Strategic distribution of the mitochondrial Ca^{2+} uniporter in cardiac mitochondria

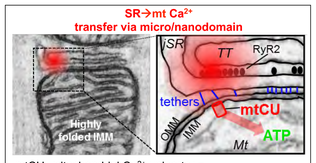
Sergio De La Fuente[1], Caitlin Vail[1], Elorm J. Agra[1], Kira Holmstrom[2], Junhui Sun[3], Jyotsna Mishra[4], Toren Finkel[2], Elizabeth Murphy[3], Shey-Shing Sheu[4], György Csordás[1]

[1]Pathology, Anatomy & Cell Biol., Thomas Jefferson University, Philadelphia, PA, USA, [2]Center for Molecular Medicine, Lab. of Molecular Biology, NIH-NHLBI, Bethesda, MD, USA, [3]Systems Biology Center, Laboratory of Cardiac Physiology, NIH-NHLBI, Bethesda, MD, USA, [4]Center for Translational Medicine, Thomas Jefferson University, Philadelphia, PA, USA

Introduction

Background:

1) Control of the mitochondrial ATP production by SR-derived Ca^{2+} signals includes local, nanodomain Ca^{2+} transfer from ryanodine receptors (RyR2) to the mitochondrial matrix (excitation-bioenergetics coupling).
2) Ca^{2+} crosses the inner mitochondrial membrane (IMM) via the mtCU, a low-affinity Ca^{2+}-activated Ca^{2+} channel complex.
3) The surface area of cardiac IMM is extensively enhanced by cristae folding; however, mitoplast patch clamp studies showed mtCU current density the lowest amongst a range of tissues (Fieni 2012. *Nat Commun*).

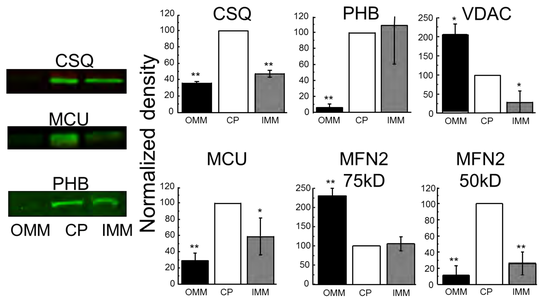


mtCU, mitochondrial Ca^{2+} uniporter = MCU (pore) + MICUs (Ca^{2+} sensors) + EMRE (assembly) + other regulators (MCUb, MCUR1)

4) One study using immunofluorescence (IF) suggested uniform distribution of MCU over the mitochondria of cardiomyocytes (Lu 2013 *Circ Res*) but a bleed-through from the mitochondrial counter-stain (MitoTracker) might have been a major factor in those results (Fig. 3)

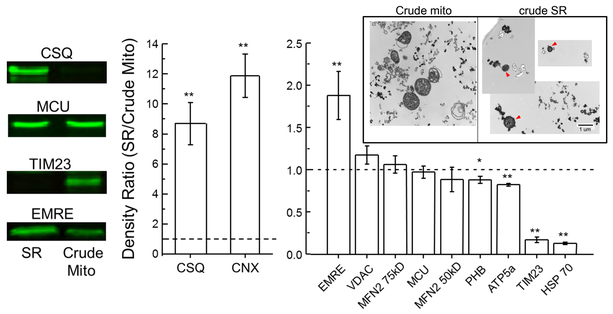
Objective:

Here we test the hypothesis that mtCU distribution is strategically biased towards mito-SR associations in the heart to support effective excitation-bioenergetics coupling.



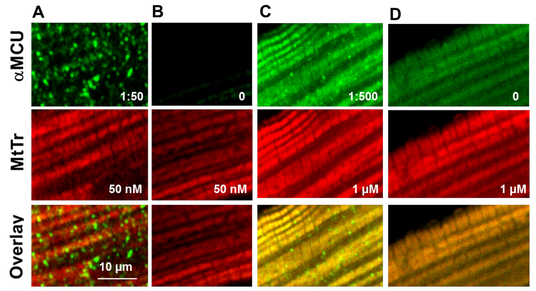
1. MCU and SR markers are most enriched in the contact points (CP) subfraction of cardiac mitochondria.

WB analysis of mitochondrial membrane subfrations obtained by osmotic and ultrasonic disruption, followed by separation on a sucrose gradient (Garcia-Perez 2011 *AJP Heart*). Based on enzyme activity and marker protein profiles three protein-rich fractions were examined: OMM, enriched in OMM and devoid of IMM markers; CP, enriched in both OMM and IMM markers; IMM, heavier fraction than CP containing high levels of IMM markers and low levels of OMM markers. In addition to calsequestrin (CSQ) and a light ~50kDa mitofusin 2 band (MFN2 50kD) that we have observed before (Garcia-Perez 2011) MCU is also enriched in the CP subfraction. On the other hand, prohibitin, another IMM protein, is almost equally distributed between the CP and the IMM subfractions.



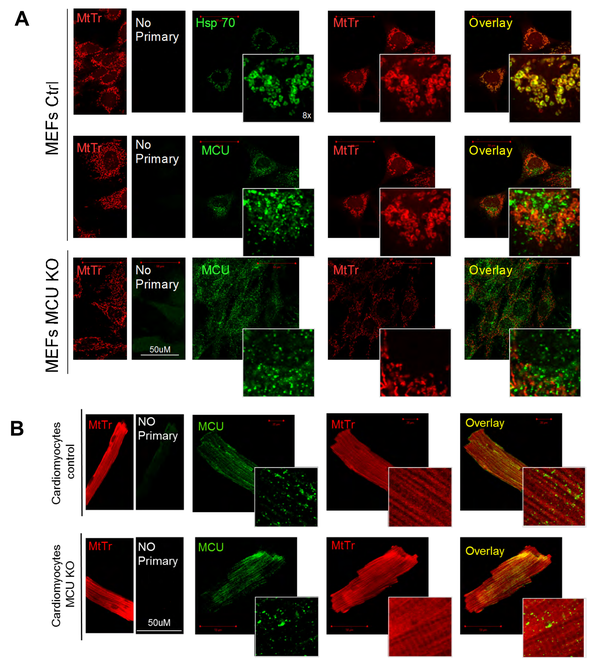
2. Mitochondria in the SR fraction have different protein profile from the crude mitochondrial fraction of rat heart and are enriched in MCU and EMRE.

Crude SR fraction (45,000"g post mitochondrial pellet) is frequently "contaminated" with mitochondria or mitochondrial fragments that are presumably small, hence the SR they are tethered to can significantly decrease their density. If mtCU was biased towards mt-SR associations, these "SR contaminant" mitochondria would be particularly rich in MCU. To this end the relative abundance of a range of mitochondrial membrane and SR membrane proteins was compared between crude rat heart mitochondria (crude mito) and crude SR. The graphs show the relative abundance as the ratio of band densities in the SR over crude mito. A value of 1 effectively reflects already an enrichment owing to the "higher dilution" of mitochondrial proteins by non-mitochondrial ones in the SR fraction. Note that the relative abundance of proteins involved in SR-mt Ca2+ transfer (VDAC, mtCU components EMRE, MCU and mt-SR tether MFN2) is ≥1. Interestingly, EMRE, an essential component of mtCU to function as a channel and be regulated by MICUs is prominently enriched in the SR fraction. Proteins associated with mitochondrial protein import (TIM23, mtHSP70) on the other hand are diminished in the SR-associated mitochondria. The inset shows a comparison between the size of the mitochondria present in the SR fraction and the cRHM



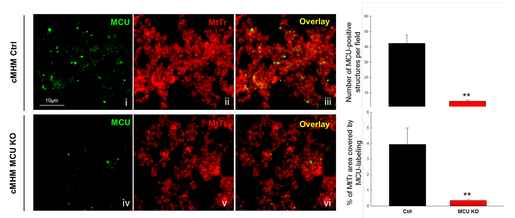
3. Optimization of αMCU and MitoTracker (MtTr) concentrations for IF in primary cardiomyocytes. Strong MtTr Red loading crosstalks to the green channel.

αMCU (Sigma) was diluted either 1:50 in the range suggested by manufacturer (**A**) or 1:500 (as published by Lu et al. 2013, **C**). 2º antibody (ab) was conjugated with Alexa 488 (green fluorescence). Before fixation the cells were loaded with MtTr Red 50 nM (**A,B**) or 1 μM (as published by Lu et al. 2013 **C,D**). The images show the green (top) and red (middle) channels separately as well as overlaid (bottom). Controls were incubated with goat serum instead of 1º ab (**B,D**). Note the 'patchy' pattern for the MCU fluorescence signal at 1:50 ab dilution vs. the homogenous mitochondrial signal indistinguishable from that of MtTr at 1:500 dilution. Furthermore, the control with MtTr 50 nM shows no green fluorescence (**B** top), even though mitochondria still show up in the red channel (**B** middle) whereas at MtTr 1μM mitochondria still show up in the green channel (**D** top) with a perfect overlap with the pattern in the red channel (**D** middle and bottom).



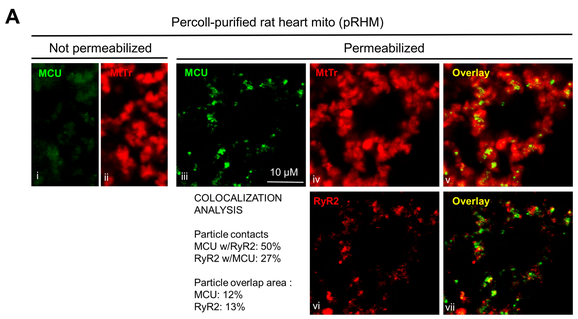
4. αMCU antibody validation in MCU KO MEFs and mouse cardiomyocytes – significant nonmitochondrial (nuclear, cytoplasmic) cross-reactions.

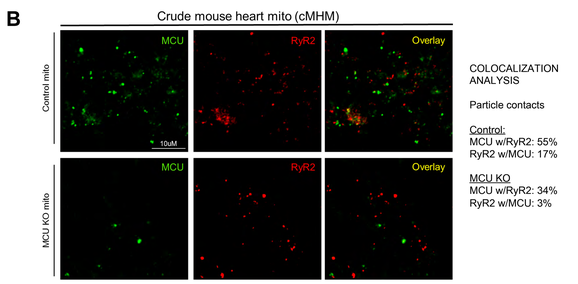
Further validation of the commercial αMCU antibody for IF was performed by comparing MCU KO to control MEFs and mouse cardiomyocytes. **A** A well-established ab against the matrix resident mtHSP 70 (top row) was used as reference and mitochondria were counterstained by MtTr Red pre-loading. Note the extensive overlap between the IF and MtTr signals (overlay on the right). Middle and bottom panels show MCU labeling and MitoTracker fluorescence from Control and MCUKO MEFs respectively. Note the significant extramitochondrial labeling in both type of cells, suggesting substantial off-target αMCU ab binding. **B** The same test was made in adult mouse cardyomiocytes with identical result. The MCU labeling in both, control and MCU KO cardiomyocytes was founded quite similar, without any decrease in the MCU signal.



5. Mitochondrial fraction isolated from mouse heart is mostly clear of non-specific MCU immunofluorescence

To isolate mitochondrial labeling by αMCU, immunofluorescence was examined using isolated mitochondria (mtIF) from mouse heart (both control and MCU KO) and attached to CellTak coated coverlips and loaded with MtTr Red. The amount of labeled MCU particles found per field in the control Mouse Heart Mitochondria (cMHM) was significantly higher in the control sample than in the MCU KO sample (close to 40 for controls and near 5 in KO). Moreover, the percentage of the Mito Tracker staining covered by MCU labeling is more than 10 times higher in the control than in the MCU KO.





6. High level of colocalization between MCU and RyR2 in isolated rat heart mitochondria.

A Percoll-purified mitocondria still have SR fragments attached to them (Garcia-Perez 2008 *JBC*). Extramitochondrial and mitocondrial outer surface binding was assessed in the absence of membrane permeabilization, and it was found to be minimal (compare i, ii vs iii, iv). Permeabilization after fixation revealed a clustered αMCU labeling distribution over the mitocondria (iii-v). Co-labeling with αRyR2 and the overlap with αMCU is shown in the bottom images (vi-vii). Colocalization analysis was based on threshold mask overlap quantitation (in FIJI). The overlap area cutoff was at 0.05 μm^2. 50% of the MCU-positive structures colocalized with RYR2, which is larger than what would be predicted from uniform, unbiased distribution. Note, that the fraction of area overlapping between the MCU and RyR2 positive particles is relatively small (12-13%) indicating that the antibodies labeled different structures. **B** The same preparation was made with cMHM in control and MCU KO mice. As it is shown in the figure 57% of the MCU positive structures colocalize with RyR2. We are actually planning to establish a rat MCU KO witch would be more relevant than mouse. More over this rats could provide higher amount of sample, making easier future studies.

References:

- Fieni F et al. Activity of the mitochondrial calcium uniporter varies greatly between tissues. Nat Commun. 2012;3:1317.
- Lu X et al. Measuring local gradients of intramitochondrial [ca(2+)] in cardiac myocytes during sarcoplasmic reticulum ca(2+) release. *Circ Res*. 2013;112:424-431
- Garcia-Perez C et al. Physical coupling supports the local ca2+ transfer between sarcoplasmic reticulum subdomains and the mitochondria in heart muscle. *J Biol Chem*. 2008;283:32771-32780
- García-Pérez C et al. Alignment of sarcoplasmic reticulum-mitochondrial junctions with mitochondrial contact points. Am J Physiol Heart Circ Physiol. 2011 Nov;301

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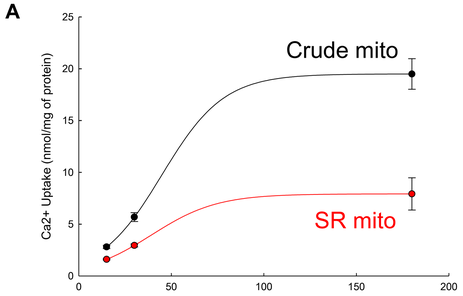

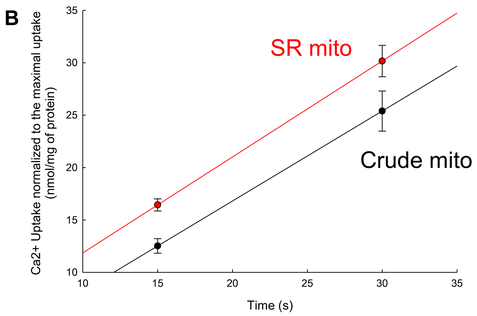

7. More effective Ca2+ uptake in mitochondria of the SR fraction than in the crude mitochondria.

Mitochondrial Ca^{2+} uptake was tested in suspensions of crude mito and crude SR using the radioactive isotope $^{45}Ca^{2+}$, and measuring the amount of specific radioactivity accumulated by the mitochondria after 15, 30 and 180 seconds. To isolate mitochondrial Ca^{2+} uptake, SERCA was blocked by thapsigargin, the Na^+ dependent mitochondrial Ca^{2+} extrusion by CGP37157 and the permeability transition pore by cyclosporine A. **A** time courses of the mitochondrial Ca^{2+} uptake upon rising the extraluminal [Ca^{2+}] to ~1.5 μM (7μM $CaCl_2$), in both crude mito and SR mito fractions. Note the large difference in the saturation levels between cRHM and crude SR, consistently with the smaller mitochondrial pool in the SR fraction. In order to correct to the pool size difference, we normalize the uptake to the maximum uptake→**B** Normalized initial Ca^{2+} uptake for both fractions. Note that the Ca^{2+} uptake by the crude SR at the same time is significantly higher than in the cRHM.

Conclusions

1. **Both, our biochemical (WB of cardiac membrane fractions) and microscopic visualization detection results are all in line with a strategic location bias of mtCU towards IMM regions more likely to be exposed to high [Ca2+]c micro/nanodomains.**

2. **The subset of mitochondria or mitochondrial segments closely associated with SR are more effective in taking up Ca2+ than the 'canonical' heavy (crude) mitochondria. Accordingly, these SR-fraction mitochondria display markedly different relative abundance of a range of mitochondrial proteins; including higher levels of MCU but most remarkably EMRE, a subunit reportedly required for mtCU channel function.**

3. **The commercially available antibody against MCU (rabbit polyclonal produced by Atlas Antibodies) that is used by most researchers has to be used with particular caution for IF/ICC owing to cross-reactions revealed in the MCUKO cells that can be minimized when labeling isolated mitochondria.**