

Aging and Endothelium-mediated Vascular Dysfunction: The Role of the NADPH Oxidases

Oh Sung Kwon, Sung Gi Noh, Soung Hun Park, Robert H. I. Andtbacka, John R Hyngstrom, and Russell S Richardson
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Daniel W Trott (Referee #2)

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Dear Dr Kwon,

Re: JP-RP-2022-283208 "Aging and Endothelium-mediated Vascular Dysfunction: The Role of the NADPH Oxidases" by Oh Sung Kwon, Sung Gi Noh, Soung Hun Park, Robert H. I. Andtbacka, John R Hyngstrom, and Russell S Richardson

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

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

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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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- You must start the Methods section with a paragraph headed [Ethical Approval](#). If experiments were conducted on humans, confirmation that informed consent was obtained, preferably in writing, that the studies conformed to the standards set by the latest revision of the Declaration of Helsinki, and that the procedures were approved by a properly constituted ethics committee, which should be named, must be included in the article file. If the research study was registered (clause 35 of the Declaration of Helsinki) the registration database should be indicated, otherwise the lack of registration should be noted as an exception (e.g. The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database.). For further information see: <https://physoc.onlinelibrary.wiley.com/hub/human-experiments>
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- 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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EDITOR COMMENTS

Reviewing Editor:

Thank you for submitting your paper, and I apologize for the delay in reviews. As you see, both reviewers were generally positive about your study and provided detailed feedback. I encourage you to consider all the points raised in detail in a revised submission. I also recommend you revise the journal's statistical policy and comply with it in the revised version of the manuscript.

The paper does not comply with the statistical policy of the journal, as it rather than standard deviation includes the SEM. Also, no precise p-value was stated in the figures or throughout the results section. As this is the initial version and no statistical summary was attached to the submission files, I recommend the authors revise the journal's statistical policy and comply with it in the revised version of the manuscript.

In addition, the ethics approval reference number needs to be provided in the Methods section.

REFEREE COMMENTS

Referee #1 (Please also see comments in attached file):

Overview

This study determine the isoform-specific role of NADPH oxidases (NOX) in the endothelium-mediated vascular dysfunction. Endothelium-dependent (flow- and acetylcholine (ACh)-induced) vasodilation in human skeletal muscle feed arteries (SMFAs) of young, middle aged, and old subjects was assessed, in vitro, with and without the inhibition of NOX1 (ML090), NOX2 (gp91), and NOX4 (plumbagin). The role of nitric oxide (NO) bioavailability in age related vascular decline was further assessed using L-NMMA; NOX1, NOX2, and NOX4 expression was determined by Western blot. Endothelium-dependent vasodilatory dysfunction was evident in young, middle and old subjects and declined with age. NOX1 inhibition had no effect on SMFA vasodilation. NOX2 inhibition restored flow- and ACh-induced vasodilation in the middle aged and old SMFAs; a similar but non significant trend was observed with NOX4 inhibition. L-NMMA negated the restorative effects of NOX2 and NOX4 blockade. NOX2 and NOX4 protein expression was greater in the two older groups and inversely related to vascular

function. In summary, NOX2 and to a lesser extent, NOX4 appear to play an important, likely NO-mediated, role in age-related endothelial dysfunction.

General Comments

This is an interesting human study examining the role of NOX in age related vascular decline. The work builds on the rich tradition of animal work and is reasonably complete, a nice accomplishment for human related work. The use of human tissues provide significant novelty to the work and the authors should be commended for their attention to detail. The reviewer provides a series of suggestions to improve the completeness of the work and the presentation of results.

Specific Comments

1. Please provide a more detailed description of the myography experiments. What was the initial pressure at which vessels were set? How did the authors verify the pressure differential across vessel segments to induced flow mediated changes. Please be clear on how the vessels were precontracted.
2. It would be nice to included a table outlining arterial characteristics by age grouping (average diameter, wall thickness, etc). Further, what was the dilatory range of these vessel in absolute terms for each dilatory perturbation?
3. The authors should use Standard Deviation rather than Standard Error. Please apply to all data presented in tables and figures. Please state in the figure legends that you are plotting mean and standard deviation.
4. A little more use of color in the line graphs would help the authors highlight key points and make the figures more appealing.
5. The authors to provide representative traces of their vasomotor response to flow and ACh. Of particular note would comparator traces between a young and old human vessels at rest and with the addition of a NOX2 inhibitor.
6. It is peculiar that each stimuli (flow, ACh, and nitroprusside) produced dilatory responses that were linear rather than sigmoidal. Do the authors have an explanation for this unusual phenomenon? Were the isolated vessel fully viable? Could they have been compromised by the dissection?
7. The wording in the discussion could be tighten. Judicious trimming of text would help the authors highlight their main findings.
8. There was an issue with the immunohistochemistry. It appears that background labelling in Figure D and F, is higher than for the young controls (Figures C and E). Do the authors have an explanation?
9. In regards to the Western blot data, is their an age related change in GAPDH expression? It is hard to discern from the blot.
10. Please provide a clear description of the statistical comparisons/methods in each figure legend.
11. Minor concern: % max dilation is fine; one can remove the work "possible" from figures and the methods section.

Referee #2:

This study sought to determine the role of specific NADPH oxidases in age-related endothelial dysfunction. Overall this is a well conducted study that provides important insight to the specific role of NADPH oxidase isoforms in impairments in endothelium dependent dilation with age. Of major importance, these studies provide mechanistic insight that NOX2 and potentially NOX4 contribute to blunted EDD in human arterioles. Despite the overall strong study, there are some issues to be addressed.

Introduction: reactive in the first mention of ROS is misspelled. The sentence referring to the Trott 2011 study is confusing, in that study, the authors found that gp91phox was elevated and EDD was blunted in old arteries and that apocynin restored dilation. It currently reads as if apocynin played a role in modulating gp91phox expression. There is a similar issue with the next sentence.

Methodology: Does incubation with NOX for an hour have any impact on starting vessel diameter compared to the control? If so, that may change the interpretation of the results. What was the control vessel allowed to incubate in? Although mentioned in the results, it should also be mentioned that the vessels were precontracted with phenylephrine in the methods section.

Results: Generally, please specify exact figure (1A) not (1A-C) in the text of the results. Presentation of a panel comparing Y, MA and O flow and ACh induced dilation at baseline would be helpful to demonstrate age-related impairments in EDD. Larger symbols and larger and/or bolded X and Y axis labels of all vessel data panels would enhance presentation. Please present L-NMMA data as figure 2 and SNP data as figure 3, it allows for more linear story telling, i.e. EDD, NO-bioavailability, smooth muscle function. For the immunofluorescence images it would be helpful to present DAPI only or

negative NOX antibody control images to help the reader interpret which staining is NOX specific. Lastly, the authors should revise figures to conform with J Physiol guidelines, ie. Presenting standard deviations rather than standard errors and individual data points rather than bar graphs. Lastly, It is this reviewers opinion that the correlation figures repeat the already presented data and do not significantly contribute to the overall findings of the study and could be omitted.

Discussion: The discussion focuses heavily on the NOX isoforms. This makes sense as it's based on the data in the present manuscript. In addition to discussion of increased mechanisms of ROS production, there should be some discussion of ROS scavenging capacity with age. In addition, the authors should discuss the present findings in light of their previous findings that mitoTEMPOL can also restore EDD in skeletal muscle arteries from older adults (Park 2018).

END OF COMMENTS

Confidential Review

12-Apr-2022

Overview

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Specific Comments

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3. The authors should use Standard Deviation rather than Standard Error. Please apply to all data presented in tables and figures. Please state in the figure legends that you are plotting mean and standard deviation.
4. A little more use of color in the line graphs would help the authors highlight key points and make the figures more appealing.
5. The authors to provide representative traces of their vasomotor response to flow and ACh. Of particular note would comparator traces between a young and old human vessels at rest and with the addition of a NOX2 inhibitor.
6. It is peculiar that each stimuli (flow, ACH, and nitroprusside) produced dilatory responses that were linear rather than sigmoidal. Do the authors have an explanation for

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7. The wording in the discussion could be tightened. Judicious trimming of text would help the authors highlight their main findings.
8. There was an issue with the immunohistochemistry. It appears that background labelling in Figure D and F, is higher than for the young controls (Figures C and E). Do the authors have an explanation?
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In addition, the ethics approval reference number needs to be provided in the Methods section.

RESPONSE: First, now I too must apologize for the delay in sending the revised manuscript and the response to reviewers. As I explained to the Journal of Physiology Editorial Office, as I had family issues in South Korea, I was out of the country and pre-occupied for a long time. Thank you, and the Journal, for your understanding. Next, we would like to, sincerely, thank you and the reviewers for insightful comments and the opportunity to resubmit this manuscript. Further, we appreciate the reviewers, generally, positive feedback about the manuscript. We hope that, by responding to their comments making the appropriate edits, we have satisfied the reviewer concerns. Finally, as requested, we have added the ethics approval reference number to the Methods section (IRB# 32786) and dealt with the noted statistical requirements, including converting all variance data from SEM to SD.

REFEREE COMMENTS

Referee #1 (Please also see comments in attached file):

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RESPONSE: Thank you for your positive review and kind words, both are much appreciated as is your time and effort expended in the review of our work. Below we have responded to each of your comments and, where appropriate, made changes to the original manuscript.

Specific Comments

1. Please provide a more detailed description of the myography experiments. What was the initial pressure at which vessels were set? How did the authors verify the pressure differential across vessel segments to induced flow mediated changes. Please be clear on how the vessels were precontracted.

RESPONSE: Based upon your comments we have added additional details to the description of the vasodilation assessments (e.g. intraluminal pressure (≈ 60 mmHg), clarified the process to induce flow, and how the vessels were pre- constricted). Of note, we utilize 60 mmHg both because this seems reasonable based upon the position of these vessels in the arterial tree and because our experience has been that a greater pressure tends to distort the majority of samples to what appears to be greater than expected physiological levels (i.e. a ballooning effect). Additionally, intraluminal pressure was not verified, per se, but is the, inevitable, consequence of the height of the reservoirs attached to both ends of the vessel and is easily calculated.

2. It would be nice to included a table outlining arterial characteristics by age grouping (average diameter, wall thickness, etc). Further, what was the dilatory range of these vessel in absolute terms for each dilatory perturbation?

RESPONSE: As requested we have added an additional table (Table2), in which we now provide, by age group and treatment, the average outer diameter at baseline (rest and following incubation with the different NOX inhibitors), with phenylephrine (PE), with flow (45 ul/min), and with acetylcholine (ACh) (10^{-3} M). Thus, this table now provides the dilatory range of these vessels, in absolute terms, for each dilatory perturbation. Due to the thickness, and therefore opaqueness, of these vessels during trans illumination, we were unable to measure wall thickness.

3. The authors should use Standard Deviation rather than Standard Error. Please apply to all data presented in tables and figures. Please state in the figure legends that you are plotting mean and standard deviation.

RESPONSE: As requested, we now present SD rather than SEM in all tables and figures. Thank you.

4. A little more use of color in the line graphs would help the authors highlight key points and make the figures more appealing.

RESPONSE: We agree with this suggestion and, hence, have, in the revised manuscript, used different colors in the line graphs to make them a little easier to interpret.

5. The authors to provide representative traces of their vasomotor response to flow and ACh. Of particular note would comparator traces between a young and old human vessels at rest and with the addition of a NOX2 inhibitor.

RESPONSE: Unlike wire myography, with pressure myography there are, actually, no continuous “traces” collected during such studies. This is because, as illustrated in Figures 1-3, discrete measurements of vessel diameter, with edge detection software, were performed at each level of the stimuli (flow, ACh, and SNP) and, therefore, we cannot provide “representative tracings”.

6. It is peculiar that each stimuli (flow, ACh, and nitroprusside) produced dilatory responses that were linear rather than sigmoidal. Do the authors have an explanation for this unusual phenomenon? Were the isolated vessel fully viable? Could they have been compromised by the dissection?

RESPONSE: This is an astute observation, and we do not have a complete explanation for the linear, rather than sigmoidal, dilatory responses. However, it should be noted that this, and our previous work in this area (PMID: 28493603, PMID: 30192630, PMID: 30690728, PMID: 32022597), which also revealed linear responses, is unique in that the vessels studied are human SMFAs and it may be that these vessel exhibit a different dilatory response compared to animal vessels. In terms of vessel functionality, we have no reason to believe that any of the vessels studied were not fully viable. In fact, the vessels were very carefully harvested including fat and nerves to minimize any damage during the actual collection and then were, extremely, carefully dissected in a cooled dissection plate using a well-lit dissection microscope.

7. The wording in the discussion could be tighten. Judicious trimming of text would help the authors highlight their main findings.

RESPONSE: Based upon your suggestion, we have attempted to better highlight the main findings of the study, in the Discussion, by trimming some of text. Thank you.

8. There was an issue with the immunohistochemistry. It appears that background labelling in Figure D and F, is higher than for the young controls (Figures C and E). Do the authors have an explanation?

RESPONSE: We readjusted the pixels and brightness for these immunohistochemistry images, and added DAPI alone figures, however, the background labelling in panel F and H still looks brighter than for the young controls (panels E and G). Thus, due to these issues, and the original lack of clarity for the message conveyed by these images, we have removed this figure from the revised manuscript. Thank you for your understanding.

9. In regards to the Western blot data, is there an age related change in GAPDH expression? It is hard to discern from the blot.

RESPONSE: Although visually there does appear to be greater GAPDH in the older subjects, there is, actually, not significantly different between the three subject groups.

10. Please provide a clear description of the statistical comparisons/methods in each figure

legend.

RESPONSE: As requested, in the revised manuscript we now provide a clear description of the statistical comparisons/methods in each figure legend, as per The Journal's instructions.

11. Minor concern: % max dilation is fine; one can remove the work "possible" from figures and the methods section.

RESPONSE: Based upon this comment, we have removed "possible" from the figures and the Methods section.

Referee #2:

This study sought to determine the role of specific NADPH oxidases in age-related endothelial dysfunction. Overall this is a well conducted study that provides important insight to the specific role of NADPH oxidase isoforms in impairments in endothelium dependent dilation with age. Of major importance, these studies provide mechanistic insight that NOX2 and potentially NOX4 contribute to blunted EDD in human arterioles. Despite the overall strong study, there are some issues to be addressed.

RESPONSE: Thank you for your positive review and kind words, both are much appreciated as is your time and effort expended in the review of our work. Below we have responded to your comments and, where appropriate, made changes to the original manuscript.

Introduction: reactive in the first mention of ROS is misspelled. The sentence referring to the Trott 2011 study is confusing, in that study, the authors found that gp91phox was elevated and EDD was blunted in old arteries and that apocynin restored dilation. It currently reads as if apocynin played a role in modulating gp91phox expression. There is a similar issue with the next sentence.

RESPONSE: Thank you for catching this misspelling of the word "reactive", it has been corrected. Additionally, thank you for pointing out the two poorly worded sentences that describe the animal studies that used apocynin to restore age-related vascular dysfunction in the vasculature. These have been re-worded to be more clear and accurate.

Methodology: Does incubation with NOX for an hour have any impact on starting vessel diameter compared to the control? If so, that may change the interpretation of the results. What was the control vessel allowed to incubate in? Although mentioned in the results, it should also be mentioned that the vessels were precontracted with phenylephrine in the methods section.

RESPONSE: In the revised manuscript, in a new table (Table 2) we now provide the average vessel outer diameter at rest and following incubation in the different NOX inhibitors. As now documented in this table, incubation in the different NOX inhibitors did not impact baseline vessel diameter. The control vessels were also "incubated" in the PPS for 1 hour and we have now noted this in the Methods, thank you. Based upon your suggestion, we have corrected the omission of the preconstruction with phenylephrine in the Methods section. Thank you.

Results: Generally, please specify exact figure (1A) not (1A-C) in the text of the results.

RESPONSE: As suggested, where appropriate, we have not abbreviated the figure panels, but, instead, list them all individually.

Presentation of a panel comparing Y, MA and O flow and ACh induced dilation at baseline would be helpful to demonstrate age-related impairments in EDD.

RESPONSE: As requested, but in a Table rather than a figure, we now provide the Young, Middle aged, and Old flow, and ACh-induced vasodilation at baseline and with the addition of the different NOX inhibitors. (Table 2).

Larger symbols and larger and/or bolded X and Y axis labels of all vessel data panels would enhance presentation.

RESPONSE: As suggested, in the revised manuscript, we have utilized larger symbols and both larger and bolded labels on the X and Y axis of the figures.

Please present L-NMMA data as figure 2 and SNP data as figure 3, it allows for more linear story telling, i.e. EDD, NO-bioavailability, smooth muscle function.

RESPONSE: Based upon your request, in the revised manuscript, we now present the figure illustrating the effect of L-NMMA on vascular function with and without NOX blockade as Figure 2. This does make for a more linear story, thank you.

For the immunofluorescence images it would be helpful to present DAPI only or negative NOX antibody control images to help the reader interpret which staining is NOX specific.

RESPONSE: Based upon your helpful comment we added young and old DAPI only images, however, we were still, somewhat, disappointed by the overall ease of interpretation of this supplemental figure and so we have decided to remove it from the revised manuscript. Thank you for your understanding.

Lastly, the authors should revise figures to conform with J Physiol guidelines, ie. Presenting standard deviations rather than standard errors and individual data points rather than bar graphs.

RESPONSE: As requested, we have revised the figures to conform with The Journal's guidelines. Specifically, we now use standard deviation and have added the individual data to the bar graphs in Figure 5. Thank you.

Lastly, It is this reviewers opinion that the correlation figures repeat the already presented data and do not significantly contribute to the overall findings of the study and could be omitted.

RESPONSE: We appreciate your rational for suggesting the omission of the correlation figures, however, we firmly contend that the correlation figures will help the reader to appropriately interpret which NOX isoforms affect vasodilatory function, the role of NO, and the effect of aging. Additionally, these analyses document the individual data, which would not be possible in some of the other Figures without significantly compromising clarity. Therefore, in our opinion, as the correlation figures greatly strengthen the findings of this study, we would prefer not to omit them. Thank you for your understanding.

Discussion: The discussion focuses heavily on the NOX isoforms. This makes sense as it's

based on the data in the present manuscript. In addition to discussion of increased mechanisms of ROS production, there should be some discussion of ROS scavenging capacity with age.

RESPONSE: Although not wanting to expand the, already rather long Discussion, as suggested, to add some balance to the otherwise "ROS heavy" text, we now make mention of the increased ROS scavenging, by antioxidants, that is typically associated with aging. Thank you.

In addition, the authors should discuss the present findings in light of their previous findings that mitoTEMPOL can also restore EDD in skeletal muscle arteries from older adults (Park 2018).

RESPONSE: In our previous study (Park 2018) we did not, actually use mitoTEMPOL, but rather the mitochondrial targeted antioxidant, MitoQ. Regardless, based upon your comment, in the Discussion, we have now included a mention of this work in relation to redox balance. Thank you.

Dear Dr Kwon,

Re: JP-RP-2022-283208R1 "Aging and Endothelium-mediated Vascular Dysfunction: The Role of the NADPH Oxidases" by Oh Sung Kwon, Sung Gi Noh, Soung Hun Park, Robert H. I. Andtbacka, John R Hyngstrom, and Russell S Richardson

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

Congratulations on an excellent revision and contribution to the field. Both reviewers were satisfied with your final version,

except for a comment that should be addressed before the final acceptance of this paper.

REFeree COMMENTS

Referee #1:

A nice, near complete study using human arteries. The authors responded well to my previous queries.

Referee #2:

The authors have been highly responsive to the reviewer critiques. I have a few remaining questions.

In the new Table 2 it refers to "outer diameter". Is this correct? From my understanding, pressure myography experiments usually record and calculate dilation using inner diameter. The authors should clarify and justify the reasoning for using outer diameter in the methods section of the manuscript if this is the case.

In the revision the authors show starting diameters for all conditions in Table 2, but my original request was for a figure with Ach and flow induced dilation in young, middle aged and old with no inhibitors so that the reader can visualize the degree of impairment in endothelium dependent dilation with age. My wording in the original review was not completely clear so this may have caused some confusion.

END OF COMMENTS

1st Confidential Review

09-Oct-2022

EDITOR COMMENTS

Reviewing Editor:

Congratulations on an excellent revision and contribution to the field. Both reviewers were satisfied with your final version, except for a comment that should be addressed before the final acceptance of this paper.

RESPONSE: We really appreciate you and the reviewers and the time and effort expended in reviewing our manuscript. Below we have responded to the remaining comments.

Referee #1:

A nice, near complete study using human arteries. The authors responded well to my previous queries.

RESPONSE: We thank for your helpful comments which have greatly enhanced the quality of the current manuscript.

Referee #2:

The authors have been highly responsive to the reviewer critiques. I have a few remaining questions.

In the new Table 2 it refers to "outer diameter". Is this correct? From my understanding, pressure myography experiments usually record and calculate dilation using inner diameter. The authors should clarify and justify the reasoning for using outer diameter in the methods section of the manuscript if this is the case.

RESPONSE: We thank for your time and effort expended in reviewing our manuscript. Also your comments have greatly enhanced the quality of the current manuscript, thank you. We have responded to your comments and, where appropriate, made changes to the revised manuscript.

Yes, correct. Due to the thickness, and, therefore, the opaqueness, of the SMFAs, during trans illumination, we were unable to measure wall thickness and so measured dilation and constriction from the outer diameters. Based upon your suggestion we have added this specific note to the Methods section.

In the revision the authors show starting diameters for all conditions in Table 2, but my original request was for a figure with Ach and flow induced dilation in young, middle aged and old with no inhibitors so that the reader can visualize the degree of impairment in endothelium dependent dilation with age. My wording in the original review was not completely clear so this may have caused some confusion.

RESPONSE: As we now understand your previous request, as you suggested we have added a new figure that clearly illustrates the age-dependent changes in endothelial dependent vasodilation, uncomplicated by the series of inhibitors employed to determine the role of the NADPH oxidases. This is nice addition to the manuscript, thank you.

Dear Dr Kwon,

Re: JP-RP-2022-283208R2 "Aging and Endothelium-mediated Vascular Dysfunction: The Role of the NADPH Oxidases" by Oh Sung Kwon, Sung Gi Noh, Soung Hun Park, Robert H. I. Andtbacka, John R Hyngstrom, and Russell S Richardson

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EDITOR COMMENTS

Reviewing Editor:

Congratulations on this excellent addition to the field.

