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UCPs – Unlikely Calcium Porters

Paul S. Brookes^{1,*}, Nadeene Parker², Julie A. Buckingham², Antonio Vidal-Puig³, Andrew P. Halestrap⁴, Thomas E. Gunter⁵, David G. Nicholls⁶, Paolo Bernardi⁷, John J. Lemasters⁸, and Martin D. Brand^{2,6}

¹Department of Anesthesiology, University of Rochester Medical Center, Rochester NY, USA

²MRC Dunn Human Nutrition Unit, Cambridge, UK

³Department of Clinical Biochemistry, Institute of Metabolic Science, University of Cambridge, UK

⁴Department of Biochemistry, Bristol University, UK

⁵Department of Biochemistry & Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY USA

⁶Buck Institute for Age Research, Novato CA, USA

⁷Dipartimento di Scienze Biomediche Sperimentali, Università degli Studi di Padova, Padova, Italy

⁸Department of Pharmaceutical and Biomedical Sciences, Center for Cell Death Injury and Regeneration, Medical University of South Carolina, Charleston, SC, USA

The mitochondrial Ca^{2+} uniporter (MCU) has been characterized for several decades [1], but the molecule responsible for this transport activity is currently unknown. Therefore, a recent *Nature Cell Biology* paper by Trenker *et al.* [2] reporting a role for uncoupling proteins (UCP2/3) in mitochondrial Ca^{2+} uniport generated much excitement in the field. Subsequently, the authors contend that Ca^{2+} transport accounts for most physiologic effects assigned to UCPs [3].

The defining characteristic of mitochondrial Ca^{2+} uptake is that mitochondrial matrix Ca^{2+} ($[\text{Ca}^{2+}]_m$) responds to changes in cytosolic Ca^{2+} at a rate dependent on both the driving force (the electrochemical gradient comprising membrane potential ($\Delta\psi_m$) plus Ca^{2+} concentration gradient), and the transporter activity [4]. The steady-state free $[\text{Ca}^{2+}]_m$ depends on the balance between Ca^{2+} uptake and efflux [4]. Trenker *et al.* report that UCP overexpression increased the free $[\text{Ca}^{2+}]_m$ attained upon agonist stimulation (Fig. 1c in [2]), apparently without effect on the rate of Ca^{2+} uptake. When mitochondrial Ca^{2+} efflux via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was blocked using CGP37157, and the driving force for Ca^{2+} entry ($\Delta\psi_m$) was collapsed by oligomycin plus FCCP, agonist-stimulated free $[\text{Ca}^{2+}]_m$ was again higher in UCP overexpressing cells, with no discernible effect on Ca^{2+} uptake rate (Fig. 1f in [2]). In such a system, where both Ca^{2+} uptake and efflux are inactive, an effect of UCP expression on steady-state free $[\text{Ca}^{2+}]_m$ levels cannot be due to changes in Ca^{2+} transport. Since overexpression of UCPs can sometimes result in a non-functional uncoupled phenotype thought to be due to protein misfolding [5], and the apparent lack of effect of UCP overexpression on $\Delta\psi_m$ (supplement Fig. 1d in [2]) suggests non-functional protein, no role for native functional UCPs in mitochondrial Ca^{2+} uniport can be inferred from these overexpression experiments. Since no

*To whom correspondence should be addressed: Paul S. Brookes, PhD, Department of Anesthesiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA. Tel: 585-273-1626. Fax: 585-273-2652. E-mail: paul_brookes@urmc.rochester.edu.

quantitative or statistical analysis was applied to the effects of siRNA on UCP expression (Fig. 2 of [2]), the significance of these data is also difficult to assess.

Alternative to the MCU, the $\text{Na}^+/\text{Ca}^{2+}$ or $\text{H}^+/\text{Ca}^{2+}$ exchangers could serve a mitochondrial Ca^{2+} uptake function, in which UCPs may play a role. However, the V_{max} of these systems is ~2 or 18 nmol $\text{Ca}^{2+}/\text{min}/\text{mg}$ protein for liver or heart mitochondria respectively, i.e. 2–3 orders of magnitude lower than the V_{max} of the MCU (1700 nmol $\text{Ca}^{2+}/\text{min}/\text{mg}$ protein) [6,7]. Thus UCPs cannot significantly affect mitochondrial Ca^{2+} uptake through such mechanisms.

In Fig. 3 of [2], Ca^{2+} uptake by isolated murine liver mitochondria was only partially inhibited by 100 nM ruthenium red (RR), although it is not clear whether crude RR was used (IC_{50} ~1 μM for MCU activity) or its purified derivative Ru360 (IC_{50} 5 nM) [4,8]. Using this experimental system, a decrease in Ca^{2+} uptake by liver mitochondria of *Ucp2*^{-/-} mice was observed relative to wild-type controls. However, liver mitochondria contain exceedingly small amounts of UCP2 (<1 ng UCP2/mg protein, vs. 25 or 75 ng UCP2/mg protein in mouse kidney or spleen mitochondria respectively; V. Azzu and M.D. Brand unpublished observations). Furthermore, the mitochondrial inner membrane surface area is $5 \times 10^{10} \mu\text{m}^2/\text{mg}$ protein [9] and each μm^2 contains 10–40 (say 25) MCU channels [1]. Thus 1 mg mitochondrial protein contains 1.25×10^{12} MCU channels. By contrast 1 ng of UCP2 (21.3 fmols) equates to only 1.9×10^{10} UCPs/mg protein. Thus UCP2 density in liver mitochondria is 2 orders of magnitude lower than MCU density, raising questions about the choice of liver to investigate a novel function for UCP2, and the interpretation of these data.

To investigate the link between native UCPs and mitochondrial Ca^{2+} transport more thoroughly, we first examined RR-sensitive Ca^{2+} uptake in mitochondria isolated from rat liver and heart, using the Ca^{2+} sensitive dye Arsenazo III [10]. Although heart mitochondria contain significantly more UCP2/3 than liver [11], the Ca^{2+} transport rates of heart and liver mitochondria were similar (Fig. 1 herein). This is consistent with previous reports on similar Ca^{2+} uptake in mitochondria of different tissues [7], but difficult to reconcile with a role for UCPs in Ca^{2+} uniport, given their differential tissue expression. Furthermore, Ca^{2+} transport in mitochondria of either tissue was insensitive to the pan-UCP inhibitor guanosine diphosphate (GDP) [12] or the UCP2 inhibitor genipin [13], both of which inhibit H^+ transport by UCPs.

While GDP and genipin controls were absent from [2], the putative Ca^{2+} transport function of UCPs may be unrelated to inhibitor-sensitive H^+ transport. Thus, to unequivocally examine the role of UCPs in mitochondrial Ca^{2+} transport, we tested skeletal muscle (SkM) mitochondria from wild-type (WT) and *Ucp3*^{-/-} mice [14]. All mice were genotyped by PCR, and lack of UCP3 in knockouts was confirmed by western blot. Furthermore, *Ucp3*^{-/-} mouse SkM does not exhibit compensatory expression of other UCPs [15]. As shown in Fig. 2A and 2B herein, no difference in Ca^{2+} transport was observed between WT and *Ucp3*^{-/-} SkM mitochondria, inconsistent with a role for UCP3 in mitochondrial Ca^{2+} uniport.

Electrophoretic mitochondrial Ca^{2+} uptake results in transient $\Delta\psi_{\text{m}}$ depolarization. However, upon measuring the effects of Ca^{2+} addition on $\Delta\psi_{\text{m}}$ using a TPMP⁺ electrode [5,12], no difference in Ca^{2+} -induced depolarization was observed between WT and *Ucp3*^{-/-} mitochondria (Supplement Fig. 1). As a control, the uncoupler FCCP fully depolarized both WT and *Ucp3*^{-/-} mitochondria and resulted in full Ca^{2+} release (Fig. 2A).

To investigate a role for UCP2 in mitochondrial Ca^{2+} uniport, we also examined liver and kidney mitochondria from WT and *Ucp2*^{-/-} mice. As with UCP3 (and in direct contradiction of the results in Fig. 3 of [2], using *Ucp2*^{-/-} mice from the same source), there was no effect of UCP2 knockout on Ca^{2+} uptake kinetics (Fig. 2E,F) or $\Delta\psi_{\text{m}}$ depolarization (not shown).

In summary, the appropriately controlled Ca^{2+} uptake data presented here, from two independent laboratories (PSB and MDB), utilizing mitochondria from 4 different tissues (liver, heart, kidney, SkM) and 2 different knockout mice (*Ucp2*^{-/-} and *Ucp3*^{-/-}), are all inconsistent with the results of Trenker *et al.* [2]. Taking our results together with concerns over the appropriateness of tissues chosen for UCP genetic ablation experiments, and observations on free $[\text{Ca}^{2+}]_m$ that are incompatible with established knowledge about mitochondrial Ca^{2+} transport, we suggest that the results of Trenker *et al.* [2] be regarded with caution. It is currently premature to assign any role in mitochondrial Ca^{2+} uniport to UCPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Ca^{2+} uptake by liver and heart mitochondria; effect of UCP inhibitors

Mitochondria were isolated from rat heart or liver as described in the supplemental information, and extra-mitochondrial $[\text{Ca}^{2+}]$ was measured using the Ca^{2+} sensitive dye Arsenazo III. Mitochondria were energized with succinate (complex II), with rotenone present to inhibit complex I. **a.** Representative Ca^{2+} traces for liver mitochondria. Each arrow represents addition of a Ca^{2+} bolus (20 μM final) to the incubation, resulting in an increase in extra-mitochondrial $[\text{Ca}^{2+}]$ followed by a decrease as Ca^{2+} is sequestered into mitochondria. Where indicated, inhibitors were present as follows: 1mM GDP (UCP inhibitor), 50 μM genipin (Gen, UCP2 inhibitor), 1 μM crude RR (MCU inhibitor). **b.** Quantitation of liver mitochondrial Ca^{2+} uptake rate (i.e. initial downward slope following Ca^{2+} spike), from experiments as shown in a. The

average of second and third Ca^{2+} spikes from each trace was used for quantitation. Data are means $\pm$ SEM, N=3 (each N was an independent mitochondrial preparation on a separate day).
c. As in b, data from heart mitochondria.

Figure 2. Ca^{2+} uptake in SkM mitochondria from wild-type (WT) and $Ucp3^{-/-}$ mice, plus liver and kidney mitochondria from wild-type and $Ucp2^{-/-}$ mice

Mitochondria were isolated and Ca^{2+} uptake measured as described in the supplemental information. **a.** Representative Ca^{2+} uptake traces in mitochondria from WT (blue) and $Ucp3^{-/-}$ (red) mouse SkM. Ca^{2+} (10 μM), FCCP (1 μM), and EGTA (1 mM) were added as indicated by arrows. Each trace is an average of 3 repeats on 1 preparation (N=1). **b.** Ca^{2+} uptake rates calculated from initial downward slopes of Ca^{2+} traces in a. The average of first and second Ca^{2+} spikes from each trace was used for quantitation. Data are means $\pm$ SEM, N=3 (where each N was an independent mitochondrial preparation on a separate day). There was no significant difference between WT and $Ucp3^{-/-}$ (Student's *t* test). **c.** Ca^{2+} uptake studies in mitochondria isolated from WT (blue) and $Ucp2^{-/-}$ (red) mouse liver. Each trace is an average of 3 repeats on 1 preparation (N=1). This experiment is essentially identical in design to that shown in Figure 3 of [2], but gives a different result. **d.** Ca^{2+} uptake studies in mitochondria isolated from WT (blue) and $Ucp2^{-/-}$ (red) mouse kidney. Each trace is an average of 3 repeats on 1 preparation (N=1). **N.B.** in all panels, open/filled symbols represent the absence/presence, respectively, of crude RR (10 μM).