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Background: Mitochondrial Ca$^{2+}$ uptake affects energy metabolism and insulin secretion. Knockdown of 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 permeabilized 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 hyperpolarization of the electrical gradient was not altered. In permeabilized 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.

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 permeabilized 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 hyperpolarization of the electrical gradient was not altered. In permeabilized 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.

Pancreatic β-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 (∆ψ_mito membrane potential) and chemical (∆pH_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)$^2$ cycle and mitochondrial metabolite shuttles. Thus, the metabolic status of the β-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 Ca$^{2+}$ concentration ([Ca$^{2+}$]_mito) is a key activator of mitochondrial metabolic function (1, 6, 7). The [Ca$^{2+}$]_mito activates several matrix enzymes including α-ketoglutarate dehydrogenase in the TCA cycle (8). The ATP synthase is also directly activated by a rise in [Ca$^{2+}$]_mito (9). In pancreatic β-cells [Ca$^{2+}$]_mito is strictly required for ATP synthase-dependent respiration stimulated by glucose (10). Given its importance, mitochondrial Ca$^{2+}$ uptake has been a research focus for

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2 The abbreviations used are: TCA, tricarboxylic acid; GSIS, glucose-stimulated insulin secretion; LETM, leucine zipper-EF hand-containing transmembrane protein; MCU, mitochondrial Ca$^{2+}$ uniporter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; COX, cytochrome c oxidase; OCR, oxygen consumption rate.
decades, starting with the functional characterization in isolated mitochondria. Nevertheless, it took 50 years to elucidate the molecular identity of the mitochondrial Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter (MCU) (11, 12). Mitochondrial Ca\textsuperscript{2+} uptake through MCU is regulated by a number of recently discovered proteins, including mitochondrial Ca\textsuperscript{2+} uptake 1 and 2 (MICU1/2) (13–15), mitochondrial Ca\textsuperscript{2+} uniporter regulator 1 (MCUR1) (16), and essential MCU regulator (EMRE) (17). Especially MICU1/2 negatively regulate MCU activity under resting cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cyt}). At stimulating [Ca\textsuperscript{2+}]\textsubscript{cyt}, (\textgreater 10 \textmu M), however, MICU1 activates MCU activity, implying that the regulatory subunits of the MCU complex modulate mitochondrial Ca\textsuperscript{2+} loads of \( \Delta \Psi \text{mito} \)-driven Ca\textsuperscript{2+} uptake without perturbing the important signal propagation from ER to mitochondria (13, 18, 19).

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

In addition to [Ca\textsuperscript{2+}]\textsubscript{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 pH\textsubscript{mito} under resting conditions. Nutrient stimulation causes matrix alkalization without any marked cytosolic pH change (29). Preventing the resulting nutrient-induced increase of the \( \Delta \Psi \text{mito} \) 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 \( \Delta \Psi \text{mito} \) 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 [Ca\textsuperscript{2+}]\textsubscript{mito} rise and second phase ATP/ADP increase (26). Knockdown of either MCU or MICU1 diminishes insulin secretion associated with defects in mitochondrial Ca\textsuperscript{2+} uptake (32). Mice lacking MCU show a significant reduction of [Ca\textsuperscript{2+}]\textsubscript{mito} and Ca\textsuperscript{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 \( \beta \)-cell metabolism-secretion coupling. In this study, we observed that reduced mitochondrial Ca\textsuperscript{2+} uptake following silencing of MCU significantly attenuated respiratory chain activity and \( \Delta \Psi \text{mito} \) 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\textsuperscript{2+} uptake for the establishment of the \( \Delta \Psi \text{mito} \) in metabolism-secretion coupling. We also provide evidence for a novel role of the putative Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter leucine zipper-EF-hand-containing transmembrane protein 1 (LETM1) as a Ca\textsuperscript{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\textsubscript{2} in a complete medium composed of RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen), 1 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES, 100 units/ml penicillin, and 100 \mu g/ml streptomycin (HyClone, Thermo Fisher Scientific Inc., Lafayette, CO). Experiments were performed with cells of passage number 80–120. Most chemicals were purchased from Sigma except JC-1 from Molecular Probes (Eugene, OR).

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

**Permeabilization with \textalpha-Hemolysin Toxin**—INS-1E cells were seeded and cultured onto well-plates or coverslips coated with 804G matrix. Cells were washed with Ca\textsuperscript{2+}-free Krebs-Ringer bicarbonate (KRB) solution (mM; 140 NaCl, 3.6 KCl, 0.5 NaH\textsubscript{2}PO\textsubscript{4}, 0.5 MgSO\textsubscript{4}, 1.5 CaCl\textsubscript{2}, 2 NaHCO\textsubscript{3}, 5.5 glucose, pH 7.4 titrated with NaOH) and then incubated for 10 min at 37 °C with 1 \mu g/ml of *Staphylococcus aureus* \textalpha-hemolysin toxin (Sigma) in an intracellular buffer (mM; 140 KCl, 5 NaCl, 7 MgSO\textsubscript{4}, 1 KH\textsubscript{2}PO\textsubscript{4}, 20 HEPES, 10.2 EGTA, 1.65 CaCl\textsubscript{2}, 0.1 ATP, pH 7.0 with KOH), which has about 120 nm of free Ca\textsuperscript{2+} concentration. After \textalpha-toxin permeabilization, 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 four different targets of each gene (SMART pool, Dharmacon).

**Quantitative Real-time PCR**—Total RNA was isolated from cells 72 h after siRNA transfection using the RNeasy kit (Cat. 74134, Qiagen GmbH, Hilden, Germany). First strand cDNA was synthesized from 1 \mu g of total RNA with a reverse transcription kit (Applied Bioscience, Foster City, CA) using oligo-dT. Quantitative PCR was performed using sequence-specific primers for rat MCU (forward: 5’-GAAAGTGGTTGAGGGTTCCA-3’, reverse: 5’-AGGAAAAGCGGAAGAGGAC-3’), and LETM1 (forward: 5’-GGCTGACTTGGACACCTCTAT-3’, reverse: 5’-CAGGGATCCACTTCGAGGTC-3’). Rat \beta-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 (Cat. 204143, Qiagen GmbH). Data were analyzed following \( \Delta \Delta C\textsubscript{T} \) method (34).
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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, Cat. HPA016480, Sigma), LETM1 (1:500, Cat. sc134672, Santa Cruz Biotechnology, Dallas, TX), complex I NDUFA9 (1:2000 dilution, Cat. 459100, Invitrogen, Carlsbad, CA), complex III UQCRCC2 (1:2500, Cat. MS504, Mitosciences, Abcam), complex IV subunit I (1:1000, Cat. 459600, Invitrogen), complex V (1:5000, Cat. MS604-300, Mitosciences, Abcam), TOM20 (1:1000, Cat. sc11415, Santa Cruz Biotechnology), and β-actin (1:5000, Cat. ab6276, Abcam, Cambridge, UK) were used. Horseradish peroxidase (HRP)-conjugated secondary antibody against either mouse or rabbit IgG (Cat. sc2004 and sc2005, Santa Cruz Biotechnology) was used. Horseradish peroxidase (HRP)-conjugated secondary antibody against either mouse or rabbit IgG (Cat. sc2004 and sc2005, Santa Cruz Biotechnology) was used.

Measurement of Mitochondrial Matrix Ca2+—To measure mitochondrial matrix Ca2+ level, we used a mitochondria-targeted ratio-pericam (RPmit) plasmid, generously provided by Prof. Roger Tsien (UC San Diego). Cells were transfected with siRNA, and 24 h after, transfected with RPmit using X-tremEGENE (Roche Diagnostics GmbH, Mannheim, Germany). Fluorescence imaging of Ca2+ 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). Intact or permeabilized cells on the confocal microscope were perfused with KB solution or intracellular buffer, respectively, and fluorescence images (435 nm excitation and 535 nm emission) were acquired every 10 s and analyzed using Metafluor 6.3 software (Universal Imaging, Molecular Devices).

Measurement of Cytosolic ATP and Insulin—Cells plated onto 48 well-plates (2 × 105 cells/well) were permeabilized with α-toxin and incubated for 5 or 15 min in an intracellular buffer containing ADP (10 μM) or without succinate (1 μM). To determine mitochondrial ATP release, the supernatant was harvested after incubation, and ATP level was measured by using the microplate reader (SynergyTM2, BioTeK Instruments Inc., Winooski, VT) with a bioluminescence assay kit (kit II, Roche Diagnostics, Mannheim, Germany).

For static insulin secretion measurement, cells in a 804G-coated 24 well-plate (1.5 × 105 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 permeabilized 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 (lauryl maltoside, 2.5 mM). Traces were started with the addition of reduced cytochrome c (25 mM 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 Biosciences, GE Healthcare Biosciences, Pittsburgh, PA). COX activity was expressed as moles of oxidized cytochrome c per min. Cytosolase activity was measured with a citrate synthase assay kit (Sigma) based on the manufacturer’s instructions.

For the MTT assay, siRNA-transfected cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (50 μg/well) for 2 h and then treated with dimethyl sulfoxide (100 μL/well). The absorbance (A570 − A630) of each well was measured by using a microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Oxygen Consumption—Cellular oxygen consumption rate (OCR) was determined by Extracellular Flux Analyzer (XF-24, Seahorse Bioscience, North Billerica, MA). Cells (2 × 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 h 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 were recorded.

Measurement of Mitochondrial Matrix pH—Mitochondrial matrix pH (pHmito) was measured using adenovirus expressing mtAlpHi (Ad-tetON-mAlpHi) as described previously (29). Intact or permeabilized 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 (ΔΨmito), cells seeded onto black-walled 96-well plates (5 × 103 cells/well) were loaded with JC-1 (500 nM) for 20 min of and then treated with dimethyl sulfoxide (100 μL/well). The absorbance (A570 − A630) of each well was measured by using a microplate reader (Molecular Devices, Sunnyvale, CA).

For the MTT assay, siRNA-transfected cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (50 μg/well) for 2 h and then treated with dimethyl sulfoxide (100 μL/well). The absorbance (A570 − A630) of each well was measured by using a microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis—Values are presented as mean ± S.E. 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.
Role of MCU for Mitochondrial pH Gradient

FIGURE 1. Effects of MCU knockdown on mitochondrial Ca\(^{2+}\) 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 h after transfection with siRNA against MCU (siMCU). Relative mRNA levels of the gene were compared with those in cells transfected with non-targeting siRNA (siControl) (n = 3). B and C, reduced protein levels of MCU were demonstrated by Western blots using primary antibodies against LETM1 and its densitometric analysis 72 h 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 h after siRNA transfection. After 48 h of incubation, cells were permeabilized with α-hemolytic toxin and fluorescence intensities from RPmit, reflecting mitochondrial Ca\(^{2+}\) level ([Ca\(^{2+}\)\(_{\text{mito}}\)]), were measured by using the confocal microscope system. Averaged traces ([Ca\(^{2+}\)]\(_{\text{mito}}\)), resulted in significantly attenuated GSIS (6, 7). To further investigate the role of [Ca\(^{2+}\)]\(_{\text{mito}}\) in energy metabolism we studied succinate-dependent ATP synthesis in permeabilized cells (n = 6) and (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 = 3). *, **, and *** denote p < 0.05, < 0.01, and < 0.001, respectively.

RESULTS

Effects of MCU Knockdown on Mitochondrial Ca\(^{2+}\) Uptake—To understand the role of mitochondrial Ca\(^{2+}\) 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 h 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 with siControl-treated cells. Western blot analysis also revealed a strong siRNA-induced reduction of the MCU protein by 82.3 ± 2.3% (Fig. 1, B and C).

To examine the impact of MCU knockdown on mitochondrial Ca\(^{2+}\) uptake, we determined the effect of extramitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{ex}}\)) on [Ca\(^{2+}\)]\(_{\text{mito}}\) in α-toxin-permeabilized INS-1E cells expressing mitochondria-targeted ratiocam (RPmit). When the cells were perfused with intracellular buffer containing ~10 nM Ca\(^{2+}\) and 3 mM succinate, switching [Ca\(^{2+}\)]\(_{\text{ex}}\) to 120 mM gradually increased [Ca\(^{2+}\)]\(_{\text{mito}}\) (Fig. 1D). The [Ca\(^{2+}\)]\(_{\text{mito}}\) declined slowly after returning to 10 nM [Ca\(^{2+}\)]\(_{\text{ex}}\). Addition of 500 mM [Ca\(^{2+}\)]\(_{\text{ex}}\), resulted in more rapid and marked increase in [Ca\(^{2+}\)]\(_{\text{mito}}\), 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 permeabilized 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\(_{\text{1}}\)F\(_{\text{0}}\)-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-en-
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Knockdown of MCU decreased respiratory chain protein levels and mitochondrial enzyme activities. A and B, total cellular protein was extracted 72 h 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, and TOM20 (n = 4–8). C–G, MTT intensities (n = 7) (C), COX activity (n = 6) (D and E), and citrate synthase activity (n = 8) (F and G) were measured 72 h after siRNA transfection and compared between siControl- and siMCU-treated cells. *, **, and *** denote p < 0.05, < 0.01, and < 0.001, respectively.

Nigericin-induced Mitochondrial Hyperpolarization Was Lowered in MCU-silenced Cells—The mitochondrial electrical gradient (ΔΨmito) is the main driving force for ATP synthesis as well as Ca2+ transport through the MCU. MCU knockdown in turn may alter the ΔΨmito. We therefore measured the ratio of fluorescence intensities (red/green) after loading with JC-1, which reflects the ΔΨmito (35). The hyperpolarizing response to succinate in control and MCU knockdown cells, was not significantly different (Fig. 4A). Glucose-induced hyperpolarization in intact cells was also not different between the two groups, which was measured by using the fluorescence probe TMRM in a non-quenching redistribution mode to measure ΔΨmito (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 ΔΨmito in insulin-secreting cells.

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

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

Addition of extramitochondrial Ca2+ to succinate-stimulated mitochondria resulted in mitochondrial matrix acidification in permeabilized 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. 5, A 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 depolarizes the ΔΨmito. We measured the effect of Ru360 on ΔΨmito in intact cell during high glucose stimulation. As shown in Fig. 6, B and C, Ru360 further hyperpolarized the ΔΨmito by blocking MCU-mediated inward depolarizing 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 pHmito upon extramitochondrial addition of Ca2+ was measured in control or LETM1-silenced INS-1E cells. After 72 h 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...
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The alteration in mitochondrial pH (Fig. 7A) and LETM1 protein (78.9 ± 0.9% reduction, Fig. 7, B and C) compared with siControl-treated cells. In α-toxin-permeabilized LETM1 knockdown cells, succinate-induced matrix alkalization was not significantly altered (Fig. 7, D and E). In contrast, Ca²⁺ (500 nm)-elicted pH_mito acidification was abolished in LETM1 knockdown cells, similar to our results in MCU knockdown cells (Fig. 7, F). Moreover, the increase in [Ca²⁺]mito by extramitochondrial Ca²⁺ export (Fig. 7, G and H). Consistent with these findings the [Ca²⁺]mito rise following K⁺-induced Ca²⁺ influx in intact cells was also enhanced in LETM1 knockdown cells (Fig. 7, I–L). These results strongly suggest that LETM1 mediates at least one important component of Ca²⁺ efflux in insulin secreting cells. In MCU knockdown cells, extramitochondrial Ca²⁺ is taken up inefficiently, and therefore LETM1-mediated Ca²⁺ efflux is strongly reduced. As a consequence the acidifying response of pH_mito to extramitochondrial Ca²⁺ is abrogated.

We further investigated the functional consequences of LETM1 silencing on mitochondrial bioenergetics and metabolism-secretion coupling. Even though the stimulus-induced mitochondrial Ca²⁺ response was augmented in LETM1 knockdown cells, glucose-induced insulin secretion and hyperpolarization of the ΔΨ_mito were attenuated (Fig. 8, A and B). Reduction of LETM1 expression also lowered protein levels of subunits of respiratory chain complexes.

**FIGURE 5.** Knockdown of MCU markedly impaired nutrient-stimulated mitochondrial matrix alkalization. INS-1E cells were transfected with non-targeting siRNA (siControl) or siRNA against MCU (siMCU) followed by infection with an adenovirus carrying mtAlpHi 24 h after siRNA transfection. After 48 h of further incubation, mitoAlpHi fluorescence was recorded by using the confocal microscope system in α-toxin permeabilized cells (A) or intact cells (B) and expressed as mitochondrial matrix pH (pH_mito) based on the subsequent pH titration. For titrations of mitoAlpHi fluorescence, mitochondrial pH was clamped to the defined pH with ionophores. Succinate-induced alkalization (C) and extramitochondrial Ca²⁺ (500 nm)-induced acidification (D) were compared between control (clear bar) and MCU knockdown cells (gray bar) (n = 6–28). Alkalization of the pH_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.

**FIGURE 6.** Acute blocking of MCU-mediated Ca²⁺ 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 (ΔΨ_mito) by Ru360 were measured with JC-1 fluorescence dye. * denotes p < 0.05.
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FIGURE 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 h after transfection with siRNA against LETM1 (siLETM1). Relative mRNA levels of the gene were compared with 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 LETM1 and its densitometric analysis 72 h 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 h 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 h 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 mM and 500 mM) in α-toxin-permeabilized 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.

DISCUSSION

Increases in cytosolic Ca\textsuperscript{2+} stimulate numerous energy consuming processes, including muscle contraction and neurotransmitter release. In particular, elevated mitochondrial matrix Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}\textsubscript{mito}] is a key stimulator of energy provision. The rise in [Ca\textsuperscript{2+}\textsubscript{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\textsuperscript{2+} waves from the cytosol to the mitochondrial matrix through Ca\textsuperscript{2+} transporters is necessary. MCU has been suggested to be the main communicating channel linking cytosolic and mitochondrial Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 ΔΨ\textsubscript{mito} (5) does not affect ΔΨ\textsubscript{mito}, 6) reduces nutrient-stimulated ATP generation, and 7) impairs glucose-stimulated insulin secretion. We provide strong evidence that mitochondrial Ca\textsuperscript{2+} uptake through MCU is a prerequisite for the establishment of the ΔΨ\textsubscript{mito} and activation of...
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mitochondrial energy metabolism. The critical role of the ΔpH_{mito} in mitochondrial ATP synthesis and insulin secretion has been demonstrated previously (29, 30). It is inferred, therefore, that the reduced [Ca^{2+}]_{mito} together with impaired ΔpH_{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 Ψ_{mito} generated by the respiratory chain (39). Because of high ΔΨ_{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 despite 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 alkalinization 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 extracelluar Ca^{2+}-free condition did not affect nutrient-stimulated alkalinization 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 ΔpH_{mito} generation and ATP synthesis, leading to impaired nutrient-stimulated insulin secretion.

Nigericin-induced hyperpolarization of Ψ_{mito} reflects the preexisting ΔpH_{mito}. Therefore, the reduced hyperpolarizing response in MCU-silenced cells suggests reduction of the pre-existing ΔpH_{mito} (Fig. 4, C and 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. 4, A and 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 depolarization 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 depolarization 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 depolarization of Ψ_{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 permeabilized 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 Liang 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_{mito} acidification after knockdown of LETM1. Similar to the response in MCU knockdown cells, the pH_{mito} in LETM1 knockdown cells was not acidified, instead slight alkalinization by extramitochondrial Ca^{2+} was observed (Fig. 6C). Furthermore, the [Ca^{2+}]_{mito} rises by extramitochondrial Ca^{2+} addition were increased in LETM1-silenced cells (Fig. 6F). Our findings demonstrate that LETM1 mediates Ca^{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^{+}/1 Ca^{2+} antiport when reconstituted in artificial liposomes (44). Thus, in intact cells, Ca^{2+} efflux via LETM1 is preferred, which is driven by the H^{+} gradient across the inner mitochondrial membrane (45).

We suggest that Ca^{2+} influx through MCU is coupled to LETM1-mediated Ca^{2+} efflux with proton uptake favored by high [Ca^{2+}]_{mito} and alkaline pH_{mito}. In MCU-silenced cells, Ca^{2+} influx via LETM1 was prevented or even reversed to Ca^{2+} influx due to reduced Ca^{2+} and pH gradients, explaining the disappearance of pH_{mito} acidification.

Why LETM1 knockdown has negative effects on the expression of subunits of the respiratory chain, glucose-stimulated Ψ_{mito} hyperpolarization and insulin secretion is not clear. A recent publication presented evidence that LETM1 holo-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^{2+} uptake is essential for the respiratory chain activity and the generation of ΔpH_{mito} in insulin-releasing cells. This chemical
gradient (ΔpHmito) 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 definitive metabolism-secretion coupling by MCU knockdown. In addition, mitochondrial Ca2+ uptake regulates cytosolic Ca2+ signaling and contributes to prevent local Ca2+ overload in the cytosol (37). On the contrary, accumulation of Ca2+ in the mitochondrial matrix induces permeability transition (PT) pore opening and apoptosis. To maintain Ca2+ homeostasis, there is an interactive operation of mitochondrial transporters involved in Ca2+ influx and efflux pathways. Further research focusing on the comprehensive understanding of mitochondrial Ca2+ 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 Ca2+ transport plays a key role in metabolism-secretion coupling, dysfunction of which leads to the development of type 2 diabetes.

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Bioenergetics:
Essential Role of Mitochondrial Ca\textsuperscript{2+} Uniporter in the Generation of Mitochondrial pH Gradient and Metabolism-Secretion Coupling in Insulin-releasing Cells

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