The Mitochondrial Calcium Uniporter Matches Energetic Supply with Cardiac Workload during Stress and Modulates Permeability Transition

Graphical Abstract

**Highlights**

- The MCU is dispensable for baseline homeostatic cardiac function.
- Deletion of Mcu protects against myocardial IR injury by reducing MPTP activation.
- The MCU is required to match energetics with contractile demand during stress.
- A slow MCU-independent uptake mechanism may maintain basal matrix \( mCa^{2+} \) content.

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**In Brief**
Luongo et al. show, using a conditional knockout mouse model, that the mitochondrial Ca\(^{2+}\) uniporter (MCU), although dispensable for homeostatic function, is necessary for the cardiac “fight-or-flight” contractile response and a significant contributor to mitochondrial permeability transition during ischemia-reperfusion injury.

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The Mitochondrial Calcium Uniporter Matches Energetic Supply with Cardiac Workload during Stress and Modulates Permeability Transition

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SUMMARY

Cardiac contractility is mediated by a variable flux in intracellular calcium (Ca^{2+}), thought to be integrated into mitochondria via the mitochondrial calcium uniporter (MCU) channel to match energetic demand. Here, we examine a conditional, cardiomyocyte-specific, mutant mouse lacking Mcu, the pore-forming subunit of the MCU channel, in adulthood. Mcu^{-/-} mice display no overt baseline phenotype and are protected against mCa^{2+} overload in an in vivo myocardial ischemia-reperfusion injury model by preventing the activation of the mitochondrial permeability transition pore, decreasing infarct size, and preserving cardiac function. In addition, we find that Mcu^{-/-} mice lack contractile responsiveness to acute β-adrenergic receptor stimulation and in parallel are unable to activate mitochondrial dehydrogenases and display reduced bioenergetic reserve capacity. These results support the hypothesis that MCU may be dispensable for homeostatic cardiac function but required to modulate Ca^{2+}-dependent metabolism during acute stress.

INTRODUCTION

The cardiomyocyte is unique in that a large and variable flux of intracellular calcium (Ca^{2+}) must occur to mediate and regulate contraction. Thus, a complex system has evolved to regulate Ca^{2+} transport to maintain homeostatic conditions (Bers, 2008). Numerous genetic components have been shown to mediate the passage of Ca^{2+} across the sarcolemma and sarcoplasmic reticulum (SR), and, while great strides have been made toward understanding the temporal and spatial relationship of Ca^{2+} in regards to excitation-contraction (EC) coupling, our understanding of other sub-cellular Ca^{2+} domains, including the components of mitochondria Ca^{2+} (mCa^{2+}) exchange, remains elementary.

The dynamic Ca^{2+} environment of the heart requires that cardiac mitochondria possess an exchange system capable of dealing with the variable changes in Ca^{2+} load. Ca^{2+} enters the mitochondrial matrix via the mitochondria calcium uniporter (MCU). The MCU is an inward rectifying, low-affinity, high-capacity channel whose uptake is mediated by mitochondrial membrane potential (Δψ = approximately –180 mV) generated by the electron transport chain (ETC) (Kirichok et al., 2004). The recent identification of the gene encoding the channel-forming portion of the uniporter, formerly named CCDC109A now known as MCU, has opened the field to genetic gain-loss-of-function studies to determine experimentally the true role of mCa^{2+} signaling in the regulation of numerous proposed cellular processes (Baughman et al., 2011; De Stefani et al., 2011). To date, multiple reports have confirmed MCU as being required for acute mCa^{2+} influx into the matrix. However, numerous outstanding questions remain in regards to the molecular regulation of the MCU and the physiological function of mCa^{2+}, particularly in excitable cells such as cardiomyocytes.

The high metabolic demand of contractility makes it essential that an efficient and tightly controlled system be in place to regulate energy production. Oxidative Phosphorylation (OxPhos) is the largest contributor to myocardial metabolism and as such the mitochondria represents a central control point to ensure that energy demands are met. Simultaneous measurements of mCa^{2+} and NADH have correlated increased mCa^{2+} load with increased oxidative phosphorylation and ATP production (Brandes and Bers, 2002; Unitt et al., 1989). Thus, Ca^{2+} is proposed to be the link between EC coupling (ECC) and OxPhos and has been shown to modulate mitochondrial metabolism through numerous mechanisms including the activation of Ca^{2+}-dependent dehydrogenases and modulation of ETC complexes (Glancy and Balaban, 2012).

In contrast to the aforementioned pro-survival metabolic signaling, numerous studies have implicated mCa^{2+} overload in the activation of apoptosis and necrosis (Rasola and Bernardi, 2011). mCa^{2+} is known to cause outer-mitochondrial membrane (OMM) permeability prompting the release of apoptogens. Ca^{2+} is also thought to be the major priming event in the opening of the
mitochondrial permeability transition pore (MPTP) causing the collapse of $\Delta \psi$ and loss of ATP production resulting in necrotic cell death. This mechanism of cellular demise is believed to significantly contribute to the initiation and progression of myocardial infarction and heart failure (Foo et al., 2005). In addition, it has been speculated that mitochondria in close contact to the sarcoplasmic reticulum (SR) may buffer Ca$^{2+}$ cycling and thereby play a direct role in modulating EC coupling, providing yet another layer of potential regulation (Drago et al., 2012; Rizzuto et al., 1998).

To begin to unravel how mCa$^{2+}$ signaling modulates in vivo physiology, a group at the NHLBI recently generated a Mcu gene-trap mouse (Pan et al., 2013). As expected, mitochondria isolated from this global Mcu-null mouse failed to take up Ca$^{2+}$. However, while they did find alterations in some aspects of skeletal muscle work capacity, they did not find a significant cardiac phenotype. Particularly intriguing, they found no change in myocardial infarct size in an ex vivo global ischemia model even though in vitro indices of MPTP opening appeared to be completely absent. These surprising results have spurred the field to question the true role of mCa$^{2+}$ signaling in the normal and diseased heart.

To advance our understanding of mCa$^{2+}$ uptake in the heart, in collaboration with the Molkentin lab, we generated a conditional, loss-of-function mouse model (MCu$^{fl/fl}$) and coupled with a tamoxifen-inducible, cardiomyocyte-specific Cre recombinase transgenic line, deleted Mcu in adulthood (Kwong et al., 2015 [this issue of Cell Reports]). Here, we report that loss of Mcu ablates mCa$^{2+}$ uptake and activity (IMCU) and protects against cell death in an in vivo ischemia-reperfusion (IR) injury model by preventing the activation of the mitochondrial permeability transition pore (MPTP). In addition, we found that Mcu-null mice lacked in vivo contractile responsiveness to beta-adrenergic receptor (βAR) stimulation and in parallel were unable to activate mitochondrial dehydrogenases and meet energetic demand. Further experimental analysis confirmed a lack of energetic responsiveness to acute sympathetic stress, supporting the hypothesis that the physiological function of the MCU is to match Ca$^{2+}$-dependent contractile demands with mitochondrial metabolism during the “fight-or-flight” response.

RESULTS

Generation of a Mcu Conditional Knockout Mouse Model and Validation of Functionality

The Mcu targeting construct was designed with loxP sites flanking the critical exons 5–6, which encode the DIME motif, an evolutionarily conserved sequence hypothesized to be necessary for Ca$^{2+}$ transport (Bick et al., 2012; Kwong et al., 2015, this issue). Three independent mutant ES cell lines were confirmed and subjected to morula aggregation and subsequent embryos transplanted into pseudo-pregnant females. Two of the three mutant ES cell lines produced germine mutant mice, which were crossed with ROSA26-FLPe mice for removal of the FRT-flanked neomycin cassette (Figure 1A). Cre-mediated deletion of exons 5–6 results in a frameshift and early termination of translation causing complete loss of MCU protein in all cells expressing Cre recombinase. Homozygous “floxed” mice (MCu$^{fl/fl}$) were interbred, and mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos. MEFs were infected with adenovirus expressing Cre recombinase (Ad-Cre) or βgal control virus and cells were lysed for western blot analysis of MCU protein expression 6 days later. Ad-Cre treatment resulted in a dose-dependent loss of MCU (Figure 1B). COXIV was used as a mitochondrial loading control. (Expression of additional mCa$^{2+}$ exchange associated proteins can be seen in Figure S1A). ETC complex expression served as a mito loading control (Figure S1B). Mcu$^{fl/fl}$ Ad-Cre or Ad-βgal treated MEFs were subsequently infected with AAV-mitycam (mito-targeted genetic Ca$^{2+}$ sensor) and cells imaged 48 hr later to monitor mCa$^{2+}$ exchange. ATP treatment (purinergic, IP3-mediated Ca$^{2+}$ release) elicited a rapid decrease in mitycam fluorescent signal in Mcu$^{fl/fl}$ Ad-βgal MEFs (mito is an inverse reporter, data shown as 1-F/Fo). Cells treated with Ad-Cre displayed almost complete loss of the acute mCa$^{2+}$ transient (Figure 1C). This difference was not attributable to a decrease in the Ca$^{2+}$ transient (Figure S1C). Quantification of mitycam amplitude immediately following ATP treatment found an ~75% decrease in mCa$^{2+}$ (Figure 1D). It should be noted that we did consistently observe that Mcu-KO MEFs continued to slowly take up Ca$^{2+}$ and eventually reached levels equivalent to control cells. Next, Mcu$^{fl/fl}$ Ad-Cre- or Ad-βgal-infected MEFs were examined for mCa$^{2+}$ uptake capacity by loading digitonin permeabilized cells with the Ca$^{2+}$ sensor, Fura-FF, and the membrane potential sensitive dye, JC-1 for simultaneous ratiometric recording. Cells were treated with thapsigargin to inhibit SERCA and block ER Ca$^{2+}$ uptake. Upon reaching a steady-state membrane potential, cells were exposed to seven consecutive pulses of 5 μM Ca$^{2+}$ (Figures 1E and 1F). A decrease in Fura signal after each bolus of bath Ca$^{2+}$ represents mCa$^{2+}$ uptake. Quantitative analysis after exposure to 10 μM Ca$^{2+}$ (a concentration where MCU is fully activated in non-excitable cells) revealed Mcu-null MEFs to have a near complete loss of mCa$^{2+}$ uptake compared to control MEFs (Figure 1G). Analysis of $\Delta \psi$ revealed no difference between groups at baseline or after delivery of 10 μM Ca$^{2+}$, confirming the observed change in uptake was not a result of an alteration in the driving force for mCa$^{2+}$ uptake (Figure 1H). Incremental increases in mCa$^{2+}$ eventually led to a decrease in membrane potential in βgal control MEFs, a phenomenon not observed in Mcu-null MEFs even after substantial Ca$^{2+}$ challenge (Figure 1I). It should be noted that in an attempt to make a MEF Mcu$^{-/-}$ cell line, we crossed Mcu$^{fl/fl}$ mice with a transgenic germline-Cre model (B6.CMV-Cre, JAX Mice) to generate Mcu$^{fl/fl}$/C24 mice for subsequent interbreeding. However, heterozygous mating (more than six litters) failed to yield Mcu$^{+/--}$ pups, suggesting homozygous deletion results in embryonic lethality.

Genetic Deletion of Mcu Results in the Complete Loss of Unipporter Ca$^{2+}$ Uptake in ACMs

Mcu$^{fl/fl}$ mice were crossed with the well-characterized αMHC-Cre transgenic mouse model to yield cardiomyocyte-specific loss of Mcu (Figure 2A). Adult cardiomyocytes (ACMs) were isolated from wild-type (WT), αMHC-Cre, Mcu$^{fl/fl}$, and Mcu$^{fl/fl}$ x αMHC-Cre mice at 8–12 weeks of age. Western blot assessment found an ~80% reduction in MCU protein compared to all controls; in accordance with previous reports of the mosaicism of
the αMHC-Cre transgenic strain (Figure 2B) (Oka et al., 2006). No expression changes in ETC complex subunits were found (Figure S2A). To examine baseline Ca2+ content, ACMs were loaded with the ratiometric Ca2+ reporter, Fura-2, and treated with Ru360 (MCU inhibitor), CGP37157 (mNCX inhibitor), thapsigargin (SERCA inhibitor) and permeabilized with digitonin to block all Ca2+ flux. During spectrofluorometric recording the protonophore, FCCP, was injected to dissipate Δψ allowing the release of all matrix free-Ca2+ (Figure 2C). Quantification of these data by calibration of the Fura-2 reporter in our experimental system (Figure S2B) found no change in matrix Ca2+ content in Mcu knockout (KO) ACMs (Figure 2D). Next, Ca2+ uptake capacity was evaluated in ACMs isolated from both Mcufl/fl and Mcufl/fl × αMHC-Cre mice (Figures 2E and 2F). The simultaneous recording of Ca2+ uptake and membrane potential discovered that Mcu KO ACMs were completely refractory to high Ca2+ challenge and failed to take up Ca2+, quantified after the second 10 μM Ca2+ pulse (Figure 2G). Mcufl/fl × αMHC-Cre ACMs displayed a slightly higher baseline mitochondrial membrane potential, although not reaching statistical significance, confirming that the lack of Ca2+ uptake was not due to a decrease in Δψ (Figure 2H). Further, Mcu-null ACMs were entirely resistant to Ca2+-overload loss of Δψ as observed in control cells. In fact, nine repeated boluses of 10 μM Ca2+ failed to elicit mitochondrial depolarization in Mcu KO ACMs (Figure 2I).

**Figure 1. Generation of a Conditional Mcu Knockout Mouse Model and Confirmation of Functionality**

(A) Schematic of Mcu targeting construct. LoxP sites (red triangles) flank exons 5–6. A neomycin (Neo) selection cassette is flanked by FRT sites (green half-circles). Mutant mice were crossed with ROSA26-FLPe mice for removal of Neo. Floxed mice (conditional allele) were crossed with cardiomyocyte-specific Cre recombinase driver lines resulting in deletion of Mcu.

(B) Mouse embryonic fibroblasts (MEFs) were isolated from Mcufl/fl mice at E13.5. MEFs were infected with adenovirus expressing Cre recombinase (Ad-Cre) or the experimental control β-galactosidase (Ad-βgal). 6 days post-infection with Ad-Cre, cells were lysed and MCU protein expression was examined by western blot. COXIV was used as a mitochondrial loading control.

(C) Mcufl/fl MEFs were treated with Ad-Cre or Ad-βgal and subsequently infected with Adeno encoding mitycam, a Ca2+ sensor, 48 hr prior to imaging. Baseline was recorded, and a single pulse of 1 mM ATP was delivered to liberate Ca2+ stores.

(D) Signal means shown as solid lines with dashed lines displaying ± SEM Ca2+ amplitude (peak intensity immediately after ATP – baseline).

(E) Mcufl/fl MEFs were treated with Ad-βgal and loaded with the Ca2+ sensor (Fura-FF), and the Δψ sensor (JC-1) was permeabilized with digitonin and treated with thapsigargin (SERCA inhibitor) for simultaneous ratiometric monitoring during repetitive additions of 5 μM Ca2+ (blue arrows). FCCP was used as a control to collapse Δψ at the conclusion of each experiment.

(F) Mcufl/fl MEFs were treated with Ad-Cre and subjected to identical experimental conditions.

(G) Percentage of Ca2+ uptake versus Ad-βgal control cells following 10 μM Ca2+ (second pulse).

(H) JC-1-derived Δψ prior to Ca2+ additions.

(I) JC-1-derived Δψ following seven pulses of 5 μM Ca2+.

All data shown as mean ± SEM. ***p<0.001 vs. βgal control.
To confirm that deletion of the Mcu gene results in loss of MCU channel activity (IMCU), we isolated ACMs, generated mitoplasts, and employed the whole-mitoplast voltage-clamping technique developed by the Clapham group that first established the uniporter as the prototypical uptake channel (Kirichok et al., 2004). IMCU was absent in Mcu-null mitoplasts subjected to a ramping protocol from $-160$ mV to 80 mV (Figure 2J). Quantitative analysis revealed a decrease in current density (Figure 2K), and likewise the current-time integral (area under the curve) was minimal (Figure 2L). These data are in agreement with initial and subsequent reports of MCU channel biophysical activity (Chaudhuri et al., 2013; Fieni et al., 2012; Kirichok et al., 2004). Collectively, these experiments corroborate that Mcu is necessary for rapid $\mu$Ca$^{2+}$ uptake in cardiomyocytes.

**MCU-Mediated $\mu$Ca$^{2+}$ Uptake Is a Significant Contributor to Myocardial IR Injury**

Given the well-substantiated role of Ca$^{2+}$ in activating the MPTP and the numerous reports that MPTP inhibition is a potent therapeutic strategy to reduce necrotic cell death (Rasola and...
TUNEL+ nuclei in the infarct boarder zone of demarcate cell death. We found a significant reduction in
We also examined DNA fragmentation by TUNEL staining, to
Mcu
diac troponin-I (cTnI) ELISA was performed as a secondary
cohort of mice was collected 24 hr after reperfusion, and a car-
3B and 3C). To corroborate this result, serum from the same
tory alterations in the expression of MPTP components, we im-
mice post-IR (Figures S3E–S3I).
Echocardiographic assessment of LV function 24 hr post-IR
and measurements of LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and percentage of fractional shortening (FS%) were
(Figure 3I) Changes in swelling quantified by measuring the area under the curve (AUC) and correcting to control.
All in vivo experiments minimum of n = 7 for all groups; data shown as mean ± SEM, *p < 0.05, **p < 0.01.
Bernardi, 2011), we next assessed genetic loss of Mcu in an
in vivo model of myocardial IR injury. Mcu flox, αMHC-MerCreMer (MCM), and Mcu flox × αMHC-MCM (Mcu cKO) mice (aged 10–12 weeks) were all injected intraperitoneally (i.p.) for 5 consecutive
days with 40 mg/kg tamoxifen (see Figures S3A and S3B for mCa2+ exchange associated proteins and ETC complex expres-
AAR was similar between all groups (Figures 3C and 3D).
To further examine the resistance of Mcu-null cardiomyo-
cytes to mitochondrial depolarization during Ca2+ overload as reported above in Figure 2I, we next isolated mitochondria from hearts and employed the classical mitochondrial-swelling assay to examine MPTP opening. Mitochondria isolated from Mcu-KO hearts failed to swell in response to increasing bath Ca2+, signified by a decrease in absorbance, in striking contrast to control mitochondria (Figure 3H, red versus black line). For these experiments, we utilized a substantial Ca2+ bolus (500 μM), such that the CypD inhibitor cyclosporine A (CsA) only had a partial inhibitory effect on swelling (gray line) in comparison to Mcu deletion. These data are quantified in Figure 3I as percentage of change in area under the curve versus control. It has previously been reported that MPTP opening occurs independent of CypD at high Ca2+ loads similar to those utilized here (Baines et al., 2005). To account for possible compensatory alterations in the expression of MPTP components, we immu
45% reduction in infarct size (INF) per area at risk (AAR)
changes in swelling (decreased absorbance at 540 nm = increase in volume) were
assessed ±2 μM CsA. Swelling was initiated by injec-

mCa2+ Uptake Is Necessary to Match Energetic Supply with β-Adrenergic Contractile Demand
Numerous studies have suggested that ECC Ca2+ cycling is inte-
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Figure 3. Genetic Ablation of Mcu Protects against Myocardial IR Injury
(A) Mcu flox, αMHC-Mer-Cre-Mer (αMHC MCM), and Mcu flox × αMHC-Mer-Cre-Mer mice were treated with tamoxifen (40 mg/kg/d) for 5 days to induce cardiomyocyte-restricted Cre expression and allowed to rest for 3 weeks prior to 40 min of ischemia and 24-hr reperfusion.
(B) Representative mid-ventricular cross sections of TTC-stained hearts. (Evans’ blue-stained area, non-ischemic zone; remaining area, area-at-risk; white area, infarcted tissue; red area, viable myocardium.)
(C) Planimetry analysis of infarct size by quantifying TTC-stained hearts. (Evans’-blue-stained area, non-TTC-stained area = infarct (INF).
(D) 24 hr after reperfusion, serum was collected, and cardiac troponin-I (cTnI) was measured by ELISA. (E-G) Mice were analyzed by echocardiography, and measurements of LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and percentage of fractional shortening (FS%) were acquired.
(H) Mitochondria were isolated from hearts of adult mice, and changes in swelling (decreased absorbance at 540 nm = increase in volume) were assessed ±2 μM CsA. Swelling was initiated by injection of 500 μM Ca2++.
content, we next induced acute cardiac stress using an adrenergic agonist to elevate the Ca$^{2+}$ load in an attempt to unmask the physiological function of the MCU. Mcu$^{+/+}$, α-MHC-MCM, and Mcu cKO mice were injected i.p. for 5 consecutive days with 40 mg/kg tamoxifen, and 10 days later we measured LV hemodynamic parameters during intravenous (i.v.) infusion of isoproterenol (Iso) (Figure 4A). Mcu cKO mice failed to increase LV contractility (dp/dt max) in response to β-adrenergic stimulation as compared to control mice (Figure 4B). In addition, there was a noted, although less dramatic, impairment in LV relaxation (dp/dt min, Figure 4C). There was no significant difference in heart rate (HR) between groups over the course of Iso infusion (Figure 4D).

Following 10 min of Iso infusion, we snap-froze ventricular tissue for metabolic analysis. We first evaluated the status of the pyruvate dehydrogenase complex (PDH), the prototypical mCa$^{2+}$-dependent enzyme that converts pyruvate into acetyl-CoA for use in the tricarboxylic acid (TCA) cycle. PDH is a central component linking glycolysis to OxPhos and also a contributor to the physiological function of the MCU.

We also examined the NAD$^+$/NADH ratio and again found no difference at baseline, acute Iso stimulation revealed an ~2-fold difference in Mcu cKO hearts versus controls (Figure 4I). We also examined the NADP$^+$/NADPH ratio and found no difference at baseline but did find a trend of increased NADP$^+$/NADPH ratio in Mcu cKO hearts during Iso infusion (Figure S4E). This was somewhat surprising since we thought NADPH generation was primarily extra-mitochondrial via the pentose phosphate pathway. However, mitochondrial enzymes such as malic enzyme, NADP-linked isocitrate dehydrogenase, and mitochondrial methylenetetrahydrofolate dehydrogenase are other significant sources of NADPH production (Fan et al., 2014; Huang and Colman, 2005; Palmieri et al., 2015; Yang et al., 1996). It is intriguing to think that this may be another metabolic consequence of altering the mCa$^{2+}$-microdomain during stress, be it direct or indirect modulation.

To further examine the hypothesis that MCU-Ca$^{2+}$ uptake is necessary to increase myocardial energy production in response to acute sympathetic signaling, we employed a cellular system to monitor energetic changes in real-time. ACMs were isolated necessary to increase myocardial energy production in response to acute sympathetic signaling, we employed a cellular system to monitor energetic changes in real-time. ACMs were isolated.
from Mcu<sup>fl/fl</sup> and Mcu<sup>GFP</sup> × αMHC-MCM mice 10 days after administration of tamoxifen. We first monitored iCa<sup>2+</sup> transients at both baseline and during Iso delivery to rule out the possibility of decreased βAR responsiveness in our Mcu<sup>CKO</sup> cells (Figure S5). We found Mcu<sup>CKO</sup> ACMs to have no impairment in Iso-mediated augmentation of iCa<sup>2+</sup> signaling during pacing. Next, ACMs were monitored for changes in NADH autofluorescence intensity (Figure 5A). While we found no difference in basal NADH levels between groups (Figure 5B), the administration of Iso (10 μM) elicited a significant increase in NADH production in control ACMs, while Mcu<sup>-KO</sup> myocytes were unresponsive with NADH consumption being greater than production. Quantification of these data with correction to control ACMs can be seen in Figure 5C. To examine maximal NADH production in the presence of Iso, we next inhibited complex I of the ETC (NADH dehydrogenase) with rotenone. Mcu<sup>-KO</sup> ACMs displayed a 50% reduction in maximal NADH production, as compared to control (Figure 5D). To evaluate whether the lack of NADH responsiveness correlated with an alteration in OxPhos capacity, we measured ACM oxygen consumption rates (OCR) using a Seahorse extracellular flux analyzer. Corroborating our previous data showing no change in baseline NADH, there was no difference in baseline respiration between groups (Figure 5E). Next, we examined maximal respiratory capacity (maxOCR, FCCP treatment) in the presence of Iso or vehicle (veh). Mcu-null ACMs displayed a significant reduction in maxOCR, as compared to controls, and were completely refractory to Iso-mediated increases in mitochondrial respiration (Figure 5F). In summation, these results support the concept of metabolic failure due to an inability to increase reducing equivalents during acute stress.

**DISCUSSION**

Since the 1970s, it has been apparent that mitochondria contained a protein capable of inducing an inward rectifying Ca<sup>2+</sup> current (Sottocasa et al., 1972). The subsequent identification of a pharmacological inhibitor, of the channel, ruthenium red (RR), allowed investigators to begin to probe the cellular function of iCa<sup>2+</sup> exchange (Moore, 1971). Various studies employing RR or a derivative have implicated iCa<sup>2+</sup> in numerous cellular processes, most notably the regulation of metabolism, cell death, and buffering of cytosolic Ca<sup>2+</sup> signaling (Hoppe, 2010). However, subsequent studies have found a multitude of cation channels that are inhibited by RR derivatives. Thus, off-target effects of these pharmacological agents may account for the conflicting results that have fueled the debate as to the true biological function of this microdomain. Further impeding causative experimentation was the unknown genetic identity of the constituents that comprise the iCa<sup>2+</sup> exchange machinery. Reports from two independent laboratories identified MCU as the channel-forming component of the MCU complex and documented its requirement for Ca<sup>2+</sup> uptake (Baughman et al., 2011; De Stefani et al., 2011).
While knocking out significant gene changes must have occurred prenatally in their no discernable baseline phenotype, supports the notion that survival signaling provides protection against necrotic cell death. This result is not greaters but decreases susceptibility to MPTP activation and thereby prevents mitochondrial entry of Ca²⁺ in contrast to control cells; and (3) cardiac mitochondria isolated from Muco null cardiomyocytes were completely resistant to swelling. Together these data suggest deletion of Muco greatly decreases susceptibility to MPTP activation and thereby provides protection against necrotic cell death. This result is not surprising given the numerous reports implicating Ca²⁺ load as a fundamental contributor to MPTP open probability (Rasola and Bernardi, 2011). Moreover, studies have shown that MPTP inhibition is potently cytoprotective, particularly in IP injury, indicating a clinical trial evaluating the efficacy of cyclosporine-A (MPTP inhibitor) administration during reperfusion of the ischemic myocardium (Elrod and Molkentin, 2013; Plot et al., 2008). It is likely that MPTP inhibition was not the sole protective mechanism, as decreasing Ca²⁺ load is also associated with decreased reactive oxygen species (ROS) generation during stress. Supporting this concept, we found a significant decrease in mitochondrial superoxide levels in Muco null cells following hypoxia/reoxygenation (Figures S1D and S1E).

However, our IR injury results are contradictory to those recently reported by Pan et al. (2013). Disparities in methodology likely account for the different results observed here. The previous study used a gene-trap approach with germline gene inactivation, versus our conditional, cardiomyocyte-specific deletion in the adult mouse. Therefore, compensatory pathways, induced by the loss of Muco during development, may have allowed for the entry of Ca²⁺ into the matrix in sufficient quantity, independent of MCU, to activate mitochondrial-dependent death pathways or alternatively mitochondrial-independent cell-death pathways may be upregulated in this mouse. Our finding that germline deletion of Muco in our model system was embryonically lethal, while knocking out Muco after birth or in adulthood resulted in no discernable baseline phenotype, supports the notion that significant gene changes must have occurred prenatally in their model to support viability. Further, it may be that deletion of Muco in other cell types in the heart, such as fibroblasts and endothelial cells, actually magnified injury by reducing the Ca²⁺-buffering capacity in non-myocytes and thereby masked the protective effect of loss of Muco in cardiomyocytes. Supporting this concept, we found that Muco-null MEFs displayed an increase in Ca²⁺ transient amplitude following IP3R stimulation (Figure S1C). Yet another possible reason is the disparity in ischemic models. The Pan et al. study employed an ex vivo Langendorff global hypoxia model compared to our in vivo LCA ligation IR model. There are major differences between these methodologies, and, while unlikely, perhaps the ex vivo model somehow lessens the contribution of MCU-dependent Ca²⁺ uptake in cardiomyocyte death. Our data do fit with previous reports of ruthenium red derivatives (MUC inhibitors) providing protection against IR injury (Zhang et al., 2006; Zhao et al., 2013).

The other major difference from the Pan et al. study is that we found no change in resting Ca²⁺ content in Muco-null cells, in contrast to their finding of ~70% reduction in skeletal muscle Ca²⁺. Our results suggest a MCU-independent mechanism of Ca²⁺ entry is not reached under homeostatic conditions in adult cardiomyocytes and that an alternative slow Ca²⁺ uptake mechanism must play a significant role. Direct evidence that MCU-independent Ca²⁺ uptake exists can be seen in our experiment examining real-time flux in MEFs (Figure 1C). Although we observed complete loss of the acute and rapid MCU-like Ca²⁺ uptake, we detected Ca²⁺ content continued to slowly rise with sustained Ca²⁺ load and eventually reached a level equivalent to WT cells. It is possible that the lower Ca²⁺ content previously reported in Pan et al. can be explained by methodological differences. We discovered that the slightest perturbation in either extracellular or sarcoplasmic Ca²⁺ stores in WT cells induced an increase in intracellular Ca²⁺ loading. We found that any Ca²⁺ liberated during our experimental procedure, be it from mitochondrial isolation or SERCA inhibition, was immediately taken up by WT mitochondria in a Muco-dependent fashion. Therefore such a perturbation elevates Ca²⁺ content in control cells and may lead to a false interpretation of decreased content in Muco KO cells. This phenomenon can be seen in Figure S2C where, in control cells after permeabilization and addition of thapsigargin, we see a decrease in the Fura ratio prior to FCCP treatment signifying reduced Ca²⁺ uptake, whereas in Muco-deleted cells we observe a rise in extra-sarcoplasmic Ca²⁺ levels. The addition of the MCU inhibitor, Rou360, and Na⁺,K⁺-ATPase inhibitor, C8P37157, prior to experimentation alleviated this problem. Summarizing the first part of our study, in a clinically relevant model of IR injury, we provide evidence that Muco-mediated Ca²⁺ uptake is a significant mechanism driving MPTP-mediated cardiomyocyte cell death and cardiac dysfunction. Further, we hypothesize that the Muco-exchange system possess a great deal of plasticity and that alternative uptake mechanisms maintain matrix Ca²⁺ content during homeostasis. A more detailed examination of this phenomenon in future studies may aid the discovery of novel exchangers and pathways that account for the observed “slow Ca²⁺ uptake.” The heart is an aerobic organ that must constantly match energy supply with demand. The contractile function of the normal
heart changes significantly during normal activities. This has led to the theory that \( \text{Ca}^{2+} \) cycling is integrated with mitochondria on a beat-to-beat basis to match ATP production with contractile demand as a real-time regulator of oxidative metabolism (Glancy and Balaban, 2012). However, our current findings suggest that rapid MCU-dependent \( \text{Ca}^{2+} \) uptake is dispensable for homeostatic cardiac function, as ablating \( \text{Mcu} \) had little effect on baseline function for all measured indices, including little to no change in LV function, structure, and cellular energetics. We found cardiomyocyte resting \( \text{Ca}^{2+} \) content to be \( \sim 200 \) nM, and we did not detect appreciable mitochondrial uptake until concentrations of \( \sim 8 \) \( \mu \)M were reached (control ACMs displayed only \( \sim 17\% \) uptake in response to a 10-\( \mu \)M-\( \text{Ca}^{2+} \) load). Both of these values fit nicely within the range of previous studies examining cardiac MCU function that were recently summarized in eloquent fashion by Williams et al. (2013). These data also agree with recent work proposing MICU1 binds MCU to inhibit uptake until a given threshold or set point of \( \text{Ca}^{2+} \) is overcome (Cserdás et al., 2013; Mallilankaraman et al., 2012). Since it is assumed global ECC \( \text{Ca}^{2+} \) cycling does not reach such levels in the homeostatic beating heart, we hypothesize that a slow MCU-independent influx mechanism must account for homeostatic maintenance of matrix \( \text{Ca}^{2+} \), aided by balanced \( \mu \)NCX efflux rates. It should be noted that \( \text{Ca}^{2+} \) levels of this magnitude might occur in discrete microdomains where a sub-population of mitochondria are tethered in close proximity to SR/T-tubule junctions (Chen et al., 2012). There are a number of mechanisms that theoretically could contribute to a slow MCU-independent \( \mu \text{NCX} \) uptake including: \( \mu \)Na/PyR, LETM1 (\( \text{H}^+ / \text{Ca}^{2+} \) exchanger), reverse-mode \( \mu \)NCX, or an as of yet unknown exchanger(s) (Beutner et al., 2001; Jiang et al., 2009; Palty et al., 2010). Additional evidence supporting MCU-independent uptake can be seen in a recent biophysical report describing a second “\( \mu \)R-insensitive” voltage-dependent inward rectifying current (Michels et al., 2009). We hope that our future experiments will aid the identification of this MCU-independent uptake mechanism.

While our data do not support a significant role for the MCU in basal cardiac physiology, cardiomyocyte-specific deletion did result in a striking inability to increase contractile function in response to the classic \( \beta \)-agonist, isoproterenol. Since a study published by Howell and Duke in 1906, it has been appreciated that \( \text{Ca}^{2+} \) is required for the “augmenting influence of the sympathetic upon the heart” (Howell and Duke, 1906). Our understanding has continued to evolve over the last century, and the various molecular mechanisms of how \( \text{IAR} \) signaling regulates changes in excitation-contraction coupling (ECC) have been defined (Bers, 2008). Our data extend these pathways to include MCU-dependent \( \text{Ca}^{2+} \) uptake as a mechanism necessary to upregulate energetic signaling to match ATP production with contractile demand during periods of acute adrenergic stress. In addition, our findings support a pathological role for MCU \( \text{Ca}^{2+} \) influx driving mitochondrial depolarization and cell death during IR injury. While much work remains to fully elucidate all the molecular constituents of the MCU complex and their mechanistic function, our current study provides a fundamental framework to aid our understanding of \( \mu \text{NCX} \) uptake in health and disease.

**EXPERIMENTAL PROCEDURES**

Please see the Supplemental Information for detailed experimental procedures.

**Generation of Mcu Conditional Knockout Mice**

The gene targeting strategy in embryonic stem cells to generate the Mcu-loxP mice that we used here is described in Kwong et al. (2015). In short, a Mcu conditional knockout mouse by recombinant insertion of a targeting gene construct containing loxP sites flanking exons 5–6 of the Mcu gene (ch10: 58930544–58911529) in mouse ES cells. Three independent mutant ES cell lines were confirmed and subjected to morula aggregation, and subsequent embryos were transplanted into pseudo-pregnant females. Two of the three mutant ES cell lines produced germline mutant mice, which were crossed with ROSA26-FLPe knockin mice for removal of the FRT-flanked neomycin cassette. Resultant Mcu \( ^{MHC} \) mice were crossed with cardiac specific-Cre transgenic mice, \( \mu \text{MHC}-\text{Cre}, \) and \( \mu \text{MHC-MCM} \), to generate cardiomyocyte-specific Mcu knockouts. B6.CMV-Cre transgenic mice (Jackson Laboratory, stock # 006054) were used for germline deletion. For temporal deletion of Mcu using the MCM model, Mcu \( ^{MHC} \) and \( \mu \text{MHC-MCM} \), and Mcu \( ^{MHC} \times \mu \text{MHC-MCM} \) were injected with i.p. 40 mg/kg/day of tamoxifen for 5 consecutive days. For all experiments, mice were 10–14 weeks of age. All mutant lines were maintained on the C57/B6 background, and all experiments involving...
animals were approved by Temple University’s IACUC and followed AAALAC guidelines.

Western Blot Analysis
All procedures were carried out as previously reported (Elrod et al., 2010).

Isolation of ACMs
ACMs were isolated from ventricular tissue as described previously (Zhou et al., 2000). All cells were used within 4 hr of isolation.

Evaluation of \( \Delta \psi \) Uptake and Content
To evaluate \( \Delta \psi \) content, permeabilized ACMs were treated with RU380 and CFP-37157 to inhibit \( \Delta \psi \) flux. Fura2 (Invitrogen) was added to monitor extra-mitochondrial Ca\(^{2+}\), FCCP was added to uncouple the \( \Delta \psi \) and release matrix free-Ca\(^{2+}\). To measure \( \Delta \psi \) uptake capacity, ACMs were permeabilized and Fura-FF (Invitrogen) was added to monitor extra-mitochondrial Ca\(^{2+}\). JC-1 (Enzo Life Sciences) was added to monitor \( \Delta \psi \). and Fura were monitored on a PTI spectrofluorometer. All details are previously reported (Malillankaraman et al., 2012).

Mitochondria Isolation and Swelling Assay
Hearts were excised from mice and mitochondria were isolated as reported (Frezza et al., 2007). For the swelling assay, mitochondria were diluted in assay buffer, and absorbance (abs) was recorded at 540 nm every 5 s. (Frezza et al., 2007). 500 \( \mu \)M CaCl\(_2\) was injected to induce swelling \( \pm \) 2 \( \mu \)M Cyclosporin A (CsA) (Elrod et al., 2010).

ACM, Ca\(^{2+}\) Transients
Isolated ACMs were loaded with Fluo-4 AM (Invitrogen) and placed in a 37 °C heated chamber on an inverted microscope stage. ACMs were perfused with Tyrode’s buffer and paced at 0.5 Hz. After baseline recordings, cells were perfused with Tyrode’s containing 100 nM Iso. Ca\(^{2+}\) transients were analyzed using Clamplt software.

Mitoplast Patch-Clamp Analysis of MCU Current
Following mitochondrial isolation, mitoplasts were prepared for patch-clamp studies. \( \Delta \psi \) was recorded as previously described in detail (Kirichok et al., 2004).

Metabolic Assays
Metabolomic analyses were carried out by metabolite profiling of ventricular tissue by LC-MS/MS as described (Jain et al., 2012). NAD/NADH and NADP/NADPH ratios were quantified using luminescence assays (Promega). PDH activity was quantified using a fluorometric assay (Mitosciences). NAD/NADH and NADP/NADPH ratios were quantified using luminescence assays (Promega). Metabolomic analyses were carried out by metabolite profiling of ventricular tissue by LC-MS/MS as described (Jain et al., 2012). NAD/NADH and NADP/NADPH ratios were quantified using luminescence assays (Promega). Metabolomic analyses were carried out by metabolite profiling of ventricular tissue by LC-MS/MS as described (Jain et al., 2012). NAD/NADH and NADP/NADPH ratios were quantified using luminescence assays (Promega).

LV Echocardiography and Hemodynamics
Trans thoracic echocardiography of the LV was performed and analyzed on a Vevo 2100 imaging system as previously reported (Elrod et al., 2007). Invasive hemodynamic measurements in anesthetized mice was performed using a pressure catheter inserted into the right carotid artery and guided into the LV. Right jugular vein catheterization allowed delivery of lidocaine during recording.

Myocardial IR Injury
LCA ligation and reperfusion was performed as previously described in Gao et al. (2010). Infarct size was measured as previously reported (Elrod et al., 2007). Serum was collected from mice after 24 hr R to measure cTnI using the Life Diagnostics ELISA kit. A TUNEL detection kit (Roche) was used to label DNA fragmentation in the infract border zone of fixed heart sections.

MEF Isolation
Embryos were collected from Mucr\(^{fl/fl}\). mice at E13.5 and MEFs isolated as previously reported (Baines et al., 2009). MEFs were treated with Ad-Cre or Ad-fgfr2 for 24 hr. 6-day post-infection cells were used for experiments.

\( \text{Ca}^{2+} \) and \( \text{Ca}^{2+} \) Flux in MEFs
MEFs were infected with AAV-mitycam to measure \( \text{Ca}^{2+} \) exchange or loaded with the \( \text{Ca}^{2+} \) indicator, Fluo-4-FF. Data were collected every 3 s and analyzed on Zen software.

Hypoxia/Reoxygenation
MEFs were plated on 35-mm glass plates and, after culturing for 24 hr, loaded with 5 \( \mu \)M MitoSOX Red (Invitrogen). Cells were placed in ischemic medium for 1 hr, reoxygenated with Tyrode’s buffer, and imaged 5 min later to evaluate mitochondrial superoxide production.

Statistics
All results are presented as mean ± SEM. Statistical analysis was performed using Prism 6.0 software (GraphPad). Where appropriate column analyses were performed using an unpaired, two-tailed t test for two groups or one-way ANOVA with Bonferroni correction for groups of three or more. For group comparisons, either multiple unpaired t test with correction for multiple comparisons using the Holm-Sidak method or, where appropriate, two-way ANOVA with Tukey post hoc analysis was performed. p values <0.05 were considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.017.

AUTHOR CONTRIBUTIONS

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