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

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.
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 (Ca2+), 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 mCa2+ 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 Ca2+-dependent metabolism during acute stress.

INTRODUCTION
The cardiomyocyte is unique in that a large and variable flux of intracellular calcium (Ca2+) must occur to mediate and regulate contraction. Thus, a complex system has evolved to regulate Ca2+ transport to maintain homeostatic conditions (Bers, 2008). Numerous genetic components have been shown to mediate the passage of Ca2+ across the sarcolemma and sarcoplasmic reticulum (SR), and, while great strides have been made toward understanding the temporal and spatial relationship of Ca2+ in regards to excitation-contraction (EC) coupling, our understanding of other sub-cellular Ca2+ domains, including the components of mitochondria Ca2+/mCa2+ exchange, remains elementary.

The dynamic Ca2+ environment of the heart requires that cardiac mitochondria possess an exchange system capable of dealing with the variable changes in Ca2+ load. Ca2+ 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) (Kirchok 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 mCa2+ 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 mCa2+ influx into the matrix. However, numerous outstanding questions remain in regards to the molecular regulation of the MCU and the physiological function of mCa2+, 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 mCa2+ and NADH have correlated increased mCa2+ load with increased oxidative phosphorylation and ATP production (Brandes and Bers, 2002; Unitt et al., 1989). Thus, Ca2+ 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 Ca2+-dependent dehydrogenases and modulation of ETC complexes (Glancy and Balaban, 2012).

In contrast to the aforementioned pro-survival metabolic signaling, numerous studies have implicated mCa2+ overload in the activation of apoptosis and necrosis (Rasola and Bernardi, 2011). mCa2+ is known to cause outer-mitochondrial membrane (OMM) permeability prompting the release of apoptogens. Ca2+ 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 \( \mathrm{mCa}^{2+} \) signaling modulates in vivo physiology, a group at the NHLBI recently generated a \( \text{Mcu} \) gene-trap mouse (Pan et al., 2013). As expected, mitochondria isolated from this global \( \text{Mcu} \)-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 \( \mathrm{mCa}^{2+} \) signaling in the normal and diseased heart.

To advance our understanding of \( \mathrm{mCa}^{2+} \) uptake in the heart, in collaboration with the Molkentin lab, we generated a conditional, loss-of-function mouse model (\( \text{Mcu}^{+/\text{fl}} \)) and coupled with a tamoxifen-inducible, cardiomyocyte-specific Cre recombinase transgenic line, deleted \( \text{Mcu} \) in adulthood (Kwong et al., 2015 [this issue of Cell Reports]). Here, we report that loss of \( \text{Mcu} \) ablates \( \mathrm{mCa}^{2+} \) uptake and activity (\( \text{Mcu}_{\text{ICU}} \)) 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 \( \text{Mcu} \)-null mice lacked in vivo contractile responsiveness to \( \beta \)-adrenergic receptor (\( \beta \)AR) 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 \( \text{Mcu} \) is to match \( \text{Ca}^{2+} \)-dependent contractile demands with mitochondrial metabolism during the "fight-or-flight" response.

RESULTS

Generation of a \( \text{Mcu} \) Conditional Knockout Mouse Model and Validation of Functionality

The \( \text{Mcu} \) 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 \( \text{Ca}^{2+} \) 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 germline mutant mice, which were crossed with ROSA26-\( \text{FLPe} \) mice for removal of the FRT-flanked neoycin 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 (\( \text{Mcu}^{+/\text{fl}} \)) were interbred, and mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos. MEFs were infected with adenovirus expressing Cre recombinase (Ad-Cre) or \( \text{rtTA} \) 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 \( \mathrm{Ca}^{2+} \) exchange associated proteins can be seen in (Figure S1A). ETC complex expression served as a mito loading control (Figure S1B). \( \text{Mcu}^{+/\text{fl}} \) Ad-Cre or Ad-\( \text{rtTA} \) treated MEFs were subsequently infected with AAV-mitycam (mito-targeted genetic \( \mathrm{Ca}^{2+} \) sensor) and cells imaged 48 hr later to monitor \( \mathrm{mCa}^{2+} \) exchange. ATP treatment (purinergic, IP3-mediated \( \mathrm{Ca}^{2+} \) release) elicited a rapid decrease in mitycam fluorescent signal in \( \text{Mcu}^{+/\text{fl}} \) Ad-\( \text{rtTA} \) MEFs (mitycam is an inverse reporter, data shown as 1-F/F0). Cells treated with Ad-Cre displayed almost complete loss of the acute \( \mathrm{mCa}^{2+} \) transient (Figure 1C). This difference was not attributable to a decrease in the \( \mathrm{Ca}^{2+} \) transient (Figure S1C). Quantification of mitycam amplitude immediately following ATP treatment found an ~75% decrease in \( \mathrm{mCa}^{2+} \) (Figure 1D). It should be noted that we did consistently observe that Mcu-KO MEFs continued to slowly take up \( \mathrm{Ca}^{2+} \) and eventually reached levels equivalent to control cells. Next, \( \text{Mcu}^{+/\text{fl}} \) Ad-Cre- or Ad-\( \text{rtTA} \)-infected MEFs were examined for \( \mathrm{mCa}^{2+} \) uptake capacity by loading digitonin permeabilized cells with the \( \mathrm{Ca}^{2+} \) 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 \( \mathrm{Ca}^{2+} \) uptake. Upon reaching a steady-state membrane potential, cells were exposed to seven consecutive pulses of 5 \( \mu \)M \( \mathrm{Ca}^{2+} \) (Figures 1E and 1F). A decrease in Fura signal after each bolus of bath \( \mathrm{Ca}^{2+} \) represents \( \mathrm{mCa}^{2+} \) uptake. Quantitative analysis after exposure to 10 \( \mu \)M \( \mathrm{Ca}^{2+} \) (a concentration where MCU is fully activated in non-excitable cells) revealed \( \text{Mcu} \)-null MEFs to have a near complete loss of \( \mathrm{mCa}^{2+} \) uptake compared to control MEFs (Figure 1G). Analysis of \( \Delta \psi \) revealed no difference between groups at baseline or after delivery of 10 \( \mu \)M \( \mathrm{Ca}^{2+} \), confirming the observed change in uptake was not a result of an alteration in the driving force for \( \mathrm{mCa}^{2+} \) uptake (Figure 1H). Incremental increases in \( \mathrm{mCa}^{2+} \) eventually led to a decrease in membrane potential in \( \text{rtTA} \) control MEFs, a phenomenon not observed in \( \text{Mcu} \)-null MEFs even after substantial Ca\(^{2+}\) challenge (Figure 1I). It should be noted that in an attempt to make a MEF \( \text{Mcu}^{+/\text{fl}} \)-cell line, we crossed \( \text{Mcu}^{+/\text{fl}} \) mice with a transgenic germline-Cre model (B6.CMV-Cre, JAX Mice) to generate \( \text{Mcu}^{+/\text{fl}} \) for subsequent interbreeding. However, heterozygous mating (more than six litters) failed to yield \( \text{Mcu}^{+/\text{fl}} \) pups, suggesting homzygous deletion results in embryonic lethality.

Genetic Deletion of \( \text{Mcu} \) Results in the Complete Loss of Unipporter \( \mathrm{Ca}^{2+} \) Uptake in ACMs

\( \text{Mcu}^{+/\text{fl}} \) mice were crossed with the well-characterized 2\( \mathrm{MMHC} \)-Cre transgenic mouse model to yield cardiomyocyte-specific loss of \( \text{Mcu} \) (Figure 2A). Adult cardiomyocytes (ACMs) were isolated from wild-type (WT), 2\( \mathrm{MMHC} \)-Cre, \( \text{Mcu}^{+/\text{fl}} \), and \( \text{Mcu}^{+/\text{fl}} \times 2\mathrm{MMHC} \)-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 Muco knockout (KO) ACMs (Figure 2D). Next, mCa2+ uptake capacity was evaluated in ACMs isolated from both Mucofl/fl and Mucofl/fl x αMHC-Cre mice (Figures 2E and 2F). The simultaneous recording of mCa2+ uptake and membrane potential discovered that Muco 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). Mucofl/fl x 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, Muco-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 Muco KO ACMs (Figure 2I).
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 /C0 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 mCa2+ uptake in cardiomyocytes.

**MCU-Mediated mCa2+ Uptake Is a Significant Contributor to Myocardial IR Injury**

Given the well-substantiated role of Ca2+ in activating the MPTP and the numerous reports that MPTP inhibition is a potent therapeutic strategy to reduce necrotic cell death (Rasola and...
Bernardi, 2011), we next assessed genetic loss of Mcu in an in vivo model of myocardial IR injury. Mcufl/fl, αMHC-MerCreMer (MCM), and Mcufl/fl x 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 expression post-tamoxifen) and 3 weeks later subjected to left coronary artery (LCA) ligation for 40-min and 24-hr reperfusion (Figure 3A). The evaluation of left ventricle (LV) infarct size by TTC staining and Evan’s blue dye perfusion revealed Mcu cKO mice to have a ~45% reduction in infarct size (INF) per area at risk (AAR) versus controls; AAR was similar between all groups (Figures 3B and 3C). To corroborate this result, serum from the same cohort of mice was collected 24 hr after reperfusion, and a cardiac troponin-I (cTnI) ELISA was performed as a secondary marker of cardiomyocyte cell death. Mcu-deleted mice displayed an ~65% reduction in cTnI versus controls (Figure 3D). We also examined DNA fragmentation by TUNEL staining, to mark nuclei in the infarct boarder zone of Mcu cKO hearts as compared to controls (Figures S3C and S3D).

Echocardiographic assessment of LV function 24 hr post-IR revealed a significant preservation of LV end-systolic diameter (LVEDD) and percentage of fractional shortening (FS%) in Mcu knockout mice (Figures 3E–3G). Additional M-mode echocardiographic data can be seen in Table S1. To account for differences in regional wall motion due to variances in infarct size, we utilized speckle-tracking of B-mode echocardiographic recordings and likewise found an improvement in LV function in Mcu cKO mice post-IR (Figures S3E–S3I).

To further examine the resistance of Mcu-null cardiomyocytes 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+, as compared to control mitochondria (Figure 3H, red versus black line). For these experiments, we utilized a subvital 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 immunoblotted for CypD, ANT, and VDAC (Figure S3J). We found no differences in expression between Mcu cKO and control hearts. These results support the hypothesis that the loss of Mcu prevents Ca2+ from entering the matrix and activating the MPTP and thereby reduces IR-mediated cardiomyocyte cell death.

**mCa2+ Uptake Is Necessary to Match Energetic Supply with β-Adrenergic Contractile Demand**

Numerous studies have suggested that ECC Ca2+ cycling is integrated into mitochondria to match ATP production with workload (Williams et al., 2015). Given that we did not find a significant difference in baseline cardiac function or resting mCa2+
content, we next induced acute cardiac stress using an adrenergic agonist to elevate the \( \text{Ca}^{2+} \) load in an attempt to unmask the physiological function of the MCU. \( \text{Mcu}^{+/+} \), \( \alpha \)-MHC-MCM, and \( \text{Mcu}^{+/+} \) 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). \( \text{Mcu}^{+/+} \) cKO mice failed to increase LV contractility (dp/dt max) in response to \( \beta \)-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 \( \text{Ca}^{2+} \)-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 NADH pool. \( \text{Ca}^{2+} \) is reported to increase PDH phosphatase activity (PDHPT), which, in turn, dephosphorylates the S293 residue on the E1 subunit resulting in increased PDH enzymatic activity. There was no change in the baseline expression of phospho-PDH, total PDH complex (Figure 4E), or other proposed \( \text{Ca}^{2+} \)-regulated dehydrogenases (\( \alpha \)-ketoglutarate dehydrogenase and isocitrate dehydrogenase; Figure S4A). However, expression analysis of post-Iso samples revealed a substantial decrease in phosphorylation of S293-E1 in control hearts versus \( \text{Mcu} \) cKO samples (Figure 4F, top panel). There was no change in total protein expression for any of the PDH subunits post-Iso (Figure 4F, bottom panel). Quantification of phospho/total E1-PDH revealed \( \text{Mcu} \)-KO hearts to have greater than a 3-fold difference in phosphorylation versus controls, signifying a failure to activate PDH during adrenergic stimulation (Figure 4G). This result was confirmed by our observation of an \(~50\%\) decrease in Iso-stimulated PDH enzymatic activity in \( \text{Mcu} \) cKO hearts (Figures 4H and S4B). To examine baseline energetics in more detail and rule out any compensatory changes in our \( \text{Mcu} \) cKO model, we employed metabolomics to measure the levels of several prominent TCA intermediates (Figures S4C and S4D). Mass spectrometry of ventricular tissue found no difference in any of the metabolites assayed.

Next, we measured the NAD\(^+\)/NADH ratio, and, while we found no difference at baseline, acute Iso stimulation revealed an \(~2\)-fold difference in \( \text{Mcu} \) cKO hearts versus controls (Figure 4I). We also examined the NADP\(^+\)/NADPH ratio and again found no difference at baseline but did find a trend of increased NADP\(^+\)/NADPH ratio in \( \text{Mcu} \) 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 \( \text{Ca}^{2+} \)-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...
from Mcu<sup>fl/fl</sup> and Mcu<sup>fl/fl</sup> × αMHC-MCM mice 10 days after administration of tamoxifen. We first monitored Ca<sup>2+</sup> transients at both baseline and during Iso delivery to rule out the possibility of decreased βAR responsiveness in our Mcu cKO cells (Figure S5). We found Mcu cKO ACMs to have no impairment in Iso-mediated augmentation of Ca<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>-null</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>-null</sup> ACMs displayed a 50% reduction in maximal NADH production, as compared to controls (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, 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 Ca<sup>2+</sup> exchange (Moore, 1971). Various studies employing RR or a derivative have implicated Ca<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 Ca<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).
With this discovery, the race was on to generate a loss-of-function mouse model for comprehensive study to begin to put into context the vast and often controversial literature regarding how the dynamic flux of Ca\(^{2+}\) into and out of the mitochondrial matrix may regulate (patho)physiology. A recent report from Pan et al. details the phenotype of a Mcu-null mouse generated using a gene trap strategy (Pan et al., 2013). While the authors reported a complete loss acute \(\text{mCa}^{2+}\) uptake in various cell types, the physiological results of the study were quite surprising. Perhaps most striking was that Mcu ablation had little effect on cardiac function, structure, or cell death. These results have prompted the field at large to question the relevance of cardiomyocyte \(\text{mCa}^{2+}\) flux. Beyond this report, a number of other questions remained unresolved regarding the impact of \(\text{mCa}^{2+}\) signaling in cardiomyocyte function.

Using a conditional knockout approach to specifically delete Mcu in cardiomyocytes in adult mice coupled with in vivo experimental methodologies, we were able to document how acute \(\text{mCa}^{2+}\) uptake impacts cardiac physiology. We found (1) a reduction in infarct size assessed both histologically by TTC staining and TUNEL and biochemically by cTnI levels coupled with in vivo LV functional data, that all support the notion that Mcu-mediated \(\text{mCa}^{2+}\) uptake contributes to IR-induced cardiomyocyte cell death; (2) Mcu KO cells displayed a greater resistance to Ca\(^{2+}\) overload, capable of maintaining \(\Delta \psi\) following numerous pulses of Ca\(^{2+}\) in contrast to control cells; and (3) cardiac mitochondria isolated from Mcu-null cardiomyocytes were completely resistant to swelling. Together these data suggest deletion of Mcu greatly decreases susceptibility to MPTP activation and thereby provides protection against necrotic cell death. This result is not surprising given the numerous reports implicating \(\text{mCa}^{2+}\) 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 IR injury, including a clinical trial evaluating the efficacy of cyclosporine-A (MPTP inhibitor) administration during reperfusion of the ischemic myocardium (Eilrod and Molkentin, 2013; Plot et al., 2008). It is likely that MPTP inhibition was not the sole protective mechanism, as decreasing \(\text{mCa}^{2+}\) 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 Mcu-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 Mcu during development, may have allowed for the entry of Ca\(^{2+}\) 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 Mcu in our model system was embryonically lethal, while knocking out Mcu 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 Mcu in other cell types in the heart, such as fibroblasts and endothelial cells, actually magnified injury by reducing the \(\text{mCa}^{2+}\)-buffering capacity in non-myocytes and thereby masked the protective effect of loss of Mcu in cardiomyocytes. Supporting this concept, we found that Mcu-null MEFs displayed an increase in \(\text{iCa}^{2+}\) 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 Ca\(^{2+}\) 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 \(\text{mCa}^{2+}\) content in Mcu-null cells, in contrast to their finding of \(\sim 70\%\) reduction in skeletal muscle \(\text{mCa}^{2+}\). Our results suggest a MCU-independent mechanism of \(\text{mCa}^{2+}\) uptake is a significant contributor to homeostatic \(\text{mCa}^{2+}\) levels. We hypothesize that the threshold for MCU-mediated Ca\(^{2+}\) entry is not reached under homeostatic conditions in adult cardiomyocytes and that an alternative slow \(\text{mCa}^{2+}\) uptake mechanism must play a significant role. Direct evidence that MCU-independent \(\text{mCa}^{2+}\) 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 \(\text{mCa}^{2+}\) uptake, \(\text{iCa}^{2+}\) content continued to slowly rise with sustained \(\text{Ca}^{2+}\) load and eventually reached a level equivalent to WT cells. It is possible that the lower \(\text{mCa}^{2+}\) content previously reported in Pan et al. can be explained by methodological differences. We discovered that the slightest perturbation in either extracellular or \(\text{iCa}^{2+}\) stores in WT cells induced an increase in \(\text{mCa}^{2+}\) loading. We found that any Ca\(^{2+}\) liberated during our experimental procedure, be it from mitochondrial isolation or SERCA inhibition, was immediately taken up by WT mitochondria in a Mcu-dependent fashion. Therefore such a perturbation elevates \(\text{iCa}^{2+}\) content in control cells and may lead to a false interpretation of decreased content in Mcu 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 \(\text{mCa}^{2+}\) uptake, whereas in Mcu-deleted cells we observe a rise in extra-mitochondrial Ca\(^{2+}\) levels. The addition of the MCU inhibitor, Ru360, and \(\text{mNCX}\) inhibitor, CGP37157, 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 Mcu-mediated Ca\(^{2+}\) uptake is a significant mechanism driving MPTP-mediated cardiomyocyte cell death and cardiac dysfunction. Further, we hypothesize that the \(\text{mCa}^{2+}\) exchange system possess a great deal of plasticity and that alternative uptake mechanisms maintain matrix Ca\(^{2+}\) 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 \(\text{mCa}^{2+}\) 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 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 Ca$^{2+}$ uptake is dispensable for homeostatic cardiac function, as ablating 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 mCa$^{2+}$ content to be $\sim 200$ nM, and we did not detect appreciable mitochondrial uptake until concentrations of $\sim 8$ μM were reached (control ACMs displayed only $\sim 17\%$ uptake in response to a 10-μM-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 Ca$^{2+}$ is overcome (Csordás et al., 2013; Mallilankaraman et al., 2012). Since it is assumed global ECC 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 Ca$^{2+}$, aided by balanced mNCX efflux rates. It should be noted that 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 mCa$^{2+}$ uptake including: memPyR, LETM1 (H$^+/Ca^{2+}$ exchanger), reverse-mode mNCX, 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 “RR-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 β-agonist, isoproterenol. Since a study published by Howell and Duke in 1906, it has been appreciated that 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 IAR signaling regulates changes in excitation-contraction coupling (ECC) have been defined (Bers, 2008). Our data extend these pathways to include MCU-dependent Ca$^{2+}$ uptake as a mechanism necessary to upregulate energetic support increases in cardiac contractility during acute sympathetic stress. Catecholamine signaling as occurs with the fight-or-flight response, strenuous exercise, or in the failing heart, elicits a marked increase in mCa$^{2+}$ levels. Specifically, isoproterenol has been shown to dramatically increase peak mCa$^{2+}$ and SR Ca$^{2+}$ load/release to levels sufficiently beyond those we show here are required for MCU-dependent uptake (Carran et al., 2007). This large increase in Ca$^{2+}$ is integrated into mitochondria to directly impact cellular energetics at multiple control points. mCa$^{2+}$ increases the activity of three matrix dehydrogenases that are rate-limiting in the tricarboxylic (TCA) cycle (Denton, 2009). Most notably, matrix Ca$^{2+}$ has been shown to indirectly activate pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA for entry into the TCA cycle and as such also links glycolysis with OxPhos (McCormack and England, 1983). We found a marked decrease in PDH E1 phosphorylation following Iso treatment in control cells, indicative of increased mCa$^{2+}$-dependent phosphatase activity and subsequent PDH enzymatic activation. In contrast, dephosphorylation of PDH was completely lacking in Mcu-KO hearts and PDH activity during isoproterenol administration was reduced by $\sim 50\%$. In both in vivo and in vitro experiments, we discovered that loss of Mcu ablated Iso-mediated increases in NADH and OxPhos capacity. Generally, our metabolic findings are in agreement with Pan et al., which found similar alterations in skeletal muscle metabolism and work capacity in Mcu$^{-/-}$ mice subjected to starvation (Pan et al., 2013). Similarly, our study found no change in baseline metabolic function or metabolite levels. However, our finding that HR was not altered in Mcu cKO mice does differ from a recent report by the Anderson group where they reported that an MCU-dominant-negative mouse model lacked chronotropic responsiveness to β-adrenergic stimulation (Wu et al., 2015). This may be due to a difference in methodology, as we did not examine HR with implantable telemeters in conscious mice void of anesthesia. Overall, our model does support their hypothesis of MCU-mediated Ca$^{2+}$ entry playing a significant role in the cardiac fight-or-flight response.

In summary, we show that the physiological function of MCU-mediated Ca$^{2+}$ uptake in the heart is to augment mitochondrial 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 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 mCa$^{2+}$ 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 (ch10:58930544-58911529) in mouse ES cells. Three independent mutant ES cell lines were maintained on the C57/BL6 background, and all experiments involving Mcu$^{+/C0}$ mice subjected to temporal deletion of Mcu using the MCM model. Mcu$^{+/C0}$, Mcu$^{-/}$, and Mcu$^{+/}$ x Mcu$^{-/}$ 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 C57BL/6 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 "Ca^{2+}" Uptake and Content**
To evaluate "Ca^{2+}" content, permeabilized ACMs were treated with RU3806 and CGP-37157 to inhibit "Ca^{2+}" flux. Fura2 (Invitrogen) was added to monitor extra-mitochondrial Ca^{2+}. FCCP was added to uncouple the Δψ and release matrix free-Ca^{2+}. To measure "Ca^{2+}" 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 Δψ and Fura were monitored on a PTI spectrofluorometer. All details are previously reported (Mallilankaraman 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. 500 μM CaCl₂ was injected to induce swelling ≥ 2 μM 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 Clampfit software.

**Mitoplast Patch-Clamp Analysis of MCU Current**
Following mitochondrial isolation, mitoplasts were prepared for patch-clamp studies. IMCU 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).

**LV Echocardiography and Hemodynamics**
Transathoracic 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 Iso 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 cTn using the Life Diagnostics ELISA kit. A TUNEL detection kit (Roche) was used to label DNA fragmentation in the infarct border zone of fixed heart sections.

**MEF Isolation**
Embryos were collected from Muc1^fl/fl mice at E13.5 and MEFs isolated as previously reported (Baines et al., 2005). MEFs were treated with Ad-Cre or Ad-tgfl for 24 hr. 6-day post-infection cells were used for experiments.

"Ca^{2+}" and "Cu^{2+}" Flux in MEFs
MEFs were infected with AAV-mitycam to measure "Ca^{2+}" exchange or loaded with the "Ca^{2+}" indicator, Fluor4-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 μM 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, multiple 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 grouped analyses, 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**
J.W.E. and T.S.L. wrote the manuscript; J.W.E., T.S.L., J.P.L., A.Y., X.Z., P.G., J.S., S.S., E.G., and M.J. performed experiments. J.W.E., S.R.H., W.J.K., J.Y.C., and M.M., provided experimental oversight. J.W.E. and T.S.L. designed experiments, and J.W.E., M.M., and S.R.H. interpreted data.

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