Peer Review File
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Reviewer A

Comment 1: The author applied TRPA1/TRPV1 antagonist in the acute ozone-exposed model. One can expect that antagonism of these proteins might lead to the reduction or suppression of their function, as has been shown in the manuscript. However, the protein level of TRPA1 and TRPV1 was also significantly reduced in both A967079 and AMG9810 treatments, respectively. Is the reduction of TRPA1 expression after A967079 or TRPV1 expression after AMG9810 secondary to suppression of ozone effects, or a standalone effect of the antagonist?

Reply 1: We thank the reviewer's good comment. TRPA1 and TRPV1 are co-expressed in nociceptive C fibers innervating the airways, and are also found in airway epithelial cells (AECs) and airway smooth muscle(ASM) cells[1], and both channels can be activated by exogenous environmental irritants, such as diesel exhaust particles (DEP), cigarette smoke, PMs and polycyclic aromatic hydrocarbons (PAH) [1,2]. Moreover, TRPV1 and TRPA1 have significant interactions and synergy [2]. A recent study has demonstrated that Liquiritin inhibited capsaicin- and allylisothiocyanate-evoked TRPV1 and TRPA1 whole-cell currents, and suppressed LPS-induced increases in TRPV1 and TRPA1 protein expression in the lung tissue. TRPA1 and TRPV1 antagonists HC030031 and capsazepine reduced TRPV1 and TRPA1 expression at both protein and mRNA levels in THP-1 monocytes [3]. Our recent study using PM2.5-induced lung inflammation and BHR in mouse showed that these two antagonists inhibited the protein levels of TRPV1 and TRPA1 in lung tissue [4]. The activation of TRPA1 and TRPV1 leads to neurogemic inflammation, while inhibition of neurogenic inflammation by the channel inhibitor may reduce the protein expression of channels. From our study, we see that treatment with TRPA1/TRPV1 antagonist not only inhibits the channel's activity, but also suppresses the channel's expression in the ozone model.

1.Dietrich A, Steinritz D, Gudermann T: Transient receptor potential (TRP) channels as molecular targets in lung toxicology and associated diseases. Cell Calcium 2017, 67:123-137.

2.Akopian AN, Fanick ER, Brooks EG. TRP channels and traffic-related environmental pollution-induced pulmonary disease. Semin Immunopathol. 2016 May;38(3):331-8.

3.Liu Z, Wang P, Lu S, Guo R, Gao W, Tong H, Yin Y, Han X, Liu T, Chen X, Zhu MX, Yang Z. Liquiritin, a novel inhibitor of TRPV1 and TRPA1, protects against LPS-induced acute lung injury. Cell Calcium. 2020,88:102198.

4.Xu M, Zhang Y, Wang M, Zhang H, Chen Y, Adcock IM, Chung KF, Mo J, Zhang Y, Li F. TRPV1 and TRPA1 in Lung Inflammation and Airway Hyperresponsiveness Induced by Fine Particulate Matter (PM2.5). Oxid Med Cell Longev. 2019, 2019: 7450151.

Changes in the text: *see Page 21, line445-463.*

Comment 2: On determining the dose of TRPA1/TRPV1 antagonist (both at 30 mg/kg), was there any preliminary data or previously published data supporting the utility of this dose?
Reply 2: Thanks for the comment. The doses of TRPA1 and TRPV1 antagonist were determined from our preliminary results and previous publication. Xu M, Zhang Y, Wang M, Zhang H, Chen Y, Adcock IM, Chung KF, Mo J, Zhang Y, Li F. TRPV1 and TRPA1 in Lung Inflammation and Airway Hyperresponsiveness Induced by Fine Particulate Matter (PM2.5). Oxid Med Cell Longev. 2019, 2019: 7450151. This is now added in Methods section.
Changes in the text: see Page 8, line160.

Comment 3: Histological evaluations of the lung slice might be more straightforward and helpful in determining lung inflammation post-ozone exposure. In addition to the mRNA level of inflammatory cytokines in lung tissue, I suggest that the authors should add histological images to reflect the inflammatory responses of the lung.
Reply 3: We thank the reviewer for his/her suggestion. The effect of ozone-induced airway/lung inflammation with histology has been reported. Sunil et al showed the H&E tissue sections of rats after ozone exposure reflecting ozone-induced injury and lung inflammation [1]. Our previous study showed inflammatory response in lung tissue as indicated by increased inflammatory scores in H&E lung sections (images provided) in single ozone exposure and multiple ozone exposure [2]. A similar finding was also reported later by other group [3]. The inhibitory effect of A967079 and AMG9810 on lung inflammation has been demonstrated in PM2.5-induced model in our previous work [4]. It is our omission that we have not performed H&E staining as we did not include this protocol in our research plan. We would keep this in mind and would provide such histological images in the future studies.
1.Sunil VR, Vayas KN, Massa CB, Gow AJ, Laskin JD, Laskin DL. Ozone-induced injury and oxidative stress in bronchiolar epithelium are associated with altered pulmonary mechanics. Toxicol Sci. 2013,133(2):309-19.
2. Li Feng, Zhang Pengyu, Zhang Min, Bao Aihua, Chen Yuqing, Tang Yueqin, Zhou Xin. The Effects of Acute and Chronic Ozone Exposure on Inflammation,Structure and Function in Murine Lung. Chinese Journal of Respiratory and Critical Care Medicine, 2014, 13(3): 295-299.
3.Michaudel C, Fauconnier L, Julé Y, Ryffel B. Functional and morphological differences of the lung upon acute and chronic ozone exposure in mice. Sci Rep. 2018,8(1):10611.
4.Xu M, Wang L, Wang M, Wang H, Zhang H, Chen Y, Wang X, Gong J, Zhang JJ, Adcock IM, Chung KF, Li F. Mitochondrial ROS and NLRP3 inflammasome in acute ozone-induced murine model of airway inflammation and bronchial hyperresponsiveness. Free Radic Res. 2019,53(7):780-790.

Reviewer B

Comment 1: The title of Results section should be a sentence which is a brief summary of that part.
Reply 1: We thank the reviewer for his/her very helpful comment. We revised each sub-title in Results section as suggested.
Changes in the text: see Page11-16, line237，259，288，305，317，354.

Comment 2: The protein levels of inflammatory cytokines should be shown for they may show different trends with that of mRNA expressions.
Reply 2: We agree with the reviewer that the mRNA levels of inflammatory cytokines could be different from the protein levels. We reported that single ozone exposure to mice induced increases in protein levels of KC, TNF-α, IL-6 and IL-1β in BAL fluid with ELISA in 2015[1]. Our recent study also showed that acute ozone exposure increased BALF inflammatory cytokines including IL-1α, IL-1β, KC, and IL-6 in BAL fluid with ELISA [2]. We fully understand that it would help convince the reviewer if we measured the protein levels of such cytokines with ELISA kits. Due to the lockdown of metropolitan Shanghai for COVID-19, the reagents shipment disrupted, our lab is closed, and we presently could not obtain these ELISA kits for measurement. We would recognize this as a limitation and would provide such data in the future studies.
1.Zhang P, Li F, Wiegman CH, Zhang M, Hong Y, Gong J, Chang Y, Zhang JJ, Adcock I, Chung KF, Zhou X. Inhibitory effect of hydrogen sulfide on ozone-induced airway inflammation, oxidative stress, and bronchial hyperresponsiveness. Am J Respir Cell Mol Biol. 2015,52(1):129-37.
2.Xu M, Wang L, Wang M, Wang H, Zhang H, Chen Y, Wang X, Gong J, Zhang JJ, Adcock IM, Chung KF, Li F. Mitochondrial ROS and NLRP3 inflammasome in acute ozone-induced murine model of airway inflammation and bronchial hyperresponsiveness. Free Radic Res. 2019,53(7):780-790.

Comment 3: Pathological images such as H＆E stain of lung sections of ozone-induced and A967079/AMG9810 treated mice should be shown.
Reply 3: We heartfully thank the reviewer's suggestion. The effect of ozone-induced airway/lung inflammation with hitology has been reported. Sunil et al showed the H&E tissue sections of rats after ozone exposure reflecting ozone-induced injury and lung inflammation [1]. Our previous study showed inflammatory response in lung tissue as indicated by increased inflammatory scores in H&E lung sections (images provided) in single ozone exposure and multiple ozone exposure [2]. A similar finding was also reported later by other group [3]. It is our omission that we have not performed H&E staining as we did not include this protocol in our research plan. We would recognize this as a limitation and would provide such histological images in future studies.
1.Sunil VR, Vayas KN, Massa CB, Gow AJ, Laskin JD, Laskin DL. Ozone-induced injury and oxidative stress in bronchiolar epithelium are associated with altered

pulmonary mechanics. Toxicol Sci. 2013,133(2):309-19.
2. Li Feng, Zhang Pengyu, Zhang Min, Bao Aihua, Chen Yuqing, Tang Yueqin, Zhou Xin. The Effects of Acute and Chronic Ozone Exposure on Inflammation,Structure and Function in Murine Lung. Chinese Journal of Respiratory and Critical Care Medicine, 2014, 13(3): 295-299.
3.Michaudel C, Fauconnier L, Julé Y, Ryffel B. Functional and morphological differences of the lung upon acute and chronic ozone exposure in mice. Sci Rep. 2018,8(1):10611.

Comment 4: It showed that A967079 and AMG9810 seemed to have similar effects on ozone-induced airway inflammation and hyperresponsiveness. Please explain the reason for using these two inhibitors. In other words, how do TRPA1/TRPV1 serve as a 'pathway' in this study?

Reply 4: We thank the reviewer's good comment. TRPA1 and TRPV1 are co-expressed in nociceptive C fibers innervating the airways, and are also found in airway epithelial cells (AECs) and airway smooth muscle(ASM) cells[1]. Acute ozone exposure directly could stimulate TRPA1 cation channels and then activate bronchopulmonary C-fibers, which is an important trigger of airway inflammation and BHR [2,3]. It has been reported that ozone selectively stimulates TRPA1 not TRPV1[3]. However, TRPV1 is an important mediator for ozone-exacerbated allergic asthma in mice [4,5]. In the previous study, we found that both TRPV1 and TRPA1 channels played important roles in PM2.5-induced lung inflammation and BHR [6] and both mediated CSE-induced bronchial and alveolar epithelial cells injury via modulation of oxidative stress, inflammation and mitochondrial [7]. To further examine if TRPA1 and TRPV1 play important roles in single ozone-induced airway inflammation and BHR, we used these two inhibitors in our study.

1.Dietrich A, Steinritz D, Gudermann T: Transient receptor potential (TRP) channels as molecular targets in lung toxicology and associated diseases. Cell Calcium 2017, 67:123-137.
2.Lambert JA, Song W. Ozone-induced airway hyperresponsiveness: roles of ROCK isoforms. American journal of physiology Lung cellular and molecular physiology. 2015,309(12):L1394-L7.
3.Taylor-Clark TE, Undem BJ. Ozone activates airway nerves via the selective stimulation of TRPA1 ion channels. J Physiol. 2010, 588(Pt 3):423-33.
4.Li J, Chen Y, Chen QY, Liu D, Xu L, Cheng G, Yang X, Guo Z, Zeng Y. Role of transient receptor potential cation channel subfamily V member 1 (TRPV1) on ozone-exacerbated allergic asthma in mice. Environ Pollut. 2019, 247:586-594.
5.Chen Y, Wu X, Yang X, Liu X, Zeng Y, Li J. Melatonin antagonizes ozone-exacerbated asthma by inhibiting the TRPV1 channel and stabilizing the Nrf2 pathway. Environ Sci Pollut Res Int. 2021,28(42):59858-59867.
6.Xu M, Zhang Y, Wang M, Zhang H, Chen Y, Adcock IM, Chung KF, Mo J, Zhang Y, Li F. TRPV1 and TRPA1 in Lung Inflammation and Airway Hyperresponsiveness Induced by Fine Particulate Matter (PM2.5). Oxid Med Cell Longev. 2019, 2019:

7450151.
7.Wang M, Zhang Y, Xu M, Zhang H, Chen Y, Chung KF, Adcock IM, Li F. Roles of TRPA1 and TRPV1 in cigarette smoke -induced airway epithelial cell injury model. Free Radic Biol Med. 2019,134:229-238.
Changes in the text: see Page 6-7, line131-137

Comment 5: The work of Taylor-Clark TE and Undem BJ showed that ozone directly stimulates TRPA1(J Physiol. 2010 Feb 1;588(Pt 3):423-33. doi:10.1113/jphysiol.2009.183301.), which would be better to be cited and discussed in the Discussion section.
Reply 5: We thank the reviewer. We cited this reference as suggested.
Changes in the text: see Page21, line445-449.

Comment 6: Such numbers of figures is not needed. It would be better to merge the related images together to make it 5-6 figures.
Reply 6: We thank the reviewer for his/her comment and suggestion. We combined and revised the figure as suggested. There are six figures now.
Changes in the text: see Figure 1-Figure 6.

Comment 7: In the Methods part of Abstract section, the description of detailed dose and concentration is not needed.
Reply 7: We deleted the dose of inhibitor in the abstract as suggested.
Changes in the text: see Page3, line51-53.

Comment 8: Please indicate the numbers of mice investigated in the legends of Figure 1 and 2.
Reply 8: We added the number of mice in Figure legend 1 and 2 as suggested.
Changes in the text: see the legends of Figure 1 and 2.
Comment 9: Please unify the format of in the manuscripts and Figure legends.
Reply9: Thanks for the comment. We revised the format of 'RL' throughout our manuscript as suggested.
Changes in the text: see Page8, line168-169; Page11, line231, 238; Page12, line247-253; Page18, line390; Page28, line645-653.

Comment 10: The strain of mice should be written as 'C57BL/6'.
Reply 10: Thanks for the comment. We revised it as 'C57BL/6' throughout our manuscript as suggested.
Changes in the text: see Page 3, line51; Page 7, line150.

Comment 11: Some abbreviations are not listed as full names when they first appear.
Reply 11: Thanks for the comment. We revised our text as advised.
Changes in the text: see Page6, line113-114,118-119; Page9, line194-196;

Comment 12: Pay attention to the correct use of grammer and tense. The English

grammar and style correction is recommended.
Reply 12: Thanks for the comment. We revised our manuscript as suggested.
Changes in the text: see Page3, line47-65; Page3, line96-107; Page6-7, line131-142,152-153; Page8, line160; Page10, line207-208; Page11, line224-231; Page13, line267-286; Page14, line297-297; Page15, line311-313; Page18, line376-394; Page19, line397, 402, 410; Page20, line419, 428, 429, 434.

Reviewer C

Comment 1: The manuscript was poorly written, with too many grammatical errors and typos, such as "The expression of key inflammatory mediators was assessed by RT-qPCR, mitochondrial proteins by Western blot analysis and of oxidative stress/antioxidant markers using commercial assays"; "…. antagonist in in vivo in mice immediately before or after ozone exposure…."; "…. Briefly, lung tissues were homogenized to extract protein and MRC complex activity detected…."; "although there was a greater range in the response…".
Reply 1: We have revised the manuscript as advised with checking the grammar and typos.
Changes in the text: see Page3, line47-65; Page3, line96-107; Page6-7, line131-142,152-153; Page8, line160; Page10, line207-208; Page11, line224-231; Page13, line267-286; Page14, line297-297; Page15, line311-313; Page18, line376-394; Page19, line397, 402, 410; Page20, line419, 428, 429, 434.

Comment 2: The study lacked mechanistical study about how the inhibitors worked in vivo. What effects of these inhibitor on smooth muscle cells and lung epithelial cells in vitro? What their effects on cytoplasmic calcium levels in cells? What signaling pathways were involved?
Reply 2: We thank the reviewer for the comment and suggestion. In our previous study, both TRPA1 and TRPV1 inhibitors prevented CSE-induced injury in A549 and Beas-2B cells via modulation of oxidative stress, inflammation and mitochondrial damage. We did observe that CSE-induced increases in Ca2+ levels in A549 and Beas-2B cells were inhibited by pretreatment with A967079 or AMG9810 alone and their combination [1]. A recent study showed TRPA1 inhibitor (HC-030031) inhibited acute CSE-induced Ca2+ influx in primary human ASM cells [2]. Our previous work demonstrated that in PM2.5-induced lung inflammation and BHR murine model, mitochondrial dynamics and the TLR4/NF-κB and NLRP3/caspase-1 pathways could be involved in the effects of TRPA1/TRPV1 inhibitors [3]. MAPK/NF-κB signaling may be involved in the activation of TRPA1 induced by LPS, resulting in lung inflammation [4]. We agree with the reviewer that an in vitro study using airway smooth muscle cells or lung epithelial cells and RNA-Seq could provide more information on the signaling pathways. Our preliminary data showed that ozone exposure activates steroid biosynthesis, PI3K-Akt and coagulation cascade pathways in Beas-2B cells via RNA-Seq. We recognize this lack of mechanistic studies as a limitation and would plan them in the future.

1.Wang M, Zhang Y, Xu M, Zhang H, Chen Y, Chung KF, Adcock IM, Li F. Roles of TRPA1 and TRPV1 in cigarette smoke -induced airway epithelial cell injury model. Free Radic Biol Med. 2019,134:229-238.
2.Lin J, Taggart M, Borthwick L, Fisher A, Brodlie M, Sassano MF, Tarran R, Gray MA. Acute cigarette smoke or extract exposure rapidly activates TRPA1-mediated calcium influx in primary human airway smooth muscle cells. Sci Rep. 2021,11(1):9643.
3.Xu M, Zhang Y, Wang M, Zhang H, Chen Y, Adcock IM, Chung KF, Mo J, Zhang Y, Li F. TRPV1 and TRPA1 in Lung Inflammation and Airway Hyperresponsiveness Induced by Fine Particulate Matter (PM2.5). Oxid Med Cell Longev. 2019,2019: 7450151.
4.Ko HK, Lin AH, Perng DW, Lee TS, Kou YR. Lung Epithelial TRPA1 Mediates Lipopolysaccharide-Induced Lung Inflammation in Bronchial Epithelial Cells and Mice. Front Physiol. 2020,11:596314.

Comment 3: Methods and statistics were not well described in figure legends, such as n number, statistics in Figure 1, 2 and other figures. The blots in Figure 5 and 6 were not labeled for sample ID, protein marker and protein size.
Reply 3: We understand the reviewer's concern. N numbers were added to Figure 1 and Figure 2. As each dot represents a number (n=8 in all figures), we would not like to add n number to every figure legend. Except for Figure 1A and 1B in which two-way ANOVA was used, one-way ANOVA was used throughout all figures. The statistics has been stated in Statistics section. It is not necessary to add statistics to every figure legend. Protein markers and protein size are added in figure5.
Changes in the text: see figure legend 1 and 2, Figure 4 and 5

Comment 4: There was no discussion about how these altered proteins were related and affected the lung inflammation and acute lung injury.
Reply 4: We thank the reviewer for this constructive suggestion. We understand that mitochondrial dysfunction induced by ROS could lead to the cleavage of fusion proteins and upregulation of fission proteins, which would further generate more mitochondrial ROS, aggravating airway inflammation and lung injury [1]. We also understand that the activation of DRP1 triggered by mitochondrial dysfunction induces PINK1–PARK2-mediated mitophagy [2], and enhanced mitophagy induced by cigarette smoke would worsen airway epithelial cells and mitochondria injury [3].
We have added some discussion in the text as suggested by the reviewer.
1.Cid-Castro C, Hernandez-Espinosa DR, Moran J. ROS as Regulators of Mitochondrial Dynamics in Neurons. Cell Mol Neurobiol. 2018,38(5):995-1007.
2. Mizumura K, Cloonan SM, Nakahira K, Bhashyam AR, Cervo M, Kitada T, Glass K, Owen CA, Mahmood A, Washko GR, Hashimoto S, Ryter SW, Choi AM. Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD. J Clin Invest. 2014,124(9):3987-4003.
3. Zhang M, Shi R, Zhang Y, Shan H, Zhang Q, Yang X, Li Y, Zhang J. Nix/BNIP3L-dependent mitophagy accounts for airway epithelial cell injury induced

by cigarette smoke. J Cell Physiol. 2019,234(8):14210-14220.

Changes in the text: see Page22, line 467-483

Comment 5: Why did TRPA1/TRPV1 inhibitor suppressed their expression? How to test that their activities were suppressed? Any evidence in intro study? Immunostaining should be provided for the expression of TRPA1/TRPV1 and other proteins in cells and lung tissues.

Reply 5: We thank the reviewer's good comment. TRPA1 and TRPV1 are co-expressed in nociceptive C fibers innervating the airways, and are also found in airway epithelial cells (AECs) and airway smooth muscle(ASM) cells[1], and both channels can be activated by exogenous environmental irritants, such as diesel exhaust particles (DEP), cigarette smoke, PM and polycyclic aromatic hydrocarbons (PAH) [1,2]. Indeed, TRPV1 and TRPA1 have significant interactions and synergy [2]. A recent study has demonstrated that Liquiritin inhibited capsaicin- and allylisothiocyanate-evoked TRPV1 and TRPA1 whole-cell currents, and suppressed LPS-induced increases in TRPV1 and TRPA1 protein expression in the lung tissue. TRPA1 and TRPV1 antagonists HC030031 and capsazepine reduced TRPV1 and TRPA1 expression at both protein and mRNA levels in THP-1 cells [3]. Our previous study showed that these two antagonists inhibited the protein levels of TRPV1 and TRPA1 in lung tissue in PM2.5-induced lung inflammation and BHR in mouse [4]. The activation of TRPA1 and TRPV1 leads to neurogenic inflammation, while inhibition of neurogenic inflammation by the channel inhibitor may reduce the protein expression of channels. From our study, we see that treatment with TRPA1/TRPV1 antagonist suppresses the channel's expression in the ozone model.

We understand that calcium flux assay using whole-cell voltage clamp recordings was used to measure the functional activities of TRPA1 and TRPV1. In our previous work, we did observe that CSE-induced increases in Ca2+ levels in A549 and Beas-2B cells were inhibited by pretreatment with A967079 or AMG9810 alone and their combination [5].

We agree with the reviewer that the immunostaining is an important way of measuring and localizing the protein in tissue. In our previous study, we have performed IHC staining of TRPA1 and TRPV1 in mouse lung tissue [4].

As pointed out by the reviewer, there are limitations in the study, i.e. lack of in vitro model and lack of lung section analysis, though these have been performed separately in our previous work[4,5]. We recognize and state these limitations at the end of the manuscript. We will make a careful research plan for the future study.

1.Dietrich A, Steinritz D, Gudermann T: Transient receptor potential (TRP) channels as molecular targets in lung toxicology and associated diseases. Cell Calcium 2017, 67:123-137.

2.Akopian AN, Fanick ER, Brooks EG. TRP channels and traffic-related environmental pollution-induced pulmonary disease. Semin Immunopathol. 2016,38(3):331-8.

3.Liu Z, Wang P, Lu S, Guo R, Gao W, Tong H, Yin Y, Han X, Liu T, Chen X, Zhu MX, Yang Z. Liquiritin, a novel inhibitor of TRPV1 and TRPA1, protects against LPS-induced acute lung injury. Cell Calcium. 2020,88:102198.

4.Xu M, Zhang Y, Wang M, Zhang H, Chen Y, Adcock IM, Chung KF, Mo J, Zhang Y, Li F. TRPV1 and TRPA1 in Lung Inflammation and Airway Hyperresponsiveness Induced by Fine Particulate Matter (PM2.5). Oxid Med Cell Longev. 2019, 2019: 7450151.
5. Wang M, Zhang Y, Xu M, Zhang H, Chen Y, Chung KF, Adcock IM, Li F. Roles of TRPA1 and TRPV1 in cigarette smoke -induced airway epithelial cell injury model. Free Radic Biol Med. 2019,134:229-238.
Changes in the text: see Page21, line 445-463