Peer Review File

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Reviewer A

The study by Yue Dai et al. Investigates the possible induction of pyroptosis in rat aortic vascular smooth muscle cells (VSMCs) by angiotensin II (Ang II) and the role of the NLRP3 signaling pathway in this process. In addition, the authors reviewed the studies on the effects and underlying mechanisms of recombinant tissue factor signaling inhibitor (rTFPI) in Ang II-induced VSMC pyroptosis. The results suggest that Ang II can trigger pyroptosis in VSMCs through activation of NLRP3, while rTFPI shows the ability to inhibit this process. The assumption used to design the study is very simple and the simplistic view is also evident in the introduction, which is very superficial in the way it summarizes the molecular mechanisms underlying vascular remodeling and the pathology associated with hypertension. Additionally, several relevant papers were missed.

1. Could the authors discuss the use of specific concentrations of Ang II, MCC950 and TFPI? It might be interesting to use an Ang II inhibitor for the same experiment.
Reply 1: We have completed the exploration of Ang II, MCC950 and TFPI concentrations in the previous pre-experiments, so we directly gave the effective concentrations in this paper. We can add relevant data on the selection of Ang II, MCC950 and TFPI concentrations if needed; It is a good proposition to use angiotensin inhibitors in trials ,we can continue to complete in future if we have the opportunity.
Changes in the text: None

2. Figure 5 D). In the Westron blot quantified of AngII+ MCC950, the band intensity may need to be reanalyzed.
Reply 2: Thank you for your suggestion. We re-analyzed the AngII+MCC950 protein band and get the same conclusions, and our final data are the final result of multiple trials, and the conclusion of the NLRP3 blot and GAPDH ratio is in line with the conclusion.
Changes in the text: None

3. The immunostaining images should be acquired at a higher resolution for better clarity.
Reply 3:Thank you for your advice. We have changed some of the immunostaining image.
Changes in the text:We re-selected a GSDMD-N immunofluorescence staining image in the hope that it would meet the requirements.(see Figure 4)

Reviewer B

This is an interesting paper showing that Ang II may induce pyroptosis in rat VSMCs by activating the inflammasome. Additionally the authors show that rTFPI can inhibit Ang II induced pyroptosis in VSMCs and this could be by inhibiting the NLRP3 pathway.

I have one main concern:

The authors have used 5 different experiment groups but this should have been 6. The NLRP3 inhibitor was used on it's own (a control) and with Ang II. Data is only shown for rTFPI + Ang II, there should be another control group with just rTFPI. Were these experiments carried out? If so please include the data in the graphs.
Reply 4：We have done many researches on low-dose rTFPI in the past experiments, and found that rTFPI separately has little effect on cells without any stimulation, so we did not add a separate rTFPI group in the follow-up experiment.
Changes in the text: None

This data was from rat VSMCs's, have you done these experiments in human cells? Would you expect the same result?
Reply 5：Thank you for your suggestion. We are also very interested in doing these experiments in human cells, but we didn't do it this time since we used the TFPI protein from human. We think it should be the same result. Meanwhile if we have the chance, we could do the experiments in future.
Changes in the text: None

Minor points:

1 For figures 1 and 5 please include n numbers used.
Reply 6：For figures 1 and 5， our trial was repeated at least 4 times, n=4.
Changes in the text: we added n=4 in the figure legends. (Please see Page12, line216 and Page19 , line317)

2 Please include arrows on Figure 4 to indicate the myofilaments, EM and various organelles mentioned in the text.
Reply 7:Thank you for advice. We have modified our images by adding annotations about myofilaments, endoplasmic reticulum, and Golgi apparatus.
Changes in the text: Please see figure 6 in page17 line14.

Reviewer C

Yue Dai et al. present a body of data to explore the effect of Angiotensin II on mediating pyroptosis in rat aortic SMCs. They define pyroptosis by using PI staining to identify cell membrane permeabilization and by quantifying key proteins involved in pyroptosis including pro-caspase 1, GSDMD-N, and IL-1B. The authors study the effect of the NLRP3 inflammasome in AngII-mediated pyroptosis using the pharmacological NLRP3 inhibitor MCC950. Finally, the significance of Tissue Factor Pathway Inhibitor in regulating AngII-mediated pyroptosis was assessed by adding recombinant TFPI. Overall, the experimental design of this manuscript is very sound and the authors do a good job explaining their rationale for their experiments. Mostly, the experiments address if AngII is directly inducing pyroptosis in vSMCs, and the addition of inhibitors for NLRP3 or rTPFI is a valid way to assess the requirement of these pathways in mediating the phenotypic changes in SMCs treated with AngII. Although the overall body of this manuscript shows interesting findings, this reviewer feels that major revisions are needed to more thoroughly substantiate the conclusions of the

manuscript.

Major Revisions Recommended:

1. Figure 1: the authors define cell viability using the CCK8 assay. They show that AngII leads to increased cell viability as compared to control, and AngII + MCC950 or AngII + rTFPI leads to decreased cell viability. While describing cell viability is satisfactory, the authors conclude in the Results Section for Figure 1 that AngII alone increases cell proliferation and AngII + MCC950 or AngII + rTFPI decrease cell proliferation. Cell proliferation is not directly measured by the CCK8 assay. If the authors wish to directly make conclusions regarding cell proliferation, it is recommended that alternate assays (BrdU or EdU staining, Ki67, MTT) be used. Further, Figure 1 would benefit from brighfield microsopy images to show the confluency of the vSMCs to support changes in proliferation.

Reply 8:Thank you for your advice! We supplemented the MTT test to detect cell proliferation, which was consistent with our overall test conclusions.

Changes in the text:Please see figure 2 in page12 line218.

2. Figure 3: The authors employ immunofluorescent staining for GSDMD-N in the experimental groups to directly test how AngII, AngII + MCC950, and AngII + rTFPI affect GSDMD-N protein expression. The red channel in Figure 4A is quite dim and makes it difficult for the audience to ascertain the specificity of the anti-GSDMD-N antibody used for this study. The red-staining in the AngII + rTFPI group looks like artifact. It is recommended to include a panel comparing AngII-stimulated SMCs stained with the primary GSDMD-N antibody vs AngII-stimulated SMCs stained with IgG isotype control, each then stained with the Alexa-594 secondary antibody to confirm the signal shown in Figure 4A is specific.

Reply 9: Thank you for your kind suggestion. Since the first author has graduated, we are unable to supplement the experiments. We were sure that our trials were authentic. We have re-selected the images, especially for the immunofluorescence of the AngII+rTFPI group.

Changes in the text: Please see figure 4 in page15 line 261.

Minor Revisions:

1. The first sentence of the second paragraph of the Introduction can be written more clearly. It is informal to end a sentence with "etc.".

Reply 10:Thank you for your advice. We have revised the sentence.

Changes in the text: we have modified our text as advised (see Page 5, line 78).

2. The Western Blot protocol in the methods section should be more thoroughly written. The authors should include the protocol they used for centfiguation of the protein lysate. The incubation time for the blocking step should be included. The dilution and incubation time of the secondary HRP-conjugated antibody should be included. The mass of protein lysate in micrograms should be included for how much protein was loaded into the acrylamide gel.

Reply 11:Thank you for your suggestion. We have enriched the western blot protocol in the methods section.

Changes in the text: Please see Page 9-10.

3. Figure 4: This figure would benefit from arrows being added to point out organelles,

myofilaments, and secreted products so that the audience has more assistance to analyze the data. Scale bars should be added that are easily readable. The authors should consider using ELISA assay to quantify secreted IL-1B.
Reply 12:Thank you for your suggestion. We added arrows to point out organelles, myofilaments, and secreted products. Scale bars are at the bottom of the image and can be zoomed in. We agree that using ELISA assay to quantify secreted IL-1B. Since the first author has graduated, we are unable to supplement the experiments. If given the opportunity, we will do that.
Changes in the text: Please see figure 5 in page16.

4. In the Discussion on line 297, the sentence describes pyroptosis as a "new" type of cell death. This sentence should be re-phrased as pyroptosis is not new (it has always been there), just newly characterized.
Reply 13: Thank you for your advice. We have revised the sentence.
Changes in the text: Please see page19 line331-332.

5. The authors should explicitly state if the data showed in Figures 1, 2, 3, and 5 were normally distributed. The authors use the ANOVA test to compare means, but it is not stated if the data shown in each figure achieved normal distribution. If the data are not normally distributed, nonparametric tests should be used. To that end, it is recommended that the data in all graphs are shown with single data points so readers can directly evaluate the spread of the data and see each individual data point.
Reply 14: Thank you for your suggestion. Our data in Figures were normally distributed. We have revised in the method.
Changes in the text: Please see page 10 line 195.