Author’s response to reviews

Title: Molecular mechanisms of hydrogen sulfide against uremic accelerated atherosclerosis through cPKCβII/Akt signal pathway

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Version: 1 Date: 24 Jul 2019

Author’s response to reviews:

Dear editor,

We would like to thank you and the reviewers for the support you’ve shown us, your constructive suggestions and detailed corrections for our manuscript entitled “Molecular mechanisms of hydrogen sulfide against uremic accelerated atherosclerosis through cPKCβII/Akt signal pathway” (No.: BNEP-D-19-00336) are very helpful. In order to reach the quality for publication, we have asked a native English speaker to review this manuscript. The detailed modifications and point-by-point responses are listed below.

Best regards,

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Sebastjan Bevc (Reviewer 1):

1. Authors should explain the abbreviation also in the abstract.

Response: Thanks for your constructive suggestion. We have added the explain of the abbreviations to the abstract in the revised manuscript. As shown in the abstract, cPKCβII is the abbreviation of conventional protein kinase C βII, UAAS is the abbreviation of uremia accelerated atherosclerosis and CSE is the abbreviation of cystathionine-γ-lyase.

2. Authors should avoid repeating the text; like in first paragraph of background and discussion.

Response: Thanks for your valuable suggestion. We have gone through the content of the manuscript carefully and deleted the duplicate parts.

3. Authors should present the exact values of "p-values", especially those which were statistically significant.

Response: Thanks for your precious opinion. We have presented the exact values of "p-values", in the revised manuscript.

4. The part "mice aortas were disarticulated from the root to the radial artery bifurcation" should be rephrased or more clearly written.

Response: Thanks for your valuable suggestion. We have rephrased this part in the revised manuscript.

5. In figure 3 why p-Akt level (%) is presented only?

Response: Thanks for your precious opinion. Studies have shown that Akt activation manifested as a change in phosphorylation levels. It was shown that the level of Akt phosphorylation was changed during the development and progression of atherosclerosis (Circ Res. 2017 Oct 27;121(10):1153-1167). In this study, protein levels of Akt, phosphorylated-Akt (p-Akt) of total protein in mice aorta were analyzed by Western blot. Therefore, p-Akt level (%) is presented in this manuscript.
Marco Colucci (Reviewer 2):

**MAJOR COMMENTS**

1. The reviewer strongly advises deep review of the whole Materials and Methods section: the study design is not described; no reason is given for the various groups, and moreover treatment with various substances is not explained (NaHS? PPG?), nor these substances are mentioned in the introduction. It is stated that these substances were injected intraperitoneally after surgery; how long after surgery? Did they receive single or multiple injection? What dosage of the various substances was administered? The administration of these substances did happen before or after the 6 weeks of high fat diet? Moreover, it is stated that "When the modeling was completed, mice were sacrificed": when the modelling was considered complete? Please review.

Response: Thank you very much for your precious comments and suggestions. We have deeply reviewed of the whole Materials and Methods section and described more details of the materials and methods in the revised manuscript. NaHS (a donor of H2S) and L-cys (a precursor of H2S generation) were used as a source of H2S, and PPG is a selective inhibitor of CSE, which is an important H2S-synthesizing enzyme. After the operation, L-cys(50mg/kg/d), NaHS(56μmol/kg/d) and PPG(37.5mg/kg/d) were intraperitoneally injected in UAAS+L-cys group, UAAS+NaHS group, and UAAS+PPG group respectively for 6 weeks. The mice were given a high-fat diet during the operation and intraperitoneal injection. After the intraperitoneally injection for 6 weeks, the modeling was completed and mice were sacrificed.

2. The review recommends general revision of written English for the whole paper. Some suggestions are made as minor comments; however the whole text would benefit a revision, in order to enhance its accessibility and enabling the reader to focus on the good job done.

Response: Thank you very much for your precious comment. In order to reach the quality for publication, we have asked a native English speaker to review this manuscript. Extensive revisions were made to our previous manuscript.

3. The study performed seems very interesting, with noteworthy results; however, extensive revisions are recommended before the manuscript could be considered for publishing.

Response: Thank you for your valuable suggestion. In order to reach the quality for publication, we have asked a native English speaker to review this manuscript. Meanwhile, we examined the content of this article carefully and made extensive revisions to the manuscript.
MINOR COMMENTS

1. Page 3, line 25-26: "The damage of arterial endothelium is recognized as initial factor for cardiovascular, and vascular endothelial damage plays an important role in the occurrence and development of cardiovascular diseases". Please review and reformulate the sentence.

Response: Thanks for your precious opinion. We have reviewed and reformulated the sentence in the revised manuscript. This sentence was modified to "The damage of arterial endothelium is recognized as initial factor for atherosclerosis and plays an important role in the occurrence and development of cardiovascular diseases".

2. Page 3, line 30: As CSE/H2S system seems to be protective against UAAS, the reviewer suggests to modify the sentence as follows: "Our group found that there was a relationship between endogenous cystathionine-γ-lyase/hydrogen sulfide (CSE/H2S) system and the risk of cardiovascular disease in maintenance hemodialysis patients".

Response: Thanks for your precious comment and suggestion. We have modified the sentence according to your suggestion.

3. Please provide more information about the role of Akt phosphorylation in the Introduction.

Response: Thanks for your constructive suggestion. We have provided more information about Akt phosphorylation in the Introduction part in the revised manuscript.

4. In the Introduction, the Authors stress the role of PKC activation in favouring UAAS; however, it is also stated that "PKC inhibitors reduce the expression of endothelial nitric oxide synthase (eNOS)", which seems to be pretty in contrast to what has been previously explained. Please provide further explanations or address this topic in the Discussion section.

Response: Thanks for your precious comment. In the Introduction section, we stated that "PKC inhibitors reduce the expression of endothelial nitric oxide synthase (eNOS)" according to the study conducted by Tabit CE et al. In the study conducted by Tabit CE et al, the subjects were people with diabetes mellitus, and it was found that inhibition of PKCβ with LY379196 reduced basal eNOS activation but improved insulin-mediated eNOS activation. We have reformulated the sentence in the revised manuscript. The conclusion of this article is not in contrast to what we have explained in the Introduction section and the results of our study. In our study, we found that the activation of cPKCβII in UAAS group was higher than sham group and the eNOS activation in UAAS group was lower than sham group. Meanwhile, we provided further explanations in the Discussion section. In the study conducted by Das SK et al, it was also shown
that cPKCβII could decrease eNOS activation in the wounded tissues of diabetic mice, which is consistent with our results.

5. Please state the aim of the study at the end of Introduction paragraph, before starting describing methods.

Response: Thanks for your precious suggestion. The aim of this study was to identify the possible molecular mechanism of the CSE/H2S system and cPKCβII/Akt signaling pathway on atherosclerosis development in UAAS mice. We have stated the aim in the revised manuscript.

6. Please review and reformulate the description of animal surgery, as by now it seems more an operative instruction than a method description.

Response: Thanks for your precious comment. We have reviewed and reformulated the description of animal surgery. At 8 weeks of age, the mice were anesthetized with sodium pentobarbital by intraperitoneal injection (0.06g/kg). A longitudinal incision of approximately 1cm was made at the lower lateral of the left costovertebral angle of the mice. Then the left kidney was exposed and the renal capsule was isolated. A total of 2/3 of the kidney tissue was removed from the upper and lower poles of the left kidney, then a gelatin sponge was used to compress and stop bleeding. After that, the muscles and skin were sutured. After 2 weeks, the right kidney was exposed in the same way, and the right renal pedicle was ligated. After confirming complete ligation, the right kidney was excised. In the sham group, the kidneys were only exposed during the two operations and no surgical resection was performed.

7. Page 5, line 27: correct with "stored".

Response: Thanks for your valuable suggestion. We have corrected with "stored" in the revised manuscript.

8. Page 5, line 31: what kind of tissue debris?

Response: Thanks for your precious comment. It should be "aortic tissue debris". We have corrected it in the revised manuscript.
9. Methods, Western blot: please review and reformulate, as it seems more an operative instruction than a method description.

Response: Thanks for your valuable suggestion. We have reviewed and reformulated the Western blot part.

10. Please correct the 3.1, 3.2, 3.3 and 3.4 paragraph titles with "aorta" or "aortic tissue".

Response: Thanks for your precious suggestion. We have corrected the titles with "aorta" in the revised manuscript.

11. Page 7, line 7: please correct with "statistical significance".

Response: Thanks for your valuable suggestion. We have corrected with "statistical significance" in the revised manuscript.

12. Please review the whole 3.3 paragraph, which is currently quite difficult to understand (also because of the lack of Methods description, maybe).

Response: Thanks for your constructive suggestion. We have reviewed the whole 3.3 paragraph. We added more information about the role of Akt phosphorylation in the Introduction and Western blot parts. In this study, protein levels of Akt, phosphorylated-Akt (p-Akt) of total protein in mice aorta were analyzed by Western blot. It was shown that the levels of Akt phosphorylation were different between groups.

13. Page 7, line 58: "End-stage renal disease patients exist high morbidity and high mortality in cardiovascular disease". Please reformulate the sentence.

Response: Thanks for your valuable suggestion. We have reformulated the sentence to "Patients with end-stage renal disease have increased cardiovascular morbidity and mortality".

14. Please provide p-values of statistically significant results.

Response: Thanks for your precious opinion. We have presented the exact values of "p-values", with statistically significant in the revised manuscript.