Activation of Endothelial NLRP3 Inflammasomes associated with Acid Sphingomyelinase-dependent Formation of Membrane Raft Redox Signaling Platforms

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ABSTRACT

There is evidence that endothelial NLRP3 inflammasomes are activated by atherogenic stimuli such as cholesterol crystals (CHC), 7-ketocholesterol (7- Keto) and visfatin. However, the mechanism by which endothelial NLRP3 inflammasomes remains poorly understood. The present study hypothesized that acid sphingomyelinase (ASM)-mediated ceramide and associated membrane redox signaling platforms may be one of the triggering mechanisms of NLRP3 inflammasomes. We first demonstrated that 7-Keto or CHC markedly increased the formation and activation of NLRP3 inflammasomes in mouse carotid arterial endothelial cells (CAECs), as shown by increased colocalization of NLRP3 with ASC or caspase-1, caspase-1 activity and IL-1β levels. This NLRP3 inflammasome formation and activation were markedly attenuated by caspase-1 inhibitor YVAD, ASC siRNA, ASM inhibitor, amitriptyline or its siRNA and CAECs from ASM-/-. Furthermore, in CAECs with NLRP3 inflammasome formation, we found that membrane raft (MR) clustering with NADPH oxidase subunits was remarkably increased as shown by more than 10 folds enhancement of CTXB and gp91phox or p47phox, indicating the formation of MR redox signaling platforms, which was further confirmed by inhibitory effects of lipid raft disruptor (MCD), ROS scavenger (TEMPOL+catalase) and TXNIP inhibitor (verapamil) on 7-Keto or CHC-induced increase in caspase-1 activity and IL-1β production. These results provide the first direct evidence showing that the activation of endothelial NLRP3 inflammasomes by 7-Keto or ChC may be triggered by ROS derived from MR redox signaling platforms. (supported by NIH grants HL057244, HL-75316 and DK54927).

BACKGROUND

- Endothelial NLRP3 inflammasomes activation has been considered as an early cellular responses to intiating cellular injury or inflammation in response to the atherogenic stimuli such as cholesterol crystals (CHC), 7-ketocholesterol (7- Keto). However, the mechanism by which endothelial NLRP3 inflammasomes are activated remains poorly understood.

- The present study was designed to test the hypothesis that acid sphingomyelinase (ASM)-mediated ceramide production and associated membrane redox (MR-Redox) signaling platforms are one of the triggering mechanisms to activate NLRP3 inflammasomes.

METHODS

Mouse Coronary Arterial Endothelium Primary Culture. C57BL/6J mice or ASM KO mice (8-12 weeks of age) were anesthetized and the carotid arteries were isolated and cut pieces into complete mouse endothelial cell medium (Cellbiologics). After 5 days, the half culture medium was changed fresh medium every two days for a week, then once or twice each week until the cells grow to confluence..

Immunofluorescent staining. Cells were grown on cover slips and fixed in PBS containing 4% paraformaldehyde for 10 minutes. Donkey serum (5%) (Sigma, St. Louis, MO) was included in all blocking and primary and secondary antibody buffers. Coverslips were incubated with primary antibodies overnight at 4°C. Secondary antibodies were Alexa Fluor-conjugated (Invitrogen, Inc., Grand Island, NY). Coverslips were mounted in Vectashield reagent containing DAPI (Vector Laboratories, Inc., Burlingame, CA). The fluorescent images were obtained using a confocal laser scanning microscope (Fluoview 1000, Olympus, Japan) and then analyzed using Image J software.

FLICA, Caspase-1 activity, and IL-1β production. Cell lysate was collected for FLICA assay and caspase-1 activity assay (Caspase-1 Colorimetric Assay Kit, BioVision). Cultured medium was collected for IL-1β production assay by using commercially available kits (Bender Medsystems; R&D Systems).

RESULTS

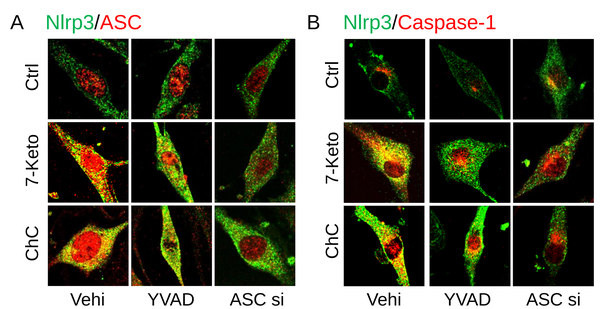

Figure 1. Co-localization of ASC, Caspase-1 and Nlrp3 in MECs. (A) Representative confocal fluorescent images showed 7-Keto or ChC stimulation induced the increases and co-localization Nlrp3 and ASC. In addition, the co-localization between Nlrp3 and Caspase-1 was also increased by those stimulators. However, caspase-1 inhibitor Z-YVAD-FMK (YVAD) or silencing ASC gene by AC siRNA transfection (ASCsi) attenuated the inflammasomes complex formation.

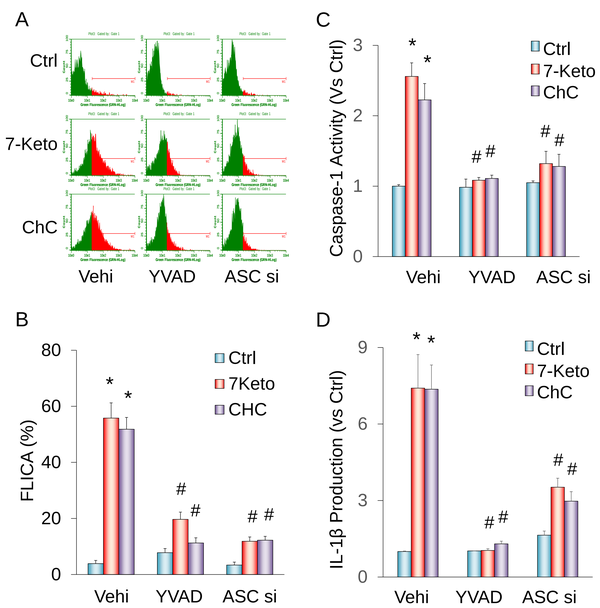

Figure 2. Inflammasome activation by 7-Keto and ChC in MECs. (A) Representative flow cytometry histogram for FLICA assay (active capase-1 assay) and the summery data (B) showed that capspase-1 activity was significantly enhanced after 7-Keto or ChC treatment. Likewise, the similar result was found by caspase-1 colorimetric assay (C). However, such enhanced caspase-1 activity was inhibited by the pretreatment of YVAD or ASC siRNA. Furthermore, a large amount of IL-1β was secreted after those stimulations and it was blocked by YVAD or ASC siRNA. n=6 batches of cells, * P<0.05 vs. Ctrl Vehi group; # P<0.05 vs. 7-Keto or ChC Vehi groups..

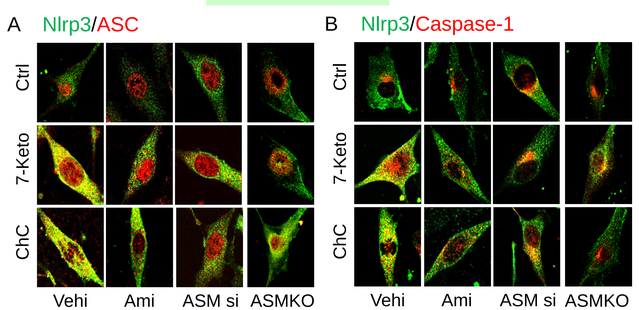

Figure 3. Effect of ASM on Nlrp3 inflamasome complex formation in MECs. (A, B) Representative confocal fluorescent images showed that 7-Keto or ChC-induced co-localization between Nlrp3 and ASC or Nalpr3 and caspase was attenuated by ASM inhibitor amitriptyline (Ami) or ASM siRNA. Furthermore, in isolated coronary endothelial cells from ASM KO mice, the co-localization among those inflammasome components was also abolished.

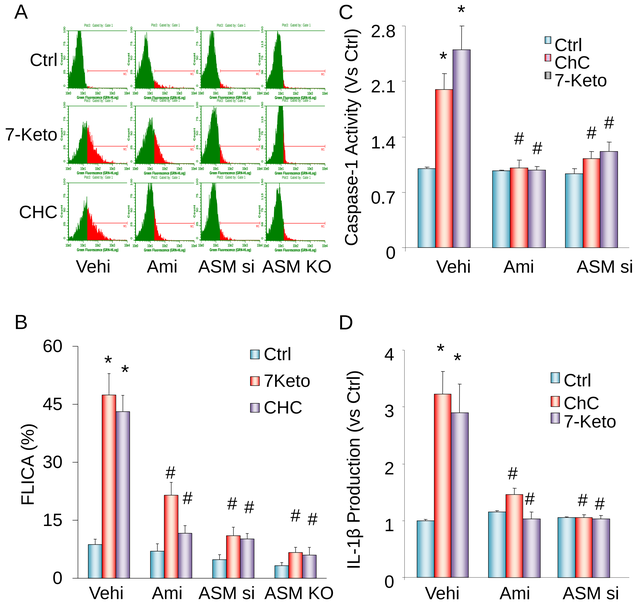

Figure 4. 7-Keto and ChC induced-Nlrp3 inflamasome activation is mediated by ASM in MECs. (A) Representative flow cytometry histogram for FLICA assay and the summery data (B) revealed that inhibition of ASM by Ami, ASM siRNA or ASM gene knock-out attenuated the Nlr3 inflammasomes activation by 7-Keto or CHC. In addition, Measurement of caspase-1 activity (C) and IL-1β production (D) showed reduced inflammasome activation by 7-Keto or ChC in the presence of amitriptyline (Ami) or ASM siRNA. n=6 batches of cells, * P<0.05 vs. Ctrl Vehi group; # P<0.05 vs. 7-Keto or ChC Vehi groups.

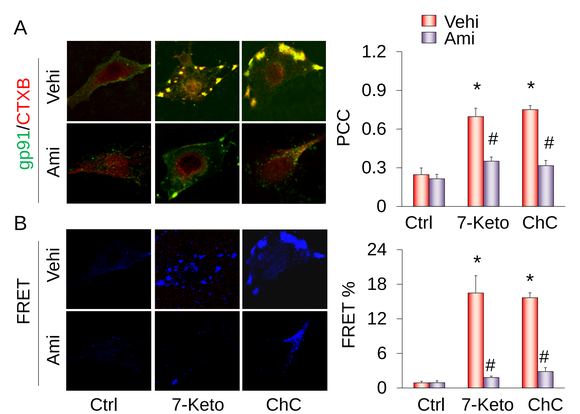

Figure 5. MR-Redox signaling by 7-Keto and ChC in ECs. Confocal microscopy analysis (A) and FRET (B) revealed that the co-localization between lipid raft (CTXB-Alexa 488) and gp91conjugated with Alexa 555 was significantly enhanced on the plasma membrane by 7-Keto or ChC and it was blocked by ASM inhibitor amitriptyline. n=4 batches of cells, * P<0.05 vs. Ctrl Vehi group; # P<0.05 vs. 7-Keto or ChC Vehi groups.

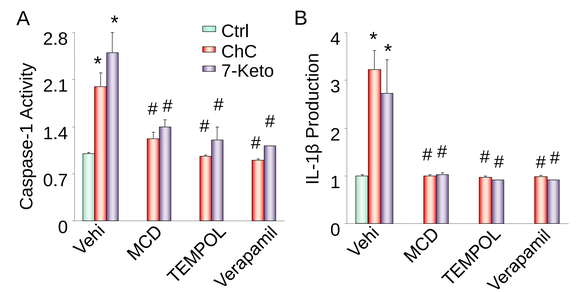

Figure 5. Effect of MR-Redox signaling blockade on inflammasome activation by 7-Keto and ChC in ECs. Measurement of caspase-1 activity (A) and IL-1β production (B) showed that 7-Keto or ChC-induced inflammasome activation was attenuated in the presence of lipid raft disruptor MCD (100 μM), SOD mimetic TEMPOL (100 μM) and TXNIP inhibitor, verapamil (50 μM). n=4 batches of cells, * P<0.05 vs. Ctrl Vehi group; # P<0.05 vs. 7-Keto or ChC Vehi groups.

CONCLUSION

These results suggest that 7-Keto or CHC-induced NLRP3 inflammasome activation in ECs may be triggered by ROS derived from MR redox signaling platforms associated with ceramide derived from ASM.