The Journal of Physiology

Insulin potentiates the response to capsaicin in dorsal root ganglion neurons in vitro and muscle afferents ex vivo in normal healthy rodents

Amane Hori, Norio Hotta, Ayumi Fukazawa, Juan A Estrada, Kimiaki Katanosaka, Kazue Mizumura, Jun Sato, Rie Ishizawa, Han Kyul Kim, Gary A Iwamoto, Wanpen Vongpatanasin, Jere H Mitchell, Scott A. Smith, and Masaki Mizuno
DOI: 10.1113/JP282740

Corresponding author(s): Masaki Mizuno (masaki.mizuno@utsouthwestern.edu)

The referees have opted to remain anonymous.

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Senior Editor: Harold Schultz

Reviewing Editor: Beth Habecker

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 Dr Mizuno,

Re: JP-RP-2021-281874 "Insulin potentiates the response to capsaicin in dorsal root ganglion neurons in vitro and muscle afferents ex vivo in normal healthy rodents" by Amane Hori, Norio Hotta, Kimiaki Katanosaka, Kazue Mizumura, Jun Sato, Rie Ishizawa, Han Kyul Kim, Gary A Iwamoto, Wanpen Vongpatanasin, Jere H Mitchell, Scott A. Smith, and Masaki Mizuno

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-2021-281874..." 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,

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EDITOR COMMENTS

Reviewing Editor:

The study is solid and the observation is interesting, if somewhat similar to the recent JP publication showing insulin-induced mechanical sensitization of small DRG neurons that might lead to sympathoexcitation. The difference in this study is the focus on insulin-induced chemical sensitization. This is a logical follow-up to the earlier study, but has somewhat less impact since the observation that insulin can sensitize peripheral DRG neurons is no longer novel.

Please address all points raised by reviewers including whether TRPV1 and insulin receptors are co-localized in the cells you examined, why the various statistical tests were selected, and how your dosing compares with physiological insulin levels in these tissues. Please be more careful in the language around sympathoexcitation since sympathetic parameters aren't being measured.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:

Actual p values must be stated

Comments to the Author:

The study was deemed to be minorly incremental to the investigators' previous work and marginally novel. Addition of functional studies to assess insulin sensitization of sympatho-exitatory responses to CAPS stimulation of muscle afferents, or relation of present study to TRPV1 muscle afferent responses in a diabetic model would enhance impact of the study.

REFEREE COMMENTS

Referee #1:

I read with great interest the present manuscript. Hori et al. clearly demonstrated that insulin potentiates TRPV1 responsiveness to capsaicin at the DRG and muscle tissue levels, possibly contributing to insulin-induced sympathoexcitation. Please see my comments below.

Could the authors provide any discussion/information if TRPV1 and insulin receptors are colocalized on nociceptors and/or metabolically sensitive skeletal muscle receptors? The lack of this information may be a major limitation. Please quote.

Another concern is related to the statistical approach used. For example, is there any rationale/calculation for using 40 small DRG neurons? Please, provide.

The authors have used both parametric and non-parametric approaches without providing any criteria for that. Please fix it.

Minor:

Throughout the manuscript (ABSTRACT and lines 350-351, 389) the authors state: "insulin-induced sympathoexcitation". Please be specific about the condition? Is it resting baseline? Is it exercise? IT is tempting to state this, and I understand that. However, there is no sympathetic measures in the present study. Please tone this down.

Referee #2:

The authors investigated whether insulin sensitizes skeletal muscle afferents and capsaisin sensitive TPRV1 receptors in dorasal root ganglion cells which could thereby support that insulin could elicit exaggerated sympathoactivation during exercise. The manuscript is well written and the results are straight forward.

I offer only the following relatively minor comments for the authors to consider.

There is little if any discussion of the physiologically relevance of the levels of insulin utilized either in the bathing media or injected into the muscle. This would improve the relevance of the manuscript. No dose response effects are described. This could also improve the results.

Sjo¨strand, Mikaela,Agneta Holma¨ng, and Peter Lo¨nnroth. Measurement of interstitial insulin in human muscle. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E151-E154, 1999

Specific Comments:

Line 43 "sympathoexcitatory actions but the mechanisms underlying this observation is unknown" should be changed to These observations are unknown.

ADDITIONAL FORMATTING REQUIREMENTS:

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-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.

-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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Confidential Review **04-May-2021**

Manuscript#: JP-RP-2021-281874 R1

Insulin potentiates the response to capsaicin in dorsal root ganglion neurons *in vitro* and muscle afferents *ex vivo* in normal healthy rodents

Reviewing Editor

The study is solid and the observation is interesting, if somewhat similar to the recent JP publication showing insulin-induced mechanical sensitization of small DRG neurons that might lead to sympathoexcitation. The difference in this study is the focus on insulin-induced chemical sensitization. This is a logical follow-up to the earlier study, but has somewhat less impact since the observation that insulin can sensitize peripheral DRG neurons is no longer novel.

Author response: First, we would like to thank the reviewing editor, senior editor, and reviewers for their thoughtful comments and suggestions. We believe that the revised manuscript is much improved following the incorporation of the suggested revisions. We have addressed all comments fully in the responses to reviewers. All changes made to the manuscript are indicated in red font in the red-lined version of the manuscript. In addition, all references to specific pages, paragraphs and lines in the responses to reviewers refer to the red-lined version submitted.

1) Please address all points raised by reviewers including whether **(i)** TRPV1 and insulin receptors are co-localized in the cells you examined, **(ii)** why the various statistical tests were selected, and **(iii)** how your dosing compares with physiological insulin levels in these tissues. **(iv)** Please be more careful in the language around sympathoexcitation since sympathetic parameters aren't being measured.

Author response:

(i) As pointed out by reviewer#1, we have performed an additional experiment where we examined whether the insulin receptor co-localizes with TRPV1 in small DRG neurons labelled with or without isolectin B4 using immunohistochemistry (details are in Lines 241-263). Consistent with previous studies (PMID: 17492627; 29955950), we have demonstrated co-localization between TRPV1 and insulin receptors in 28.7% of small DRG neurons. (Details are in Lines 408-413).

(ii) We confirmed data normality by using the Shapiro-Wilk test and then used parametric or non-parametric analysis according to the normality of the data. Therefore, we have modified some statistical tests and revised the "Statistical analysis" section of the revised manuscript in

Lines 326-337.

(iii) We have added commentary about the physiological relevance of the levels of insulin chosen for the study using the reference (PMID: 9886961) in the discussion section of the revised manuscript (Lines 500-505).

(iv) To address this issue, we have performed additional *in vivo* experiments using decerebrated rats. We measured renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to injection of capsaicin into the arterial supply of the hindlimb in decerebrate rats before and after intramuscularly injecting saline solution (vehicle) or insulin (5U/mL) (details are in Lines 265-323). We have observed that the RSNA response to capsaicin after injecting insulin was significantly greater than after injection of saline. Further, the MAP response to capsaicin administration was significantly augmented by intramuscular injection of insulin (Details are in Lines 415-444).

Senior Editor

1) Comments for Authors to ensure the paper complies with the Statistics Policy: Actual p values must be stated

Author response: Following the statistics policy of the *Journal of Physiology*, we have reported all p values in the manuscript (lines 347-348, 358-359, 382, 388, and 397), as well as in Figures 2 and 5. In addition, in some sections, variability was incorrectly characterized by reporting standard error. This has been corrected such that only standard deviations are now reported (lines 358-359, and 382).

Comments to the Author

2) The study was deemed to be minorly incremental to the investigators' previous work and marginally novel. Addition of functional studies to assess insulin sensitization of sympatho-exitatory responses to CAPS stimulation of muscle afferents, or relation of present study to TRPV1 muscle afferent responses in a diabetic model would enhance impact of the study.

Author response: As suggested, we have performed additional *in vivo* experiments using decerebrated rats. We measured renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to injection of capsaicin into the arterial supply of the hindlimb in decerebrate rats before and after intramuscularly injecting saline solution (vehicle) or insulin (5U/mL) (details are in Lines 265-323). We observed that the RSNA response to capsaicin after injecting insulin was significantly greater than after injection of saline. Further, the MAP response to capsaicin administration was significantly augmented by intramuscular injection of insulin (Details are in Lines 415-444).

Referee #1

I read with great interest the present manuscript. Hori et al. clearly demonstrated that insulin potentiates TRPV1 responsiveness to capsaicin at the DRG and muscle tissue levels, possibly contributing to insulin-induced sympathoexcitation. Please see my comments below.

1) Could the authors provide any discussion/information if TRPV1 and insulin receptors are colocalized on nociceptors and/or metabolically sensitive skeletal muscle receptors? The lack of this information may be a major limitation. Please quote.

Author response: A suggested, we have performed additional immunohistochemical experiments examining whether the insulin receptor co-localizes with TRPV1 in small DRG neurons labelled with or without isolectin B4 (details are in Lines 241-263). Consistent with previous studies (PMID: 17492627; 29955950), we have demonstrated co-localization between TRPV1 and the insulin receptor in 28.7% of small DRG neurons. (Details are in Lines 408-413).

2) Another concern is related to the statistical approach used. For example, is there any rationale/calculation for using 40 small DRG neurons? Please, provide.

Author response: In the present study, we adhered to the principles of the 3Rs (replacement, reduction and refinement) in the *Journal of Physiology* and *Experimental Physiology* (PMID: 26095019). Based on these principles, to minimize the use of animals, we terminated experiments when statistically significant differences were obtained.

3) The authors have used both parametric and non-parametric approaches without providing any criteria for that. Please fix it.

Author response: We firstly confirmed data normality by using the Shapiro-Wilk test and then performed parametric or non-parametric analysis as appropriate based on the presence or absence of a normal data distribution. Therefore, we have modified some statistical tests and revised the "Statistical analysis" section of the manuscript to enhance clarity in Lines 326-337. Thank you very much again for pointing out this important point.

4) Minor:

Throughout the manuscript (ABSTRACT and lines 350-351, 389) the authors state: "insulin-induced sympathoexcitation". Please be specific about the condition? Is it resting baseline? Is it exercise? IT is tempting to state this, and I understand that. However, there is no sympathetic measures in the present study. Please tone this down.

Author response: We thank the reviewer for this insightful comment. Sympathoexcitation is expected during physical exercise. Thus, we have revised the last sentence of the abstract (Line 67) as well as the last sentence of the conclusion (Lines 554-544). As suggested by the other reviewers, to determine whether insulin augments sympathoexcitation, we have performed additional *in vivo* experiments using decerebrated rats. We measured renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to injection of capsaicin into the arterial supply of the hindlimb in decerebrate rats before and after intramuscularly injecting saline solution (vehicle) or insulin (5U/mL) (details are in Lines 265-323). We observed that the RSNA response to capsaicin after injecting insulin was significantly greater than after injection of saline. Further, the MAP response to capsaicin administration was significantly augmented by intramuscular injection of insulin (Details are in Lines 415-444).

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1) There is little if any discussion of the physiologically relevance of the levels of insulin utilized either in the bathing media or injected into the muscle. This would improve the relevance of the manuscript. No dose response effects are described. This could also improve the results.
Sjo¨strand, Mikaela,Agneta Holma¨ng, and Peter Lo¨nnroth. Measurement of interstitial insulin in human muscle. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E151-E154, 1999

Author response: Thank you for bringing this to our attention. We have added commentary about the physiological relevance of the levels of insulin utilized in the investigation using the reference (PMID: 9886961) in the revised discussion section of the manuscript (Lines 500-505). We acknowledge that concentration dependency should be studied. We did not examine insulin concentration dependency in the present study as our intent was to first clearly establish that insulin maintains the potential to augment the response to chemical stimuli in small DRG neurons and thin fiber muscle afferents, possibly resulting in sympathoexcitation. That being said, lack of concentration dependency is an acknowledged study limitation that we have described in the discussion section of the revised manuscript (Lines 500-505).

2) Specific Comments:
Line 43 "sympathoexcitatory actions but the mechanisms underlying this observation is unknown" should be changed to These observations are unknown.
Author response: We agree with your comment and have revised the manuscript accordingly (Line 47).

Dear Dr Mizuno,

Re: JP-RP-2021-282740X "Insulin potentiates the response to capsaicin in dorsal root ganglion neurons in vitro and muscle afferents ex vivo in normal healthy rodents" by Amane Hori, Norio Hotta, Ayumi Fukazawa, Juan A Estrada, Kimiaki Katanosaka, Kazue Mizumura, Jun Sato, Rie Ishizawa, Han Kyul Kim, Gary A Iwamoto, Wanpen Vongpatanasin, Jere H Mitchell, Scott A. Smith, and Masaki Mizuno

Thank you for resubmitting your revised Research Article to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

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

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- Statistical Summary Document

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

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-We invite you to include a Translational Perspective paragraph in your manuscript. This should be included in the main body of the manuscript after the Acknowledgements. It should describe the wider translational implications of the work, in plain English, for a broad scientific audience. Please use the following guidelines to prepare a Translational perspective of your paper https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#authortranspersp The Translational perspective should not exceed 250 words in total and should be presented as a single paragraph. Abbreviations and technical terms must be defined as briefly and simply as possible the first time they are used, unless they are generally/easily understood, e.g. ECG, HIV/AIDS, K+ channel. Use language that can be understood by scientists or clinicians with a general knowledge of the topic addressed. Ensure the paragraph includes the hypothesis tested in the paper and accurately reflects the findings of the paper and the implications for future research. State the word count of the Translational perspective paragraph.

-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.

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

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-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

-Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can

assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

This revised study addressed the issues raised in the previous review and includes significant new data that increase the impact of the work. The new data show co-localization of insulin receptor and TRPV1 in a subset of DRG neurons and, more importantly, show increased renal sympathetic nerve activity and mean arterial pressure responses to injection of capsaicin into the arterial supply of the hindlimb in decerebrate rats after intramuscularly injecting insulin. Changes to the text, including the discussion, do a nice job of addressing issues raised by reviewers.

Senior Editor:

We thank the authors for careful revision of their manuscript, which has been markedly improved and deemed highly relevant and influential. The manuscript is now acceptable for publication. However, there are a minor point to address concerning format. Please state in the table/figure legend, the statistical used in the table/figure. I believe this refers to Table 1, Figure 2,5 and 8. Thank you for submitting this outstanding work to our Journal.

REFEREE COMMENTS

Referee #1:

No further comments! Congratulations on this outstanding contribution! Very impressive!

_____

END OF COMMENTS

1st Confidential Review **15-Dec-2021**

Manuscript#: JP-RP-2021-281874X R1

Insulin potentiates the response to capsaicin in dorsal root ganglion neurons *in vitro* and muscle afferents *ex vivo* in normal healthy rodents

Reviewing Editor:

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Senior Editor:

We thank the authors for careful revision of their manuscript, which has been markedly improved and deemed highly relevant and influential. The manuscript is now acceptable for publication. However, there are a minor point to address concerning format. Please state in the table/figure legend, the statistical used in the table/figure. I believe this refers to Table 1, Figure 2, 5 and 8. Thank you for submitting this outstanding work to our Journal.

Referee #1:

No further comments! Congratulations on this outstanding contribution! Very impressive!

Author response: Again, we appreciate the reviewing editor, senior editor, and reviewers for their thoughtful comments and suggestions. We have addressed all formatting concerns (i.e., captions for Table 1, Figure 2, 5 and 8), and provided a figure for the abstract (uploaded) as well as a Translational Perspective (Lines 579-596). All changes made to the manuscript are indicated in red font in the red-lined version of the manuscript. Thank you!

Dear Dr Mizuno,

Re: JP-RP-2021-282740XR1 "Insulin potentiates the response to capsaicin in dorsal root ganglion neurons in vitro and muscle afferents ex vivo in normal healthy rodents" by Amane Hori, Norio Hotta, Ayumi Fukazawa, Juan A Estrada, Kimiaki Katanosaka, Kazue Mizumura, Jun Sato, Rie Ishizawa, Han Kyul Kim, Gary A Iwamoto, Wanpen Vongpatanasin, Jere H Mitchell, Scott A. Smith, and Masaki Mizuno

Thank you and congratulations on a fine study.

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,

Harold D Schultz
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2nd Confidential Review **22-Dec-2021**