Essential Role of Mitochondrial Ca$^{2+}$ Uniporter in the Generation of Mitochondrial pH Gradient and Metabolism-Secretion Coupling in Insulin-releasing Cells

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Running Head: Role of MCU for mitochondrial pH gradient

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Background: Mitochondrial Ca$^{2+}$ uptake affects energy metabolism and insulin secretion

Results: Knockdown of mitochondrial Ca$^{2+}$ uniporter decreases respiratory chain activity and mitochondrial pH gradient generation

Conclusion: Mitochondrial Ca$^{2+}$ uptake via uniporter is essential for oxidative phosphorylation and metabolism-secretion coupling

Significance: The present study identifies mechanisms of action and bioenergetic consequences of mitochondrial Ca$^{2+}$ transporters in insulin-releasing cells.

ABSTRACT

In pancreatic β-cells, ATP acts as a signaling molecule initiating plasma membrane electrical activity linked to Ca$^{2+}$ influx, which triggers insulin exocytosis. The mitochondrial Ca$^{2+}$ uniporter (MCU) mediates Ca$^{2+}$ uptake into the organelle, where energy metabolism is further stimulated for sustained second phase insulin secretion. Here, we have studied the contribution of the MCU to the regulation of oxidative phosphorylation and metabolism-secretion coupling in intact and permeabilised clonal β-cells as well as rat pancreatic islets. Knockdown of MCU with siRNA transfection blunted matrix Ca$^{2+}$ rises, decreased nutrient-stimulated ATP production as well as insulin secretion. Furthermore, MCU knockdown lowered the expression of respiratory chain complexes, mitochondrial metabolic activity, and oxygen consumption. The pH gradient formed across the inner mitochondrial membrane following nutrient stimulation was markedly lowered in MCU-silenced cells. In contrast, nutrient-induced hyperpolarisation of the electrical gradient was not altered. In permeabilised cells, knockdown of MCU ablated matrix acidification in response to extramitochondrial Ca$^{2+}$. Suppression of the putative Ca$^{2+}$/H$^+$ antiporter leucine zipper-EF hand-containing transmembrane protein 1 (LETM1) also abolished Ca$^{2+}$-induced matrix acidification. These results demonstrate that MCU-mediated Ca$^{2+}$ uptake is essential to establish a nutrient-induced mitochondrial pH gradient which is critical for sustained ATP synthesis and metabolism-secretion coupling in insulin-releasing cells.

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Pancreatic \( \beta \)-cells maintain blood glucose homeostasis by adapting insulin secretion to the changes in circulating nutrients. A major signaling molecule in this metabolism-secretion coupling linking nutrient metabolism to insulin secretion is cytosolic ATP most of which is synthesized from oxidative phosphorylation. Mitochondrial ATP synthesis is driven by the electrical (\( \Delta \Psi_{\text{mito}} \), membrane potential) and chemical (\( \Delta p \text{H}_{\text{mito}} \)) gradients across the mitochondrial inner membrane. These gradients are established as a result of electron transport and the associated export of protons mediated by the respiratory chain. Reducing equivalents in mitochondrial matrix are mainly produced by the tricarboxylic acid (TCA) cycle and mitochondrial metabolite shuttles. Thus, the metabolic status of the \( \beta \)-cell mitochondria critically controls ATP synthesis and insulin secretory activity (1). Accumulating evidence suggests that defective mitochondrial function results in impaired glucose-stimulated insulin secretion (GSIS) and may contribute to the development of type 2 diabetes (2-5).

The matrix \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \])_{\text{mito}}\) is a key activator of mitochondrial metabolic function (1,6,7). The [\( \text{Ca}^{2+} \])_{\text{mito}} activates several matrix enzymes including \( \alpha \)-ketoglutarate dehydrogenase in the TCA cycle (8). The ATP synthase is also directly activated by a rise in [\( \text{Ca}^{2+} \])_{\text{mito}}\) (9). In pancreatic \( \beta \)-cells [\( \text{Ca}^{2+} \])_{\text{mito}}\) is strictly required for ATP synthase-dependent respiration stimulated by glucose (10). Given its importance, mitochondrial \( \text{Ca}^{2+} \) uptake has been a research focus for decades, starting with the functional characterization in isolated mitochondria. Nevertheless, it took 50 years to elucidate the molecular identity of the mitochondrial \( \text{Ca}^{2+} \) uniporter (MCU) (11,12). Mitochondrial \( \text{Ca}^{2+} \) uptake through MCU is regulated by a number of recently discovered proteins, including mitochondrial \( \text{Ca}^{2+} \) uptake 1 and 2 (MICU1/2) (13-15), mitochondrial \( \text{Ca}^{2+} \) uniporter regulator 1 (MCUR1) (16), and essential MCU regulator (EMRE) (17). Especially MICU1/2 negatively regulate MCU activity under resting cytosolic \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \])_{i}\). At stimulating [\( \text{Ca}^{2+} \])_{i} (> 10 \text{ \( \mu \text{M} \))\), however, MICU1 activates MCU activity, implying that the regulatory subunits of the MCU complex modulate mitochondrial \( \text{Ca}^{2+} \) loads of \( \Delta \Psi_{\text{mito}} \)-driven \( \text{Ca}^{2+} \) uptake without perturbing the important signal propagation from ER to mitochondria (13,18,19).

Mitochondrial \( \text{Ca}^{2+} \) homeostasis is maintained by balanced \( \text{Ca}^{2+} \) influx and efflux. Mitochondrial \( \text{Ca}^{2+} \) export is mediated by antiporters exchanging \( \text{Ca}^{2+} \) for \( H^+ \) or \( Na^+ \) (20). Two mitochondrial antiporters promoting \( \text{Ca}^{2+} \) efflux have been identified: The leucine zipper-EF hand-containing transmembrane protein 1 (LETM1) and the mitochondrial sodium calcium exchanger (NCLX). LETM1, which is defective in Wolf-Hirschhorn syndrome (WHS), works as a \( K^+/H^+ \) exchanger in yeast mitochondria (21) or mammalian ER (22). LETM1 was also shown to mediate \( \text{Ca}^{2+}/H^+ \) exchange in mitochondria with a \( [\text{Ca}^{2+}]_{\text{mito}}\)-dependent biphasic mode (23). NCLX was confirmed as an electrogenic \( Na^+/Ca^{2+} \) antiporter (exchanging 3 or 4 \( Na^+ \) per \( Ca^{2+} \)) (24). Inhibition of NCLX in pancreatic \( \beta \)-cells increases [\( \text{Ca}^{2+} \])_{mito}, accelerates mitochondrial oxidative metabolism and GSIS (25-28).

In addition to [\( \text{Ca}^{2+} \])_{mito}, the matrix pH has been identified as a regulator of mitochondrial energy metabolism in \( \beta \)-cells. In contrast to other cell types, pancreatic \( \beta \)-cells have acidic \( \text{pH}_{\text{mito}} \) under resting conditions. Nutrient stimulation causes matrix alkalinization without any marked cytosolic pH change (29). Preventing the resulting nutrient-induced increase of the \( \Delta \text{pH}_{\text{mito}} \) changes using ionophores abrogated proton-coupled mitochondrial ion/metabolite transport, ATP synthesis, and GSIS regardless of elevated \( \Delta \Psi_{\text{mito}} \) (29-31). Therefore, pathogenic conditions causing a reduction of \( \Delta \text{pH}_{\text{mito}} \) may seriously deteriorate ATP generation and insulin secretion in pancreatic \( \beta \)-cells.

Several recent reports demonstrate the functional role of MCU in pancreatic \( \beta \)-cells (26,32). MCU mediates glucose-stimulated [\( \text{Ca}^{2+} \])_{mito} rise and second phase ATP/ADP increase (26). Knockdown of either MCU or MICU1 diminishes insulin secretion associated with defects in mitochondrial \( \text{Ca}^{2+} \) uptake (32). Mice lacking MCU show a significant reduction of [\( \text{Ca}^{2+} \])_{mito} and \( \text{Ca}^{2+} \)-stimulated oxygen consumption in muscle mitochondria, without changes in the basal respiration in embryonic
fibroblasts (33). It remains unclear, however, how reduced MCU activity attenuates mitochondrial signal generation in pancreatic β-cell metabolism-secretion coupling. In this study, we observed that reduced mitochondrial Ca\(^2^+\) uptake following silencing of MCU significantly attenuated respiratory chain activity and \(\Delta p\text{H}_{\text{mito}}\) increase in permeabilized as well as in intact insulin-secreting cells. These defects lead to impaired ATP synthesis and insulin secretion, demonstrating the crucial role of mitochondrial Ca\(^2^+\) uptake for the establishment of the \(\Delta p\text{H}_{\text{mito}}\) in metabolism-secretion coupling. We also provide evidence for a novel role of the putative Ca\(^2^+\)/H\(^+\) antiporter leucine zipper-EF hand-containing transmembrane protein 1 (LETM1) as a Ca\(^2^+\) efflux route in insulin secreting cells, the role of which is altered in the absence of MCU.

**EXPERIMENTAL PROCEDURES**

**Cell culture and drugs**

Rat insulinoma INS-1E cells were cultured in a humidified atmosphere (37°C) containing 5% CO\(_2\) in a complete medium composed of RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (HyClone, Thermo Fisher Scientific Inc., Lafayette, CO, USA). Experiments were performed with cells of passage number 80–120. Most chemicals were purchased from Sigma (St. Louis, MO, USA) except JC-1 from Molecular Probes (Eugene, OR, USA).

Pancreatic islets were isolated from 200–300g male Sprague-Dawley rats (Orient Bio, Seongnam, Korea) by collagenase (Sigma) digestion (29) and dispersed by a brief incubation with trypsin (Gibco). Islet cells were seeded on multi-well-plates coated with 804G matrix and cultured in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (7).

**Permeabilisation with α-hemolysin toxin**

INS-1E cells were seeded and cultured onto well-plates or coverslips coated with 804G matrix. Cells were washed with Ca\(^2^+\)-free Krebs-Ringer bicarbonate (KRB) solution (mM; 140 NaCl, 3.6 KCl, 0.5 NaH\(_2\)PO\(_4\), 0.5 MgSO\(_4\), 1.5 CaCl\(_2\), 10 HEPES, 2 NaHCO\(_3\), 5.5 glucose, pH 7.4 titrated with NaOH) and then incubated for 10 min at 37°C with 1 μg/ml of *Staphylococcus aureus* α-hemolysin toxin (Sigma) in an intracellular buffer (mM; 140 KCl, 5 NaCl, 7 MgSO\(_4\), 1 KH\(_2\)PO\(_4\), 20 HEPES, 10.2 EGTA, 1.65 CaCl\(_2\), 0.1 ATP, pH 7.0 with KOH) which has about 120 nM of free Ca\(^2^+\) concentration. After α-toxin permeabilisation, cells were washed once with 0.5% bovine serum albumin (BSA) containing intracellular buffer and used for experiments (29).

**siRNA transfection**

Cells were transfected with non-targeting or target-specific small interfering RNA (siRNA) using DharmaFECT1 (Dharmacon, Thermo Fisher Scientific Inc.). The target-specific siRNAs for rat MCU and LETM1 were purchased from Dharmacon which is composed of siRNAs for 4 different targets of each gene (SMARTpool, Dharmacon).

**Quantitative real time PCR**

Total RNA was isolated from cells 72 hrs after siRNA transfection using the RNeasy kit (Catalog # 74134, Qiagen GmbH, Hilden, Germany). First strand cDNA was synthesized from 1 μg of total RNA with a reverse transcription kit (Applied Bioscience, Foster City, CA, USA) using oligo-dT. Quantitative PCR was performed using sequence-specific primers for rat MCU (forward: 5'-GAAGTAGGTGACCGGTTCCA-3', reverse: 5'-AGGAAAGCGGAGAAGAGGAC-3'), and LETM1 (forward: 5'-GGCTGGACTTGCACCTGTA-3', reverse: 5'-CAGAGCGCCGGCAGAGGAC-3'). Rat β-actin was used as the reference control. For the analysis of each gene expression, experiments were conducted in a triplicate in the real time PCR system (7900HT, Applied Bioscience) using SYBR Green PCR Master Mix (Catalog # 204143, Qiagen GmbH). Data were analyzed following ΔΔCT method (34).
Western blot

The protein level of MCU or LETM1 was determined in a cell extract using Western blotting as described previously (31). Primary antibodies for MCU (1:1000, Catalog # HPA016480, Sigma), LETM1 (1:500, Catalog # sc134672, Santa Cruz Biotechnology, Dallas, TX, USA), complex I NDUFA9 (1:2000 dilution, Catalog # 459100, Invitrogen, Carlsbad, CA, USA), complex III UQCRC2 (1:2500, Catalog # MS304, Mitosciences, Abcam), complex IV subunit I (1:1000, Catalog # 459600, Invitrogen), complex V (1:5000, Catalog # MS604-300, Mitosciences, Abcam), TOM20 (1:1000, Catalog # sc11415, Santa Cruz Biotechnology), and β-actin (1:5000, Catalog # ab6276, Abcam, Cambridge, UK) were used. Horseradish peroxidase (HRP)-conjugated secondary antibody against either mouse or rabbit IgG (Catalog # 31450 and 31460, Thermo Scientific) was incubated for 1 hr at room temperature. The bands were visualized with a UVP Biospectrum-600 imaging system using enhanced chemiluminescence (ECL) solution (Luminata Forte, Millipore Corporation, Billerica, MA, USA).

Measurement of mitochondrial matrix Ca\(^{2+}\)

To measure mitochondrial matrix Ca\(^{2+}\) level, we used a mitochondria-targeted ratio-pericam (RPmit) plasmid, generously provided by Prof. Roger Tsien (UC San Diego, USA). Cells were transfected with siRNA, and 24 hrs after, transfected with RPmit using X-tremeGENE (Roche Diagnostics GmbH, Mannheim, Germany). Fluorescence imaging of Ca\(^{2+}\) was performed by using an inverted microscope (IX-81, Olympus, Tokyo, Japan) with an array laser confocal spinning disk (CSU10, Yokogawa Electric Corporation, Tokyo, Japan) and a cooled charge-coupled device (CCD) camera (Cascade 512B, Photometrics, Tucson, AZ, USA). Intact or permeabilised cells on the confocal microscope were perfused with KRB solution or intracellular buffer, respectively, and fluorescence images (435 nm excitation and 535 nm emission) were acquired every 10 sec and analysed using Metafluor 6.3 software (Universal Imaging, Molecular Devices).

Measurement of cytosolic ATP and insulin

Cells plated onto 48 well-plates (2 X 10\(^5\) cells/well) were permeabilised with α-toxin and incubated for 5 or 15 min in an intracellular buffer containing ADP (10 μM) with or without succinate (3 mM). To determine mitochondrial ATP release, the supernatant was harvested after incubation and ATP level was measured by using the microplate reader (Synergy™2, BioTek Instruments Inc., Winooski, VT, USA) with a bioluminescence assay kit (HS II, Roche Diagnostics, Mannheim, Germany).

For static insulin secretion measurement, cells in a 804G-coated 24 well-plate (1.5 x 10\(^5\) cells/well) were transfected with siRNA and grown for 72 h. After deprivation of glucose for 1 h, cells were preincubated for 30 min with a KRB solution containing 2.8 mM glucose and 0.1% BSA. Then, cells were washed and incubated for 30 min with 2.8 mM or 16.7 mM glucose-containing KRB solution. Supernatant was collected for estimation of insulin release. Cellular insulin contents were determined in acid-ethanol extracts. Insulin levels were measured by using an insulin ELISA kit (Shibayagi Co., Gunma, Japan).

Mitochondrial enzyme activity and MTT assay

For cytochrome c oxidase (COX) activity measurement, INS-1E cells were permeabilised by freeze-thaw cycle three times and mixed with isotonic solution (10 mM KH2PO4, 250 mM sucrose, 0.1% BSA, pH 6.5) with detergent (laurylmaltoside, 2.5 mM). Traces were started with the addition of reduced cytochrome c (25 nM; with a tiny amount of sodium hydrosulfite) and the enzymatic activity of COX was estimated by measuring absorbance at 550 nm continuously with a spectrophotometer (Amersham, GE healthcare Biosciences, Pittsburgh, PA, USA). COX activity was expressed as moles of oxidized cytochrome c per min. Citrate synthase activity was measured with citrate synthase assay kit (Sigma) based on the manufacturer's instructions.

For MTT assay, siRNA-transfected cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (50 μg/well) for 2 hrs and then treated with dimethylsulfoxide.
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The absorbance \((A_{570} - A_{630})\) of each well was measured by using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Measurement of oxygen consumption**

Cellular oxygen consumption rate (OCR) was determined by Extracellular Flux Analyzer (XF-24, Seahorse Bioscience, North Billerica, MA, USA). Cells (2 x 10⁴ cells/well) seeded on 24 well-plates (Seahorse Bioscience) were transfected with siRNA and cultured for 72 h. On the experiment day, cells were incubated for 1 hr at 37°C with KRB solution containing 2.8 mM glucose prior to 20 min of basal OCR measurement. Then, glucose (16.7 mM), oligomycin (3 μg/ml), FCCP (3 μM), and antimycin A (3 μM) were added consecutively and the changes in OCR analysed.

**Measurement of mitochondrial matrix pH**

Mitochondrial matrix pH \((pH_{mito})\) was measured using adenovirus expressing mtAlpHi (Ad-tetON-mAlpHi) as described previously (29). Intact or permeabilised cells on the confocal microscope were perfused with KRB solution (pH 7.4) or intracellular buffer (pH 7.0), respectively, and the fluorescence signals (488 nm excitation and 535 nm emission) were recorded. Titration of the mitochondrial pH was performed by clamping the matrix pH with high K⁺ buffer (mM) (125 KCl, 5 NaCl, 1 NaH₂PO₄, 1 MgSO₄, 10 HEPES) of defined pH containing the ionophores nigericin (5 μM) and monensin (5 μM) (31).

**Measurement of mitochondrial membrane potential**

To measure the mitochondrial membrane potential \(\Delta \Psi_{mito}\), cells seeded onto black-walled 96 well-plates (5 x 10⁴ cells/well) were loaded with JC-1 (500 nM, Invitrogen) for 30 min and then permeabilised with a-toxin. The ratio of red (540 nm excitation and 590 nm emission) over green (490 nm excitation and 540 nm emission) fluorescence intensity was monitored from permeabilised cells in the presence of intracellular buffer containing JC-1 (500 nM) using a multi-well fluorescence reader (FlexStation, Molecular Devices) (35).

As an alternative method to measure the mitochondrial membrane potential, cells seeded on coverslips were loaded with 5 nM TMRM for 20 min, and perfused with KRB solution containing TMRM (5 nM) on the inverted microscope. Fluorescence imaging with 514 nm excitation and 560 nm emission were recorded with the array laser confocal spinning disk microscopic system and analyzed by using Metamorph 6.1 software.

**Statistical analysis**

Values are presented as mean ± S.E.M. and \(N\) is the number of independent experiments. \(P\)-values were obtained by Student's \(t\)-test or one-way ANOVA and < 0.05 was considered to be significant.

**RESULTS**

**Effects of MCU knockdown on mitochondrial Ca²⁺ uptake**

In order to understand the role of mitochondrial Ca²⁺ transport in metabolism-secretion coupling, we transfected non-targeting siRNA (siControl) or siRNA selectively targeted to MCU (siMCU) in INS-1E cells, and assessed the effect of silencing after 72 hrs using quantitative real-time PCR and Western blotting. Application of siMCU efficiently reduced the transcript levels of MCU (73.8 ± 5.3% reduction, Fig. 1A) compared to siControl-treated cells. Western blot analysis also revealed a strong siRNA-induced reduction of the MCU protein by 82.3 ± 2.3% (Fig. 1B and C).

To examine the impact of MCU knockdown on mitochondrial Ca²⁺ uptake, we determined the effect of extramitochondrial Ca²⁺ ([Ca²⁺]₀) on [Ca²⁺]mito in a-toxin-permeabilised INS-1E cells expressing mitochondria-targeted ratiopericam (RPmit). When the cells were perfused with intracellular buffer containing approximately 10 nM Ca²⁺ and 3 mM succinate, switching [Ca²⁺]₀ to 120 nM gradually increased [Ca²⁺]mito (Fig. 1D). The [Ca²⁺]mito declined slowly after returning to 10 nM [Ca²⁺]₀. Addition of 500 nM [Ca²⁺]₀ resulted in more rapid and marked increase in [Ca²⁺]mito, which slowly decreased again after cessation of the stimulus. In MCU-silenced cells,
the responses to both Ca\(^{2+}\) concentrations were markedly reduced by 61.8% and 58.2%, respectively (Fig. 1E). These results demonstrate that MCU contributes to mitochondrial Ca\(^{2+}\) uptake at physiological Ca\(^{2+}\) concentrations.

**Effect of MCU Knockdown on ATP synthesis and insulin secretion**

It is well known that [Ca\(^{2+}\)]\(_{\text{mito}}\) amplifies metabolism-secretion coupling in β-cells and reduction of [Ca\(^{2+}\)]\(_{\text{mito}}\) inhibits GSIS (6,7). To further investigate the role of [Ca\(^{2+}\)]\(_{\text{mito}}\) in energy metabolism we studied succinate-dependent ATP synthesis in permeabilised cells. In the absence of substrate, silencing of MCU did not affect basal ATP formation (Fig. 1F). However, time-dependent ATP synthesis stimulated by succinate was markedly lowered in siMCU-treated cells. Consistent with the role of ATP as a signaling molecule for insulin exocytosis, GSIS in intact INS-1E cells was also dramatically decreased in MCU knockdown cells (76.3% inhibition, Fig. 1G). The role of MCU in metabolism-secretion coupling in pancreatic β-cells, was assessed following siMCU transfection of isolated rat pancreatic islets. Successful silencing of MCU in dispersed islet cells (Fig. 1H), resulted in significantly attenuated GSIS (Fig. 1I). Our data emphasize the importance of MCU-dependent mitochondrial Ca\(^{2+}\) uptake in metabolism-secretion coupling of pancreatic β-cells.

**Effects of MCU knockdown on mitochondrial respiratory function**

Mitochondrial ATP synthesis by the F\(_{1}\)F\(_{0}\)-ATPase (complex V) is driven by the proton electrochemical gradient across the inner mitochondrial membrane, which is generated by the proton pumping activity of the respiratory complexes I, III, and IV. Protein expression of selected subunits of complex I, III, IV, and V was examined using Western blot analysis. MCU knockdown markedly reduced complex I (NDUFA9, nuclear DNA-encoded, -47.9%), complex III (UQCRCC2, nuclear DNA-encoded, -47.5%), complex IV (subunit I, mitochondrial DNA-encoded, -67.1%), and complex V (ATP5A, nuclear DNA-encoded, -29%) (Fig. 2A and B). Consistently, the enzyme activity of Complex IV was reduced by 16.6% after MCU knockdown (Fig. 2D and E). Other mitochondrial functions such as citrate synthase activity (TCA cycle) or the TOM20 protein expression (subunit of mitochondrial protein import) were not altered when lowering MCU (Fig. 2F and G)(Fig. 2A and B). These results demonstrate that lowering mitochondrial calcium uptake selectively affect the expression of respiratory chain complexes.

Knockdown of MCU causes neither cell loss nor alteration in total soluble proteins (data not shown). Given these findings, we used the MTT assay as a read-out of mitochondrial reductive activity (36). Using an identical number of cells, silencing of MCU lowered the ability of cells to reduce MTT by 15% (Fig. 2C).

Finally, we investigated the effect of MCU knockdown on oxygen consumption rate (OCR). Under basal conditions (2.8 mM glucose), the OCR was modestly reduced in MCU knockdown cells (19.3% inhibition; Fig. 3A and B). The OCR induced by high glucose (16.7 mM) was strongly impaired following MCU knockdown (35.0% inhibition; Fig. 3A and C), suggesting that the activation of mitochondrial respiration is highly dependent on MCU activity. Taken together, in insulin-secreting cells, mitochondrial Ca\(^{2+}\) uptake via MCU is necessary for mitochondrial functions at multiple levels from nutrient oxidation to ATP synthesis.

**Nigericin-induced mitochondrial hyperpolarisation was lowered in MCU-silenced cells**

The mitochondrial electrical gradient (ΔΨ\(_{\text{mito}}\)) is the main driving force for ATP synthesis as well as Ca\(^{2+}\) transport through the MCU. MCU knockdown in turn may alter the ΔΨ\(_{\text{mito}}\). We therefore measured the ratio of fluorescence intensities (red/green) after loading with JC-1 which reflects the ΔΨ\(_{\text{mito}}\) (35). The hyperpolarising response to succinate in α-toxin permeabilised control and MCU knockdown cells, was not significantly different (Fig. 4A). Glucose-induced hyperpolarisation in intact cells was also not different between the two groups, which was measured by using the fluorescence probe TMRM.
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in a non-quenching redistribution mode to measure ΔΨ_{mito} (Fig. 4B) (on the figure it says JC-1 not TMRM). We also confirmed these findings by using JC-1 dye (data not shown), showing that silencing of MCU does not significantly alter ΔΨ_{mito} in insulin-secreting cells.

The K⁺/H⁺ electroneutral ionophore nigericin, dissipates the ΔpH_{mito} across the inner mitochondrial membrane. This results in a compensatory elevation of ΔΨ_{mito} in order to maintain the total proton motive force (30). Therefore, hyperpolarisation of ΔΨ_{mito} by nigericin is proportional to the pH gradient prior to the addition of the ionophore. Interestingly, hyperpolarisation by nigericin was markedly decreased (53.0 % inhibition) following knockdown of MCU in permeabilised INS-1E cells (Fig. 4C and D). This result suggests that MCU-silenced cells have defects in the establishment of a ΔpH_{mito} gradient but not ΔΨ_{mito}, in response to nutrients.

**Knockdown of MCU impaired nutrient-generated pH gradient**

The ability of β-cells to elevate their ΔpH_{mito} following glucose stimulation is important for mitochondrial energy metabolism and thereby metabolism-secretion coupling (1). To directly assess the effect of MCU knockdown on pH_{mito}, we expressed the mitochondria-targeted pH sensitive protein mtAlpHi in siMCU-treated INS-1E cells. As shown in Figure 5A and C, succinate-induced alkalinisation of pH_{mito} in MCU-silenced cells was blunted compared to control cells (44.7% reduction). In intact MCU-silenced cells, glucose-induced matrix alkalinisation was also strongly decreased (52.4% inhibition; Fig. 5B and E). These results show that suppression of MCU-dependent mitochondrial Ca²⁺ uptake also prevents the establishment of the nutrient-generated ΔpH_{mito}.

Addition of extramitochondrial Ca²⁺ to succinate-stimulated mitochondria resulted in mitochondrial matrix acidification in permeabilised control cells. Interestingly, Ca²⁺-induced matrix acidification was not observed in MCU knockdown cells, indicating that this acidification is a secondary consequence of MCU-mediated Ca²⁺ uptake (Fig. 5A and D). Ca²⁺ loading via MCU may be followed by Ca²⁺ efflux in exchange for H⁺ in energized mitochondria causing the observed net acidification.

**Acute Blocking of MCU did not affect metabolism-secretion coupling**

Gene silencing with siMCU transfection reduces the protein expression of MCU slowly over a time-course of several days. In order to understand the acute effects of blocking mitochondrial calcium import, we performed insulin measurement with the specific MCU blocker, Ru360. Pretreatment with Ru360 did not affect glucose-stimulated insulin secretion (Fig. 6A). MCU is a selective Ca²⁺ channel mediating inward current and this Ca²⁺ influx through MCU depolarises the ΔΨ_{mito}. We measured the effect of Ru360 on ΔΨ_{mito} in intact cell during high glucose stimulation. As shown in Fig. 6B and C, Ru360 further hyperpolarised the ΔΨ_{mito} by blocking MCU-mediated inward depolarising currents. These results also confirm the effectiveness of Ru360 in intact cells. Taken together, long term reduction of mitochondrial Ca²⁺ uptake leads to down-regulation of mitochondrial bioenergetics and metabolism-secretion coupling, which is not reproduced by acute pharmacological blocking of MCU.

**LETM1 participated as a mitochondrial Ca²⁺-H⁺ antiporter in INS-1E cells**

We hypothesized that the Ca²⁺/H⁺ antiporter LETM1 may be required for the observed matrix acidification triggered by Ca²⁺ (23). To elucidate the role of LETM1 on Ca²⁺-coupled pH regulation, the change in pH_{mito} upon extramitochondrial addition of Ca²⁺ was measured in control or LETM1-silenced INS-1E cells. After 72 hrs of siRNA treatment, the knockdown effect was evaluated by using quantitative real-time PCR and Western blotting. Application of siLETM1 efficiently reduced the transcript (78.0 ± 5.7% reduction, Fig. 7A) and LETM1 protein (78.9 ± 0.9% reduction, Fig. 7B and C) compared to siControl-treated cells. In α-toxin-permeabilised LETM1 knockdown cells, succinate-induced matrix alkalinisation was not significantly altered.
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(Fig. 7D and E). In contrast, Ca\(^{2+}\) (500 nM)-elicited pH\(_{\text{mito}}\) acidification was abolished in LETM1 knockdown cells, similar to our results in MCU knockdown cells (Fig. 7D and F). Moreover, the increase in [Ca\(^{2+}\)]\(_{\text{mito}}\) by extramitochondrial Ca\(^{2+}\) was enhanced in LETM1-silenced cells (2.1 fold increase at 120 nM Ca\(^{2+}\), 1.4 fold at 500 nM Ca\(^{2+}\)), likely caused by impaired Ca\(^{2+}\) export (Fig. 7G and H). Consistent with these findings the [Ca\(^{2+}\)]\(_{\text{mito}}\) rise following K\(^{+}\)-induced Ca\(^{2+}\) influx in intact cells was also enhanced in LETM1 knockdown cells (Fig. 7I-L). Very similar results were obtained using the two different mitochondrial calcium probes ratiopericam (Fig. 7I and J) and Rhod-2 (Fig. 7K and L). These results strongly suggest that LETM1 mediates at least one important component of Ca\(^{2+}\) efflux in insulin secreting cells. In MCU knockdown cells, extramitochondrial Ca\(^{2+}\) is taken up inefficiently, and therefore LETM1-mediated Ca\(^{2+}\) efflux is strongly reduced. As a consequence the acidifying response of pH\(_{\text{mito}}\) to extramitochondrial Ca\(^{2+}\) is abrogated.

We further investigated the functional consequences of LETM1 silencing on mitochondrial bioenergetics and metabolism-secretion coupling. Even though the stimulus-induced mitochondrial Ca\(^{2+}\) response was augmented in LETM1 knockdown cells, glucose-induced insulin secretion and hyperpolarisation of the ΔΨ\(_{\text{mito}}\) were attenuated (Fig. 8A and B). Reduction of LETM1 expression also lowered protein levels of subunits of respiratory chain complexes., These changes at the level of the electron transport chain likely explain defective insulin secretion in LETM1-silenced cells (Fig. 8C and D).

**DISCUSSION**

Increases in cytosolic Ca\(^{2+}\) stimulate numerous energy consuming processes, including muscle contraction and neurotransmitter release. In particular, elevated mitochondrial matrix Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{mito}}\)) is a key stimulator of energy provision. The rise in [Ca\(^{2+}\)]\(_{\text{mito}}\) activates TCA cycle dehydrogenases and ATP synthase leading to accelerated mitochondrial ATP production (6). For the proper coupling between energy demand and supply, propagation of Ca\(^{2+}\) waves from the cytosol to the mitochondrial matrix through Ca\(^{2+}\) transporters is necessary. MCU has been suggested to be the main communicating channel linking cytosolic and mitochondrial Ca\(^{2+}\) signaling driven by the mitochondrial electrical gradient. Since the discovery of the molecular identity of MCU by two groups (11,12), several investigations on the role of MCU have been conducted. These studies consistently showed that MCU mediates the main mitochondrial Ca\(^{2+}\) uptake route in HeLa cells (15), cardiac myocytes (37), neuronal cells (38), and pancreatic β-cells (26,32). In the latter cell type, the regulatory role of matrix Ca\(^{2+}\) in mitochondrial ATP synthesis is therefore not limited to the provision of cellular energy, but also plays a key role as a signal in metabolism-secretion coupling.

In this study, we aimed to better understand the role of MCU as a regulator of mitochondrial metabolism and bioenergetics in pancreatic islet cells and clonal β-cells. Our findings demonstrate that silencing of MCU in insulin-releasing cells 1) decreases mitochondrial Ca\(^{2+}\) uptake, 2) down-regulates electron transport chain proteins and enzyme activities, 3) reduces glucose-stimulated oxygen consumption, 4) impairs the generation of nutrient-stimulated ΔpH\(_{\text{mito}}\), 5) does not affect ΔΨ\(_{\text{mito}}\), 6) reduces nutrient-stimulated ATP generation and 7) impairs glucose-stimulated insulin secretion. We provide strong evidence that mitochondrial Ca\(^{2+}\) uptake through MCU is a prerequisite for the establishment of the ΔpH\(_{\text{mito}}\) and activation of mitochondrial energy metabolism. The critical role of the ΔpH\(_{\text{mito}}\) in mitochondrial ATP synthesis and insulin secretion has been demonstrated previously (29,30). It is inferred, therefore, that the reduced [Ca\(^{2+}\)]\(_{\text{mito}}\) together with impaired ΔpH\(_{\text{mito}}\) generation are the main reasons for defective metabolism-secretion coupling in MCU-silenced cells.

MCU is an inwardly rectifying, highly Ca\(^{2+}\)-selective ion channel driven by the negative ΔΨ\(_{\text{mito}}\) generated by the respiratory chain (39). Because of high ΔΨ\(_{\text{mito}}\) (assumed as 180mV), energized mitochondria have the ability to capture cytosolic Ca\(^{2+}\) over a wide range of concentrations (40,41).
We observed that knockdown of MCU significantly diminished mitochondrial Ca^{2+} influx at relatively low concentrations of extramitochondrial Ca^{2+} (< 500 nM). These data imply that the MCU is the main mediator of mitochondrial Ca^{2+} uptake from the cytosol under physiological conditions in spite of the negative regulation by other subunits of this complex such as MICU1/2 (18). Independent of the Ca^{2+} source, either release from the ER or influx from the extracellular space, MCU works as the main Ca^{2+} transport route into mitochondria (42). In MCU-silenced insulinoma cells, [Ca^{2+}]_{mito} rises following either ER Ca^{2+} release or high K^+-induced Ca^{2+} influx are reduced (32).

We found a close functional connection between mitochondrial Ca^{2+} uptake and ΔpH_{mito} regulation, not previously observed. Mitochondria in pancreatic β-cells are of relatively high volume density, facilitating nutrient metabolism and signal generation (35). Indeed, detection of plasma glucose levels is strictly dependent on mitochondrial oxidative phosphorylation in the β-cells (2). In this context, matrix alkalinisation by glucose is a distinctive characteristic of β-cell mitochondria (29). In contrast, glycolytic cells such as HeLa cells or HepG2 cells have high resting pH_{mito} and do not respond to nutrient stimulation (29,43). Our previous studies in insulin-secreting cells showed that short term attenuation of [Ca^{2+}]_{mito} rises in an extracellular Ca^{2+} free condition did not affect nutrient-stimulated alkalinisation of pH_{mito}. Conversely, a [Ca^{2+}]_i transient caused by tolbutamide, a K_{ATP} channel blocker, also did not affect ΔpH_{mito} (29).

In the present study, however, continuous suppression of calcium uptake after knockdown of MCU had profound effects on matrix pH and oxidative phosphorylation. As strong evidence for impaired mitochondrial metabolism following MCU knockdown, we observed that protein levels and function of electron transport chain complexes, mitochondrial enzyme activities, and oxygen consumption rate were all reduced. Our findings are consistent with an earlier study which showed that effective buffering of matrix Ca^{2+} lowered NAD(P)H levels, oxygen consumption, and ATP synthesis in hormone secreting cells (7).

We propose that persistent inhibition of [Ca^{2+}]_{mito} rises perturbs Krebs cycle and electron transport chain activities, which in turn causes defective ΔΨ_{mito} generation and ATP synthesis, leading to impaired nutrient-stimulated insulin secretion.

Nigericin-induced hyperpolarisation of ΔΨ_{mito} reflects the preexisting ΔpH_{mito}. Therefore, the reduced hyperpolarising response in MCU-silenced cells suggests reduction of the preexisting ΔpH_{mito} (Fig. 4C & D). This effect on ΔpH_{mito} was confirmed using the mitochondrial pH sensitive probe mtAlpHi (Fig. 5). On the other hand, the mitochondrial electrical gradient, the main component of the proton motive force, was not affected by MCU silencing (Fig. 4A & B). This finding is similar to those observed in other cell types (11,12). It is not clear why there is a selective defect in ΔpH_{mito} generation without alterations in ΔΨ_{mito}. Ca^{2+} influx through MCU uses the electrical gradient as a driving force therefore rapid Ca^{2+} influx elicits depolarisation of ΔΨ_{mito} (40,41). In MCU-silenced cells, mitochondrial Ca^{2+} inward currents are reduced contributing to the preservation of ΔΨ_{mito}. Another mechanism contributing to the maintenance of ΔΨ_{mito} could be lower activity of the mitochondrial Na^+/Ca^{2+} exchanger (NCLX) because the amplitude of [Ca^{2+}]_{mito} is decreased in MCU-silenced cells. Ca^{2+} efflux through NCLX leads to depolarisation of ΔΨ_{mito} because of its electrogenic property (24). Taken together, we can infer that reduced Ca^{2+} influx and efflux in MCU-silenced cells prevent depolarisation of ΔΨ_{mito}, which may counteract the effect of attenuated respiratory chain activity.

An interesting finding in this study is the acidification of pH_{mito} by extramitochondrial Ca^{2+} addition to energized mitochondria of permeabilised INS-1E cells. This pH_{mito} acidification was dependent on mitochondrial Ca^{2+} uptake via MCU. Mitochondrial Ca^{2+} transport is coupled with H^+ through a Ca^{2+}/H^+ exchanger, the molecular identity of which is not clear. In a genome-wide RNA interference screen Jiang et al. identified LETM1 to mediate this exchange in mitochondria (23). To investigate the role of LETM1 on Ca^{2+}/H^+ coupled transport in insulin-secreting cells, we tested whether there is...
an alteration in pH\textsubscript{mito} acidification after knockdown of LETM1. Similar to the response in MCU knockdown cells, the pH\textsubscript{mito} in LETM1 knockdown cells was not acidified, instead slight alkalinisation by extramitochondrial Ca\textsuperscript{2+} was observed (Fig. 6C). Furthermore, the [Ca\textsuperscript{2+}]\textsubscript{mito} rises by extramitochondrial Ca\textsuperscript{2+} addition were increased in LETM1-silenced cells (Fig. 6F). Our findings demonstrate that LETM1 mediates Ca\textsuperscript{2+} efflux from mitochondria of insulin-secreting cells working in parallel with NCLX. A recent publication shows that purified human LETM1 mediates electroneutral 2 H\textsuperscript{+}/1 Ca\textsuperscript{2+} antiport when reconstituted in artificial liposomes (44). Thus, in intact cells, Ca\textsuperscript{2+} efflux via LETM1 is preferred, which is driven by the H\textsuperscript{+} gradient across the inner mitochondrial membrane (45). We suggest that Ca\textsuperscript{2+} influx through MCU is coupled to LETM1-mediated Ca\textsuperscript{2+} efflux with proton uptake favored by high [Ca\textsuperscript{2+}]\textsubscript{mito} and alkaline pH\textsubscript{mito}. In MCU-silenced cells, Ca\textsuperscript{2+} efflux via LETM1 was prevented or even reversed to Ca\textsuperscript{2+} influx due to reduced Ca\textsuperscript{2+} and pH gradients, explaining the disappearance of pH\textsubscript{mito} acidification.

Why LETM1 knockdown has negative effects on the expression of subunits of the respiratory chain, glucose-stimulated \(\Psi\textsuperscript{mito}\) hyperpolarisation and insulin secretion is not clear. A recent publication presented evidence that LETM1 haplo-insufficiency (+/-) increases mitochondrial superoxide levels which is responsible for mitochondrial dysfunction (46). We propose that oxidative stress in LETM1-silenced insulin-releasing cells may negatively affect mitochondrial bioenergetics, respiratory activity and metabolism-secretion coupling.

Our findings herein demonstrate that MCU-mediated Ca\textsuperscript{2+} uptake is essential for the respiratory chain activity and the generation of \(\Delta p\textsubscript{H\textsubscript{mito}}\) in insulin-releasing cells. This chemical gradient (\(\Delta p\textsubscript{H\textsubscript{mito}}\)) is critically required for the substrate transport into the mitochondrial matrix, including pyruvate and inorganic phosphate (31) and ATP synthesis (29). Therefore, evidence in the present study suggests the bioenergetic mechanism to explain the defective metabolism-secretion coupling by MCU knockdown. In addition, mitochondrial Ca\textsuperscript{2+} uptake regulates cytosolic Ca\textsuperscript{2+} signaling and contributes to prevent local Ca\textsuperscript{2+} overload in the cytosol (37). On the contrary, accumulation of Ca\textsuperscript{2+} in the mitochondrial matrix induces permeability transition (PT) pore opening and apoptosis. To maintain Ca\textsuperscript{2+} homeostasis, there is an interactive operation of mitochondrial transporters involved in Ca\textsuperscript{2+} influx and efflux pathways. Further research focusing on the comprehensive understanding of mitochondrial Ca\textsuperscript{2+} transporters may lead to the identification of novel therapeutic targets to improve mitochondrial energy metabolism and to prevent cytotoxicity. This may be especially relevant in insulin-releasing cells, where mitochondrial Ca\textsuperscript{2+} transport plays a key role in metabolism-secretion coupling, dysfunction of which leads to the development of type 2 diabetes.
REFERENCES


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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effects of MCU knockdown on mitochondrial Ca2+ uptake, ATP synthesis, and insulin secretion. (A) Efficiency of siRNA for MCU was validated by quantitative real time PCR. Total RNAs were isolated from INS-1E cells 72 hrs after transfection with siRNA against MCU (siMCU). Relative mRNA levels of the gene were compared to those in cells transfected with non-targeting siRNA (siControl) (N=5). (B and C) Reduced protein levels of MCU were demonstrated by Western blots using primary antibodies against MCU and its densitometric analysis 72 hrs after transfection with siControl or siMCU (N=3). β-actin was used as the reference control. INS-1E cells were transfected with ratiometric-pericam (RPmit) plasmid 24 hrs after siRNA transfection. After 48 hrs incubation, cells were permeabilised with α-hemolytic toxin and fluorescence intensities from RPmit, reflecting mitochondrial Ca2+ level ([Ca2+]mito), were measured by using the confocal microscope system. Averaged traces (D) and quantitative analysis (E) of [Ca2+]mito signals from cells transfected with siControl (black line, N=13) and siMCU (gray line, N=7) upon stimulation with 120 nM and 500 nM extramitochondrial Ca2+ ([Ca2+]o). (F) Succinate-induced ATP production in permeabilised cells (N=6) (G) insulin secretion by glucose (16.7 mM) stimulation in intact INS-1E cells (N=6). Transfection with siRNA was performed in dispersed rat pancreatic islets. Protein level of MCU and glucose-stimulated insulin secretion were compared between pancreatic islet cells treated with siControl (N=4) and siMCU (N=5). *, ** and *** denote p < 0.05, < 0.01 and < 0.001, respectively.

Fig. 2. Knockdown of MCU decreased respiratory chain protein levels and mitochondrial enzyme activities. (A and B) Total cellular protein was extracted 72 hrs after transfection with non-targeting siRNA (siControl) or siRNA against MCU (siMCU). (A) Western blots and (B) densitometric analyses for respiratory chain complex I, III, IV, V, and TOM20 (N=4~8). (C~G) MTT intensities (N=7) (C), cytochrome c oxidase (COX) activity (N=6) (D and E), and citrate synthase activity (N=8) (F and G) were measured 72 hrs after siRNA transfection and compared between siControl- and siMCU-treated cells. *, **, and *** denote p < 0.05, < 0.01, and < 0.001, respectively.

Fig. 3. Knockdown of MCU reduced basal and glucose-stimulated oxygen consumption. INS-1E cells (2 x 10⁴ cells/well) were seeded onto 24-well plates and transfected with non-targeting siRNA (siControl) or siRNA against MCU (siMCU). After 72 hrs, culture medium was replaced with 2.8 mM glucose KRB solution and incubated for 1 hr at 37°C prior to measurement of basal oxygen consumption rate (OCR) for 20 min. Subsequently, glucose (16.7 mM) was applied to the incubation solution, followed by the addition of mitochondrial inhibitors; oligomycin (3 μg/ml), FCCP (3 μM), and antimycin A (3 μM). The levels of basal (B) and glucose-stimulated OCR (C) were compared between control and MCU-silenced cells. Values of OCR are presented as picomoles of consumed oxygen per min per μg protein. N=14, * and ** denote p < 0.05 and < 0.01, respectively.

Fig. 4. Knockdown of MCU did not affect mitochondrial membrane potential but decreased the hyperpolarising response induced by dissipating the pH gradient with ionophore. (A) Mitochondrial membrane potential (Ψmito) in α-toxin-permeabilised INS-1E cells was measured in the presence of JC-1, a potential-sensitive fluorescence probe (500 nM). Increased JC-1 ratio (red/green) reflects hyperpolarisation of the Ψmito. (B) Intact cells were loaded and perfused with another potential-sensitive fluorescence dye, TMRM (5 nM), and the Ψmito was measured in a nonquenching redistribution mode. Hyperpolarisation of the Ψmito by succinate (3 mM) in permeabilised cells (N=15) (A) and by high glucose (16.7 mM) in intact cells (N=6) (B) were compared between control and MCU-silenced cells. In α-toxin-permeabilised cells, nigericin (500 nM)-induced hyperpolarisation of the Ψmito as compensation for the collapsed mitochondrial pH gradient (ΔpHmito) was compared between control and MCU-silenced
cells (N=6) (C and D). * denotes p < 0.05.

**Fig. 5. Knockdown of MCU markedly impaired nutrient-stimulated mitochondrial matrix alkalisation.** INS-1E cells were transfected with non-targeting siRNA (siControl) or siRNA against MCU (siMCU) followed by infection with an adenovirus carrying mtAlpHi 24 hrs after siRNA transfection. After 48 hrs of further incubation, mitoAlpHi fluorescence was recorded by using the confocal microscope system in α-toxin permeabilised cells (A) or intact cells (B) and expressed as mitochondrial matrix pH (pH\textsubscript{mito}) based on the subsequent pH titration. For titrations of mitoAlpHi fluorescence, mitochondrial pH was clamped to the defined pH with ionophores. Succinate-induced alkalinisation (C) and extramitochondrial Ca\textsuperscript{2+} (500 nM)-induced acidification (D) were compared between control (clear bar) and MCU knockdown cells (gray bar) (N=6–28). Alkalinisation of the pH\textsubscript{mito} by high glucose (16.7 mM) in intact cells was also compared between control (clear bar) and MCU knockdown cells (gray bar) (N=8) (E). ** and *** denote p < 0.01 and < 0.001, respectively.

**Fig. 6. Acute blocking of MCU-mediated Ca\textsuperscript{2+} uptake did not affect metabolism-secretion coupling.** (A) Effect of a selective MCU blocker, Ru360, on glucose-stimulated insulin secretion were analyzed in INS-1E cells (N=4). (B and C) Changes in mitochondrial membrane potential (Ψ\textsubscript{mito}) by Ru360 were measured with JC-1 fluorescence dye. * denotes p < 0.05.

**Fig. 7. Knockdown of LETM1 abolished acidification of mitochondrial matrix pH upon extramitochondrial Ca\textsuperscript{2+} addition.** (A) Efficiency of siRNA for LETM1 was validated by quantitative real time PCR. Total RNA was isolated 72 hrs after transfection with siRNA against LETM1 (siLETM1). Relative mRNA levels of the gene were compared to those in cells transfected with non-targeting siRNA (siControl) (N=5). (B and C) Reduced protein levels of LETM1 were validated by Western blots using primary antibodies against MCU and its densitometric analysis 72 hrs after transfection with siControl or siLETM1 (N=3). β-actin was used as the reference control. INS-1E cells were transfected with non-targeting siRNA (siControl) or siRNA against LETM1 (siLETM1) and infected with an adenovirus carrying mtAlpHi or transfected with a ratiometric-pericam plasmid 24 hrs after siRNA transfection. (D–F) Changes in mitochondrial matrix pH (pH\textsubscript{mito}) in response to succinate (3 mM) or extramitochondrial Ca\textsuperscript{2+} (500 nM) were measured after 48 hrs of further incubation and compared between control (clear bar) and LETM1 knockdown cells (gray bar) (N=14–21). (G and H) Increases in mitochondrial matrix Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{mito}) by the addition of extramitochondrial Ca\textsuperscript{2+} (120 nM and 500 nM) in α-toxin-permeabilised cells were compared between two groups (N=5–12). The changes in [Ca\textsuperscript{2+}]\textsubscript{mito} by the application of high K\textsuperscript{+} (30 mM) in intact cells were measured using ratiopericam probe (N=5) (I and J) or Rhod-2 dye (K and L). ** and *** denote p < 0.01 and < 0.001, respectively.

**Fig. 8. Down-regulation of LETM1 impaired metabolism-secretion coupling.** (A) 72 hrs after transfection with siRNA against LETM1 (siLETM1) or control (siControl), insulin secretion by glucose (16.7 mM) stimulation was analyzed in intact INS-1E cells (N=6). (B) In α-toxin-permeabilised cells, mitochondrial membrane potential was measured with TMRM (5 nM) fluorescence dye. Total cellular protein was extracted 72 hrs after siRNA transfection and performed Western blots (C) and densitometric analyses (D) for respiratory chain complexes (N=4). * and ** denote p < 0.05 and < 0.01, respectively.
Fig. 1. Quan et al.
Fig. 2. Quan et al.

A) Western blot analysis showing protein levels of complexes I, III, IV, and V, and TOM 20 in siControl and siMCU cells. 

B) Graph showing normalized protein levels of complexes I, III, IV, V, and TOM 20 in siControl and siMCU cells. 

C) Graph showing MTT intensity (% of control) in siControl and siMCU cells. 

D) Graph showing the relative absorbance (550 nm) of cytochrome c over time in siControl and siMCU cells. 

E) Graph showing cytochrome c oxidase activity in nmole/min/mg protein in siControl and siMCU cells. 

F) Graph showing absorbance at 412 nm in siControl and siMCU cells over time. 

G) Graph showing citrate synthase activity in umole/min/mg protein in siControl and siMCU cells.
Fig. 3. Quan et al.

A) Graph showing OCR (pmol/min/µg protein) over time (min) with glucose 16.7 mM and Antimycin/Oligomycin treatment.

B) Bar graph comparing baseline OCR (pmol/min/µg protein) between siControl and siMCU conditions.

C) Bar graph comparing glucose-stimulated ΔOCR (pmol/min/µg protein) between siControl and siMCU conditions.
Fig. 4. Quan et al.

A) Succinate 3 mM

B) FCCP

C) Antimycin

D) Nigericin-induced $\Delta \Psi_{mito}$
Fig. 5. Quan et al.

A) Mitochondrial matrix pH ($pH_{mito}$) changes with [Ca$^{2+}$], Succinate, and SiControl vs. SiMCU.

B) Glucose 16.7 mM effect on mitochondrial matrix pH ($pH_{mito}$) with SiControl vs. SiMCU.

C) Suc-induced alkalinisation ($\Delta pH$) with SiControl vs. SiMCU.

D) Ca$^{2+}$-induced acidification ($\Delta pH$) with SiControl vs. SiMCU.

E) High glucose-induced alkalinisation ($\Delta pH_{mito}$) with SiControl vs. SiMCU.
Fig. 6. Quan et al.
Fig. 8. Quan et al.

**Insulin release**:
- siControl
- siLETM1

**Glucose**
- 2.8 mM
- 16.7 mM

**Normalized TMRM intensity**
- Antimycin
- Succinate 3 mM
- siControl
- siLETM1

**Protein levels**
- Complex I
- Complex III
- Complex IV
- β-actin

Normalized protein levels:
- siControl
- siLETM1

**Statistical significance**
- "**" indicates significant difference.