The Journal of Physiology

Phytochemical compound PB125 attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline

Robert Vincent Musci, Kendra M Andrie, Maureen A. Walsh, Zackary John Valenti, Melissa A. Linden, Maryam F. Afzali, Sydney Bork, Margaret Campbell, Taylor Johnson, Thomas E. Kail, Richard Martinez, Tessa Nguyen, Joseph Sanford, Sara Wist, Meredith D Murrell, Joe McCord, Brooks Hybertson, Qian Zhang, Martin A Javors, Kelly S. Santangelo, and Karyn L Hamilton
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The referees have opted to remain anonymous.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Musci,

Re: JP-RP-2021-282273 "Phytochemical Nrf2 activator attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline" by Robert Vincent Musci, Kendra M Andrie, Maureen A. Walsh, Zackary John Valenti, Melissa A. Linden, Maryam F. Afzali, Sydney Bork, Margaret Campbell, Taylor Johnson, Thomas E. Kail, Richard Martinez, Tessa Nguyen, Joseph Sanford, Sara Wist, Meredith D Murrell, Joe M McCord, Brooks Hybertson, Benjamin F Miller, Qian Zhang, Martin A Javors, Kelly Santangelo, and Karyn L Hamilton

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 Referees and the reports are copied below.

I regret to say that the manuscript has not been accepted for publication.

Some positive comments were made on the manuscript. Unfortunately, they did not outweigh the more serious criticisms which led the Reviewing Editor to recommend rejection.

I am sorry to have to pass on this disappointing news, and hope it will not discourage you from making future submissions of new work to The Journal of Physiology.

However, we believe your manuscript is worthy of further consideration and suggest that you transfer your manuscript to Physiological Reports (https://physoc.onlinelibrary.wiley.com/hub/journal/2051817X/aims-and-scope/read-full-aims-and-scope), a peer-reviewed, open access, interdisciplinary journal, jointly owned by the American Physiological Society and The Physiological Society.
To transfer your manuscript to Physiological Reports, the corresponding author must send authorization within 120 days of receipt of this letter. Please use this link Transfer to Physiological Reports to send an authorization email to transfer your manuscript. If your manuscript does not require additional peer review, the editors of Physiological Reports will aim to give you an initial decision within 3 working days. In fact, >80% of transferred submissions are accepted for publication. Please note, of course, that we cannot guarantee final acceptance.

I hope you will take advantage of the opportunity to allow the editors of Physiological Reports to evaluate your manuscript.

You may be able to publish Open Access with no direct cost to yourself. You can check your eligibility here https://secure.wiley.com/openaccess?

Yours sincerely,

Michael C. Hogan
Senior Editor
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EDITOR COMMENTS

Reviewing Editor:

While both of the reviewers found the study interesting and an important addition to the field, methodological considerations make the study results seem too preliminary for publication in JP.

In particular, the lack of strong evidence for activation of Nrf2 with the given dose of PB125 is a concern and the results are therefore difficult to interpret.

However, given the novel insights on the mechanistic benefits of pharmaceutical Nrf2 activation provided by your study, we hope that you will revise the manuscript for publication in Physiological Reports.

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REFEREE COMMENTS

Referee #1:

I read, with much interest, the manuscript submitted by Musci and colleagues. Their study evaluated the effects of an Nrf2 activator, PB125, on mitochondrial respiration and reactive oxygen species production, muscle protein synthesis rates, and mobility in a Hartley guinea pigs, a model of osteoarthritis. This is a well written and robust study that provides insights on the mechanistic benefits of pharmaceutical Nrf2 activation. However, there are a few major points that detract from the quality of this work in its current form. I have detailed these major points below, along with several minor comments for the authors to consider.

Major comments:

Lines 213-215: The concentration of PB125 used in this study was selected based on the data in Supplementary Figures 1A-C. While this is a robust approach for selecting the dose of PB125, there is minimal change in the plasma concentration of Luteolin, Carnosol, or Withaferin A over the 120 minute time course at 8mg/kg. Thus, I am unsure why the authors selected 8mg/kg rather than 40mg/kg, which showed clear increases in the plasma concentration of Luteolin, Carnosol, and Withaferin A. Please clarify why the lower concentration was selected given the lack of change in the plasma concentration of the active ingredients. Further, did the authors test for any sex-related differences in the plasma concentrations of the active ingredients that might help explain some of the sex differences in the outcome variables? At a minimum, the authors should clarify why the lower dose was administered in the methods and then discuss the potential benefit of using higher doses in the discussion section of the manuscript.

Supplementary Figure 1E: There is a lot of variability in Nrf2 content of the gastrocnemius in the control group. Indeed, the Nrf2 content was higher in some of the controls than the treatment group. Might this result be due to the minimal change in plasma concentration of the active ingredients? If so, this point should be acknowledged in the discussion.

Figures 8 & 9: PB125 clearly mitigated some of the effects of age/disease on fractional synthesis rates in the soleus, but not in the gastrocnemius. Might this muscle-dependent effect be related to the fiber type composition of the muscle studied? It would be useful if the authors could comment on which portion of the gastrocnemius muscle was sampled (red vs. white gastrocnemius) given that this muscle is much more mixed than the mostly type I soleus. A brief discussion of the potential fiber-type differences in the effects of PB125 should be added to the discussion.

Lines 418-419: Reporting differences with $p<0.10$ to highlight potential directions for future research seems inappropriate to me, although I understand the lack of prior data on mitochondrial respiration in permeabilized fibers in Hartley guinea pigs precluded a power analysis being performed a priori. However, I think a more suitable approach would be to report the effect sizes because these are independent of sample size (PMID: 23997866). The authors should remove statements about statistical significance where $p>0.05$ but <0.10, and instead report the effect sizes throughout the manuscript.

Minor comments:

Lines 59-60: "Targeting the mitochondrial dysfunction and impaired protein homeostasis may mitigate the progressive musculoskeletal decline." Please clarify if this pertains to age or disease-related declines in mitochondrial function and impaired proteostasis, or both.

Lines 82-83: Please provide a reference for the number of US adults affected by osteoarthritis.

Line 94: "Type I fibers increase" is unclear. Please clarify if this is an increase in type I fiber area or the percentage of type I fibers.

Lines 383-392: The authors should clarify in the methods section whether or not the time of day for the mobility testing was consistent between groups, and what time of day the testing was performed.

Line 447: The authors use the terms mitochondrial respiration and mitochondrial function interchangeably throughout the manuscript. Because mitochondria are complex organelles with many functions (e.g., calcium homeostasis, reactive oxygen species handling), it would be more accurate to refer to the specific mitochondrial function in each context (i.e., mitochondrial respiration or mitochondrial ROS production).

Lines 448-450: Please clarify which group had higher coupled and uncoupled respiration compared with the 15 month old male and female guinea pigs.

Lines 458-461: Nrf2a did not enhance coupled respiration, but increased ETS capacity. Please provide some insight into the in vivo relevance of increased ETS capacity in the absence of improved coupled respiration.

Line 473: Please refer to Figure 5A rather than 4A here as the ADP Vmax data are shown in Figure 5.

Line 482: Please replace "decrease" with "increase" as the data in Figure 5 show that Nrf2a increased Km in males.

Line 582: Since mitochondrial density was not measured in this study, please replace the word 'density' with 'content' to more accurately reflect the measures used in this study.

Lines 797-802: This section of the conclusions detracts from the bottom-line of the current work. I suggest moving this section to the discussion to help strengthen the impact of the conclusions.

Figure 2: Because this is the first work to have measured mitochondrial respiration from Hartley guinea pigs in permeabilized muscle fibers, it would be nice to include Figures with the respiration from each step of the SUIT protocols in the supplementary data. This would allow others in the field to see the state 2 respiration in addition to the state 3 and RCR data.

Figure 10: Please add a horizontal line at 50% on the y-axis of each panel.

Supplementary Table 1. The authors do a really nice job of detailing their SUIT protocols. However, it would be useful if cytochrome C was added to this Table to more clearly reflect the protocol that was used in this study. Additionally, it is unclear how the cytochrome C control factor was calculated. Please add this calculation to Supplementary Table 1. Lastly, because cytochrome c is a redox-active substance it is considered to be incompatible with the Amplex Red system for detection of mitochondrial ROS production. Please clarify whether cytochrome C was used in the SUIT protocol for determining ROS production.

Supplementary Figure 3: Please add a vertical line to the x-axis of Panel A at 0.25 control factor so readers can more easily see which data were excluded. Additionally, it is unclear to me if Panels A and C represent the SUIT protocols 1 and 2 presented in Supplementary Table 1, respectively. It would be useful if the headings above each Panel read "SUIT protocol 1" and "SUIT protocol 2" to align with the information in supplementary Table 1.

Supplementary Figure 5. Please remove panels A-D as they are presented in Figure 5A and 5B of the main manuscript (i.e., these Figures appear to be duplicates).

Supplementary Figure 6: It would be useful to see a representative western blot image in addition to the group data.

The p-values are reported to a different number of decimal places in places throughout the manuscript and Figures. I recommend the authors be consistent with the number of decimal places for the p values throughout the text and Figures.

The respiration data from SUIT protocol 2 are not presented. These data should be included in addition to the respiration data from SUIT protocol 1 because this provides additional information relating to fatty acid-supported mitochondrial respiration.

Referee #2:

The study "Phytochemical Nrf2 activator attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline" set out to test the effects of PB125 on mitochondrial respiration, muscle proteostasis, and mobility in a model of OA progression. This is an interesting study that uses both a new model of skeletal muscle degeneration and a new natural supplement. The study proposes to test the relationship between Nrf2 and mitochondrial dysfunction and loss of proteostasis in the guinea pig model and how changes in these parameters affect mobility. The overall goals of the study are important to identify potential targets for therapeutic intervention. Unfortunately, there are several limitations to the study and the report. The major concern is that the results do not match the conclusions. The authors enthusiasm for the effects of this PB125 compound should be more tempered given the results reported and lack of additional measures of Nrf2 activation. Furthermore, there are some concerns of study design that bring the interpretation of results into question. Furthermore, the authors acknowledge this was part of a larger study. This suggests there are other data, and perhaps these data would strengthen the other, and vice versa with a larger single publication. As it stands, there are a collection of measurements making the overall hypothesis of the investigation unclear and subject to post hoc motivated reasoning. Below are some specific comments that will hopefully improve the quality of this manuscript.

The authors call this an disease/aging model throughout the paper, their rationale being that they cannot discern between aging effects or disease effects at the ages studied. However, by criteria listed in the manuscript, I think it could be argued quite effectively that this is not an aged model for the following reasons: 1) 15 months is only 25% of the average animal lifespan, 2) if the animals are "still experiencing long bone growth at 10 mo of age", couldn't it be argued that they are still developing and aren't even fully mature yet? By these two criteria, it would be more appropriate that the authors are testing whether Nrf2a treatment is preventing the onset or progression of the OA disease, or perhaps the effects of PB125 on developmental processes, but not aging. Therefore the "age" term is misleading and should not be used in this manuscript. Only disease and or OA progression should be used.

There are some major issues with the design and claims surrounding PB125 and related Nrf2 activation, which are as follows:

1) Why was 8.0mg/kg used for supplement dosage? The authors state from line 213-215 - "Based on the analysis conducted at the NSC Analytical Pharmacology Core (Supplemental Figure 1A - C), we selected a dosage of 8 mg/kg of bodyweight, which corresponds to 250 PPM, about 2.5x the dose of PB125 mice in the NIA ITP receive." Both the 24mg/kg and 40mg/kg had larger increases in plasma while 8mg/kg had hardly any increase in plasma from the PK study (Suppl. Fig 1A-C). The decision to use the 8.0mg/kg as the dosage for long term supplementation does not make sense in the context of the data presented for the PK study in this report. Since this dose was used, it is questionable that any sufficient increases in plasma were seen and calls into question whether PB125 could activate Nrf2 or any other signal transduction cascade that

converges on Nrf2 activation within skeletal muscle in vivo. In other words, how could it have a direct effect (or an indirect effect) on Nrf2 in skeletal muscle if there isn't any of the 3 compounds in the PB125 supplement showing up in plasma?

2) Perhaps over the long term, repeated supplementation would gradually increase PB125 content. However, it is impossible to know because PB125 content wasn't measured at the end of the study. Why wasn't the PB125 content measured in plasma and skeletal muscle after long term treatment of PB125? I think it is prudent to perform the PK experiments over the entire treatment period in addition to short term PK study, especially given the chosen dose didn't produce any significant increases in plasma over the short term. Measuring PB125 in plasma before starting supplementation, and after completion of supplementation, and comparing PB125 content within skeletal muscle compared to controls at the end of the study would give reasonable confidence that it can get into plasma/tissues and activate Nrf2 in vivo. Furthermore, if there were data from other sources on in vivo PK results with long term supplementation, I think that would suffice here. But the citations given for PB125 mechanisms in the intro (Hybertson et al., 2019, McCord et al., 2021) are an in vitro study, and a review, which do not inform readers about how this compound behaves in vivo which is absolutely necessary, in my opinion, if the claim is that this compound activates Nrf2 in vivo. The lack of increases in plasma at 8mg/kg, and without any data on plasma or skeletal muscle PB125 content measured after the long-term supplementation, it is not clear that PB125 activates Nrf2 in vivo (directly or indirectly). Furthermore, it is possible that Nrf2 gradually increases in response to the stress induced by OA progression (higher basal activity) rather than any increase induced by the PB125 supplement. Increases in basal Nrf2 have been shown in other models of aging (PMID: 28863281) and in aging humans PMID: 32866619. Furthermore, the decrease in Nrf2 basally allows for increased inducibility in response to exercise. This has also been shown in mouse models of exercise training (PMID: 27471236).

3) The authors state that QPCR/ other Nrf2 endpoints were not done because the tissues were collected 24 hours after the last treatment was given, preventing the assessment of acute effects. The rationale here is flawed. If Nrf2 is elevated, and this increase is physiologically relevant, you should also see elevations in the downstream protein content of Nrf2 regulated genes because these proteins will have reached a new elevated steady state (regardless of mRNA content 24 hours after last supplementation given) with repeated transient activations of Nrf2 from supplementation of PB125. Measuring protein content of NQO1 or some other protein that is primarily regulated be Nrf2 would strengthen the evidence for activation of Nrf2 in the long term supplementation.

Taken together, the above concerns surrounding rationale for 8.0mg/kg PB125 supplementation dosage, lack of PB125 content measurements in plasma or skeletal muscle after the in vivo treatment regimen at 3 months and 10 months, and lack of downstream Nrf2 outcome measures, call into question the claims that this compound activated Nrf2 in vivo in any sufficient way to produce the effects claimed. The claims generally do not match the results here, and should either be tempered to match the results, or additional measures should be done such as downstream protein expression to increase confidence of Nrf2 activation in remaining skeletal muscle tissue if possible.

With regards to the functional mobility assay there are two concerns:

1) Given that there are other behavioral factors that play into the mobility assay (i.e. animals learned the environment through subsequent exposures and thus reduced exploratory behavior once the environment was learned) the assay has some fundamental limitations. The authors should address this in a limitations section.

2) Line 44-46 "These effects were not associated with statistically significant prolonged maintenance of voluntary mobility in guinea pigs but may reflect clinically meaningful improvements in mobility". Since this was not statistically significant, the conclusions drawn here do not match the data, rather it is highly speculative. Additionally, the term "clinically relevant" would be more appropriate if this study was done in a clinical human trial. Even if this were the case, given the fact that it was not statistically significant, the speculation here would not be appropriate in my opinion. This sentence should be removed entirely.

Is the decline shown with mitochondrial respiration truly an effect of disease? The authors suggest that these effects might just be a decline in mitochondrial function because the animals are exiting the growth phase (~9mo). Additionally, the authors claim: Line 616-618 "However, these data clearly demonstrate that impaired mitochondrial respiration is a characteristic of this pre-clinical model of musculoskeletal decline." This does not seem clear in my opinion given the authors previous statement regarding animal development at 9mo.

Minor comments/ concerns

Line 736-737 Add the word "in" - Interventions designed to mitigate age-related increases in oxidative stress or inflammation seem to improve...

All western blot data should have accompanying representative images (Nrf2 WB in Suppl. Figure 1E) to confirm that the appropriate band for Nrf2 is being assessed. The 55-60kDa band has been shown not to be Nrf2, but the reader does not know which band was quantified without an image and ID of the band you are measuring in the image.

Figure 2 needs a legend to identify which color corresponds to control and treated groups.

Line 479-480 "However, Nrf2a did significantly increase the apparent Km (p=0.007) indicating lower ADP sensitivity, though this is likely a consequence of increased ADP Vmax in the absence of changes in respiration rates in sub-saturating amounts of ADP (Supplemental Figure 5)" This is the definition of lower ADP sensitivity. The Vmax is controlled for in the calculation of apparent Km so regardless of the effect on Vmax it appears that the treatment actually lowers ADP Km.

Confidential Review **17-Aug-2021**

The authors wish to thank the Reviewing Editor and Referees for their interest in our study and the time they invested in providing us with feedback. We appreciate the opportunity to appeal the previous decision. We would like to take special note that in between submissions, the Journal of Physiology has changed policy such that data we previously included as supplemental data can no longer be submitted as such. Thus, we have substantially changed the organization of graphs and figures.

Additionally, we have removed some data from the supplemental dataset as we believe it does not add much to the story and would be distracting or burdensome to the reader. If the present reviewers would like to review this data or feel that it should be included, please express such and we will add it back to the manuscript.

Nonetheless we are excited to begin reporting the findings from this pre-clinical study in a unique model of musculoskeletal decline, and think that our revised manuscript is a good fit for the Special Issue "*Metabolic approaches to slow ageing and extend healthspan.*"

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REFEREE COMMENTS

Referee #1:

I read, with much interest, the manuscript submitted by Musci and colleagues. Their study evaluated the effects of an Nrf2 activator, PB125, on mitochondrial respiration and reactive oxygen species production, muscle protein synthesis rates, and mobility in a Hartley guinea pigs, a model of osteoarthritis. This is a well written and robust study that provides insights on the mechanistic benefits of pharmaceutical Nrf2 activation. However, there are a few major points that detract from the quality of this work in its current form. I have detailed these major points below, along with several minor comments for the authors to consider.

> We thank you for your interest in our study and your thoughtful critique. We have responded to each of your comments and have tried to incorporate all suggested changes. As a result, we believe the revised manuscript is much improved.

Major comments:

Lines 213-215: The concentration of PB125 used in this study was selected based on the data in Supplementary Figures 1A-C. While this is a robust approach for selecting the dose of PB125, there is minimal change in the plasma concentration of Luteolin, Carnosol, or Withaferin A over the 120 minute time course at 8mg/kg. Thus, I am unsure why the authors selected 8mg/kg rather than 40mg/kg, which showed clear increases in the plasma concentration of Luteolin, Carnosol, and Withaferin A. Please clarify why the lower concentration was selected given the lack of change in the plasma concentration of the active ingredients. Further, did the authors test for any sex-related differences in the plasma concentrations of the active ingredients that might help explain some of the sex differences in the outcome variables? At a minimum, the authors should clarify why the lower dose was administered in the methods and then discuss the potential benefit of using higher doses in the discussion section of the manuscript.

We designed the PK studies to confirm the absorption (i.e. bioavailability) of orally administered PB125. While we hypothesize that the mechanism of action of PB125 is not in the plasma, plasma concentrations of these compounds are simply used as a surrogate measurement to confirm drug delivery. The goal of these experiments was not to maximize plasma concentrations of the active ingredients of PB125. Rather, these experiments were designed to confirm that there were detectable levels of each of three primary active ingredients of PB125 acutely after oral administration. As we were not completely confident that 8 mg/kg of PB125 would achieve detectable levels of the three main components, we elected to simultaneously test higher doses. While we considered choosing a higher dose, we decided to stick to a dosage of 8 mg/kg, which was already 2.5x the dosage of PB125 administered to mice in the NIA ITP study. In addition, we wanted to determine whether or not a dose of PB125 that resulted in detectable, but modest concentrations of the active components, could have demonstrable effects on cellular function *in vivo*.

In addition, we took plasma samples from a limited number of both male and female guinea pigs. Both sexes demonstrated detectable concentrations of the active ingredients, though we were not adequately powered in this sub-experiment to test if there was a sex difference.

We have made edits to succinctly explain the purpose of measuring the active ingredients of PB125 in plasma (line 176 - 179) and why we chose the 8mg/kg dose (lines 221 - 225).

Supplementary Figure 1E: There is a lot of variability in Nrf2 content of the gastrocnemius in the control group. Indeed, the Nrf2 content was higher in some of the controls than the treatment group. Might this result be due to the minimal change in plasma concentration of the active ingredients? If so, this point should be acknowledged in the discussion.

Based on reviewer comments, we decided to repeat Western blot analyses with an expanded sample size and additional antibodies. To our surprise, a strong age x treatment interaction was present where PB125 increased Nrf2 content in 5 mo guinea pigs, but decreased Nrf2 content in 15 mo guinea pigs. It is difficult to state with certainty why this occurred, however, we have attempted to do so in the discussion:

Line 629 – 646: *"Though PB125 does stimulate Nrf2 activation (as measured by a promoter/report assay) in vitro (Hybertson et al., 2019), the changes we observed in Nrf2 content with PB125 treatment were not consistent between young and older guinea pigs. Instead, PB125 had a significant interaction with age, increasing Nrf2 content in 5 mo guinea pigs, and decreasing Nrf2 protein expression in 15 mo guinea pigs. HO-1 content showed a similar pattern. Though we have no acute in vivo data to support this, we hypothesize that PB125 activated Nrf2, as demonstrated by greater Nrf2 content in 5 mo guinea pigs. 10 months of treatment with PB125 led to consistent Nrf2 activation, leading to greater downstream antioxidant enzymes. With age, we hypothesize that greater basal levels of ROS led to a greater Nrf2 content in 15 mo CON guinea pig. We further posit that PB125 ameliorated the increase in age-related ROS, which led to significantly lower levels of Nrf2 and HO-1. This pattern is similar to aerobic exercise, which upregulates antioxidative capacity and protects from age-related increases in oxidative stress* (Muthusamy *et al.*, 2012). In humans, Nrf2 expression increases with age; however, aerobic exercise training reduces levels of Nrf2 (Ostrom & Traustadottir, 2020).

We speculate a similar pattern occurred with PB125 treatment, though further work is required to understand how PB125 affects Nrf2 activation in vivo in both young and old organisms. Similar to many drugs and supplements, it is quite possible that PB125 has widespread effects that are not mediated through a singular pathway."

Figures 8 & 9: PB125 clearly mitigated some of the effects of age/disease on fractional synthesis rates in the soleus, but not in the gastrocnemius. Might this muscle-dependent effect be related to the fiber type composition of the muscle studied? It would be useful if the authors could comment on which portion of the gastrocnemius muscle was sampled (red vs. white gastrocnemius) given that this muscle is much more mixed than the mostly type I soleus. A brief discussion of the potential fiber-type differences in the effects of PB125 should be added to the discussion.

Please note, Figures 8 & 9 have been combined to Figure 6.

Differences in fiber type compositions might explain why there was modest attenuation of the effects of disease/age on FSR in the soleus, but not gastrocnemius. We homogenized the gastrocnemius and did not discriminate between red/white sections of the muscle. Therefore, from our analyses of "mixed gastrocnemius fibers," we cannot draw conclusions about our outcomes in fast versus slow fibers.

We have added in lines 732 – 735: "*It is worth noting that the attenuation of decline in protein synthesis occurred only in the soleus. It is unclear why there is a muscle-specific effect. However, the soleus muscle is primarily comprised of type I myofibers and gastrocnemius, as indicated in your comment, is much more mixed (Musci and Walsh et al 2020)."*

We are in the progress of analyzing a muscle with a type II fiber composition as part of another study in our lab, and will then be able to draw conclusions about fiber type-specific outcomes.

Lines 418-419: Reporting differences with $p<0.10$ to highlight potential directions for future research seems inappropriate to me, although I understand the lack of prior data on mitochondrial respiration in permeabilized fibers in Hartley guinea pigs precluded a power analysis being performed a priori. However, I think a more suitable approach would be to report the effect sizes because these are independent of sample size (PMID: 23997866). The authors should remove statements about statistical significance where $p>0.05$ but <0.10, and instead report the effect sizes throughout the manuscript.

We have added effect sizes as suggested throughout the manuscript. We thank the reviewer for their suggestion. For many of the measurements where PB125 did not have a significant effect, the effect size of PB125 was "small" to "medium." (per PMID: 23997866).

Minor comments:

Lines 59-60: "Targeting the mitochondrial dysfunction and impaired protein homeostasis may mitigate the progressive musculoskeletal decline." Please clarify if this pertains to age or disease-related declines in mitochondrial function and impaired proteostasis, or both.

We have revised the statement to indicate that targeting age- and disease- related proteostasis or mitochondrial function may improve overall musculoskeletal function (e.g. gait, strength):

Lines 54 – 56: Targeting both mitochondrial dysfunction and impaired protein homeostasis (proteostasis), which contribute to the age and disease process, may mitigate the progressive decline in overall musculoskeletal function (e.g. gait, strength).

Lines 82-83: Please provide a reference for the number of US adults affected by osteoarthritis.

We have revised the reference (now line 79), which is the US Bone and Joint Initiative. We found this source from a document prepared by the CDC. (See reference 1 of Osteoarthritis: 2020 Update) https://www.cdc.gov/arthritis/docs/oaagenda2020.pdf

Line 94: "Type I fibers increase" is unclear. Please clarify if this is an increase in type I fiber area or the percentage of type I fibers.

We have revised the sentence (now lines 90 – 94): "*Similar to humans with OA (Kemmler et al., 2015; Noehren et al., 2018), skeletal muscle fiber size and density decrease and the proportion of type I fibers increases in the gastrocnemius by 15 months in these guinea pigs (Tonge et al., 2013; Musci et al., 2020). Low skeletal muscle mass in the lower limb is associated with knee osteoarthritis (Lee et al., 2016)*."

Lines 383-392: The authors should clarify in the methods section whether or not the time of day for the mobility testing was consistent between groups, and what time of day the testing was performed.

Thank you, the following statement has been added:
Lines 367 - 368 "*Mobility assessments were conducted in the mornings to minimize variance due to time of day. Additionally, cohorts containing both CON and PB125 treated guinea pigs were evaluated on the same day and time*."

Line 447: The authors use the terms mitochondrial respiration and mitochondrial function interchangeably throughout the manuscript. Because mitochondria are complex organelles with many functions (e.g., calcium homeostasis, reactive oxygen species handling), it would be more accurate to refer to the specific mitochondrial function in each context (i.e., mitochondrial respiration or mitochondrial ROS production).

We have revised the manuscript to reflect this comment. For our findings, "mitochondrial function" was replaced with "mitochondrial respiration." When citing a study that measured several aspects of mitochondrial function (Ca homeostasis, ROS handling, respiration), we maintained the use of "mitochondrial function." For a study that only measured one aspect of mitochondrial function (e.g. Ca homeostasis OR respiration), we specifically referenced that component of function (instead of broadly defining as "function").

Lines 448-450: Please clarify which group had higher coupled and uncoupled respiration compared with the 15 month old male and female guinea pigs.

Lines 425 - 427: "*15 mo male and female guinea pigs had lower coupled (State $3_{[PGM+S]}$) (Figure 3A) and uncoupled ($ETS_{[CI-CIV]}$) (Figure 3B) respiration compared to 5 mo counterparts (Cohen's d=0.731, 0.680; p=0.001, p=0.004, respectively).*"

Lines 458-461: Nrf2a did not enhance coupled respiration, but increased ETS capacity. Please provide some insight into the in vivo relevance of increased ETS capacity in the absence of improved coupled respiration.

To avoid over-speculation, we brought this observation forward in our discussion and provided a suggestion of what this finding could represent:
Line 616 - 618 "*PB125 did not augment coupled respiration despite improvements in uncoupled respiration, which may reflect enhanced reserve capacity suited to handle greater electron flux*."

Line 473: Please refer to Figure 5A rather than 4A here as the ADP Vmax data are shown in Figure 5.

Thank you for pointing out the error. We have corrected it.

Line 482: Please replace "decrease" with "increase" as the data in Figure 5 show that Nrf2a increased Km in males.

Thank you, we have corrected the statement:
Line 461 - 463: "*There was a non-significant interaction between sex and PB125 treatment (p=0.092), indicating that the PB125-mediated increase in Km (i.e. decrease in sensitivity) may have occurred only in males*."

Line 582: Since mitochondrial density was not measured in this study, please replace the word 'density' with 'content' to more accurately reflect the measures used in this study.

You are absolutely right. We corrected this.

Lines 797-802: This section of the conclusions detracts from the bottom-line of the current work. I suggest moving this section to the discussion to help strengthen the impact of the conclusions.

This has been done and can now be found on lines 690 – 696.

Figure 2: Because this is the first work to have measured mitochondrial respiration from Hartley guinea pigs in permeabilized muscle fibers, it would be nice to include Figures with the respiration from each step of the SUIT protocols in the supplementary data. This would allow others in the field to see the state 2 respiration in addition to the state 3 and RCR data.

We have added titration data in Figure 2.

Figure 10: Please add a horizontal line at 50% on the y-axis of each panel.

This figure has been revised (now Figure 7).

Supplementary Table 1. The authors do a really nice job of detailing their SUIT protocols. However, it would be useful if cytochrome C was added to this Table to more clearly reflect the protocol that was used in this study. Additionally, it is unclear how the cytochrome C control factor was calculated. Please add this calculation to Supplementary Table 1. Lastly, because cytochrome c is a redox-active substance it is considered to be incompatible with the Amplex Red system for detection of mitochondrial ROS production. Please clarify whether cytochrome C was used in the SUIT protocol for determining ROS production.

We appreciate the comment. In order to add the cytochrome C step without changing the font size, we separated the SUIT protocols into two tables.
We calculated the cytochrome C control factor according to bioblast.at https://www.bioblast.at/index.php/Cytochrome_c_control_efficiency
The specific calculation for each SUIT protocol is presented in the according table.
Finally, we did use cytochrome C in the second SUIT protocol with the Amplex Red system. However, as the reviewer pointed out, because cytochrome C is redox active, we did not make any assessment of ROS emission after adding cytochrome C. We have clarified this in our methods as well:

Lines 294 - 298*"…which is what the cytochrome C control factor approximates (Pesta & Gnaiger, 2011) (Figures 2A - C). After the addition of cytochrome C we did not make any further assessments of ROS emission as cytochrome C is a redox substrate. We added 5 µM rotenone to determine maximal coupled respiration…"*

Supplementary Figure 3: Please add a vertical line to the x-axis of Panel A at 0.25 control factor so readers can more easily see which data were excluded. Additionally, it is unclear to me if Panels A and C represent the SUIT protocols 1 and 2 presented in Supplementary Table 1, respectively. It would be useful if the headings above each Panel read "SUIT protocol 1" and "SUIT protocol 2" to align with the information in supplementary Table 1.

This is now Figure 2. Thank you for the suggestion, we have done so and it is reflected in the legend: "*Cytochrome C Control Factor Scatterplots. Scatterplots and regression line relating Cytochrome C Control Factor to coupled respiration in Suit 1* ***(A)*** *and Suit 2 before* ***(B)*** *and after* ***(C)*** *a limit of 0.25 (dotted line drawn on* ***B****) was implemented to establish O2K trials to exclude due to over permeabilization.*"

Supplementary Figure 5. Please remove panels A-D as they are presented in Figure 5A and 5B of the main manuscript (i.e., these Figures appear to be duplicates).

The figures are the same data as Figure 5. We have removed these figures as they are redundant. The intent was to present averaged curves and corresponding values to provide the reader context with how different Vmax and Km values shape a titration curve..

Supplementary Figure 6: It would be useful to see a representative western blot image in addition to the group data.

We have provided these representative western blot images in the revised manuscript. Please refer to the additional attachment for all raw western blot images.

The p-values are reported to a different number of decimal places in places throughout the manuscript and Figures. I recommend the authors be consistent with the number of decimal places for the p values throughout the text and Figures.

We have checked and the number of decimal places used throughout the manuscript and figures are consistent with the Journal's guidelines.

The respiration data from SUIT protocol 2 are not presented. These data should be included in addition to the respiration data from SUIT protocol 1 because this provides additional information relating to fatty acid-supported mitochondrial respiration.

The respiration data from protocol 2 are presented in Figure 3E - G. We have modified the text in the figure legend to make this clearer. "*Disease/Age-, Sex-, and Treatment-related differences in mitochondrial respiration from SUIT 1 & 2 protocols*"

Referee #2:

The study "Phytochemical Nrf2 activator attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline" set out to test the effects of PB125 on mitochondrial respiration, muscle proteostasis, and mobility in a model of OA progression. This is an interesting study that uses both a new model of skeletal muscle degeneration and a new natural supplement. The study proposes to test the relationship between Nrf2 and mitochondrial dysfunction and loss of proteostasis in the guinea pig model and how changes in these parameters affect mobility. The overall goals of the study are important to identify potential targets for therapeutic intervention. Unfortunately, there are several limitations to the study and the report. The major concern is that the results do not match the conclusions. The authors enthusiasm for the effects of this PB125 compound should be more tempered given the results reported and lack of additional measures of Nrf2 activation. Furthermore, there are some concerns of study design that bring the interpretation of results into question. Furthermore, the authors acknowledge this was part of a larger study. This suggests there are other data, and perhaps these data would strengthen the other, and vice versa with a larger single publication. As it stands, there are a collection of measurements making the overall hypothesis of the investigation unclear and subject to post hoc motivated reasoning. Below are some specific comments that will hopefully improve the quality of this manuscript.

> We appreciate the reviewer's thorough critique and assessment of our manuscript. We believe that addressing the comments and suggestions has improved the manuscript. The reviewer is correct that there are more data in the process of being published. However, to maintain the focus of this manuscript we hesitate to add more data from other components (e.g. brain, bone, cartilage) of the larger study.

The authors call this an disease/aging model throughout the paper, their rationale being that they cannot discern between aging effects or disease effects at the ages studied. However, by criteria listed in the manuscript, I think it could be argued quite effectively that this is not an aged model for the following reasons: 1) 15 months is only 25% of the average animal lifespan, 2) if the animals are "still experiencing long bone growth at 10 mo of age", couldn't it be argued that they are still developing and aren't even fully mature yet? By these two criteria, it would be more appropriate that the authors are testing whether Nrf2a treatment is preventing the onset or progression of the OA disease, or perhaps the effects of PB125 on developmental processes, but not aging. Therefore the "age" term is misleading and should not be used in this manuscript. Only disease and or OA progression should be used.

> We appreciate the reviewer's thoughtful critique and have spent some time addressing as many of these comments as possible.
>
> Regarding the disease/age factor, we strove to be clear that this is a point readers should take into consideration. Nonetheless, we still assert that these findings are relevant for OA/age-related declines in musculoskeletal health.
>
> Lines 160 - 162: "*Thus, any changes between 5 mo and 15 mo guinea pigs could be a result of disease progression or a maturation/aging process. We acknowledge this is a limitation."*

Lines 806 - 834: "*A limitation of the study was comparing guinea pigs still in the growth/maturation process to those that have finished growing. While Hartley guinea pigs reach sexual maturity by 4 mo, our data demonstrate they do not cease growing until 9 mo. Nonetheless, even at a relatively young age, these Hartley guinea pigs are an established model of age-related knee osteoarthritis (Santangelo 2014, Jimenez 1997). Thus, some of the comparisons made could be a consequence of comparing guinea pigs that are growing to those that are not. Regardless, the phenotype exhibited by these guinea pigs still reflects age-related OA and musculoskeletal decline in humans. The aim of our study was to determine whether long term supplementation of a phytochemical compound could ameliorate the progression of age-related OA- and musculoskeletal decline documented in this model (Musci and Walsh 2020, Elliehausen et al 2021, Minton et al 2021). Further, our group has also documented that brains of these guinea pigs exhibit hallmarks of human brain aging (Wahl 2022). Future studies should focus on comparisons in these guinea pigs after the growth phase. Such studies would reveal how the musculoskeletal system changes after maturation and while OA continues to progress, as well as provide opportunities to test potential interventions that target both aging and musculoskeletal dysfunction. However, a significant barrier to asking such questions is that OA is often so severe in Hartley guinea pigs at 18 mo, that veterinarians recommend euthanasia. However, future studies could investigate interventions between from 10 mo to 18 mo, after the growth phase has completed."*

We have conducted Western blots of both Nrf2 and HO-1, both of which are downstream targets of Nrf2 activation. To our surprise, a strong age x treatment interaction was present where PB125 increased Nrf2 content in 5 mo guinea pigs, but decreased Nrf2 content in 15 mo guinea pigs. It is difficult to state with certainty why this occurred, however, we have attempted to do so in the discussion:

Line 629 – 646: *"Though PB125 does stimulate Nrf2 activation (as measured by a promoter/reporter assay) in vitro (Hybertson et al., 2019), the changes we observed in Nrf2 content with PB125 treatment were not consistent between young and older guinea pigs. Instead, PB125 had a significant interaction with age, increasing Nrf2 content in 5 mo guinea pigs, and decreasing Nrf2 protein expression in 15 mo guinea pigs. HO-1 content showed a similar pattern. Though we have no acute in vivo data to support this, we hypothesize that PB125 activated Nrf2, as demonstrated by greater Nrf2 content in 5 mo guinea pigs. 10 months of treatment with PB125 led to consistent Nrf2 activation, leading to greater downstream antioxidant enzymes. With age, we hypothesize that greater basal levels of ROS led to a greater Nrf2 content in 15 mo CON guinea pig. We further posit that PB125 ameliorated the increase in age-related ROS, which led to significantly lower levels of Nrf2 and HO-1. This pattern is similar to aerobic exercise, which upregulates antioxidative capacity and protects from age-related increases in oxidative stress* (Muthusamy *et al.*, 2012). In humans, Nrf2 expression increases with age; however, aerobic exercise training reduces levels of Nrf2 (Ostrom & Traustadottir, 2020). *We speculate a similar pattern occurred with PB125 treatment, though further work is required to understand how PB125 affects Nrf2 activation in vivo in both young and old organisms. Similar to many drugs and supplements, it is quite possible that PB125 has widespread effects that are not mediated through a singular pathway."*

As a result, we have changed the title of our manuscript to focus more on the compound, rather than the purported pathway by which the compound exerts biological activity.

There are some major issues with the design and claims surrounding PB125 and related Nrf2 activation, which are as follows:

1) Why was 8.0mg/kg used for supplement dosage? The authors state from line 213-215 - "Based on the analysis conducted at the NSC Analytical Pharmacology Core (Supplemental Figure 1A - C), we selected a dosage of 8 mg/kg of bodyweight, which corresponds to 250 PPM, about 2.5x the dose of PB125 mice in the NIA ITP receive." Both the 24mg/kg and 40mg/kg had larger increases in plasma while 8mg/kg had hardly any increase in plasma from the PK study (Suppl. Fig 1A-C). The decision to use the 8.0mg/kg as the dosage for long term supplementation does not make sense in the context of the data presented for the PK study in this report. Since this dose was used, it is questionable that any sufficient increases in plasma were seen and calls into question whether PB125 could activate Nrf2 or any other signal transduction cascade that converges on Nrf2 activation within skeletal muscle in vivo. In other words, how could it have a direct effect (or an indirect effect) on Nrf2 in skeletal muscle if there isn't any of the 3 compounds in the PB125 supplement showing up in plasma?

We designed the PK studies to confirm the absorption (i.e. bioavailability) of PB125 as orally administered. While we hypothesize that the mechanism of action of PB125 is not in the plasma, plasma concentrations of these compounds are simply used as a surrogate measurement to confirm drug delivery. The goal of these sub-experiments was not to maximize plasma concentrations of the active ingredients of PB125. Rather, these experiments were designed to confirm that there were detectable levels of each of three primary active ingredients of PB125 acutely after oral administration.

As we were not completely confident that 8 mg/kg of PB125 would achieve detectable levels of the three main components, we elected to simultaneously test higher concentrations. However, according to our analytical chemists, 8 mg/kg of PB125 was sufficient to increase plasma concentrations of all three active ingredients in the plasma. We have revised these figures so that the scaling of the graphs does not give the impression that concentrations of the active ingredients were non-detectable.

While we considered choosing a higher dose, we decided to stick to a dosage of 8 mg/kg, which was already 2.5x the dosage of PB125 administered to mice in the NIA ITP study. In addition, we wanted to determine whether or not a dose of PB125 that had elevated to detectable, but modest concentrations of the active components, could have demonstrable effects on cellular function *in vivo*.

Of course, some of the reviewer’s concerns are addressed simply by virtue of not asserting that this is a Nrf2 activator that increases Nrf2 content.

2) Perhaps over the long term, repeated supplementation would gradually increase PB125 content. However, it is impossible to know because PB125 content wasn't measured at the end of the study. Why wasn't the PB125 content measured in plasma and skeletal muscle after long term treatment of PB125? I think it is prudent to perform the PK experiments over the entire treatment period in addition to short term PK study, especially given the chosen dose didn't produce any significant increases in plasma over the short term. Measuring PB125 in plasma before starting supplementation, and after completion of supplementation, and comparing PB125 content within skeletal muscle compared to controls at the end of the study would give reasonable confidence that it

can get into plasma/tissues and activate Nrf2 in vivo. Furthermore, if there were data from other sources on in vivo PK results with long term supplementation, I think that would suffice here. But the citations given for PB125 mechanisms in the intro (Hybertson et al., 2019, McCord et al., 2021) are an in vitro study, and a review, which do not inform readers about how this compound behaves in vivo which is absolutely necessary, in my opinion, if the claim is that this compound activates Nrf2 in vivo. The lack of increases in plasma at 8mg/kg, and without any data on plasma or skeletal muscle PB125 content measured after the long-term supplementation, it is not clear that PB125 activates Nrf2 in vivo (directly or indirectly). Furthermore, it is possible that Nrf2 gradually increases in response to the stress induced by OA progression (higher basal activity) rather than any increase induced by the PB125 supplement. Increases in basal Nrf2 have been shown in other models of aging (PMID: 28863281) and in aging humans PMID: 32866619. Furthermore, the decrease in Nrf2 basally allows for increased inducibility in response to exercise. This has also been shown in mouse models of exercise training (PMID: 27471236).

We certainly appreciate the reviewer's comments regarding PB125 and Nrf2 activation. We chose not to measure the active components of PB125 in either the plasma or muscle at the end of the study because we did not anticipate that there would be an accumulation of the active ingredients in either the plasma or the muscle tissue based on our acute dosing study. Rather, we hypothesize that the acute presence of the active ingredients of PB125 stimulate a cascade of cellular events that lead to the phenotypic outcomes we have observed.

We agree with the reviewer that future studies should consider how the compound is metabolized. However, we believe that is beyond the scope of this study, which was primarily focused on the effects of prolonged treatment with this compound.

We did take into consideration the reviewer's notion that increases in basal Nrf2 have been reported with age. Indeed, repeating our Western blot experiments, with a much larger sample number and more targets, demonstrated this pattern: there was an age-related increase in Nrf2, which could reflect an increase in "basal stress" as the reviewer suggested. Interestingly, PB125 increased Nrf2 content in 5 mo guinea pigs, but decreased Nrf2 content in 15 mo guinea pigs. We highlight this in our manuscript, particularly in the discussion (Lines 636 - 642). We thank the reviewer for the insightful comment.

We emphatically agree that more *in vivo* investigation on the pharmacokinetics and bioavailability of PB125 is necessary, in addition to the acute transcriptional and translational response to PB125. We hope to pursue these questions in future studies.

3) The authors state that QPCR/ other Nrf2 endpoints were not done because the tissues were collected 24 hours after the last treatment was given, preventing the assessment of acute effects. The rationale here is flawed. If Nrf2 is elevated, and this increase is physiologically relevant, you should also see elevations in the downstream protein content of Nrf2 regulated genes because these proteins will have reached a new elevated steady state (regardless of mRNA content 24 hours after last supplementation given) with repeated transient activations of Nrf2 from supplementation of PB125. Measuring protein content of NQO1 or some other protein that is primarily regulated be Nrf2 would strengthen the evidence for activation of Nrf2 in the long term supplementation.

We certainly appreciate this comment. At the reviewer's suggestion, we measured protein content of HO-1 and remeasured Nrf2 content, both of which are downstream targets of Nrf2 activation. As indicated in other comments, these experiments revealed that, broadly speaking, PB125 supplementation increased Nrf2 and HO-1 content in 5 mo guinea pigs, and decreased Nrf2 and HO-1 content in 15 mo guinea pigs.

Taken together, the above concerns surrounding rationale for 8.0mg/kg PB125 supplementation dosage, lack of PB125 content measurements in plasma or skeletal muscle after the in vivo treatment regimen at 3 months and 10 months, and lack of downstream Nrf2 outcome measures, call into question the claims that this compound activated Nrf2 in vivo in any sufficient way to produce the effects claimed. The claims generally do not match the results here, and should either be tempered to match the results, or additional measures should be done such as downstream protein expression to increase confidence of Nrf2 activation in remaining skeletal muscle tissue if possible.

As indicated, we have tempered our language around the usage of a "Nrf2 activator" and instead focused directly on the compound, PB125, itself. Regardless of whether or not PB125 activated Nrf2, there were still demonstrable effects of PB125 on mitochondrial function and protein synthesis.

With regards to the functional mobility assay there are two concerns:

1) Given that there are other behavioral factors that play into the mobility assay (i.e. animals learned the environment through subsequent exposures and thus reduced exploratory behavior once the environment was learned) the assay has some fundamental limitations. The authors should address this in a limitations section.

Thank you for the comment and we agree. Although the previous draft of the manuscript did not incorporate neurocognitive behavior, our group has certainly considered this a possibility.

Indeed, we have assessed changes in the brain of these guinea pigs and have demonstrated that Hartley guinea pigs exhibit transcriptomic changes similar to human Alzheimer's disease between the ages of 5 and 15 months (Wahl et al 2022). This has been reflected in our discussion (Lines 788 - 790). We are currently planning experiments to assess cognitive/memory function with behavioral evaluations.

2) Line 44-46 "These effects were not associated with statistically significant prolonged maintenance of voluntary mobility in guinea pigs but may reflect clinically meaningful improvements in mobility". Since this was not statistically significant, the conclusions drawn here do not match the data, rather it is highly speculative. Additionally, the term "clinically relevant" would be more appropriate if this study was done in a clinical human trial. Even if this were the case, given the fact that it was not statistically significant, the speculation here would not be appropriate in my opinion. This sentence should be removed entirely.

We have removed this statement. We made this statement from a translational perspective, particularly with veterinarians on the research team. Measurements such as gait are critical aspects in determining quality of life, both in humans and in animals, and are similarly used when monitoring the progression of disease, such as knee osteoarthritis. Nonetheless, at the Referee's request, we have removed this statement.

Is the decline shown with mitochondrial respiration truly an effect of disease? The authors suggest that these effects might just be a decline in mitochondrial function because the animals are exiting the growth phase (~9mo). Additionally, the authors claim: Line 616-618 "However, these data clearly demonstrate that impaired mitochondrial respiration is a characteristic of this pre-clinical model of musculoskeletal decline." This does not seem clear in my opinion given the authors previous statement regarding animal development at 9mo.

Since the initial review of this manuscript, another study team has published mitochondrial respiration data in Hartley guinea pigs at 9 mo (Elliehausen et al 2021). Though it is difficult to make comparisons between labs, following a similar SUIT protocol, the other team reported respiration values higher than our 15 mo guinea pigs. In addition, their respiration values were approximately 10-20% greater than the values we reported in 5 mo males. Thus, it is possible the respiration did peak at 9 mo. These data would suggest that the decline in respiration values we observe are not a result of exiting the growth phase. One would hypothesize that mitochondrial respiration would be greater during more rapid growth phases (i.e. 5 mo) vs a time when growth has rapidly slowed down (i.e. 9 mo). Importantly, this is mostly speculation. However, only one study, to our knowledge, has monitored mitochondrial respiration/function over the maturation process (Stolle et al., 2017) and demonstrated a decline in respiration between 6 and 12 mo in mice. Regardless, we have tempered the statement:

Lines 610 – 613: "*However, these data, as well as other literature, support the notion that impaired mitochondrial respiration is a characteristic of this pre-clinical model of musculoskeletal decline.*"

Minor comments/ concerns

Line 736-737 Add the word "in" – Interventions designed to mitigate age-related increases in oxidative stress or inflammation seem to improve…

This has been corrected as suggested.

All western blot data should have accompanying representative images (Nrf2 WB in Suppl. Figure 1E) to confirm that the appropriate band for Nrf2 is being assessed. The 55-60kDa band has been shown not to be Nrf2, but the reader does not know which band was quantified without an image and ID of the band you are measuring in the image.

We have provided these western blot images in the revised manuscript. Please refer to the additional attachment for all raw western blot images. To note, we did quantify the 55-60kDa band for Nrf2 protein content analysis. As you will find in the supplemental data, the unedited blots demonstrate that we did not consistently detect bands between 95kDa – 110kDa. This issue has been raised in other publications and other labs have reported similar experiences. From Islam et al 2020 (PMID: 31707475):

There is debate surrounding the observed molecular weight of Nrf2 and it has been suggested that Nrf2 migrates to ~ 100 kDa rather than its predicted molecular weight of 50–60 kDa in Western blots performed on cell lysates (Lau et al. 2013). We observed a single clear band corresponding to the predicted molecular weight of Nrf2 (~55 kDa) in all Nrf2 blots performed on human skeletal muscle tissue homogenate (see Fig S2 for

unedited images of all Nrf2 blots) using a validated and highly specific monoclonal antibody (D1Z9C) (Kemmerer et al. 2015). Thus, in the absence of a visible band in the ~ 100 kDa range, the ~ 55 kDa band corresponding to the predicted molecular weight of Nrf2 was used for the determination of Nrf2 protein content.

Figure 2 needs a legend to identify which color corresponds to control and treated groups.

This has been corrected as suggested.

Line 479-480 "However, Nrf2a did significantly increase the apparent Km (p=0.007) indicating lower ADP sensitivity, though this is likely a consequence of increased ADP Vmax in the absence of changes in respiration rates in sub-saturating amounts of ADP (Supplemental Figure 5)" This is the definition of lower ADP sensitivity. The Vmax is controlled for in the calculation of apparent Km so regardless of the effect on Vmax it appears that the treatment actually lowers ADP Km.

Revised, lines 461 - 463: "*There was a non-significant interaction between sex and PB125 treatment (p=0.092), indicating that the PB125-mediated increase in Km (i.e. decrease in sensitivity) may have occurred only in males*."

Dear Dr Musci,

Re: JP-RP-2022-282273R1-A "Phytochemical compound PB125 attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline" by Robert Vincent Musci, Kendra M Andrie, Maureen A. Walsh, Zackary John Valenti, Melissa A. Linden, Maryam F. Afzali, Sydney Bork, Margaret Campbell, Taylor Johnson, Thomas E. Kail, Richard Martinez, Tessa Nguyen, Joseph Sanford, Sara Wist, Meredith D Murrell, Joe McCord, Brooks Hybertson, Qian Zhang, Martin A Javors, Kelly Santangelo, and Karyn L Hamilton

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Please advise your co-authors of this decision as soon as possible.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

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I hope you will find the comments helpful and have no difficulty returning your revisions within 4 weeks.

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Yours sincerely,

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REQUIRED ITEMS:

-You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

-You must upload original, uncropped western blot/gel images (including controls) if they are not included in the manuscript. This is to confirm that no inappropriate, unethical or misleading image manipulation has occurred https://physoc.onlinelibrary.wiley.com/hub/journal-policies#imagmanip These should be uploaded as 'Supporting information for review process only'. Please label/highlight the original gels so that we can clearly see which sections/lanes have been used in the manuscript figures.

-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If n â‰¤ 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

-'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

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EDITOR COMMENTS

Reviewing Editor:

This paper is targeted to a specialized research issue of the Journal of Physiology and, as the referee noted, with appropriate revision it would make a valuable contribution to this special issue.

Senior Editor:

The single referee of this manuscript felt that this is a study that is suited for a specialized journal. It is felt that because this study is targeted to be included in a specialized issue of the Journal of Physiology, a revision will be acceptable.

REFEREE COMMENTS

Referee #2:

The authors have generally responded to the main concerns of the first review round. This study set out to test the effects of PB125 on mitochondrial respiration, muscle proteostasis, and mobility in a model of OA progression. I commend the authors for their efforts and appreciate them incorporating the feedback provided. Specifically, it appears the concerns about the PK study and PB125 dose were simply because some analytes were extremely high relative to others on the Y axis leading to the appearance of no uptake in the plasma at the 8mg/kg dose. Separating the graphs and adding secondary Y axes are very helpful in that regard. Additionally, the Nrf2/ HO1 data are helpful to provide some mechanistic insights, albeit limited. The main limitation of the study is that the results presented are incremental with modest effect sizes of the treatment, and minimal effects of the treatment in direct comparisons between the aged control and aged treated. Due to the inconsistent and small effects of the treatment, the primary impact will be the characterization of skeletal muscle mitochondria and proteostasis in a new model of OA.

Below are some minor revisions that will improve the quality of this manuscript:

Lines 764-766 - Delete the second "increase in" in the sentence: "There is an increase in some increase in systemic inflammatory mediators in these Hartley guinea pigs, which may be associated with OA (Huebner et al., 2007; Santangelo et al., 2011)."

Lines 1464-1466 - This is an incomplete sentence: "(E). Fatty acid supported respiration with 1.0 mM was higher in PB125 treated guinea pigs (Cohen's d=0.519, p=0.035 effect of Treatment), but the effect of Sex or Disease/Age (n=90)."

_____

END OF COMMENTS

1st Confidential Review **10-May-2022**

Comments to the Author

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We appreciate the editors' comments and assessments of the manuscript. We are excited for its inclusion in the special issue.

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Referee #2:

The authors have generally responded to the main concerns of the first review round. This study set out to test the effects of PB125 on mitochondrial respiration, muscle proteostasis, and mobility in a model of OA progression. I commend the authors for their efforts and appreciate them incorporating the feedback provided. Specifically, it appears the concerns about the PK study and PB125 dose were simply because some analytes were extremely high relative to others on the Y axis leading to the appearance of no uptake in the plasma at the 8mg/kg dose. Separating the graphs and adding secondary Y axes are very helpful in that regard. Additionally, the Nrf2/ HO1 data are helpful to provide some mechanistic insights, albeit limited. The main limitation of the study is that the results presented are incremental with modest effect sizes of the treatment, and minimal effects of the treatment in direct comparisons between the aged control and aged treated. Due to the inconsistent and small effects of the treatment, the primary impact will be the characterization of skeletal muscle mitochondria and proteostasis in a new model of OA.

We thank the reviewer for their re-evaluation of the manuscript. We agree that the additional Nrf2/HO1 data provide limited insight but nonetheless believe its inclusion in the manuscript is

beneficial. We appreciate the reviewer's assessment of the overall impact of the intervention and study.

Below are some minor revisions that will improve the quality of this manuscript:

Lines 764-766 - Delete the second "increase in" in the sentence: "There is an increase in some increase in systemic inflammatory mediators in these Hartley guinea pigs, which may be associated with OA (Huebner et al., 2007; Santangelo et al., 2011)."

Thank you, the change has been made as advised.

Lines 1464-1466 - This is an incomplete sentence: "(E). Fatty acid supported respiration with 1.0 mM was higher in PB125 treated guinea pigs (Cohen's d=0.519, p=0.035 effect of Treatment), but the effect of Sex or Disease/Age (n=90)."

Thank you, we have revised the sentence:

"Fatty acid supported respiration with 1.0 mM ADP was higher in PB125 treated guinea pigs (Cohen's d=0.519, p=0.035 effect of Treatment), but the effect of Sex or Disease/Age was not significant (p=0.121, 0.880 respectively) (n=90) (F)."

Dear Dr Musci,

Re: JP-RP-2022-282273R2 "Phytochemical compound PB125 attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline" by Robert Vincent Musci, Kendra M Andrie, Maureen A. Walsh, Zackary John Valenti, Melissa A. Linden, Maryam F. Afzali, Sydney Bork, Margaret Campbell, Taylor Johnson, Thomas E. Kail, Richard Martinez, Tessa Nguyen, Joseph Sanford, Sara Wist, Meredith D Murrell, Joe McCord, Brooks Hybertson, Qian Zhang, Martin A Javors, Kelly S. Santangelo, and Karyn L Hamilton

Thank you for providing the additional gel information.

Congratulations on your revision! I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

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Yours sincerely,

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