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Effect of selected fluorophores on equine skeletal muscle mitochondrial respiration

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Reviewer 1: Christopher Axelrod
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Manuscript reviewed 2023-09-27 (round 1), 2023-12-12 (round 2): *Only major points included.*

Round 1

Reviewer 1

Davis et al. examine the influence of exogenous fluorophores on parameters of mitochondrial respiratory function. To address this issue, the authors compared the effects of three dyes commonly used in conjunction with O_2 flux analysis, AmR, TMRM, and MgG, on the respiratory function of equine skeletal muscle mitochondria. The study is of interest to the energetics community in that fluorophores are now frequently added to conventional O_2 consumption studies for simultaneous evaluation of systems (ie. calcium dynamics, ROS production, ATP dynamics etc…). The following concerns are noted:

Since isolated mitochondria were used instead of tissue homogenates or fibers, quality of the isolation largely is always of concern. Based on the data alone, the mitochondria appear highly coupled (acceptor control of ~12). Please provide more information/data on the yield and quality controls for the preparation.

Authors

The reviewer highlights a major issue with regards to studies of mitochondrial physiology: how does one quantify mitochondrial abundance in a sample? There are many different proposed ways, all with major flaws (we would argue that, from a functional point of view, respiration of the sample represents the most relevant means of quantifying mitochondrial abundance). As detailed in the Methods, our final suspension was created using 80 ul of suspension media per 100 mg of parent tissue used for mitochondrial isolation. In other studies, we have used both suspension protein and citrate synthase activity assays as one means of quantifying relative yield and facilitate statistical comparison across samples. However, as described below, the experimental design – specifically, the fact that all fluorophores were tested using aliquots from a common suspension, made this unnecessary. The only quality control performed on these samples was the aforementioned respiration rates – damaged mitochondria, either due to subject disease or sample damage, would not be expected to be highly coupled.

Reviewer 1

Since isolated mitochondria were used, the most appropriate normalization factor for the experiment would be abundance of mitochondrial protein per chamber. Otherwise, the effect could be mediated by differences in protein amount. This is simply addressed by performing a BCA assay on sample recovered from the chamber (adjusted for dilution) or from sample preserved for BCA analysis.

Authors

The authors agree that, in many experimental designs, results must be normalized for possible differences in sample content. However, we point out in the methods that ALL chambers were simultaneously loaded with sample out of the same suspension of isolated mitochondria. Since the hypothesis of interest was then tested statistically with a repeated measures ANOVA, our conclusions would be unchanged by normalization – normalization would only allow for subject-to-subject comparison which is not of interest to this study.

Reviewer 1

In the SUIT protocol, the authors state that ROX was determined prior to addition of substrates, which was subtracted out from the resulting values. However, I don't think this is correct. If sample is present, then this would represent the ROUTINE state, not ROX. If sample was not present, this was just experimental background from the media. In either case, adequate inhibitors are required to account for extramitochondrial sources of oxidation. For NADH-linked respiration, this is addressed with rotenone. However, with succinate, there is no inhibitor. Thus, the succinate cannot be exclusively denoted as the OXPHOS state. To improve the rigor and reproducibility of the manuscript, I strongly encourage the authors to conduct additional studies with adequate controls and ROX measures consistent with current best practice (https://doi.org/10.26124/bec:2020-0002).

Authors

The authors respectfully disagree with the reviewer that residual oxygen consumption can only be measured through the poisoning of specific elements of the electron transfer system. In the process of sample preparation, whether it is permeabilized fibers or isolated mitochondria, the sample is repeatedly exposed to media free of substrates and adenylates in volumes several orders of magnitude greater than the volume of the sample itself. During this process, substrates and adenylates are washed out of the sample, which is in fact the basis for the validity of the measurements made early in the titration process when not all required chemicals are present. The measurement of ROX in the absence of substrates is specifically mentioned in the reference cited by the reviewer on page 33 ("Residual oxygen consumption Rox is the respiration due to oxidative side reactions in the ROX state, after application of ET inhibitors acting downstream of any fuel substrates supplied to mitochondrial preparations or cells, or in *mt-preparations incubated without substrates*."(emphasis added)). We have elected to take this approach routinely for two reasons: first, the absence of poisons which have an unknown complete spectrum of activity preserves the *in vitro* metabolic system in a state more comparable to the *in vivo* state that we are seeking to reproduce; and second, in the past when we did take the chemical inhibitor

approach, we were frequently presented with a ROX value in excess of Leak respiration, raising the question as to whether the inhibitors were completely and "cleanly" inhibiting the intended targets and only the intended targets.

Reviewer 1

Related to point number three, you have included all data points except the Imt-R state (rate of O2 consumption of isolated mitochondria with saturating O2 and endogenous substrates/adenylates corrected for ROX [antimycin sensitive rate, preferably]).

Authors

As mentioned above, this calculated value is not available since in our protocol, the proposed Imt-R state is in fact what we have designated as ROX. However, we note that Routine respiration is typically used to refer to respiration in INTACT cells, not permeabilized cells or isolated mitochondria.

Reviewer 1

For the repeated measures ANOVA, did you assess distributional assumptions? Given the smaller sample, it's likely that the distributional assumptions of the ANOVA are violated at times, which requires subsequent statistical correction.

Authors

The residuals were visually assessed using a residual plot and QQ plot and were judged to be within acceptable limits for data arising from a Gaussian distribution.

Round 2

Reviewer 1

Since isolated mitochondria were used instead of tissue homogenates or fibers, quality of the isolation largely is always of concern. Based on the data alone, the mitochondria appear highly coupled (acceptor control of ~12). Please provide more information/data on the yield and quality controls for the preparation.

Authors

We have added data regarding the citrate synthase activity of the isolated mitochondria for Study #1 as an additional indicator of yield during mitochondrial isolation. The data from Study 1 are part of a larger study in which multiple different incubation conditions were investigated, including the effect of fatiguing exercise and of aerobic conditioning. As a result, there was some change in the citrate synthase content of the isolated mitochondria suspension, likely due to changes in in vivo skeletal muscle mitochondrial content. However, these differences in mitochondria isolation yield do not affect the conclusions being drawn in this paper since the relevant statistical comparisons are across data for different fluorophores applied to aliquots of mitochondria from the same isolation batch. We agree that rigor and reproducibility are important

considerations; however, we believe that we have provided sufficient assurance of rigor and reproducibility to support the SPECIFIC conclusions in THIS paper.

Reviewer 1

Since isolated mitochondria were used, the most appropriate normalization factor for the experiment would be abundance of mitochondrial protein per chamber. Otherwise, the effect could be mediated by differences in protein amount. This is simply addressed by performing a BCA assay on sample recovered from the chamber (adjusted for dilution) or from sample preserved for BCA analysis.

Authors

See response above – rather than use mitochondrial protein mass, we elected to use citrate synthase activity to address the differences in mitochondrial isolation yield between samples. However, differences in mitochondrial isolation yield do not affect the results and conclusions of THIS study because analysis was performed WITHIN a given sample, rather than across samples.

Reviewer 1

In the SUIT protocol, the authors state that ROX was determined prior to addition of substrates, which was subtracted out from the resulting values. However, I don't think this is correct. If sample is present, then this would represent the ROUTINE state, not ROX. If sample was not present, this was just experimental background from the media. In either case, adequate inhibitors are required to account for extramitochondrial sources of oxidation. For NADH-linked respiration, this is addressed with rotenone. However, with succinate, there is no inhibitor. Thus, the succinate cannot be exclusively denoted as the OXPHOS state. To improve the rigor and reproducibility of the manuscript, I strongly encourage the authors to conduct additional studies with adequate controls and ROX measures consistent with current best practice (https://doi.org/10.26124/bec:2020-0002).

Authors

As previously stated, our approach for determining ROX and applying that correction factor is consistent with current best practice as detailed in the reference provided by the reviewer. ("Residual oxygen consumption Rox is the respiration due to oxidative side reactions in the ROX state, after application of ET inhibitors acting downstream of any fuel substrates supplied to mitochondrial preparations or cells, or in mt-preparations incubated without substrates."(emphasis added)). Thus, no additional studies are necessary.