Peer Review File

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REVIEWER A

Summary:
In this paper, the authors aim to describe the effects of hydrogen sulfide on autophagy in vascular endothelial cells. Through gain- and loss-of-function experiments the authors explore how Sirt1 regulation of FOXO1 influences autophagy, and relate this to autophagy-mediated protection against apoptosis.
Overall, the study is well carried out and needs only minor revisions.

Minor comments:
-The manuscript needs a grammatical check throughout its entire length. Specifically, there is a reocurring lack of congruence between singular/ plural forms of verbs and nouns making the manuscript difficult to read clearly. For example, p. 4, lines 3-4 of the introduction: "Autophagy is a dynamic process involving the formation of biphasic membrane autophagosomes, which isolates cytoplasmic components and fuses with lysosomes, eventually degrading the cargo in autolysosomes…."

Response:
We have tried our best to eliminate grammatical errors and colloquial, non-scientific language to improve the writing in our whole manuscript, the modified parts are marked in blue.

-The authors explore the role of a Sirt1-FOXO1-axis in H2S-mediated autophagy but do not comment on whether other FOXO transcription factors might be involved. Were any of the other FOXOs examined in their study and how can the authors rule out that the effects they see are specifically due to FOXO1 actions?

Response:
Thanks for your suggestions. Autophagy is a very complex process involving many factors and signalling pathways. Indeed, important roles of the Sirt1-FoxO axis in modulating autophagy have been documented in previous studies in cardiomyocytes and skeletal muscle cells(1-3), FoxO1 and FoxO3 regulate autophagy in skeletal and cardiac muscles by activating genes that are involved in autophagosome formation(4, 5), although the results appear controversial. Our data showed that H_2S may enhance FoxO1 functions in endothelial cells via the upregulation of Sirt1, whereas FoxO1 activation in turn promotes the autophagic response. Specific FoxO1-siRNA ensured that FoxO1 played an important role in H_2S-induced endothelial autophagy. The mechanisms by which FoxO modulates autophagy appear to be complex(1, 6), whereas transcriptional regulation of autophagic genes may not be able to fully explain the effect

of FoxO. We didn't examine other FoxO transcription factors, such as FoxO3, FoxO4 and FoxO6 in our study. More studies are needed to be further elucidate the mechanisms by which the Sirt1/FoxO pathway regulates autophagy in endothelial cells.

-When comparing the western blots in figure 2 A and B, it appears that GYY4137 treatment + scrambled siRNA induces a more robust response in both Sirt1 and LC3II protein levels than in GYY4137 + vector-treated cells, which could indicate that the scrambled siRNA itself has effects on autophagy. Did the authors include a lipofectamine-only control to ensure that there were no off-target effect of the scrambled siRNA controls?

Response:

Thanks for your reminder. The sequence of scrambled siRNA and Sirt1-siRNA were cited from Picard F's article(7), and they were used by many other papers(8), the effectiveness of Sirt1-siRNA and the off-target effect of scrambled siRNA have been validated. We also use lipofectamine-only control to detect the off-target effect of the scrambled siRNA and the effectiveness of Sirt1-siRNA. As shown in Figure S1, there was no off-target effect of Scrambled-siRNA. Moreover, Figure 2A and 2B in the manuscript were two independent experiments, which need to be analyzed independently.

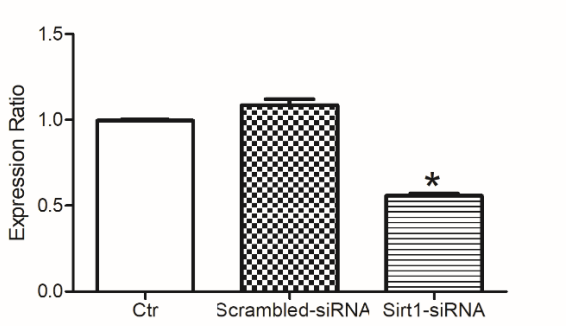

Figure S1 The off-target effect of Scrambled-siRNA and the effectiveness of Sirt1-siRNA detected by q-PCR. After treating HUVEC with lipofectamine (Ctr), Scrambled-siRNA and Sirt1-siRNA, the total RNA of the cells was extracted and q-PCR was performed. Scrambled-siRNA had no effect on Sirt1-mRNA level, Sirt1-siRNA significantly reduced Sirt-mRNA level of HUVECs (*$P < 0.05$ versus Scrambled-siRNA, Data are expressed as the mean ± S.E.M.).

Reviewer B

In this article, the authors investigated the effects of H_2S on autophagy in human vascular Endotelial Cells. Impaired autophagy has strong clinical relevance in a number of diseases, including cancer and cardiovascular disfunctions, and the possibility to modulate autophagy with chemicals/drugs represents a very intriguing therapeutic opportunity which deserves to be further explored.

In this study it has been reported a novel mechanism mediated by Sirt1/FOXO1

responsible for H2S-induced autophagy in ECs. In addition, it has been showed that H2S-induced autophagy protected ECs from Ox-LDL induced apoptosis through activation of Sirt1. In the conclusion, the authors report that H2S exerts its vascular-protective actions through Sirt1-mediated autophagy in ECs.
The study is well done, the methodology is adequate, with several functional assays and signaling study that enrich the work. However, there are some points that should be clarified and some simple experiments done before to definitively accept the paper.

Minor points:

- The role of H2S in autophagy induction through other mechanisms it has been already reported in several cellular models, and these references should be cited and shortly discussed in the introduction: DOI: 10.1002/jcp.27797; 10.1111/jcmm.13223; 10.1159/000493824.; 10.1186/s13578-016-0099-1.

Response:

Thank you for your suggestions. We added some discussion about the role of H_2S in autophagy and cited above references in the introduction.

Changes on the text: Please see page 3 line 12-17.

- Considering the above-mentioned refs, please discuss why in some time H2S exerts a protective function by inhibiting autophagy and other time occurs by over stimulating autophagy. This is very important to discuss since autophagy has often a dual role and both its inhibition/stimulation can result in cell death and cytoprotective function.

Response:

H_2S plays important roles in a wide range of physiological and pathological conditions, including glucose metabolism, energy production, ischemia-reperfusion (I/R) injury, vascular relaxation, angiogenesis, neuronal activity, and atherosclerosis. Studies indicated that endogenously produced and/or exogenously administered H_2S could exhibit two obviously opposite effects on autophagy in a number of disease models, which may be attributed to the concentration, time frame, and reaction time of H_2S, as well as the differences between disease stages or models.

Autophagy is an evolutionarily conserved process which degrades dysfunctional organelles and damaged proteins to promote cell survival under stress conditions(9). Autophagy maintains a balance between protein degradation and synthesis, which can protect or destroy cell. The effect of autophagy is determined by specific pathological processes. Beyond a certain range, it eventually results in cell death, with the excessive accumulation of autophagosomes(10). Therefore, the different effects of H_2S on autophagy when H_2S exerts its protective function may be partly dependent on the state of autophagy process during H_2S stimulation, the different influential factors, and the different levels of basal autophagy in different types of cells or animal models.

- Please detail along the text why GYY4137 is an H2S inducer. May this molecule also interfere with other processes? What it the specificity of this drug? Please provide reference.

Response:

In 2008, Moore and co-workers reported GYY4137, a Lawesson's reagent derivative, as a water-soluble H_2S donor. Since then, this compound has been widely used in the field(11). The specificity of this drug was high. GYY4137 releases H_2S very slowly and hydrolysis is believed to be the mechanism. In vitro experiments showed that 4.17 ± 0.5 nmol/25 min of H_2S was released from 1 mM of GYY4137. H_2S release from this donor is pH dependent with greater release at acidic pH (3.0) and less release at neutral or basic pH (7.4 and 8.5). Some recent studies also suggested that thiols may trigger H_2S release from GYY4137(12). The slow release of H_2S is believed to be a major advantage of GYY4137.

Almost all the research on GYY4137 is based on its nature as a H_2S donor. Other H_2S-related biological actions have also been observed for GYY4137, such as cyto-protection(13), anticancer effects both in vitro and in vivo(14), anti-inflammatory effects(15, 16), vasodilation effects in aortic rings and so on(17). The function of its by-products (after H_2S release) have not been well identified, and the by-products' activities are unclear.

- Page 12: correct the nomenclature of FOXO to "FOXO1".

Response:

We have corrected the "N.S, not significant versus FOXO1-siRNA" to "N.S, not significant versus FoxO1-siRNA" in page 18 line 14.

Changes on the text: Please see page 18 line 14.

- The authors should cite the references describing the mechanisms of nuclear translocation of FOXO1. I suggest these ones doi: 10.1042/BJ20040167; doi: 10.1042/BJ20031450

Response:

We discussed the mechanisms of nuclear translocation of FoxO1 and cited above two articles in the "Discussion" section, which were used as reference of 31 and 32.

Changes on the text: Please see page 12 line 21-24.

Major points:

- Fig. 1D: if RED puncta indicate autophagosomes + lysosomes, what is the needed to overlap the two colours (green + red)? Moreover, the image is not convincing since in the CTRL I see a lot of green puncta.

Response:

We are sorry for the confusion made by our negligence of detailed statement. The red puncta and green puncta represent different meanings in individual images and merged images. In the acidic environment of lysosomes, GFP loses its fluorescence while mRFP retains its fluorescence. So, green LC3 puncta mainly indicate autophagosomes, whereas red LC3 puncta indicate both autophagosomes and autolysosomes in the individual images. Red puncta that overlaid with the green puncta and appeared as yellow in the merged images, indicating autophagosomes, and free red puncta in the merged images indicated autolysosomes. We have added detailed illustration in the text.

Autophagy is a dynamic process involving the formation of biphasic membrane autophagosomes, which isolates cytoplasmic components and fuses with lysosomes, eventually degrading the cargo in autolysosomes, this process is termed as autophagy flux. In physiological state, there is a certain level of autophagy in the cells, so there will be a certain amount of red and green fluorescence in the cells of the control group.

Changes on the text: Please see page 9 line 1.

- FIG. 1C: Why GYY4137 + 3-MA does not induce LC-3 as occur for GYY4137 + Bafalomicin, considering that both are inhibitors of autophagy flux?

Response:

3-Methyladenine (3-MA) was a PI3K inhibitor, it was a widely used inhibitor of autophagy via its inhibitory effect on class III PI3K. 3-MA inhibits autophagy activation and reduces LC3BII formation. While when the cells were stimulated with bafilomycin, the intracellular autophagosome degradation was inhibited, and the observed changes of LC3B-II represented changes in the number of autophagosomes. After autophagy activation, LC3BII content will be further increased based on the use of bafilomycin.

- FIG. 3C: the authors show that SIRT1 over-expression promote nuclear translocation of FOXO1 in the nuclei. However, a western blot of nuclear extracts should be done in the same conditions that those used in FIG. 3C

Response:

Thanks for your suggestion. We have performed the western blot to demonstrate the nuclear translocation of FoxO1 in the same conditions that those used in Figure 3C, and we have shown that Sirt1 over-expression promote translocation of FoxO1 from cytoplasm to nuclear, and those effects were abolished when Sirt1 was downregulated by Sirt1-siRNA. We have added the western blot images, the corresponding methods and figure legends in our manuscript.

Changes on the text: Please see Figure 3, “Material and methods” section page 5 line 24-25, “Results” section page 10 line 1, “Figure legends” section page 18 line 7-9.

- A cell viability assay of GYY4137 + Baf should be required to determinate the pro-survival/pro cell death role of autophagy.

Response:

Both Baf and 3-MA were inhibitors of autophagy, we used 3-MA as an autophagy inhibitor to demonstrate the prosurvival role of GYY4137 induced autophagy in Figure 5D.

Reviewer C

This manuscript investigated the effects of H2S on autophagy in human vascular ECs.the advices are as follow：

1. The apoptosis should be detected by TUNEL and/or AnnexinV/PI；

Response:

Thanks for your suggestion. We have performed flow cytometric analysis (AnnexinV/PI) to detected the apoptosis in the same condition that those used in Figure 5A, and we got the same conclusion as Figure 5A. We have added the flow cytometric images, the corresponding methods and figure legends in our manuscript.

Changes on the text: Please see Figure 5, “Material and methods” section page 7 line 5-11, “Results” section page 10 line 24-25, “Figure legends” section page 18 line 23-25.

2.The transcriptional level activity of FOXO1 should be detected by EMSA and/or dual-luciferase reporter assay；

Response:

Thanks for your suggestion. FoxO regulates the expression of multiple autophagy-related genes, including LC3, ATG5, ATG7, ATG8, ATG12, Bnip3, and Beclin-1(2, 18, 19). Moreover, there is evidence showing that FoxO can modulate the transcription of some autophagic genes by directly binding to the promoter region, such as FoxO1 binding to ATG12 promoters in cardiomyocytes(2), in response to stress, acetylated FOXO1 (Ac-FOXO1) binds to ATG7, an E1-like protein, to induce autophagy leading to cell death(20, 21), and the interaction of Ac-FOXO1 and ATG7 was related to the induction of autophagy in rats with cerebral ischemia/reperfusion injury(22). Our data suggest that H_2S may enhance FoxO1 functions in endothelial cells via upregulation of Sirt1, whereas FoxO1 activation in turn promotes the autophagic response through modulating the protein expression of ATG5 and Beclin-1. Studies have shown that FoxO1 increased the mRNA expression levels of Beclin-1, Atg5 in endothelial cells(23), which was consistent with our results.

It is noted that the mechanisms by which FoxO modulates autophagy appear to be

complex(1, 6),whereas transcriptional regulation of autophagic genes may not be able to explain the effect of FoxO sufficiently. Therefore, more studies are needed for us to further elucidate the mechanisms by which the Sirt1/FoxO1 pathway regulates autophagy induced by H_2S in endothelial cells in the future.

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