Peer-Review Record

Retinoic acid-related orphan receptors regulate autophagy and cell survival in cardiac myocytes during hypoxic stress

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by Eryn Kirshenbaum, Huong Nguyen, Victoria Margulets, Molly Crandall, Darya Nematisouldaragh, Inna Rabinovich-Nikitin

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Academic Editor: Ali J. Marian

Reviewer 1: Anonymous

Reviewer 2: Anonymous

Reviewer 3: Anonymous

Round 1

Reviewer 1 Report

The authors report that activation of RORa protects cardiomyocytes from hypoxia (HPX)-induced damages. They propose that RORa positively regulates autophagy.

General:
The authors should clarify the primary target of NOB. For example, it can reduce oxidative stress and the effect upon mitochondria, and the effect upon autophagy and mitophagy could be secondary or vice versa. This issue should be discussed as a limitation.

The experiments regarding autophagy and mitophagy are not conducted properly. The authors should show more convincing data to show NOB increases autophagic flux. This reviewer suggests that the authors should repeat the experiments in Fig. 4B with or without a lysosome inhibitor.

Specific:
The visual abstract is confusing since it looks like that Nobiletin is induced by HPX.

Figure 1 should show p16 protein expression in cardiomyocytes. Please explain why p16 is studied here.

In Figure 4, the authors should show autophagic flux by testing the effect of lysosome

inhibitors.

In Figure 5, please explain how one can determine that mitophagy is inhibited by HPX in these experiments. The reviewer's interpretation is the opposite. HPX increases red color in representative microscope images and 560/440 in the bar graph, both of which show increases in mitophagy. On the other hand, NOB inhibits Hpx-induced increases in mitophagy.

In Figure 6, it would be hard to determine the effect of RORa upon autophagy by looking at the level of Beclin 1, p62 and LC3 proteins alone. The authors should evaluate autophagy with LC3II or puncta in the presence or absence of lysosome inhibition.

In Figure 7, the authors should evaluate autophagic flux. It would be hard to evaluate autophagy by showing the level of LC3 II alone.

Authors' Response

The authors report that activation of RORa protects cardiomyocytes from hypoxia (HPX)-induced damages. They propose that RORa positively regulates autophagy.

General:
The authors should clarify the primary target of NOB. For example, it can reduce oxidative stress and the effect upon mitochondria, and the effect upon autophagy and mitophagy could be secondary or vice versa. This issue should be discussed as a limitation.

We thank the reviewer for their comment. We added a paragraph that discusses the primary target of Nob as a limitation in the discussion:

"Nevertheless, whether the protective effect of Nobiletin is due to reduced oxidative stress which consequently signals to activation of autophagy and mitochondrial clearance, or whether Nobiletin induced activation of autophagy leads to reduced oxidative stress has not been identified in our study. Therefore, it will be interesting to explore in future studies whether Nobiletin impinges on these or other cellular targets that confer protection during hypoxic stress."

The experiments regarding autophagy and mitophagy are not conducted properly. The authors should show more convincing data to show NOB increases autophagic flux. This reviewer suggests that the authors should repeat the experiments in Fig. 4B with or without a lysosome inhibitor.

We thank the reviewer for their suggestion. We added proper autophagy flux study with chloroquine (CQ) as a lysosomal inhibitor, please see figure 4B.

Specific:
The visual abstract is confusing since it looks like that Nobiletin is induced by HPX.

We thank the reviewer for this comment. We agree with the reviewer and have modified the graphical abstract which better reflects the findings of our paper.

Figure 1 should show p16 protein expression in cardiomyocytes. Please explain why p16 is studied here.

Thank you for this comment. The reason we chose to show mRNA levels of p16 and not protein levels, is based on our previous observations that measurement of p16 mRNA in the heart is more reliable than its derived protein. This is supported by the paper by Hara et al. (DOI: 10.1128/MCB.16.3.859), showing that p16 mRNA has a very long half-life of greater than 24 hours, compared with p16 protein with half-life of 30 minutes to 3.5 hours. Given this unparalleled stability, RT-PCR assay for measuring p16 expression is more precise and reproducible compared to protein.

In addition, the reason for studying p16 is based on earlier work that demonstrated that mitochondria are the major source of ROS and cellular damage associated with aging. This notion is elaborated in the introduction and results of Figure 1, and we have further elaborated on this in the discussion, as follows:

"Indeed, excessive cellular ROS has been associated with increased aging and expression of p16(15). Furthermore, previous studies have shown that Nobiletin could delay skeletal muscle aging by improving autophagy, decreasing ROS production and reversing mitochondrial damage(16). Interestingly, our study showed that Nobiletin suppressed mitochondrial ROS production coincident with a reduction in p16 levels."

In Figure 4, the authors should show autophagic flux by testing the effect of lysosome inhibitors.

We thank the reviewer for their important comment. As recommended by the reviewer, we have conducted additional experiments to assess autophagic flux. As shown in the revised version of Figure 4, the effect of Nobiletin on autophagic flux is shown in the absence and presence of chloroquine (CQ).

In Figure 5, please explain how one can determine that mitophagy is inhibited by HPX in these experiments. The reviewer's interpretation is the opposite. HPX increases red color in representative microscope images and 560/440 in the bar graph, both of which show increases in mitophagy. On the other hand, NOB inhibits Hpx-induced increases in mitophagy.

Thank you for this comment. To better clarify the interpretation of mitophagy clearance by the dual probe Mitokeima, we added the paragraph below to the results and supported it with appropriate citations:

"For these studies, we utilized the dual emission mitophagy reporter MitoKeima that fluoresces green following lysosomal clearance of mitophagosomes (neutral pH) or fluoresces yellow/red when mitophagy is impaired (acidic pH). The size of puncta is

another parameter for the status of mitophagy as indicated by large puncta when mitophagosomes accumulate due to disrupted mitochondrial – lysosomal clearance, or smaller puncta when mitochondrial clearance is normal(4,13). As shown in Figure 5, we observed a marked reduction in mitophagy, as indicated by the presence of large red fluorescent puncta in cardiac myocytes subjected to hypoxia compared to normoxic control cells. Importantly, hypoxia-induced impairment of mitophagy was restored in cardiac myocytes treated with Nobiletin."

In Figure 6, it would be hard to determine the effect of RORa upon autophagy by looking at the level of Beclin 1, p62 and LC3 proteins alone. The authors should evaluate autophagy with LC3II or puncta in the presence or absence of lysosome inhibition.

We thank the reviewer for their important comment. As recommended by the reviewer, we have conducted additional experiments to assess autophagic flux. As shown in the revised version of Figure 6D, the effect of Nobiletin and RORα on autophagic flux is shown in the absence and presence of chloroquine (CQ).

In Figure 7, the authors should evaluate autophagic flux. It would be hard to evaluate autophagy by showing the level of LC3 II alone.

Thank you for this comment. Since we evaluated the effect of Nobiletin on autophagic flux in figures 4B and 6D and we have shown in our previous publication (DOI: 10.1080/15548627.2021.1938913) that shATG7 fails to activate autophagy, even in the presence of CQ, we did not repeat these experiments here.

Reviewer 2 Report

Autophagy is a crucial cellular process involved in maintaining cell quality control and preventing various chronic human diseases. The study focuses on the gene ROR α , which is found to be cardioprotective by regulating autophagy and clearing damaged mitochondria. The compound Nobiletin activates ROR α and enhances autophagy, reducing aging-related markers and harmful mitochondria during hypoxic conditions. Loss of ROR α activity and autophagy inhibition both undermine the protective effects of Nobiletin, emphasizing the importance of ROR α and autophagy in cardiac health and suggesting potential therapeutic applications.

Figure 1 – The p16 transcript level under hypoxia upon NOB treatment shows the reduction trend but based on the bar graph presented, highly unlikely to be a significant reduction. I suggest repeating the statistics and show the graph as dot plot/ or box and whiskers with min to max, show all points.

Figure 2A- The staining panels under HPX do not represent the difference between with and without NOB shown in graph; while the difference in the graph is less than 2 folds, the staining panels represent a much wider difference. I suggest to replace the control panel under HPX with a panel that visually match the graph.

Figure 4A- The WB for the p62 shows no clear band of protein to begin with and the quantitation graph certainly do not represent the blot shown for p62. I suggest replacing the blot or removing from the figure.

Figure 6- figure legend doesn’ t match with the panels (A and B switched).
Figure 6B and C- There is no evidence shown, either at transcript or at protein level for ROR α to be downregulated by siROR α .
Figure 6C- The graph labels do not match with the blot labels. Also, the LC3II protein bands are so faint and invisible. I suggest to improve the image intensity or replace it with a new blot and correct the quantitation accordingly.

Figure 6D- The graph labeling do not match with the panel shown for cell viability assay.
Figure 7A- The LC3II protein levels keep on changing among different blots shown in this study for the same condition and is not reproducing the data even under NMX comparing NOB to control. It seems that this is due to experimental condition that has not been optimized and confounds the final conclusion including those reported under HPX.

Authors’ Response

Autophagy is a crucial cellular process involved in maintaining cell quality control and preventing various chronic human diseases. The study focuses on the gene RORα, which is found to be cardioprotective by regulating autophagy and clearing damaged mitochondria. The compound Nobiletin activates RORα and enhances autophagy, reducing aging-related markers and harmful mitochondria during hypoxic conditions. Loss of RORα activity and autophagy inhibition both undermine the protective effects of Nobiletin, emphasizing the importance of RORα and autophagy in cardiac health and suggesting potential therapeutic applications.

Figure 1 –The p16 transcript level under hypoxia upon NOB treatment shows the reduction trend but based on the bar graph presented, highly unlikely to be a significant reduction. I suggest repeating the statistics and show the graph as dot plot/ or box and whiskers with min to max, show all points.

Thank you for this comment. As suggested, we present this graph and all other graphs in this manuscript as dot plot.

Figure 2A- The staining panels under HPX do not represent the difference between with and without NOB shown in graph; while the difference in the graph is less than 2 folds, the staining panels represent a much wider difference. I suggest to replace the control panel under HPX with a panel that visually match the graph.

We thank the reviewer for pointing this out. We have replaced the image during hypoxia to better represent the differences without and with Nobiletin treatment as shown in the histogram in revised Figure 2A.

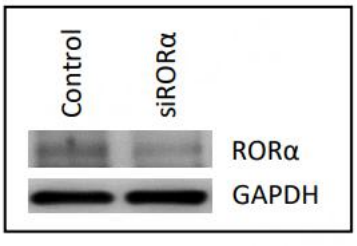
Figure 4A- The WB for the p62 shows no clear band of protein to begin with and the quantitation graph certainly do not represent the blot shown for p62. I suggest replacing the blot or removing from the figure.

We thank the reviewer for their comment. We decided to remove p62 from both figures 4A and 6C.

Figure 6- figure legend doesn't match with the panels (A and B switched).

We thank the reviewer for their comment, the figure legend has been changed accordingly.

Figure 6B and C- There is no evidence shown, either at transcript or at protein level for RORα to be downregulated by siRORα.



Thank you for this comment. The sequence for siRORα was adopted from DOI: 10.1038/s41419-021-04170-0, where its inhibitory function was well studied. However, to validate the inhibition of RORα in our study, we performed the following western blot analysis which demonstrates knock-down of RORa in the presence of siRORa.

Figure 6C- The graph labels do not match with the blot labels. Also, the LC3II protein bands are so faint and invisible. I suggest to improve the image intensity or replace it with a new blot and correct the quantitation accordingly.

Thank you for pointing out this oversight. The control labels on the histogram have been revised to match the labels on the immunoblot. In addition, we changed the intensity of the LC3II blot in order to improve the visibility of the bends.

Figure 6D- The graph labeling do not match with the panel shown for cell viability assay.

Thank you for this comment. The labels on the bar graph have been changed to fit with the labels on the cell viability assay.

Figure 7A- The LC3II protein levels keep on changing among different blots shown in this study for the same condition and is not reproducing the data even under NMX comparing NOB to control. It seems that this is due to experimental condition that has not been optimized and confounds the final conclusion including those reported under HPX.

Thank you for this comment. We replaced the western blot in Figure 6C to better match with the other western blots for LC3II.

Reviewer 3 Report

Manuscript JCA-2023-31 by Eryn Kirshenbaum et al. entitled: Retinoic Acid-Related Orphan Receptors Regulate Autophagy and Cell Survival in Cardiac Myocytes during Hypoxic Stress.

The article describes a comprehensive study and provides data supporting the role of RORalpha in promoting autophagy, cell survival, mitochondrial function, and attenuating senescence in the cell culture system. Overall, the data support the conclusions of the study. It focuses on the flavonoid compound Nobiletin (NOB), which is considered an anti-inflammatory agent. The findings are consistent with the role of RORA in enhancing

autophagy and lysosomal function and advancing the existing knowledge by providing new information.

The reviewer has the following suggestions:

One issue that the authors might wish to address is the specificity of the effects of NOB as it interacts with several other targets /pathways in addition to mitochondrial functions, such as inflammation through NFkB1, metabolism through PGC1a, and various forms of cell death. The multiplicity of the effects should be discussed.

The authors use a hypoxia model, which suppresses Rora transcript levels. Hypoxia also activates P53, which induces Rora. If data is available in the P53-RORalpha axis, it should be included. Otherwise, it should be mentioned in the discussion.

NOB and RORalpha are considered regulators of the circadian rhythm through CLOCK/BMAL, which may mediate some of the molecular effects. The authors may wish to comment.

Abstract: “RORα regulates autophagic processes linked to aging through activation with Nobiletin.” The reviewer suggests changing “through activation” to “upon activation”.

Methods section: “into an air-tight chamber continually gassed with 95% N_2, 5% CO_2, and po2 ≤ 5 mm Hg.” The conditions seem like an anoxia model rather than hypoxia. The authors might wish to provide a previse % of each gas, as with 95%N_2 and 5% CO_2, there will be zero% O_2.

Results section: Rather than myocyte age or aging, it is better to state markers of aging in cardiac myocytes or a similar term (as markers are studied).

On p16 levels, if protein data are available, it will add to the robustness of the findings.

Likewise, if other markers of cell cycle regulations or senescence are analyzed, it would strengthen the data. (optional)

Linea 240-242: For these studies, we utilized the dual emission mitophagy reporter that fluoresces green with normal mitochondrial clearance but fluoresces yellow/red when mitophagy is impaired." It is better to state what biological trait or protein the green and yellow colors represent.

In Figures 2B and C, the Y axis should indicate fluorescence signal intensity.

An increase in p62 in the HPX group treated with NOB in Figure 4A is not evident. The blot contrasts with the bar graph data. The reviewer suggests revisiting the data and if p62 is unchanged, it should be stated as such.

Whenever an intervention is done, it would be informative to include data on efficiency and specificity (for example data on knockdown of ATG7 or RORalpha). Such blots, if available, should be incorporated into Figure 6C.

Regarding LC3, it is more informative to include data on LC3-II and LC3-I, as markers of autophagosomes.

Figure 6, panel C, bar graph labeled LC3. Should it be LC3-II or is it LC3-II and LC3-I?

The reviewer suggests using the conventional nomenclature. When referring to human gene names, all capital and in italics, and if referring to mouse genes, the first letter is capitalized and then small letters and all italics. Protein names all capital, regardless of human and mouse proteins, and not italics. Also instead of alpha, A should be used in RORalpha and not alpha for the gene's name (it should be Rora (in italics)). Alpha is fine for protein.

The reviewer suggests showing the individual data as dot plots (with mean or median and SD/SE) instead of bar graphs.

The graphic abstract gives the impression that hypoxia induces NOB. It should be revised to show that NOB activates RORalpha and inhibits hypoxia-induced autophagy. (Hypoxia suppresses RORa and NOB blokes this effect of hypoxia)

Authors' Response

Manuscript JCA-2023-31 by Eryn Kirshenbaum et al. entitled: Retinoic Acid-Related Orphan Receptors Regulate Autophagy and Cell Survival in Cardiac Myocytes during Hypoxic Stress.

The article describes a comprehensive study and provides data supporting the role of RORalpha in promoting autophagy, cell survival, mitochondrial function, and attenuating senescence in the cell culture system. Overall, the data support the conclusions of the study. It focuses on the flavonoid compound Nobiletin (NOB), which is considered an anti-inflammatory agent. The findings are consistent with the role of RORA in enhancing autophagy and lysosomal function and advancing the existing knowledge by providing new information.

The reviewer has the following suggestions:

One issue that the authors might wish to address is the specificity of the effects of NOB as it interacts with several other targets /pathways in addition to mitochondrial functions, such as inflammation through NFkB1, metabolism through PGC1a, and various forms of cell death. The multiplicity of the effects should be discussed.

We thank the reviewer for their important comment. We addressed the multifunctional effects of Nobiletin in the discussion:

"In summary, Nobiletin was previously shown to have multifunctional effects by interacting with several targets/pathways, such as N-methyl-D-aspartate (NMDA) receptor, NF-kB, Pparg coactivator 1 alpha (PGC1α) and Phosphoinositide 3-kinase (PI3K)(19). Furthermore, RORa is a positive regulator of the gene Basic Helix-Loop-Helix ARNT Like 1 (BMAL1), which is one of the core circadian genes(1), suggesting a role for Nobiletin in regluing circadian rhythms. Our findings reveal a novel signaling pathway that functionally links Nobiletin mediated RORa signaling to cellular quality control mechanisms involving autophagy activation and cardiac cell survival. Given that impaired mitochondrial function and cellular quality control mechanisms are commonly associated with myocardial infarction and advanced aging, our data highlight a critical connection between RORa and autophagy regulation of mitochondrial quality control. Hence, the findings of the present study suggest that Nobiletin may be an effective therapeutic intervention for restoring cellular quality control mechanisms and cardiac function during cardiac stress conditions in the aged myocardium."

The authors use a hypoxia model, which suppresses Rora transcript levels. Hypoxia also activates P53, which induces Rora. If data is available in the P53-RORalpha axis, it should be included. Otherwise, it should be mentioned in the discussion.

Thank you for this comment. We assume that the study mentioned by the reviewer is based on the important paper published in 2011 in Molecular Cel (https://doi.org/10.1016/j.molcel.2011.09.023). In this paper, the authors demonstrated that RORα is a direct target of p53 and that during DNA damage, RORα stabilizes and activates p53 in a HAUSP/Usp7-dependent manner, leading to increased apoptosis in Drosophila model system. Although this study provides an important regulatory link between RORα and p53, it does not describe this axis during hypoxic stress or in the context of the heart. Furthermore, although we did not examine p53 levels in our study, we did demonstrate a reduction in RORα levels during hypoxia. Therefore, we hypothesize that under the conditions tested in our study, the RORα-p53 axis may not play an important regulatory role. Hence, we believe that elaborating on the possible link between RORα and p53 in the discussion, may shift the focus of our study and confuse the readers, and

therefore we did not include this possible link in the discussion.

NOB and RORalpha are considered regulators of the circadian rhythm through CLOCK/BMAL, which may mediate some of the molecular effects. The authors may wish to comment.

We thank the reviewer for their important comment. Please see response to point 1 which includes discussion on the circadian role of Nobiletin and RORα.

Abstract: "RORα regulates autophagic processes linked to aging through activation with Nobiletin." The reviewer suggests changing "through activation" to "upon activation".

Thank you for this suggestion. The sentence has been changed accordingly.

Methods section: "into an air-tight chamber continually gassed with 95% N_2, 5% CO_2, and po2 ≤ 5 mm Hg." The conditions seem like an anoxia model rather than hypoxia. The authors might wish to provide a previse % of each gas, as with 95%N_2 and 5% CO_2, there will be zero% O_2.

Thank you for this comment. The hypoxia chamber was gassed with 95% N_2 only, which allows 5% O_2 in the chamber. The 5% CO_2 refers to the conditions of the incubator. We updated this information in the methods section.

Results section: Rather than myocyte age or aging, it is better to state markers of aging in cardiac myocytes or a similar term (as markers are studied).

Thank you for this comment. We changed the term to "markers of aging" as suggested.

On p16 levels, if protein data are available, it will add to the robustness of the findings.

Thank you for this comment. The reason we chose to show mRNA levels of p16 and not protein levels, is based on our previous observations that measurement of p16 mRNA in the heart is more reliable than its derived protein. This is supported by the paper by Hara et al. (DOI: 10.1128/MCB.16.3.859), showing that p16 mRNA has a very long half-life of greater than 24 hours, compared with p16 protein with half-life of 30 minutes to 3.5 hours. Given this unparalleled stability, RT-PCR assay for measuring p16 expression is more precise and reproducible compared to protein.

Likewise, if other markers of cell cycle regulations or senescence are analyzed, it would strengthen the data. (optional)

Thank you for this excellent suggestion, however, we did not look at other markers of cell cycle in this study as we wanted to focus on autophagic markers.

Linea 240-242: For these studies, we utilized the dual emission mitophagy reporter that fluoresces green with normal mitochondrial clearance but fluoresces yellow/red when mitophagy is impaired." It is better to state what biological trait or protein the green and yellow colors represent.

Thank you for this comment. To better clarify the interpretation of mitophagy clearance by the dual probe Mitokeima, we added the paragraph below to the results section and supported it with appropriate citations:

"For these studies, we utilized the dual emission mitophagy reporter MitoKeima that fluoresces green following lysosomal clearance of mitophagosomes (neutral pH) or fluoresces yellow/red when mitophagy is impaired (acidic pH). The size of puncta is another parameter for the status of mitophagy as indicated by large puncta when mitophagosomes accumulate due to disrupted mitochondrial – lysosomal clearance, or smaller puncta when mitochondrial clearance is normal(4,13). As shown in Figure 5, we observed a marked reduction in mitophagy, as indicated by the presence of large red fluorescent puncta in cardiac myocytes subjected to hypoxia compared to normoxic control

cells. Importantly, hypoxia-induced impairment of mitophagy was restored in cardiac myocytes treated with Nobiletin."

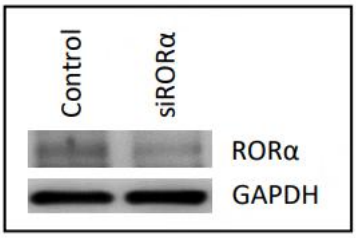
In Figures 2B and C, the Y axis should indicate fluorescence signal intensity.

We thank the reviewer for their suggestion, we changed the Y axis to indicate fluorescence signal intensity.

An increase in p62 in the HPX group treated with NOB in Figure 4A is not evident. The blot contrasts with the bar graph data. The reviewer suggests revisiting the data and if p62 is unchanged, it should be stated as such.

Thank you for pointing out to this oversight. Based on reviewer's comment, we decided to remove the p62 data from this paper.

Whenever an intervention is done, it would be informative to include data on efficiency and specificity (for example data on knockdown of ATG7 or RORalpha). Such blots, if available, should be incorporated into Figure 6C.



Thank you for this comment. The sequence for siRORα was adopted from DOI: 10.1038/s41419-021-04170-0, where its inhibitory function was well studied. However, to validate the inhibition of RORα in our study, we performed the western blot presented on the right.

Regarding validation of ATG7 knockdown, we showed the efficiency and specificity of this adenovirus in our previous publication (DOI: 10.1080/15548627.2021.1938913), which is also supported by the publication of Dr. Junichi Sadoshima who provided us with this adenovirus (https://doi.org/10.1172/JCI122035.). For the reason that the efficiency and specificity of knockdown of ATG7 and RORα is already published, we decided to exclude it from the paper.

Regarding LC3, it is more informative to include data on LC3-II and LC3-I, as markers of autophagosomes.

We agree with the reviewer that presenting both LC3-II and LC3-I could be more informative, however, since in our data LC3-I fit with the same trend as LC3-II, we decided to present LC3-II only and avoid space issues in the figures.

Figure 6, panel C, bar graph labeled LC3. Should it be LC3-II or is it LC3-II and LC3-I?

Thank you for pointing out to this oversight. We modified the label to LC3-II.

The reviewer suggests using the conventional nomenclature. When referring to human gene names, all capital and in italics, and if referring to mouse genes, the first letter is capitalized and then small letters and all italics. Protein names all capital, regardless of human and mouse proteins, and not italics. Also instead of alpha, A should be used in RORalpha and not alpha for the gene's name (it should be Rora (in italics)). Alpha is fine for protein.

We thank the reviewer for this suggestion. All gene and protein names have been modified according to the suggested nomenclature.

The reviewer suggests showing the individual data as dot plots (with mean or median and SD/SE) instead of bar graphs.

Thank you for this comment. All graphs have been changed to dot plots as suggested.

The graphic abstract gives the impression that hypoxia induces NOB. It should be revised to show that NOB activates RORalpha and inhibits hypoxia-induced autophagy. (Hypoxia suppresses RORa and NOB blokes this effect of hypoxia)

Thank you for this comment. We agree with the reviewer, we modified the graphical abstract better reflect the findings of our paper please see our response to Reviewer 1 point 1.