The Journal of Physiology

AMP-activated protein kinase activation in skeletal muscle modulates exercise-induced uncoupled protein 1 expression in brown adipocyte in mouse model

Je Kyung Seong, Hye Jin Kim, and Youn Ju Kim
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Brendan Gabriel (Referee #2)

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Senior Editor: Michael Hogan

Reviewing Editor: Bettina Mittendorfer

Transaction Report:

(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 Professor Seong,

Re: JP-RP-2021-282660 "AMP-activated protein kinase activation in skeletal muscle modulates exercise-induced uncoupled protein 1 expression in brown adipocyte" by Je Kyung Seong, Hye Jin Kim, and Youn Ju Kim

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.

Please let your co-authors know of the following editorial decision as quickly as possible.

As you will see, in its current form, the manuscript is not acceptable for publication in The Journal of Physiology. In comments to me, the Reviewing Editor expressed interest in the potential of this study, but much work still needs to be done (and this may include new experiments) in order to satisfactorily address the concerns raised in the reports.

In view of this interest, I would like to offer you the opportunity to carry out all of the changes requested in full, and to resubmit a new manuscript using the "Submit Special Case Resubmission for JP-RP-2021-282660..." on your homepage.

We cannot, of course, guarantee ultimate acceptance at this stage as the revisions required are substantial. However, we encourage you to consider the requested changes and resubmit your work to us if you are able to complete or address all changes.

A new manuscript would be renumbered and redated, but the original referees would be consulted wherever possible. An additional referee's opinion could be sought, if the Reviewing Editor felt it necessary. A full response to each of the reports should be uploaded with a new version.

I hope that the points raised in the reports will be helpful to you.

Yours sincerely,

Michael C. Hogan
Senior Editor
The Journal of Physiology
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EDITOR COMMENTS

Reviewing Editor:

The reviewers found considerable merit in the paper. However, they also noted some major concerns regarding the experimental design. These concerns can be addressed but require additional experiments.

REFEREE COMMENTS

Referee #1:

Kim et al. examined potential mechanisms mediating the effects of exercise training on UCP1 protein content and expression in white and brown adipocytes. They first demonstrated that 4 weeks of voluntary wheel running led to the "browning" of white adipose tissue and increases in UCP1 in brown adipose tissue with these changes in adipose tissue being mirrored by increases in the phosphorylation of AMPK in skeletal muscle. The authors were able to recapitulate the effects of exercise on UCP1 by treating cultured brown adipocytes with serum from trained mice or from conditioned media from C2C12 muscle cells treated with AICAR. While an interesting area of inquiry there are several major issues that dramatically dampen the enthusiasm for this manuscript.

1. The in vivo exercise experiments were performed at room temperature, not thermal neutrality, which is a more clinically translatable condition. Several groups have recently reported that the browning of white adipose is essentially non-existent when mice are housed at thermal neutrality (PMIDs: 31297830, 32265830, 32214091) and thus the current study design is severely lacking in translatability.

2. The author's report that p-AMPK is increased ~ 2-fold in mice given access to voluntary running wheels. It's not clear when and how mice were killed and thus its not clear if the increase in skeletal muscle p-AMPK is a residual effect of the last bout of exercise or if it's truly a training effect? If the latter, it is difficult to conceptualize how training would increase the activity of AMPK as this would suggest an underlying degree of energetic stress.

3. The authors use AICAR as a pharmacological tool to activate AMPK in skeletal muscle. AICAR is an indirect activator of AMPK and has off target effects. Given this, it is a stretch to conclude that the activation of AMPK in C2C12s with AICAR is mediating the effects of the conditioned media on UCP1 in cultured brown adipocytes. In order to conclude this the authors would need to complete experiments using more specific AMPK activators and preferably compliment this with studies where AMPK was knocked down in C2C12 muscle cells.

4. The data in the current manuscript demonstrating a mechanism of how conditioned media from C2C12 or serum from exercise animals induces UCP1 is sparse. Further information teasing this out is required. One approach would be to use neutralizing antibodies against IL-6.

5. The sample size in many of the experiments is very low with N's of 3-5 being reported. There is a concern that the experiments in this manuscript are underpowered.

6. The statistical analysis in some of the figure would appear to be incorrect. When more than two groups are compared an ANOVA not a T-test needs to be used. See figures 5B, 5C and 6A

Referee #2:

The authors of this manuscript sought to assess whether exercise training affects the expression of uncoupled protein 1 (UCP1) in brown adipocytes via release of different blood factors. This is an important question which may be impactful in the field. The authors have used conditioned media in an important experiment in their study. I appreciate their clear

presentation of data throughout the study and also from the cell survival assay in figure 4 B. However, I would like the authors to clarify the statistical tests performed on this data. The authors also somewhat overclaim from their data at a few points which I have highlighted below in my comments. The authors should also be aware of the relevance (or lack thereof) of a mouse model where exercise causes robust weight loss, since this is not often seen in human studies. Additionally, the relevance of thermogenesis to whole-body metabolism is well known to be very different between mice and humans, and the relevance of this to human obesity should be discussed.

Comments:

• Figure 4: A key experiment in the study is to show that the conditioned media did not affect cell survival (Figure 4B). There appears to be a trend for reduced survival in the 5% conditions (P=0.094). Could the authors clarify whether t-tests performed when assessing this assay were corrected for multiple testing.

• Due to the trend for reduced survival mentioned in Figure 4B, could the authors present the CT values for their housekeeping gene used in Figure 4E (36B4) (in supplemental data if applicable).

• In Figure 4 - Could the authors clarify why immunoblots were performed on cells treated with 5% serum and not 1%?

• Title: The title should mention that this study was conducted in mice/mouse cells.

• Key points: "Exercise promotes thermogenesis by activating uncoupling protein 1 (UCP1), which leads to a decrease in the body weight and body fat content." I don't think the data in the manuscript supports the assertion that thermogenesis was directly promoted by UCP1, or that this led to a decrease in body weight and body fat since no mechanistic studies were conducted. This statement should be tempered.

• Abstract: "Aerobic exercise is not only the most effective intervention in preventing obesity" There is little evidence to support this statement, please temper accordingly.

• Abstract: The abstract should include details of the methods and results from the mouse study.

• "Similarly, our observations suggest that activation of AMPK in skeletal muscle induces activation of brown adipocytes by promoting IL-6 release and inhibiting MCP-1 release." I don't think the author's evidence that the method of UCP-1 induction is due to IL-6 release or an inhibition of MCP-1 is very strong. It could be due to any number of factors in the CM that the author's have not tested. This sentence should be tempered.

• Could the authors discuss the relevance of their findings to human obesity as discussed above.

ADDITIONAL FORMATTING REQUIREMENTS:
Please could authors add an 'Acknowledgements'?

END OF COMMENTS

Confidential Review **30-Nov-2021**

Dear Editors,

Thank you for your consideration and the critical comments on our manuscript. Accordingly, we have carefully revised our manuscript. Furthermore, we have responded to all of the reviewers' comments in a point-by-point manner. The comments (bold font) and our responses (normal font) are given below. In particular, as requested, the revised manuscript includes the results of additional experiments with corresponding interpretation. Furthermore, we have discussed the relevance of our findings to human obesity.

According to the reviewers' suggestion, the title of the manuscript has been revised to " AMP-activated protein kinase activation in skeletal muscle modulates exercise-induced uncoupled protein 1 expression in brown adipocyte in mouse model" (Original title: AMP-activated protein kinase activation in skeletal muscle modulates exercise-induced uncoupled protein 1 expression in brown adipocyte). We believe that the revisions have strengthened the manuscript and we hope that the revised manuscript is now suitable for publication in *Journal of Physiology.*

Thank you very much for your kind consideration of this resubmitted version of our manuscript.

Sincerely,
Je Kyung Seong.

REFEREE COMMENTS

Referee #1:

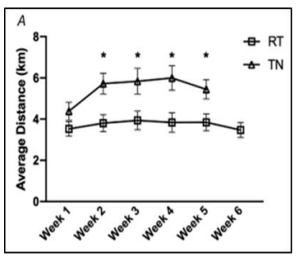
Kim et al. examined potential mechanisms mediating the effects of exercise training on UCP1 protein content and expression in white and brown adipocytes. They first demonstrated that 4 weeks of voluntary wheel running led to the "browning" of white adipose tissue and increases in UCP1 in brown adipose tissue with these changes in adipose tissue being mirrored by increases in the phosphorylation of AMPK in skeletal muscle. The authors were able to recapitulate the effects of exercise on UCP1 by treating cultured brown adipocytes with serum from trained mice or from conditioned media from C2C12 muscle cells treated with AICAR. While an interesting area of inquiry there are several major issues that dramatically dampen the enthusiasm for this manuscript.

Question 1. The in vivo exercise experiments were performed at room temperature, not thermal neutrality, which is a more clinically translatable condition. Several groups have recently reported that the browning of white adipose is essentially non-existent when mice are housed at thermal neutrality (PMIDs: 31297830, 32265830, 32214091) and thus the current study design is severely lacking in translatability.

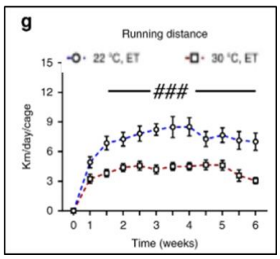
-Answer 1: Upon performing a literature search on recent research in mice, we have learnt that a thermoneutral (TN) environment yields results different from that of room temperature (RT). We agree that the TN environment is more amenable for extrapolation to human applications. As suggested by the referee, we have reviewed several papers. However, we found certain inconsistencies in previous studies, which made us question the exercise in TN.

1) Weekly running distance: Two papers showed reported contrasting running distances at RT and TN. Therefore, no conclusions could be made about the association of exercise capacity with temperature.

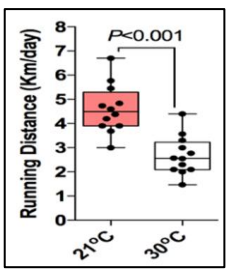
PMID:31297830



PMID: 32214091



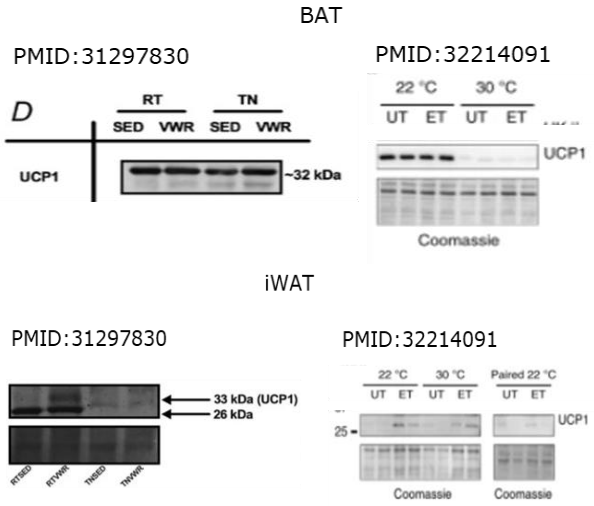
PMID:31032475



In recent reports from Goodyear's group (PMID:31032475), mice housed at thermoneutrality (30 °C) ran approximately 42% lesser than those at RT (21 °C). Their study aimed at comparing mice housed at RT with mice housed in a TN (30 °C) was not performed for this reason. Therefore, the mice were housed at RT. Although we have not shown data on this, we observed that the voluntary running distance of mice significantly decreases when the temperature rises due to air conditioning issue in the breeding room. We presume the TN state to be an environment that

discourages mice from voluntary running. Therefore, we are uncertain whether the effect of exercise could be explained using mice that exercised less. Overcoming this problem experimentally needs to be addressed in the future.

2) UCP1 expression in iWAT and BAT: In PMID:31297830, the researcher observed the effects of TN in decreasing UCP1 contents in BAT (p=0.0066) and iWAT (only detected RTVWR*)*, respectively. However, in PMID:32214091, UCP1 expression was completely abolished by TN in BAT, but not in iWAT. Exercise increased UCP1 protein expression in iWAT, despite the TN environment. These findings imply that research results on exercise-induced UCP1 levels according to temperature are still ambiguous.



In PMID:31297830, housing at thermoneutrality dampened the exercise-induced increases in mitochondrial and thermogenic markers in inguinal white adipose tissue. In PMID:32214091, mice housed at 22 °C displayed increased exercise-induced and diurnal metabolic fluctuations as well as augmented running volume compared with mice at 30 °C. Taken together, these results clearly show that exercise-induced browning effect is better in RT than in TN. Rather, there is a limitation in that the effect of exercise should be explained with a small amount of exercise in the thermoneutral state (as mentioned in the study results of Goodyear's group). Therefore, this aspect remains to be explored by future studies.

3) Roh et al., (2018) reported that during the course of warming (30°C) both BAT and iWAT displayed an increasingly white-like morphology, with the appearance of unilocular adipocytes and reduced UCP1 expression. This process seemed to stabilize between 4 and 8 weeks after exposure to thermoneutral conditions. Our study model was the fully differentiated brown adipocyte. Therefore, if we performed in TN, we should have studied in cells that lost the properties of BAT.

4) A previous study has reported that browning of white adipose and BAT activation in mice are possible under TN conditions. This study showed that young mice injected with CL-316,243 for 5days showed increased thermogenesis related gene (Ucp1, Pgc1a, Cox7a1) expressions and UCP1 protein level at TN (30°C). Furthermore, it was also showed that when mice were injected with CL-316,243 for 5days under thermal neutrality conditions, BAT and iWAT temperatures were higher

than that of the control group. These results implied that young mice possess beta-AR (adrenergic receptor) activation induced browning capacity of iWAT under TN condition.

Taken together, we agree that the TN environment is a more suitable temperature that is conductive to human applications. However, we believe that our study has no reliability concerns because it involves explaining the effect of exercise at the same temperature (RT). We believe that our study at RT was well-controlled with respect to explaining the effects of exercise in the same environment. In addition, we do not think that it is impossible to translate our experimental condition in human research, because the thermoneutrality zone of human beings in each region of the world is different and the temperature at which exercise is performed varies by season. We have mentioned this in the discussion section.

Question 2. The author's report that p-AMPK is increased ~ 2-fold in mice given access to voluntary running wheels. It's not clear when and how mice were killed and thus its not clear if the increase in skeletal muscle p-AMPK is a residual effect of the last bout of exercise or if it's truly a training effect? If the latter, it is difficult to conceptualize how training would increase the activity of AMPK as this would suggest an underlying degree of energetic stress.

Answer 2: As suggested by the reviewer, we have included additional text in the manuscript (materials and methods, animal studies) as given below.

"After the 4 weeks of training, all mice were kept sedentary for 4 h before sacrifice to abolish the last bout effect."

Laker et al., (2017) have reported skeletal muscle AMPK phosphorylation immediately after the cessation of treadmill exercise and a return to baseline values by 3h post-exercise in C57BL/6 mice aged 10-12 weeks (PMID: 28916822).

In our study, the mice were kept sedentary for 4 h before sacrifice. Therefore, our study design could explain the phosphorylation of AMPK by 4 weeks of wheel running training.

Question 3. The authors use AICAR as a pharmacological tool to activate AMPK in skeletal muscle. AICAR is an indirect activator of AMPK and has off target effects. Given this, it is a stretch to conclude that the activation of AMPK in C2C12s with AICAR is mediating the effects of the conditioned media on UCP1 in cultured brown adipocytes. In order to conclude this the authors would need to complete experiments using more specific AMPK activators and preferably compliment this with studies where AMPK was knocked down in C2C12 muscle cells.

Answer 3: We agree with the reviewer's suggestion. The UCP1 expression was reduced in both C2C12 CM transfected with AMPKα1 or AMPKα2 siRNAs in differentiated brown adipocytes treated with AICAR as well as untreated (Fig 7D).

These findings suggest that skeletal muscle AMPK plays an important role in UCP1 expression in differentiated brown adipocytes. Because the AMPK knockdown experiment was sufficient to explain our data, additional experiments were not performed (e.g. using other AMPK activators or inhibitors).

Question 4. The data in the current manuscript demonstrating a mechanism of how conditioned media from C2C12 or serum from exercise animals induces UCP1 is sparse. Further information teasing this out is required. One approach would be to use neutralizing antibodies against IL-6.

Answer 4: We did not consider this aspect during the course of the experiment. We greatly appreciate the critical comment of the reviewer. Inferring from several references, we tested whether neutralization of IL-6 in C2C12 CM could abolish the expression of UCP1 in brown adipocytes. However, CM treated brown adipocytes showed no abolishment of UCP1 expression. These results suggested that IL-6 does not primarily mediate the exercise effects on UCP1 expression. (Fig 8G)

Question 5. The sample size in many of the experiments is very low with N's of 3-5 being reported. There is a concern that the experiments in this manuscript are underpowered.

Answer 5: We agree with the reviewer’s comment. Therefore, we increased the sample size as much as possible to 7-9 in the cell study. AICAR-CM-induced UCP1 protein expression shown in Fig. 6C was supplemented with additional experiments shown in Fig 7F and Fig8G.

Question 6. The statistical analysis in some of the figure would appear to be incorrect. When more than two groups are compared an ANOVA not a T-test needs to be used. See figures 5B, 5C and 6A.

Answer 6: We agree with the reviewer’s comment. As shown in Fig. 5B, 5C and 6A, more than two groups were compared using one-way ANOVA and a post hoc test (Tukey’s test and Dunnett’s test).

Referee #2:

The authors of this manuscript sought to assess whether exercise training affects the expression of uncoupled protein 1 (UCP1) in brown adipocytes via release of different blood factors. This is an important question which may be impactful in the field. The authors have used conditioned media in an important experiment in their study. I appreciate their clear presentation of data throughout the study and also from the cell survival assay in figure 4 B. *However, I would like the authors to clarify the statistical tests performed on this data. The authors also somewhat over claim from their data at a few points which I have highlighted below in my comments. The authors should also be aware of the relevance (or lack thereof) of a mouse model where exercise causes robust weight loss, since this is not often seen in human studies. Additionally, the relevance of thermogenesis to whole-body metabolism is well known to be very different between mice and humans, and the relevance of this to human obesity should be discussed.*

Answer. We appreciate the reviewer for the time invested and the consideration of our work. We believe that the revisions made based on the insightful comments have strengthened the manuscript. We hope that the revised manuscript is now suitable for publication in *Journal of Physiology*. We have responded to the reviewers' comments in a point-by-point manner. The comments (bold font) and our responses (normal font) are given below.

Question 1. However, I would like the authors to clarify the statistical tests performed on this data.

Answer 1. Statistical analysis has been carefully re-performed, depending on the data (Figure 5B,5C, 6A, 7 and 8).

Question 2. The authors also somewhat over claim from their data at a few points which I have highlighted below in my comments. The authors should also be aware of the relevance (or lack thereof) of a mouse model where exercise causes robust weight loss, since this is not often seen in human studies. Additionally, the relevance of thermogenesis to whole-body metabolism is well known to be very different between mice and humans, and the relevance of this to human obesity should be discussed.

Answer 2. According to the reviewer's comment, additional experiments were performed and the manuscript and figures were revised. We also acknowledge the need to be more critical in pursuing animal research and in making claims about the applicability of this research to humans. Due to the limitations associated with human research, we took our best efforts to the mouse model, in light of applications to humans.

1)The C57BL/6 mice has been shown to be a good strain for exploring the human

metabolic disorder observed in obesity. In order to present a more reasonable response, we measured the UCP1 level after treating brown adipocytes with serum in each mouse strain (129, C3H/HeN, Balb/c, B6) bred at warm conditions (stored at our laboratory after 7 days of breeding in a 30℃ environment) (supplemental data 1). The results showed that C57BL/6 mice were more sensitive to obesity. Therefore, we considered the C57BL/6 mice to be a good model for exploring the human metabolic disorder observed in obesity.

PMID: 20713682

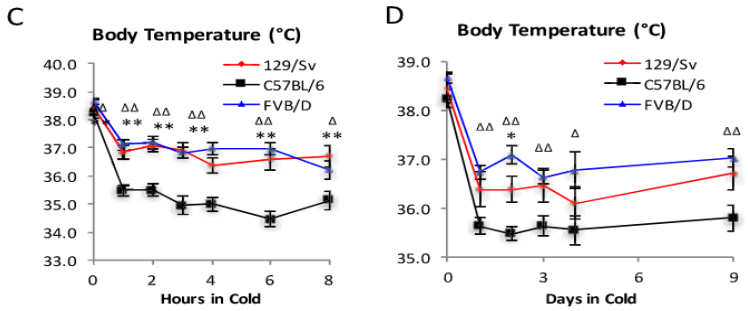
TABLE 1
Metabolic parameters in B6 vs. 129 mice at different ages and dietary conditions

	Chow diet (21% fat)				Low-fat diet (14% fat)		High-fat diet (55% fat)	
	6 weeks old		6 months old					
	B6	129	B6	129	B6	129	B6	129
Body weight (g)	21.9 ± 0.6	23.5 ± 0.5	45.5 ± 1.0	30.4 ± 1.2	41.5 ± 1.4	30.9 ± 1.2	46.4 ± 1.1	39.2 ± 1.5
Epididymal fat mass (g)	0.09 ± 0.01	0.21 ± 0.02	1.67 ± 0.14	2.14 ± 0.30	1.62 ± 0.17	0.58 ± 0.11	3.39 ± 0.33	3.92 ± 0.14
Blood glucose–random fed (mg/dl)	155.5 ± 3.1	129.3 ± 2.9	168.2 ± 12.3	106.6 ± 3.5	148.5 ± 7.4	102.5 ± 3.4	153.3 ± 6.7	105.5 ± 3.7
Insulin (ng/ml)	2.26 ± 0.48	3.12 ± 0.58	92.14 ± 25.71	3.96 ± 2.04	24.78 ± 12.51	1.29 ± 0.32	32.73 ± 12.46	3.58 ± 0.41
Leptin (ng/ml)	7.14 ± 0.70	9.00 ± 0.90	76.18 ± 3.82	16.67 ± 4.5	72.50 ± 8.06	4.97 ± 0.77	82.38 ± 7.41	46.81 ± 6.02

Values are expressed as mean ± SE of 8 animals per group.

This paper has reported that B6 mice had more obese phenotypes such as body weight, epididymal fat mass, blood glucose, insulin and leptin than 129 mice.

PMID: 27689007



Moreover, this paper has reported that B6 mice displayed greater impairment of cold tolerance capacity than that of 129 mice under cold conditions (4°C) for 9 days. This data implies that 129 mice possess a better defense of body temperature loss than that of B6 mice under cold conditions.

Comments:

Comments 1.

Figure 4: A key experiment in the study is to show that the conditioned media did not affect cell survival (Figure 4B). There appears to be a trend

for reduced survival in the 5% conditions (P=0.094). Could the authors clarify whether t-tests performed when assessing this assay were corrected for multiple testing.

Answer 1. We enhanced the MTT assay data by increasing the number of samples treated with serum, as suggested by the reviewer. In addition, a one-way ANOVA was used, as well as a post-hoc test. There was no statistical significance in the results (n=9). (Fig. 4B).

Comments 2.

Due to the trend for reduced survival mentioned in Figure 4B, could the authors present the CT values for their housekeeping gene used in Figure 4E (36B4) (in supplemental data if applicable).

Answer 2. Yes, we have presented the CT values for 36B4 and UCP1 gene in the supplemental data.

Comments 3.

In Figure 4 - Could the authors clarify why immunoblots were performed on cells treated with 5% serum and not 1%?

Answer 3. We have presented the blot performing using 1% serum according to the reviewer's comment (Fig. 4D).

Comments 4.

Title: The title should mention that this study was conducted in mice/mouse cells.

Answer 4. As suggested by the reviewer, we have inserted additional text in the title as given below.

"AMP-activated protein kinase activation in skeletal muscle modulates exercise-induced uncoupled protein 1 expression in brown adipocyte in mouse model"

Comments 5.

Key points: "Exercise promotes thermogenesis by activating uncoupling protein 1 (UCP1), which leads to a decrease in the body weight and body fat content." I don't think the data in the manuscript supports the assertion that thermogenesis was directly promoted by UCP1, or that this led to a decrease in body weight and body fat since no mechanistic studies were conducted. This statement should be tempered.

Answer 5. We appreciate your critical concern. In our study, exercise significantly increased UCP1 protein and gene expression in both iWAT and BAT (Fig.2A-D). Furthermore, exercise-trained mice significantly blunt the body weight gain and fat mass gain (Fig. 1D-E). These two results were clearly presented in the manuscript.

However, our data and explanation were insufficient to explain the first key point.

In order to investigate the relevance of our findings on body weight loss and fat loss upon UCP1 activation, we further explored whether the effects of weight loss and fat loss were due to increased oxygen uptake and heat generation (Fig.2E and 2F). We believe that this additional data included in the manuscript supports the assertion that thermogenesis is directly promoted by UCP1.

In addition, the key point revised as below.

"Exercise promotes thermogenesis by activating uncoupling protein 1 (UCP1), which leads to a decrease in the body weight gain and body fat content."

Comments 6.

Abstract: "Aerobic exercise is not only the most effective intervention in preventing obesity" There is little evidence to support this statement, please temper accordingly.

Answer 6. This sentence summarizes the results of previous studies on the beneficial effects of aerobic exercise and might have seemed exaggerated. Therefore, we revised "aerobic exercise is not only the most effective intervention in preventing obesity" to "aerobic exercise is an effective intervention in preventing obesity."

Comments 7.

Abstract: The abstract should include details of the methods and results from the mouse study.

Answer 7. We have included additional text in the abstract, as below.

"Four weeks of exercise training significantly decreased the body weight gain and fat mass gain. Furthermore, trained mice exhibit higher levels of energy expenditure and UCP1 expression compared with untrained mice. Surprisingly, treatment with serum from exercise-trained mice increased the expression of UCP1 in differentiated brown adipocytes."

Comments 8.

"Similarly, our observations suggest that activation of AMPK in skeletal muscle induces activation of brown adipocytes by promoting IL-6 release and inhibiting MCP-1 release." I don't think the author's evidence that the method of UCP-1 induction is due to IL-6 release or an inhibition of MCP-1 is very strong. It could be due to any number of factors in the CM that the author's have not tested. This sentence should be tempered.

Answer 8. 'Referee #1' has also made the same comment. Therefore, we investigated IL-6 neutralization in AICAR-induced CM using neutralizing antibodies against IL-6. As shown in Fig.8E, neutralization of IL-6 significantly reduced the activation and expression of AMPK in AICAR-induced C2C12 myotubes. However,

inhibition of IL-6 did not significantly abolish UCP1 expression. There was only a decreasing trend. These results suggested that IL-6 is not the most potent mediator of the exercise-induced effect on UCP1 expression. According to these results, we have revised the manuscript, as below.

"However, since several factors are associated with AICAR-treated CM, reasoning that the increase in IL-6 or the decrease in MCP1 had a significant effect on the expression of UCP1 would be far-fetched and could indicate a limitation. In addition, the expression of UCP1 protein was not suppressed upon treatment with IL-6 neutralized CM. Presumably, other factors contributed to the expression of UCP1. However, the inability to identify the specific factors involved is a limitation of our study."

Comments 9.

Could the authors discuss the relevance of their findings to human obesity as discussed above.

Answer 9. As suggested by reviewer, we have included additional text about the relevance of our findings to human obesity in the discussion section.

Dear Dr Seong,

Re: JP-RP-2022-282999X "AMP-activated protein kinase activation in skeletal muscle modulates exercise-induced uncoupled protein 1 expression in brown adipocyte in mouse model" by Je Kyung Seong, Hye Jin Kim, and Youn Ju Kim

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

- 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.

- Your manuscript must include a complete Additional Information section

- Please upload separate high-quality figure files via the submission form.

- 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 {less than or equal to} 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.

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In summary:

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EDITOR COMMENTS

Reviewing Editor:

The reviewers felt the manuscript has much improved. However, they were concerned about the translational value of the work.

REFEREE COMMENTS

Referee #1:

While this reviewer appreciates the additional experiments that the authors have completed, the fact remains that they are examining a condition, i.e. browning, that doesn't occur in humans. The fact that it is seen in rodents is most likely a function of an interaction between exercise and sub-thermal neutral housing temperatures. Given this point, there is no translatability of the findings to human physiology.

Referee #2:

The authors have addressed all my comments sufficiently, I congratulate them on this robust study and well-written manuscript.

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