotometer (Horiba Jobin Yvon). A calibration curve was generated using known amounts of NADPH (the product of malate reductive decarboxylation) in the reaction buffer (17). To determine the rate of NADPH oxidation via oxidative carboxylation of pyruvate, protein extracts (20 µg) were added to the reaction buffer containing 50 mM HEPES, pH 7.4, 50 µM NADPH, 10 mM pyruvate, 30 mM NaHCO₃, and 5 mM MnCl₂. To correct for the chemical instability of NADPH, the rate of enzymatic oxidation of NADPH was calculated as the difference between the rates of total NADPH oxidation and non-enzymatic NADPH oxidation, which were determined in the presence of enzyme source or vehicle (PBS), respectively.

**Construction of adenoviruses.** Recombinant, replication-deficient type 5 adenoviruses expressing either cytosolic firefly luciferase (Ad-CytoLuc) or the human cytosolic malic enzyme from OriGene (Ad-ME1-GFP) were either directly purchased from (Ad-CytoLuc) or custom-constructed (Ad-ME1-GFP) by Vector BioLabs (Philadelphia, PA). The expression of ME1 is under the control of CMV, which also directs the transcription of GFP from an internal ribosome entry site. A control virus (Ad-CV-GFP) was constructed in parallel. Viral titers were determined by the plaque formation assay.

**Immunocytochemistry.** At 48 hours post-transduction, cells were washed twice with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100 for 15 mins and probed with an mouse anti-human ME1 antibody (1:500, Abnova, Taiwan) and rabbit anti-mouse insulin antibody (1:200, Immunostar, WI) in 10% normal goat serum blocking solution for 1 hr (Zymed Labs, CA). A negative control contained only blocking serum. The primary antibodies were probed with Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 647 goat anti-rabbit IgG (1:1000, Invitrogen) for 1 hr. Cells were co-stained with the nuclear DNA marker DAPI (300 nM, Invitrogen) before imaging. Cells were imaged using a laser scanning confocal microscope (Zeiss LSM 510 Meta) with a 63X oil immersion objective (NA = 1.4; z-resolution ≈ 0.7 µm). Fluorescence images of x-y sections were recorded with 512 pixels per line. Raster point size was 60 nm with an overall lateral resolution of 0.2 µm. GFP, Alexa Fluor 546 and Alexa Fluor 647 were excited at the 488, 543 and 633 nm laser lines, respectively, with emission collected through a 488/543/633 nm dichroic mirror. DAPI was imaged with two-photon excitation (Ultrafast Ti:Sapphire; Coherent) tuned to 720 nm and emission was collected through a 700/488 dichroic mirror. Digital image overlay was performed using Adobe Photoshop (version 6; Adobe Systems).

**Ca²⁺ measurement.** Cells were loaded for 60 min with the Ca²⁺ indicator Fura Red-AM (Molecular Probes, Eugene, OR) in the presence of 0.2% Pluronic F127 in the growth medium. Cells were washed in KRB (5 mM glucose, 140 mM NaCl, 30 mM HEPES, 4.6 mM KCl, 1 mM MgSO₄, 0.15 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 2 mM CaCl₂ and 0.05 % BSA, pH
7.4) and imaged on a laser scanning confocal microscope (Zeiss LSM 510 Meta) equipped with a heated stage. Fura Red was excited using a 488 Argon laser line and emitted light was collected with a 650 long pass filter. At these settings no contamination from GFP was detected. Images were analyzed by the LSM Image Browser software to derive Ca²⁺ profiles. After the experiment, confocal dishes were fixed with 4% paraformaldehyde and β-cells were identified using rabbit anti-mouse insulin antibody (1:200, Immunostar), probed with Alexa Fluor 647 goat anti-rabbit IgG (1:1000, Invitrogen). Only insulin positive cells were included in the analysis.

**ATP levels.** Changes in cytosolic ATP levels ([ATP]c) were measured after cell infection with adenovirus carrying cytosolically targeted luciferase (Ad-CytoLuc, Vector Biolabs) using the luciferin/luciferase reaction (33). Coverslips with cells, mounted inside a 35 mm dish (MatTek, Ashland, MA) were placed directly onto the surface of the photocathode optical window of the Hamamatsu R464 photomultiplier tube housed in a 37°C heated box. After application of 100 µM luciferin, and allowing for the signal to reach a steady-state level, changes in the concentration of ATP in response to fuel addition were determined by measuring luminescence.

**Oxygen Consumption.** Oxygen consumption in single islets in response to fuel additions was measured by the self-referencing method based on an electrochemical oxygen sensor moving between the “near” and “far” position from the islet (48). The magnitude of the amperometric current used for the reduction of oxygen is proportional to the oxygen concentration at that particular point.

**Insulin secretion.** Cells were preincubated for 2 hr in the presence of 2 mM (INS-1 832/13 cells) or 4 mM (mouse islets) glucose in KRB buffer. The amount of released insulin was determined after 30 min of static incubation in the presence of secretory fuels using a radioimmunoassay kit (Linco Research, St. Charles, MO) with rat insulin as the standard. Data were normalized for protein content determined by the Micro-BCA Protein Assay kit (Pierce, Rockford, IL).

**LC/MS/MS analysis of metabolites.** Following 30 min incubation, cells were either collected by centrifugation or subjected to rapid fractionation to obtain mitochondria, using a fractionation kit (BioVision, CA). The pellets were suspended in 200 µl ice-cold acetonitrile:water (1:1) with 2 mM ammonium acetate and 10 µM d4-taurine, disrupted by sonication, and centrifuged. The supernatant was analyzed by LC/MS/MS. Metabolite levels were calculated from standard curves, with d4-taurine as the internal standard, and were normalized to DNA concentration. LC/MS/MS analysis of metabolites was performed on an Applied Biosystems API4000 QTrap interfaced to a Shimadzu HPLC (LC-20AD, SIL-20AC, CTO-20A). Metabolites were eluted from a Dionex Aclaim Polar Advantage (C16, 5 µm, 120 Å, 4.5 x 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 µl/min. Metabolite concentrations were determined by electrospray ionization monitoring of the positive product ion transition pairs of ATP (506.0/158.9), ADP (426.0/158.9), citrate (191.0/87.0), malate (133.0/71.0), and d4-taurine (127.9/80.0).

**Glucose oxidation.** Groups of 30 islets or 250,000 INS-1 832/13 cells were incubated in a 0.6 ml Eppendorf tube without a cap in the presence of 2 or 10 mM glucose and D-[U-14C]-glucose (PerkinElmer, 250 mCi/mmol). Eppendorf tube was placed upright in an airtight-sealed 20 ml scintillation vial, which contained an empty 1.5 ml Eppendorf tube without a cap. After 90 min of incubation at 37°C with agitation, reaction was terminated by the injection of 10 µM rotenone, 10 µM antimycin and 1 mg KCN. To the empty 1.5 ml tube in the scintillation vial, 500 µl of 5% KOH was injected. Following a 60 min incubation at room temperature, glucose oxidation was determined by measuring the KOH-trapped 14CO₂.

**Statistical analysis.** Data are expressed as means ± SE. Significance was determined for multiple comparisons using one-way analysis of variance (ANOVA); a P-value of <0.05 was considered significant.

**RESULTS**

**Kinetic characteristics of ME1.** To obtain a large uniform supply of highly concentrated enzyme needed for kinetic studies, and to avoid possible contamination of the source with other cytosolic enzymes, ME1 was purified from INS-1 832/13 cells. Enzyme activity following
individual purification steps are listed in Table 1. Purification resulted in an approximately 2000-fold increase in specific activity measured by both oxidative carboxylation of pyruvate and reductive decarboxylation of malate. In the final fraction, levels of oxidative carboxylation of pyruvate reached about 85% of the reductive decarboxylation of malate. \( K_m \) values determined at pH 7.4 for malate and pyruvate were 240 µM and 5.8 mM, respectively, in agreement with a previous report on ME1 from liver and skeletal muscle (52).

**Effect of ME1 over-expression on ME1 activity, GSIS, ATP/ADP ratio and malate and citrate levels.** Adenoviral-mediated expression of ME1 cDNA in INS-1 832/13 cells resulted in a significant increase in NADP+-dependent malic enzyme activity (from 8.1±2 to 115±22 µmol NADPH/mg cytosol protein/min). Similar treatment of mouse islets led to the introduction of protein-soluble NADP+-dependent malic enzyme activity (from non-detectable levels to 123±23 µmol NADPH/mg cytosol protein/min). Immunocytochemical detection of transduced ME1 protein is shown in Fig. 1. In INS-1 832/13 cells, ME1 overexpression significantly potentiated glucose- and methyl-pyruvate (MP)-, but not methyl-succinate (MS)-, mediated secretion (Fig. 2A). KCl-induced secretion remained unchanged, suggesting that a metabolic rather than ionic mechanism underlies the increase in secretion. The fuel-dependent increase in intracellular ATP level was not changed in ME1 over-expressing cells compared to controls, as determined by the measurements from the luciferin/luciferase reaction in populations of INS-1 832/13 cells co-expressing cytosolically targeted luciferase and ME1 or control (Fig. 2B). Similarly, the ratio of ATP/ADP as well as the rate of glucose oxidation (Table 2) were not affected by ME1 over-expression. ME1 over-expression resulted in increased glucose-dependent rise in malate and citrate levels (Fig. 2C and 2D) in INS-1 832/13 cells. Introduction of ME1 activity in mouse islets altered GSIS to a lesser degree than in INS-1 832/13 cells (Fig. 3A). As in INS-1 832/13 cells, ATP levels remained unaffected (Fig. 3B).

Malate is a component of the malate-pyruvate pathway, and its membrane permeable analog dimethylmalate (DMM) has been proposed to counteract lipid-induced impairment of GSIS by enhancing pyruvate cycling pathways in INS-1 832/13 cells (5). We have confirmed that DMM nearly doubles GSIS in INS-1 832/13 cells (Fig. 2A), which effect is accompanied by increase in malate and citrate levels (Fig. 2C and 2D). However, we have also demonstrated that DMM nearly doubles GSIS in isolated mouse islets (Figure 3A), where the malate/pyruvate shuttle is not operative due to the absence of ME1. To clarify the mechanism by which DMM potentiates GSIS, the effect of DMM on GSIS was tested in the INS-1 832/13 cells and mouse islets over-expressing ME1. ME1 over-expression removed the potentiating effect of DMM on glucose-mediated secretion in both cell preparations, suggesting that activation of the malate/pyruvate shuttle by malate does not directly underlie the mechanism of DMM action (Fig. 2A and 3A). In accordance with these findings, mitochondrial malate levels were increased in INS-1 832/13 cells following DMM treatment, together with increased citrate levels. The DMM-dependent increase in malate and citrate content was, however, removed following ME1 over-expression (Fig. 2C and 2D).

**Effect of ME1 on MSSIS and cytosolic Ca\(_{2+}\).** It has been hypothesized (27) that in contrast to rat islets, the lack of ME1 activity in mouse islets may explain the absence of MSSIS in mouse islets. Contrary to this hypothesis, MSSIS was not restored in whole mouse islets following ME1 expression (Fig. 3A). A group of islets infected with Ad-ME1-GFP is shown in Fig. 1B, to demonstrate that infection with adenovirus was not limited to the outer cell layer of islets. In a parallel experiment, freshly isolated mouse islets were dispersed, yielding a population of single islet cells. After overnight culture in PDL-coated culture vessels, single islet cells were infected with Ad-ME1-GFP. This approach assured that more than 95% of cells were infected. However, this approach did not change the outcome: while 10 mM glucose stimulated secretion at 289±32 % of basal secretion, MS did not elicit secretion above the basal level (98±16 % of basal, where basal secretion was 18.4±2.3 ng insulin/mg protein/hr). A rise in the cytosolic Ca\(_{2+}\) accompanies insulin secretion, and has been shown to take place during MS stimulation in rat islet cells (17). The inability of ME1-transduced mouse β-islet cells to respond...
to MS was further defined by determining the Ca\(^{2+}\) response in single mouse islets cells. Since GFP was co-expressed in cells expressing ME1, the Ca\(^{2+}\) indicator Fura Red (far red emission) was used to avoid cross-contamination with the GFP signal (14). In contrast to other Ca\(^{2+}\) indicators such as Fluo-4, the intensity of Fura Red decreases upon binding to Ca\(^{2+}\) (15). Thus, an increase in the Ca\(^{2+}\) levels will be paralleled by a decrease in the Fura Red signal. Out of 120 (ME1 over-expressing) and 134 (Control) single β-cells, none responded to MS, while 77% and 79% of cells, respectively, responded upon subsequent stimulation with 10 mM glucose. (An example of such a response is presented in Fig. 4A and 4B, respectively). These results suggest that there may be additional requirements for MSSIS besides ME1 activity, or, that MSSIS is unrelated to the ME1 activity. This notion is further supported by the fact that the mouse insulinoma cell line (MIN-6) possesses cytosolic NADP\(^{+}\)-dependent malate dehydrogenase activity, as demonstrated by MacDonald (27) and confirmed by us (Fig. 4B). However, these cells do not secrete insulin in response to MS, whereas they exhibit robust secretion in response to treatment with methylpyruvate (MP) (Fig. 4B). To further assess the effect of MS on mouse islet metabolism, oxygen consumption was measured in mouse islets exposed to MS. While rat islets demonstrated a sustained increase in oxygen consumption after MS application (Fig. 5B), mouse islets show only a transient increase (Fig. 5A). The lack of sustained increase in oxygen consumption in mouse islets was not changed by ME1 over-expression (Fig. 5C).

DISCUSSION

Evidence for and against the role of ME1 in GSIS has been previously reported (13; 39; 43). Mouse islets have been reported to have no detectable ME1 activity (2; 17; 27), however this view has been recently challenged by reports demonstrating the presence of Me1 mRNA (23) and enzymatic activity in mouse islets, which was suggested to be significantly correlated to the absence of the DTT in the homogenization buffer (51). We have tested this by preparing mouse islet extract in the absence or presence of DTT. However, we did not detect ME1 activity under either condition (data not shown). It is possible that the strain of mouse we used (CD-1) in contrast to the C57BL/6 used in study by Xu (51), is responsible for these differences. Regardless, the reported level of ME1 activity in mouse islets has been found to be significantly lower than in rat islets (51), and ME1 activity was not measured in the earlier study (23). In addition, a variety of regulatory events can mediate translation of this enzyme (6). The consequential absence or low level of malate/pyruvate and pyruvate/citrate cycling pathways was hypothesized to explain the lack of MSSIS in mouse islets (27). If this hypothesis were correct, introduction of ME1 into mouse islets would enable operation of malate/pyruvate and pyruvate/citrate pathways, increase GSIS, restore the secretory response of mouse islets to MS, and enhance GSIS. In addition, we tested the effect of ME1 over-expression on the potentiating effect of DMM on GSIS, proposed earlier to occur via activation of pyruvate cycling pathways (5).

**ME1 over-expression increases GSIS and malate and citrate levels in INS-1 832/13 cells.** ME1 over-expression increased GSIS in INS-1 832/13 cells, and was paralleled by an increase in mitochondrial malate and citrate levels. This suggests that ME1 over-expression resulted in increased anaplerosis. An increase in citrate synthesis will require an increase in malate, but the mechanism by which malate is increased after ME1 over-expression is not clear. Pyruvate carboxylation to malate in islets is typically thought to occur via enzyme pyruvate carboxylase (PC). It is possible that side-effect of ME1 over-expression is alteration in PC expression or activity, which would result in increased malate formation from pyruvate. However, ME1 is also capable of carboxylating pyruvate, as has been demonstrated in liver, muscle and brain tissue (8; 50; 52). Furthermore, we have demonstrated that ME1 purified from the pancreatic cell line INS-1 832/13 cells, is also capable of mediating this reaction. Whether the reaction catalyzed by ME1 will proceed toward malate formation (oxidative decarboxylation of pyruvate) or pyruvate formation (reductive carboxylation of malate), will be influenced by cytosolic levels and sub-compartmentalization of its reactants and products: malate and pyruvate, as well as the NADPH/NADP\(^{+}\) ratio and the concentration of CO\(_2\). Under increased cytosolic pyruvate levels
(glycolysis for example), we speculate that ME1 catalyzes the reaction that proceeds towards malate, rather than toward pyruvate, resulting in the observed elevated levels of malate found in cells overexpressing ME1. This would be further enhanced by the constitutively high NADPH/NADP⁺ ratio (17) and the presence of CO₂ (7). Cytoplasmic malate, a product of this reaction, can enter into the mitochondria and promote citrate synthesis and its export via TCC. Although we have demonstrated that ME1 overexpression increases malate levels, future studies are required to unambiguously demonstrate whether pyruvate carboxylase, ME1, or both enzymes are the principle mediators of enhanced malate and citrate formation in cells overexpressing ME1.

It is not clear at present how much cytosolic pyruvate, derived from the glycolysis, can enter the mitochondria, and how much can undergo oxidative carboxylation to form malate via ME1. Our view, however, is consistent with the notion that separate pyruvate pools (mitochondrial and extramitochondrial) exist within the β-cell (24). Furthermore, studies suggest that even within the cytosol itself, a single metabolite can be distributed within more than one pool (3; 4). In hepatocytes, data supporting the existence of at least two separate cytosolic pyruvate pools, one associated with glycolysis and lactate (the glycolytic pool), the other with mitochondrial pyruvate, has been presented (38). A higher proportion of the glycolytic pyruvate pool available for carboxylation via ME1 in INS-1 832/13 cells compared to mouse islets, could be the reason why ME1 over-expression increased GSIS to a greater extent in INS-1 832/13 cells than it did in mouse islets, and studies are underway to address this issue.

**DMM potentiates GSIS by stimulating malate entry to the mitochondria.** The role of malate import to the mitochondria was explored in studies with the membrane permeable malate analog, dimethyl-malate (DMM). If potentiation of GSIS by DMM requires ME1-dependent malate/pyruvate cycling, then DMM would not potentiate secretion in mouse islets, which lack ME1 enzymatic activity. Conversely, overexpression of ME1 would enhance the potentiating effect of DMM on GSIS. However, we observed that DMM potentiated GSIS in mouse islets to the same extent as it did in INS-1 832/13 cells, and that this effect of DMM on GSIS was abolished by ME1 over-expression in both preparations. Accordingly, DMM-dependent increases in malate and citrate levels were abolished following ME1 over-expression. In addition, the level of ME1 activity adjusted by varying viral titer was inversely correlated to the DMM potentiation of GSIS in mouse islets (data not shown). Thus, malate entry to the mitochondria seems to be an important step during DMM-mediated potentiation of GSIS.

The potentiating effect of DMM on the GSIS is likely a consequence of maintaining a balance in charges and masses of mitochondrial intermediates. In mitochondria, acetylCoA and oxaloacetate (OAA), formed from decarboxylation and carboxylation of pyruvate, respectively, condense to form citrate. It is established that mitochondrial pyruvate carboxylation and decarboxylation occur in approximately a 4:6 ratio (22; 25). Thus, malate, entering the mitochondria from the cytosol will replenish the mitochondrial OAA pool and serve as a counter-ion to promote citrate or isocitrate export on the tri-carboxylate carrier (TCC) via electroneutral exchange of malate²⁻ for tricarboxylate ²⁻ (37). Thus, the addition of DMM can promote all pyruvate cycling pathways. Application of malate in the absence of glucose will not lead to tri-carboxylate export, since acetylCoA will be depleted by condensation with OAA. Accordingly, application of DMM in the absence of stimulatory levels of glucose does not promote insulin secretion (data not shown).

When ME1 is over-expressed in INS-1 832/13 cells and introduced to mouse islets, exogenously applied malate (as DMM) will be converted to pyruvate, as the forward reaction of ME1 will be favored by abundance of this substrate. This will decrease the cytosolic malate pool available for entry to the mitochondria. Interestingly, although malate has been shown to accumulate inside the intact cells (46), and is transported out of isolated islet mitochondria (28) there is no report demonstrating malate export out of the mitochondrion of intact cells under physiological levels of stimulatory glucose. However, citrate export out of the mitochondria in permeabilized cells has been demonstrated (9). Cytosolic citrate can undergo cleavage by citrate lyase to produce...
OAA and acetylCoA. While evidence has been presented against the involvement of long-chain CoA (malonylCoA) (1; 36), short-chain acyl CoA’s (SC-CoA) have been recently implicated to play a role in insulin secretion (30; 31).

In mouse islets, the activity of isocitrate dehydrogenase has been reported to be relatively high as compared to rat and human islets, (26), perhaps to compensate for the lack of a shuttle requiring ME1 activity. It is plausible that the transport of malate, derived from DMM, to the mouse islets mitochondria, is balanced by export of isocitrate. Isocitrate can be converted to α-KG with generation of NADPH, and α-KG enters the mitochondria.

**MSSIS and Malic Enzyme.** MSSIS was not restored after introduction of ME1 in mouse islets, nor was MSSIS enhanced in INS-1 832/13 cells following ME1 over-expression. The original hypothesis linking MSSIS to presence of ME1 activity (27) is based on the observation that malate is the main metabolite transported out of isolated mitochondria from rat and mouse islets in response to pyruvate and succinate (28). In the cytosol of rat β-cells, malate would be converted to pyruvate by the action of the cytosolic ME1 and pyruvate would reenter mitochondria (26), thus completing the cycle of the malate/pyruvate pathway. In the cytosol of mouse β-cells, exported malate would not be converted to pyruvate due to the absence or a low level of Me1 activity. Differences between our results and the above described hypothesis (28) might be a result of different types of preparations. Isolated mitochondria, in contrast to intact cells, cannot account for factors that are likely to be important for transport, such as the metabolite concentration gradient between mitochondrial and cytosolic compartments (11; 45). It could be speculated, based on our results, that malate export out of the mitochondria in response to MS does not take place in intact β-cells and our data demonstrating the lack of enhancement of MSSIS in INS-1 832/13 cells support this. However, based solely on the inability of ME1 activity to restore MSSIS in the mouse β-cells, the possibility that malate is exported from the mitochondria in exchange for succinate on the dicarboxylate carrier cannot be completely ruled out (Fig. 6) (10). Differences in the activities of mitochondrial metabolite carriers between mouse and rat β-cells might also be responsible for the failure of ME1 to restore the MSSIS in mouse. Absence or a low activity of the specific dicarboxylate carrier can serve as an alternative explanation for the failure of MS to trigger insulin secretion in mouse islets as follows: Insufficient malate transport out of mitochondria would halt succinate import and eventually cause cessation of the insulin secretion response. While MS is an inefficient secretagogue in mouse islets, it does however trigger a transient increase in both the ATP/ADP ratio (17) and oxygen consumption (Fig. 5) in these islets. In addition, MS fails to stimulate insulin secretion in mouse insulinoma MIN-6 cells, which possess high levels of Me1 activity (Fig. 4C). While the important role of mitochondrial carriers in GSIS has just started to be recognized (21), there is still a lack of knowledge about the relative importance of individual carriers for the mechanism of action of a particular fuel secretagogue in the β-cell, and studies are underway to address these issues.

In summary, we have demonstrated that over-expression of ME1 augments insulin secretion in INS-1 832/13 cells and that the underlying mechanism likely involves increased citrate synthesis, although the exact mechanism is not clear. The fact that ME1 over-expression did not significantly affect GSIS in isolated mouse islets is consistent with the recent report demonstrating no effect of ME1 silencing on GSIS in primary rat islets (42). We speculate that differences in size and compartmentalization of the metabolite pools within the cell (in this case the cytosolic pyruvate pool that is available for carboxylation via the reverse reaction of ME1) between clonal insulinoma cells and primary islets might underline the failure of ME1 expression to greatly enhance GSIS in mouse islets. Transport of malate to the mitochondria likely promotes the efflux of citrate or isocitrate and activation of the corresponding cycling pathway, resulting in production of NADPH. While our view does not support the role of pyruvate/malate cycling pathway in GSIS and MSSIS, it is consistent with key features demonstrated to play a role in insulin secretion including pyruvate carboxylation, tricarboxylate efflux and the generation of NADPH. Thus, these data further the current understanding of metabolic changes underlying insulin secretion.
REFERENCES


**FOOTNOTES**
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FIGURE LEGENDS

Fig. 1. Cytosolic ME1 expression in single mouse β-cell and intact islet. A. Intracellular localization of hME1 protein was determined using an anti-hME1 antibody and Alexa fluor 546. Insulin was detected using anti-insulin antibody and Alexa Fluor 647. Nuclei were detected using DAPI. B. Example of a group of mouse islets infected with Ad-ME1-GFP. The strength of the GFP signal (ex.488/550) was used to determine infection efficiency.

Fig. 2. Effect of ME1 over-expression on insulin secretion, ATP, and metabolite levels in INS-1 832/13 cells. INS-1 832/13 cells were either untreated or transduced with Ad-CV-GFP or Ad-ME1-GFP in the absence or presence of Ad-CytoLuc at 50 MOI. A. Following a 2 hr pre-incubation period in the presence of 2 mM glucose, insulin secretion in response to 10 mM concentration of secretory fuels or KCl was measured in static incubation over 30 min. Basal secretion at 2 mM glucose was 20.5±2.2, 23.2±3.1 and 22±2.7 ng insulin/mg protein/hr in untreated, Ad-CV-GFP and Ad-ME1-GFP treated cells, respectively. Non-stimulatory 2 mM glucose was present during incubation with MP, MS and KCl. Stimulatory 10 mM glucose was present during incubation with DMM. Data are means ± SE from 5 independent experiments. B. ATP levels, measured in real time as relative light output, were determined in a population of ~0.5 x 10⁶ live cells using the luciferase/luciferin system in response to 10 mM concentration of secretory fuels. Data, expressed as % of basal secretion are means ± SE from 4 independent measurements. Malate (C) and citrate (D) levels were determined in the mitochondrial fractions using LC/MS/MS. *p<0.05, when compared Ad-ME1-GFP group with Ad-CV-GFP group. G:glucose, MP:methyl-pyruvate, MS:methyl-succinate, DMM: dimethyl-malate.

Fig. 3. Effect of ME1 over-expression on insulin secretion and ATP levels in mouse islets. Isolated mouse islets were either untreated or transduced with Ad-CV-GFP or Ad-ME1-GFP in the absence (secretion, panel A) or presence (ATP levels, panel B) of Ad-CytoLuc at 50 MOI. A. Following a 2 hr pre-incubation period in the presence of 4 mM glucose, insulin secretion was measured in response to 10 mM of secretory fuels or KCl in static incubation over 30 min period. Basal secretion at 4 mM glucose was 3.2±0.4, 3.7±0.45 and 3.6±0.5 ng insulin/10 islets/hr in untreated, Ad-CV-GFP and Ad-ME1-GFP treated islets, respectively. Non-stimulatory 4 mM glucose was present during incubation with MP, MS and KCl. Stimulatory glucose (10 mM) was present during incubation with DMM. Data are means ± SE from 3-4 independent experiments. B. ATP production, measured as relative light output, was determined as relative light output, was determined in a population of ~3.5 x 10⁶ live islets cells (obtained by dispersion of whole islets) using the luciferase/luciferin system in response to stimulatory (10 mM) concentration of fuels. Data, expressed as % of basal are means ± SE from 3 independent experiments. *p<0.05, when compared Ad-ME1-GFP group with Ad-CV-GFP group.

Fig. 4. Methyl-succinate (MS)-mediated metabolic response is not contingent upon ME1 activity. Examples of a single mouse β-cell Ca²⁺ response in cells treated with Ad-ME1-GFP (A) and control cells (B). Additions of secretory fuels at 10 mM concentration are indicated by arrows. Fura Red fluorescence is expressed as relative fluorescence units (F₁/F₀; where F₀ is mean baseline fluorescence). Note that Fura Red fluorescence intensity decreases upon the Ca²⁺ binding (15). C. MIN-6 cells do not respond to MS despite ME1 activity. Following a 2 hr pre-incubation in the presence of 2 mM glucose, MIN-6 cells were exposed to 10 mM concentration of secretory fuels. Control cells were treated with 2 mM glucose (basal
secretion). Insulin secretion was measured in static incubation over a 30 min period. Basal secretion was 56±5.5 insulin/mg protein/hr. NADP+-dependent malate dehydrogenase activities were measured in the soluble protein fraction as described in Methods. Data are means ± SE from 4-5 independent experiments.

**Fig. 5. Differential effect of MS on oxygen consumption in mouse and rat islets.** A. MS does not stimulate a sustained increase in oxygen consumption in mouse islets. B. MS elicits a sustained increase in oxygen consumption in rat islets. Additions of fuels (10 mM) are indicated by arrows. Each trace is representative of 4-6 independent measurements. C. ME1 over-expression does not restore MS-mediated oxygen consumption in mouse islets. Data are means ± SE from 3 independent experiments.

**Fig. 6. DMM enhances insulin secretion: role of malate import to the mitochondria.** At initially high levels of cytosolic pyruvate (glycolysis) and low levels of cytosolic malate, the ME1-catalyzed reaction proceeds in the direction of malate formation and cytosolic malate enters mitochondria, resulting in increased output of citrate or isocitrate and enhanced insulin secretion. However, when the level of cytosolic malate becomes high (for example, after DMM application), the ME1 dependent reaction functions in reverse, favoring pyruvate formation (dotted line), resulting in decreased citrate/isocitrate output and insulin secretion. Under the second condition, increased ME1 activity (ME1 overexpression), as compared to cells with lower endogeneous (INS-1 832/13) or low or absent (mouse islets) ME-1 activity facilitates the removal of cytosolic malate and removes DMM-potentiation of GSIS via decreased citrate/isocitrate output. OAA: oxaloacetate, TCC: tricarboxylate carrier, ICDc: Isocitrate dehydrogenase, α-KG: α-Ketoglutarate, PC: pyruvate carboxylase, PDH: pyruvate dehydrogenase, SCI: secretory coupling intermediates.

| Table 1. Purification of cytosolic malic enzyme (ME1) from INS-1 832/13 cells. |
|-----------------------------|-----------------|-----------------|
| Step                        | Specific activity (µmol NADPH/min/mg protein) | Purification (fold) |
| Total homogenate            | 0.34 ± 0.035    | 1               |
| Cytosol                     | 10 ± 1.4        | 29.4            |
| 2,’5’-ADP-agarose           | 758 ± 56 (687 ± 34)* | 2229.4          |
| * Oxidative carboxylation of pyruvate to malate. |
| Values are means ± SE form 3-4 independent experiments |

| Table 2. Effect of ME1 over-expression on ATP/ADP ratio and glucose oxidation in INS-1 832/13. |
|-----------------------------------------------|-----------------|-----------------|
| ATP/ADP                                      | Untreated       | Ad-CV-GFP       | Ad-ME1-GFP      |
| Basal                                        | 1.56 ± 0.25     | 1.69 ± 0.35     | 1.44 ± 0.09     |
| 10 mM G                                      | 2.30 ± 0.35     | 2.49 ± 0.48     | 2.27 ± 0.24     |
| Glucose oxidation                            | Untreated       | Ad-CV-GFP       | Ad-ME1-GFP      |
| Basal                                        | 4.2 ± 0.52      | 3.9 ± 0.42      | 4.0 ± 0.49      |
| 10 mM G                                      | 5.8 ± 0.74      | 5.6 ± 0.76      | 5.8 ± 0.68      |
| Values are means ± SE from 3 independent experiments. Glucose oxidation is in nmol/mg protein/90 min |
A

Untreated
Ad-CV-GFP
Ad-ME1-GFP

Insulin secretion (% of basal)

Basal | G | MP | MS | KCl | G+DMM

B

Untreated
Ad-CV-GFP
Ad-ME1-GFP

ATP levels (% of basal)

Basal | G | MP
Glucose → Pyruvate → Mitochondrial Matrix

Pyruvate → Glucose

NADPH → ME1

NADPH → OAA → Acetyl-CoA → α-KG

DMM → Malate → TCC → Citrate → Isocitrate

SCI → α-KG

NADP+ → α-KG

40% PC → NADPH

60% PDH → NADP+

OAA → Succinate → Succinyl-CoA → α-KG