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Research Paper

Endothelial NLRP3 inflammasome activation and arterial neointima formation associated with acid sphingomyelinase during hypercholesterolemia

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ABSTRACT

The NLRP3 inflammasome has been reported to be activated by atherogenic factors, whereby endothelial injury and consequent atherosclerotic lesions are triggered in the arterial wall. However, the mechanisms activating and regulating NLRP3 inflammasomes remain poorly understood. The present study tested whether acid sphingomyelinase (ASM) and ceramide associated membrane raft (MR) signaling platforms contribute to the activation of NLRP3 inflammasomes and atherosclerotic lesions during hypercholesterolemia. We found that 7-ketocholesterol (7-Keto) or cholesterol crystal (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, enhanced caspase-1 activity and elevated IL-1β levels, which were markedly attenuated by mouse *Asm* siRNA, ASM inhibitor- amitriptyline, and deletion of mouse *Asm* gene. In CAECs with NLRP3 inflammasome formation, membrane raft (MR) clustering with NADPH oxidase subunits was found remarkably increased as shown by CTXB (MR marker) and $gp91^{phox}$ aggregation indicating the formation of MR redox signaling platforms. This MR clustering was blocked by MR disruptor (MCD), ROS scavenger (Tempol) and TXNIP inhibitor (verapamil), accompanied by attenuation of 7-Keto or ChC-induced increase in caspase-1 activity. In animal experiments, Western diet fed mice with partially ligated left carotid artery (PLCA) were found to have significantly increased neointimal formation, which was associated with increased NLRP3 inflammasome formation and IL-1β production in the intima of $Asm^{+/+}$ mice but not in $Asm^{-/-}$ mice. These results suggest that *Asm* gene and ceramide associated MR clustering are essential to endothelial inflammasome activation and dysfunction in the carotid arteries, ultimately determining the extent of atherosclerotic lesions.

1. Introduction

The inflammasomes are an intracellular machinery responsible for the activation of inflammation in variety of tissues or organs [1]. Among different types of inflammasomes, the NLRP3 inflammasome has been well characterized, which consists of a proteolytic complex formed by Nlrp3, the adaptor protein ASC, and caspase-1. Caspase-1 is activated when the inflammasome complex is formed to produce active IL-1β and IL-18 by cleavage of their precursors. NLRP3 acts as the sensory component to recognize both endogenous and exogenous danger signals [2,3], when ASC and caspase-1 are recruited to form a protein complex, where caspase-1 is activated [3–7]. The active caspase-1 not only proteolytically cleaves IL-1β and/or IL-18 into their biologically active form, but also produces other uncanonical effects such as release of damaging molecules like damage-associated molecular patterns (DAMPs), cell pyroptosis and alterations of cell membrane permeability, turning on the inflammatory response and directly inducing cell dysfunction or injury. There are increasing evidences that the activation of NLRP3 inflammasomes may not only contribute to autoinflammatory disorders, but also to a number of metabolic diseases including gout, silicosis, diabetes, liver toxicity, Alzheimer's disease and atherosclerosis [5,8–15]. In macrophages, NLRP3 inflammasome activation is critical for the foam cell formation and other atherosclerotic lesions upon proatherogenic stimuli such as cholesterol crystals (ChC) [13,16]. More interestingly, some non-atherogenic endanger factors also activate NLRP3 inflammasomes including adenosine triphosphate

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(ATP), uric acid and DAMPs [4,11,15–22], which may enhance the susceptibility to atherosclerosis or other vascular diseases. However, it remains unclear how NLRP3 inflammasomes in endothelial cells (ECs) are activated by proatherogenic stimuli and how activated NLRP3 inflammasomes lead to endothelial injury and ultimate atherosclerotic lesions.

In this regard, it was shown in vascular ECs, membrane rafts (MRs) and their temporal-spatial organization within caveolae are reported to involve in signaling of vascular endothelial growth factors (VEGF) [23], nitric oxide synthase [24], H_2O_2 [25], and prostanoid receptor activation [26]. Downstream effector response to MRs clustering in these ECs includes receptor autophosphorylation, cAMP production, caspase activation, decreases or increases in nitric oxide (NO), reorganization of the actin cytoskeleton and Ca^{2+} mobilization [23,27–29]. Functionally, the MR-mediated signaling may contribute to the regulation of important endothelial functions such as endothelial barrier function [30], endothelium-dependent vasodilator or constrictor response [31–35], endothelial metabolic function [35,36], and its antithrombolic functions [37]. We have demonstrated that MRs clustering occurred in arterial ECs [35] upon stimulation of different agonists such as FasL, TNF-α, OxLDL, visfatin and endostatin induced aggregation of NADPH oxidase (NOX) subunits such as $gp91^{phox}$ and $p47^{phox}$ into MR clusters, whereby NOX activity markedly increased. This membrane MR-NOX cluster or complex that possesses redox signaling function has been referred to as an MR redox signaling platform [35,38,39]. ASM has been shown to importantly participate in the formation of this redox signaling platform in ECs [34,35,40] which is associated with lysosome trafficking and fusion to MR area via a SNARE-centered exocytic machinery [41,42]. However, the role of ASM-induced MR clustering in NLRP3 inflammasome activation remains unknown.

The present study hypothesized that ASM and its product of sphingomyeline, ceramide via the formation of MR redox signaling platforms mediate the activation of NLRP3 inflammasomes and thereby result in endothelial dysfunction and athersogenesis. To test this hypothesis, we first determined whether the NLRP3 inflammasome is activated in response to cholesterol crystal (ChC) and 7-ketocholesterol (7-Keto) in ECs from *Asm* wild type and gene knockout mice. In the in vivo animal experiments, we examined whether endothelial NLRP3 inflammasome activation associated with enhanced ceramide production via ASM contributes to atherosclerotic lesions in the carotid arteries. Our results demonstrate that ASM and ceramide-associated MR clustering with NOX subunits in Ecs is essential to endothelial inflammasome activation and dysfunction in the carotid arteries, which determines the extent of neointima formation in the carotid arteries during PLCA mouse model.

2. Material and methods

2.1. Cell culture and treatments

The mouse carotid arterial endothelial cells were isolated and characterized as described earlier [43]. For the proatherogenic stimulation, cells were treated with 7-Keto (10 ng/ml) or ChC (0.5 mg/ml) and then incubated for overnight. In case of inhibitors used, the cells were pretreated with amitriptyline (20 μmol/L), methyl-β-cyclodextrin (MCD, 1 mmol/L), Tempol (0.1 mmol/L) and verapamil (50 μmol/L) for 30 min.

2.2. RNA interference of Asm gene

Small interference RNAs (siRNAs) were purchased from Santa Cruz. The sequence for *Asm* siRNA is: 5′- CACGTGGATGAGTTTGAGGT-3′ which was confirmed to be effective in silencing *Asm* gene in different cells by the company. The scrambled small RNA (AATTCTCCGAACGTGTCACGT) has been also confirmed as non-silencing double stranded RNA and was used as control in the present study. Transfection of siRNA was performed using the siLentFect Lipid Reagent (Bio-Rad, CA, USA) according to the manufacturer's instructions.

2.3. Confocal microscopic analysis

For confocal analysis of inflammasome molecules, cultured CAECs were grown on glass coverslips, stimulated or unstimulated, fixed in 4% paraformaldehyde in phosphate-buffer saline (PFA/PBS) for 15 min. After being permeablized with 0.1% Triton X-100/PBS and rinsed with PBS, the cells were incubated overnight at 4 °C with goat anti-NLRP3 (1:200, Abcam, MA) and rabbit anti-ASC (1:50, Enzo, PA) or rabbit anti-caspase-1 (1:100, Abcam, MA). To colocalize inflammasome molecules in the mouse carotid artery, double-immunofluorescent staining was performed using frozen tissue slides. After fixation, the slides were incubated overnight at 4 °C with goat anti-NLRP3 (1:200) and rabbit anti-ASC (1:50) or anti-caspase-1 (1:100). After washing, these slides probed with primary antibodies were incubated with Alexa-488- or Alexa-555-labeled secondary antibodies for 1 h at room temperature. The slides were mounted and subjected to examinations using a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan), with photos being taken and the colocalization of NLRP3 with ASC or caspase-1 analyzed by the Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). The summarized colocalization efficiency data was expressed as Pearson correlation coefficient (PCC) as we described previously [44,45].

2.4. Immunofluorescent microscopic analysis of MR clusters

CAECs were grown on poly-L-lysine–coated glass coverslips. After fixation with 4% PFA, cells were incubated with Alexa Fluor 488 conjugated Cholera toxin B (Alexa488-CTXB, 2 μg/ml, 2 h, Molecular Probes, CA), which binds with the MR-enriched ganglioside G_{M1}. For dual-staining detection of the colocalization of MRs with $gp91^{phox}$, the cells were first incubated with Alexa488-CTXB and then with anti-$gp91^{phox}$ (1: 200, BD Biosciences, CA), respectively, which was followed by corresponding Alexa555-conjugated secondary antibodies (1: 500, Invitrogen, NY). Then, the colocalization were visualized with confocal microscopy [43,46].

2.5. Caspase-1 activity and IL-1β production assay

After 7-Keto and ChC treatment with or without prior inhibitor, cells were harvested and homogenized to extract proteins for caspase-1 activity assay using a commercially available kit (Biovision, CA). The data was expressed as the fold change compared with control cells. In addition, the cell supernatant was also collected to measure the IL-1β production by a mouse IL-1β ELISA kit (Bender Medsystems, CA) according to the protocol described by the manufacturer.

2.6. FLICA staining

During the last hour of incubation, cells were labeled with FAM-YVAD-fmk caspase-1 FLICA™ kit (Immunochemistry, Bloomington, IN), which binds activated caspase-1. Flow cytometric analysis was performed according to manufacturer's manual. In brief, cells were washed two times with PBS and then incubated with 1 × FLICA for 1 h followed by two washes and analyzed with a Guava EasyCyte (Guava Technologies, Hayward, CA).

2.7. Endothelial permeability measurement

CAECs were cultured in 24-well transwell plates and treated as indicated for 12 h. The transwell inserts were moved into non-used wells with 200 μl fresh media. 100 μl Fluorescein isothiocyanate (FITC)–dextran (10 kDa, Invitrogen) solution was added into each