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Measuring mitochondrial Ca^{2+} efflux in isolated mitochondria and permeabilized cells

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Reviewer 2

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*Only major points from review and responses included.

Reviewer 2

Liver mitochondrial isolation: since the authors used an electric Potter-Elvehjem tissue grinder I recommend including the speed of the rotation because this can change the quality of the isolated mitochondria.

Authors

We now have included this information in the manuscript (page 5, item 2.1, point 5).

Reviewer 2

It is also not clear in step 8 if it is to repeat the centrifugation in the supernatant obtained in step 6 (purifying the supernatant) or if one should resuspend the pellet obtained in step 6 and centrifugate again, combining the supernatants (used sometimes to increase the yield).

Authors

In step 7, we specified that the pellet (containing cell debris) is discarded. To further emphasize this, we now write in step 8 that the supernatant obtained must be re-centrifuged, and the pellet discarded (page 5, item 2.1, points 6-9).

Reviewer 2

There is a proportion between the weight of the liver and the volume of isolation buffer or resuspension buffer used? No information about the animals was given, for example, the age, to estimate the liver size.

Authors

We used 3-4-month-old C57BL/6NTac mice (mature adults), as now included under heading 2, page 5. For an adult mouse, liver mass is ~1.5 g, and 60 mL of buffer (either isolation or resuspension buffer) were used; in proportion, that corresponds to 2.5 % (m/v). The information is now included in the experimental description under heading 2.1 (page 5, item 2.1, point 4).

Reviewer 2

After the isolation how long do the authors wait to start the experiments and for how long is it possible to use the sample without a decrease in CRC?

Authors

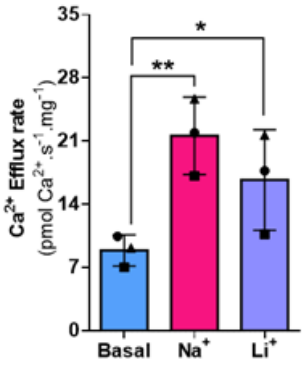
Experiments began immediately after mitochondria isolation and protein concentration estimation (~20 min). Experiments were performed up to 4 h after mitochondrial isolation. This is now included in the text (page 6, upper Note).

Reviewer 2

In figure 3, there is no difference in CsA-insensitive basal Ca^{2+} efflux with different concentrations of Ca^{2+} loads. The authors expect differences between Ca^{2+}/H_2^+ efflux and NCLX in CsA-insensitive Ca^{2+} efflux with different concentrations of Ca^{2+} loads? In other words, it was tested Na^+ and Li^+-dependent efflux with CsA?

Authors

We do not expect changes in the calcium efflux rates mediated by the Ca^{2+}/H^+ exchanger (CHE) or NCLX in the presence of CsA under the mitochondrial Ca^{2+} load conditions used, as there was no overt uncoupling and the Ca^{2+} load used is far from the maximum retention capacity. These conditions were chosen based on the lack of mtPTP induction after Ca^{2+} uptake. In preliminary experiments using the low Ca^{2+} loads shown in the presence of CsA, sodium-stimulated calcium efflux was unaltered (right).



Reviewer 2

Following the recommendations of MitoEAGLE, the states should be renamed to OXPHOS and LEAK, instead of State 3 and 4. Did you test if the ADP concentration (1 mM) was saturating in your type of cells? In case it is, you could change to OXPHOS but if this was not tested, I would recommend keeping state 3 and adding a short explanation that this is not OXPHOS state due to the non-saturating ADP concentrations. I would change

state 4 to LEAK (page 11, line 29) and "non-mitochondrial respiration" to "residual oxygen consumption (Rox)" (page 11, line 30).

In the same line, I would suggest changing RCR (figure 5) to *P-L* control efficiency: $j_{P-L} = (P-L)/P = 1-L/P$, with values from 0 to 1, since RCR is non-linear and ranges from 0 to infinity, which needs to be considered if any statistics are performed with testing for normal distribution. In your current data: *P* = state 3 and *L* = state 4.

Authors

All changes suggested were made throughout the manuscript.

Reviewer 2

How did the authors determine the best EGTA concentration to be added at the beginning of the experiment? For example, for the cells experiments, there is a range from 20-40 µM.

Authors

EGTA concentrations were titrated in preliminary experiments, as follows: first, permeabilize cells (or suspend the isolated mitochondrial sample) in experimental medium supplemented with a small amount of EGTA, i.e. 10 µM EGTA. If Ca^{2+} uptake is observed (marked by an exponential decay-shaped decrease in fluorescence), increase EGTA concentrations until uptake is no longer present. Repeat the experiment with the concentration determined previously, and verify if mitochondrial Ca^{2+} uptake is not observed since the beginning of the trace. The manuscript was modified to add this explanation (page 14, item 1.1).

Reviewer 2

Figure 4: if RuR titration is time = 0, and in experiments with Li^+ and Na^+ one should wait for 100 to 200 s to add NaCl or LiCl, the efflux Na(or Li)-dependent would only be measured starting at time ~ 200 s. This difference is explained in figure 6 legend – panel B. To be more understandable, time = 0 is not when RuR is added but after 200 s in the basal, or when Na^+ or Li^+ was added.

For isolated mitochondria I understood that to have basal, Na^+ and Li^+-dependent Ca^{2+} efflux, the experiment needs to be done 3 times, one for the basal, one adding NaCl and one adding LiCl. For the cells I understood that it is done in the same experiment (basal + Na or basal + Li), 700 s for basal but no time is mentioned for the Na(or Li)-dependent Ca^{2+} efflux after the basal (if it is done in the same experiment). If it is not in the same experiment, why do cells need longer efflux measurement times? The figure shows only the first 400 s as for imt.

Authors

Thank you for this important comment. We always compared traces at equal time points (as explained under "Measuring Na^+-dependent mitochondrial Ca^{2+} efflux" point 3, page 9, and point 3.2, page 15), but agree that the time = 0 text was confusing in this sense. Both permeabilized cells and isolated mitochondrial traces were conducted separately, as now clarified (point 3.2, page 15). We have also removed any reference to RuR addition as time 0 throughout the text and figure legends.

Reviewer 2

There are replicates from the experiments performed? Please add the information in figure legends. Please also add information about the statistical tests performed.

Authors

We now clarify in the figure legends the number of biological replicates (represented by the scatter points in the bar graphs), and statistical analysis used.