Endothelial Acid Sphingomyelinase Promotes NLRP3 Inflammasome and Neointima Formation During Hypercholesterolemia

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Abstract The NOD-like receptor pyrin domain 3 (NLRP3) inflammasome is activated during atherogenesis, but how this occurs is unclear. Here, we explored the mechanisms activating and regulating NLRP3 inflammasomes via the acid sphingomyelinase (ASM)-ceramide signaling pathway. As a neointima formation model, partial left carotid ligations were performed on endothelial cell (EC)-specific ASM transgene mice (Smpdl\(^{1\text{rg}}\)/EC\(^{\text{cre}}\)) and their control littermates (Smpdl\(^{1\text{rg}}\)/WT and WT/WT) fed on the Western diet (WD). We found neointima formation remarkably increased in Smpdl\(^{1\text{rg}}\)/EC\(^{\text{cre}}\) mice over their control littermates. Next, we observed enhanced colocalization of NLRP3 versus adaptor protein ASC (the adaptor molecule apoptosis-associated speck-like protein containing a CARD) and caspase-1 in the carotid ECs of WD-treated Smpdl\(^{1\text{rg}}\)/EC\(^{\text{cre}}\) mice but not in their control littermates. In addition, we used membrane raft (MR) marker flotillin-1 and found more aggregation of ASM and ceramide in the intima of Smpdl\(^{1\text{rg}}\)/EC\(^{\text{cre}}\) mice than their control littermates. Moreover, we demonstrated by in situ dihydroethidium staining, carotid intimal superoxide levels were much higher in WD-treated Smpdl\(^{1\text{rg}}\)/EC\(^{\text{cre}}\) mice than in their control littermates. Using ECs from Smpdl\(^{1\text{rg}}\)/EC\(^{\text{cre}}\) mice and WT/WT mice, we showed ASM overexpression markedly enhanced 7-ketocholesterol (7-Ket)-induced increases in NLRP3 inflammasome formation, accompanied by enhanced caspase-1 activity and elevated interleukin-1β levels. These 7-Ket-induced increases were significantly attenuated by ASM inhibitor amitriptyline. Furthermore, we determined that increased MR clustering with NADPH oxidase subunits to produce superoxide contributes to 7-Ket-induced NLRP3 inflammasome activation via a thioredoxin-interacting protein-mediated controlling mechanism. We conclude that ceramide from ASM plays a critical role in NLRP3 inflammasome activation during hypercholesterolemia via MR redox signaling platforms to produce superoxide, which leads to TXNIP dissociation.

Supplementary keywords carotid artery • endothelial cells • lysosomal ceramide • redox signaling • amitriptyline • NADPH • atherogenesis • 7-ketocholesterol • IL-1β • caspase-1

It has been demonstrated that activation of NOD-like receptor pyrin domain 3 (NLRP3) inflammasome is importantly involved in atherogenesis (1). During NLRP3 inflammasome activation, NLRP3 protein as a sensor protein recognizes endogenous and exogenous danger signals and recruits the accumulation of the adaptor protein ASC (the adaptor molecule apoptosis-associated speck-like protein containing a CARD) and the effector protein procaspase-1 to form a proteolytic complex. In such a complex, procaspase-1 is activated to form active caspase-1 leading to the production of mature interleukin (IL)-1β and IL-18 by cleavage of their precursors (2). Recent studies have indicated that endothelial dysfunction and consequent vascular injury and inflammation are associated with the formation and activation of NLRP3 inflammasome (3, 4). In this regard, endothelial NLRP3 inflammasome was found to be activated upon different proatherogenic stimuli such as cholesterol crystals (5), ATP (6), uric acid (7), hyperhomocysteinemia (8), and damage-associated molecular patterns (9) via different pathways. However, the precise mechanisms by which NLRP3 inflammasomes are activated and regulated remain poorly understood.

In general, NLRP3 inflammasome activation has been proposed to relate to increased ionic potassium flux, enhanced production of reactive oxygen species (ROS), and lysosomal rupture (2, 10). Our recent studies have demonstrated the crucial role of ROS-dependent NLRP3 inflammasomes in the regulation of functions of glomeruli or arterial endothelial cells (ECs) and the development of atherosclerosis during hyperlipidemia.
over, using the primary cultured ECs isolated from
in endothelial dysfunction and atherogenesis. More-
redox signaling platforms, promote the activation of
production of ceramide and the formation of MR
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activation was confirmed in cultured ECs (24). However,
the usage of the global gene knockout mouse model
could not elucidate whether the ASM-MR redox
signaling is derived from an endothelial source that
contributes to endothelial NLRP3 inflammasomes, which has been tested in the present
study.

Acid sphingomyelinase (ASM, a lysosomal hydrolase
encoded by the Smpd1 gene) has been shown to
importantly participate in the aggregation of NOX
subunits in ceramide-enriched MR microdomains pro-
miting the formation of MR redox signaling platform
in ECs (15, 19, 20). Upon stimulation, lysosome traffick
and fuses to the MR area via a SNARE-centered exo-
cytic machinery (21, 22), where ASM hydrolyzes sphin-
gomyelin into ceramide serving to reorganize and
cluster MR-associated signaling molecules (e.g., NOX
subunits) in ceramide-enriched MR microdomains (23).
Our recent study linked the ASM-MR redox signaling
with endothelial NLRP3 inflammasome activation,
which was demonstrated by the fact that deficiency of the
Smpd1 gene inhibited the activation of endothelial
NLRP3 inflammasomes and neointimal lesion forma-
tion in a mouse model of Niemann-Pick disease using
Smpd1 gene global knockout mice (24). The role of
ASM-MR redox signaling in NLRP3 inflammasome
activation was confirmed in cultured ECs (24). However,
the usage of the global gene knockout mouse model
could not elucidate whether the ASM-MR redox
signaling is derived from an endothelial source that
contributes to endothelial NLRP3 inflammasome activ-
ation and neointimal lesion formation in vivo. In this
study, we generated EC-specific Smpd1 transgenic mice
(Smpd1TgR/EC+/−) and examined whether the EC-specific
overexpression of the Smpd1 gene could enhance the
production of ceramide and the formation of MR
redox signaling platforms, promote the activation of
endothelial NLRP3 inflammasomes, and thereby result
in endothelial dysfunction and atherogenesis. More-
over, using the primary cultured ECs isolated from
Smpd1TgR/EC+/− mice, we examined whether ASM-MR
redox signaling is coupled with endothelial NLRP3
inflammasome activation via a redox sensor,
thioredoxin-interacting protein (TXNIP).

MATERIALS AND METHODS

Mice
All animal experiments were performed following the
National Institutes of Health guidelines for the care and use
of laboratory animals. The protocols were approved by the
Institutional Animal Care and Use Committee of Virginia
Commonwealth University. Eight- to 12-week-old male and
female C57BL/6J WT (WT/WT), Smpd1TgR/WT mice, and EC-
specific Smpd1 transgenic mice were used in the current study.
EC-specific Smpd1 transgenic mice were generated by cross-
breeding EC-specific Cre transgenic (Tie2 [tunica intima
endothelial kinase 2]-Cre) mice with Smpd1TgR/WT mice and
genotyped in a similar method as we described for other
tissue-specific transgenic mice with Smpd1 gene over-
expression in podocytes (25) and smooth muscle cells (26).
Mice were maintained in a controlled environment of 20°C
and 40–50% humidity, with a 12-h light/dark cycle. Mice were
separated into six groups randomly and fed with the WD
(0.21% cholesterol, DI2079, Research Diet) for 60 days.

Partial ligated carotid artery

Partial ligated carotid artery (PLCA) surgery was per-
formed as previously reported by others (27–31). The surgery
was performed after 30 days of WD treatment. Briefly, mice
were anesthetized with 2% isoflurane inhalation for 5 min
and epilated in the neck and then continued being anes-
ethetized through a nose cone. A ventral midline incision was
made in the neck and disinfected with 70% ethanol, and then
the muscle layers were separated with curved forceps
to expose the left carotid artery after blunt dissection. The
external carotid, internal carotid, and occipital artery were
ligated with a piece of 6.0 silk suture, whereas the superior
thyroid artery was left intact, which provided the sole source
for blood circulation. The right carotid artery was not ligated
and served as an internal control. After closing the incision
and disinfection, the mice were kept on a heating pad until
they gained consciousness. After 4 weeks of PLCA, mice were
sacrificed, and both their carotid arteries were perfused and
isolated for frozen sections and paraffin sections. The slides
were used for immunohistochemistry (IHC), dual fluores-
cence staining, and confocal analysis, respectively.

Morphologic examination and medial thickening
analysis

To study the morphological changes, H&E staining of ca-
rotid artery sections was used as described previously (32).
Briefly, the carotid artery was perfused with cold PBS for
5 min and 4% cold paraformaldehyde for another 5 min.
Then the carotid artery was separated and stored into 10%
neutral-buffered formalin. After at least 48 h, the tissues were
embedded in paraffin and cut into 5 μm serial sections for
histopathological evaluation. For H&E staining, the sections
were heated for 10 min at 65°C, and deparaffinization was
performed twice in 100% xylene for 10 min. The samples were
rehydrated with 100, 95, 90, and 75% ethanol to tap water and
immersed in hematoxylin and hydrochloride alcohol. Once the
nucleus turned blue, the sections were stained with eosin.
After that, the sections were rinsed with running tap water
and dehydrated with ethanol of 75, 90, 95, and 100%. Finally,
dibutyl phthalate polystyrene xylene was used to mount the
slides. Intimal-medium thickening of carotid arteries was
measured using Image-Pro Plus 6.0 software (Media Cyber-
netics, Inc, Bethesda, MD).

Immunofluorescence staining

Cells cultured on the sterilized cover slides or frozen ca-
rotid artery sections were rinsed three times for 2 min with
positive staining was examined using Image-Pro Plus 6.0. The area percentage of the hematoxylin and dehydrated as well as mounted using dibutyl phthalate-polystyrene-xylene. Finally, the sections were counterstained with tinylated secondary antibodies and a streptavidin peroxidase complex (PK-7800; Vector Laboratories, Burlingame, CA) for results in irreversible binding with the enzyme, and a fluorescent tag carboxyfluorescein reporter. After entering the cells, the FLICA reagent carboxyfluorescein-YVAD-fluoromethyl ketone becomes covalently coupled to the active caspase-1, whereas any unbound FLICA reagent diffuses out of the cell and is washed away. The remaining green fluorescent signal is a direct measure of the active caspase-1 enzyme activity in the cell or tissue samples. To detect caspase-1 activity in the carotid arterial endothelium, frozen artery section slides were first fixed in acetone and incubated overnight at 4°C with sheep anti-vWF (1200 dilution, Abcam, Waltham, MA). These slides were then costained with fluorescence-conjugated anti-sheep secondary antibody and FLICA reagent (1:10 dilution) for 15 h at room temperature, washed, mounted, visualized, and analyzed by confocal microscopy as described previously.

**Cell culture**

Isolation of mouse carotid arterial ECs was performed and characterized as previously described (15). ECs were cultured in DMEM (Gibco), supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) in humidified 100% air and 5% CO2 mixture at 37°C. ECs were primed with a low dose of lipopolysaccharide (1 ng/ml) for 3 h before any experiments. For the proatherogenic stimulation, cells were treated with 7-ketocholesterol (7-Ket; 15 μg/ml) and then incubated for 21 h. In the case of inhibitors used, the cells were pretreated with amitriptyline (Ami) (20 μM) for 30 min.

**Western blot analysis**

Western blot analysis was performed as we described previously (35). Briefly, whole-cell lysate protein concentrations were measured and resuspended to 2 μg/μl. Cell lysates were run on an SDS-PAGE gel at a voltage of 100 V for 2 h and transferred into a polyvinylidene difluoride membrane at a voltage of 100 V for 1 h. The membrane was blocked with 5% nonfat milk for 1 h, followed by incubation with the following primary antibodies: cleaved caspase-1 (1:500 dilution, Santa Cruz) overnight at 4°C. Then the secondary antibody was labeled with HRP for 1 h at room temperature, and the membrane was washed three times with Tris-buffered saline with Tween-20. The intensity of the bands was quantified using ImageJ 6.0.

**ELISA analysis of IL-1β secretions**

The fresh culture medium was collected for IL-1β product measurement with an IL-1β ELISA kit according to the manufacturer’s instructions as we described (36). In brief, at least 200 μl of the culture medium was used and incubated for 2 h at room temperature. Then, the samples were incubated with IL-1β conjugate for another 2 h at room temperature. Thorough washes were performed between and after the two incubations. About 100 μl of substrate solution was applied to generate chemiluminescence. Chemiluminescent absorbance was examined using a microplate reader at λ = 450.

**ESR analysis of O2•− production**

ESR detection of O2•− was performed as previously described (37). Briefly, cellular protein concentrations were measured in a lysis buffer and then prepared for analysis by resuspension in a modified Kreb’s-Hepes buffer containing deferoxamine (100 μM) and diethyldithiocarbamate (5 μM). NOX-dependent O2•− production was examined by the addition of 1 mM NADPH as a substrate in 20 μg protein in the

Smpdl105/EC3− mice enhance NLRP3 inflammasome activation
presence or the absence of superoxide dismutase (200 U/ml) to produce O$_{2}^\cdot$−. Then, 10 mM 1-hydroxy-3-methoxy carbonyl-2,2,5,5-tetramethylpyrrolidine, a superoxide-specific spin-trapping compound, was added to trap O$_{2}^\cdot$− before the mixture was loaded into the glass capillaries and immediately measured the O$_{2}^\cdot$− production kinetically for 10 min using a Miniscope MS200 ESR spectrometer (Magnettech, Germany). The ESR settings were as follows: biofield, 3,350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3G; 4,096 points of resolution; receiver gain, 100; and kinetic time, 10 min. Superoxide dismutase-sensitive components of ESR signals were used to calculate changes in O$_{2}^\cdot$− production or its level, which were shown as the fold changes of control.

In situ O$_{2}^\cdot$− production in mouse carotid arteries

Dihydroethidium (DHE) is a lipophilic cell-permeable dye that is one of the most widely used fluorogenic probes for the detection of intracellular superoxide (38). In brief, the frozen tissue slides were incubated with 10 mM DHE in phosphate-buffered saline at room temperature for 30 min. Then, the slides were washed, fixed, and subjected to confocal microscopic analysis (Fluoview FV1000; Olympus).

Trichrome stain in mouse carotid arteries

Trichrome stain (Abcam; catalog no: ab150686) was used to detect extracellular matrix and fibrosis following the manufacturer’s instructions. Deparaffinized sections and hydrate in distilled water. Preheat Bouin’s fluid in a water bath to 56–64°C in a fume hood or very well-ventilated area. Place slide in preheated Bouin’s fluid for 60 min followed by a 10-min cooling period. Rinse the slide in tap water until the section is completely clear. Rinse once in distilled water. Mix equal parts of Weigert’s (A) and Weigert’s (B) and stain the slide with working Weigert’s iron hematoxylin for 5 min. Rinse slide in running tap water for 2 min. Apply Biebrich Scarlet/ Acid Fuchsin solution to slide for 15 min. Rinse the slide in distilled water. Differentiate in phosphomolybdic/phosphotungstic acid solution for 10–15 min or until the collagen is not red. Without rinsing, apply Aniline Blue solution to the slide for 5–10 min. Rinse the slide in distilled water. Apply acetic acid solution (1%) to slide for 3–5 min. Dehydrate very quickly in two changes of 95% alcohol, followed by two changes of absolute alcohol. Clear in xylene or xylene substitute and mount in synthetic resin. The images are quantified using ImageJ 6.0.

ASM activity assay

ASM activity was measured using the Acid Sphingomyelinase Assay Kit (catalog no: ab190554; Abcam) following the manufacturer’s instructions. The WT/WT ECs were treated with different concentrations of Ami (Ctrl, 10, 20, 40 μM). Then cell homogenization was used for the activity assay.

Statistics

Data are presented as means ± SEM. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan’s multiple range test. The statistical analysis was performed by SigmaPlot 12.5 software (Systat Software, San Jose, CA). P < 0.05 was considered statistically significant.

RESULTS

EC-specific overexpression of the Smpd1 gene exacerbates atherosclerotic lesions in mouse carotid arteries

To induce neointima formation in carotid arteries, a PLCA model was surgically prepared in mice fed with a Western diet (WD) for 4 weeks as we described previously (3, 24, 39). Two control mice were used in our studies including WT (WT/WT) mice and floxed Smpd1 transgene (Smpd1$^{fl/fl}$/WT) mice. The EC-specific Smpd1 transgene (Smpd1$^{fl/fl}$/EC$^{cre}$) mice were generated by crossing Smpd1$^{fl/fl}$/WT mice with EC-specific Tie2-Cre mice as described (40). Tie2 promoter-driven Cre recombinase activity results in overexpression of the Smpd1 gene in ECs. We first confirmed the animal model by measuring the serum lipids (LDL-C, HDL-C, total cholesterol, and triglyceride) and found that Smpd1$^{fl/fl}$/EC$^{cre}$ mice enhanced the lipid levels induced by the WD (supplemental Fig. S3A–D). As shown in Fig. 1A, the neointima formation was not observed in PLCAs of normal diet (ND)-treated WT/WT mice or Smpd1$^{fl/fl}$/WT mice. ND-treated Smpd1$^{fl/fl}$/EC$^{cre}$ mice exhibited mild neointima thickening, but there is no statistically significant increase in the ratio of intima/media (Fig. 1A, B). In contrast, WD treatment significantly increased the neointima formation and the intima/media ratio in PLCAs of WT/WT and Smpd1$^{fl/fl}$/WT mice, which were more significantly enhanced in Smpd1$^{fl/fl}$/EC$^{cre}$ mice (Fig. 1A). These data indicate that hypercholesterolemia-induced neointimal lesions were exacerbated when the Smpd1 gene is overexpressed in ECs.

To further investigate the histological changes in the carotid arteries of all groups, Masson’s trichrome staining and α-SMA expression on the carotid wall by IHC staining was performed. We also found that WD treatment significantly showed active collagen production (Fig. 1C, D) and increased α-SMA expression (Fig. 1E, F) on the carotid media and intima layer of WT/WT and Smpd1$^{fl/fl}$/WT mice, which were more significantly enhanced in Smpd1$^{fl/fl}$/EC$^{cre}$ mice.

We also studied the effects of ASM overexpression on the endothelial injury. As shown in supplemental Fig. S1A, C, intact endothelium in ND-treated mice expresses similar higher ZO-1 and ZO-2 than in WD-treated mice; however, Smpd1$^{fl/fl}$/EC$^{cre}$ mice significantly attenuated WD-induced decrease. supplemental Fig. S1B, D showed clear changes.

EC-specific overexpression of the Smpd1 gene enhances endothelial inflammasome activation in the carotid arteries

The NLRP3 inflammasome formation in PLCAs was analyzed by examining the colocalization of NLRP3 inflammasome subunits using confocal immunofluorescence microscopy. As shown in Figure 2A, C, in WT or Smpd1$^{fl/fl}$/WT control mice, WD significantly
increased the colocalization of NLRP3 with ASC or caspase-1 compared with ND as shown by yellow spots in the intima of the carotid arteries. Moreover, the WD-induced increase in the colocalization of NLRP3 subunits was enhanced in Smpd1\textsuperscript{trg/ECcre} mice compared with WT/WT or Smpd1\textsuperscript{trg/WT} mice. The quantified colocalization coefficient data are summarized in Figure 2B, D. In addition to their formation, the activation of the NLRP3 inflammasome was analyzed by examining the caspase-1 activity using FLICA probes (Fig. 3A, B) and IL-1\textbeta expression using IHC staining (Fig. 3C, D). As shown in Figure 3A, B, there was no significant colocalization of FLICA with endothelial marker vWF in all mouse strains with ND treatment suggesting a basal level of NLRP3 inflammasome activity in these mice. In contrast, WD treatment significantly increased the FLICA/vWF colocalization in WT/WT and Smpd1\textsuperscript{trg/WT} control mice, and such WD-induced colocalization was more significantly increased in Smpd1\textsuperscript{trg/ECcre} mice. Accompanied by enhanced caspase-1 activity by WD, Smpd1\textsuperscript{trg/ECcre} mice showed a more significant increase in the levels of IL-1\textbeta expression by WD in the carotid intima region compared with that in WT/WT.
and Smplδ^{ΔS}/WT mice (Fig. 3C, D). Together, these data suggest that EC-specific Smplδ gene overexpression enhances endothelial NLRP3 inflammasome formation and activation in the carotid arteries of mice during hypercholesterolemia.

EC-specific overexpression of Smplδ gene amplifies ceramide-MR-mediated redox signaling in the carotid artery walls

Earlier reports from our laboratory have demonstrated that ASM plays an essential role in forming ceramide-enriched MR clusters in ECs, in which NOX subunits are aggregated and activated leading to redox signaling and consequent endothelial NLRP3 inflammasome activation and arterial neointima formation during hypercholesterolemia (18, 24). Here, we observed that WD treatment increased the colocalization of ASM or ceramide within flotillin (MR marker) in the intima region of PLCAs of WT/WT or Smplδ^{ΔS}/WT mice compared with ND-treated controls, whereas such increases were further enhanced in Smplδ^{ΔS}/EC^{cre} mice (Fig. 4A, C). These changes were quantified as a colocalization coefficient as summarized in Figure 4B, D. It should be noticed that under basal conditions (ND groups), Smplδ gene overexpression only caused a statistically insignificant increase in either the ASM protein expression or ceramide in MR clusters in the carotid intima of Smplδ^{ΔS}/EC^{cre} mice compared with WT/WT or Smplδ^{ΔS}/WT mice (Fig. 4A–D). Thus, our data suggest that EC-specific overexpression of the Smplδ gene sensitizes the hypercholesterolemia-induced formation of ceramide-enriched MR clusters in the carotid arterial intima.

Consistent with the increased formation of ceramide-enriched MR clusters by WD in Smplδ^{ΔS}/EC^{cre} mice, we also observed that WD induced more significant increases in the aggregation of NOX subunits gp91 or p47 in ceramide-enriched MR clusters in these mice (Fig. 5A–D). Furthermore, O_{2}•− production in situ was detected by DHE staining on the carotid arterial wall. As shown in Figure 5E, F, WD caused a more significant increase in DHE-O_{2}•− signal in Smplδ^{ΔS}/EC^{cre} mice compared with that in WT/WT or Smplδ^{ΔS}/WT mice. Together, these data suggest that hypercholesteremia-induced MR-redox signaling is amplified in the...
carotid arterial wall of mice when the Smpd1 gene is overexpressed in ECs.

**Smpd1 gene overexpression enhances NLRP3 inflammasome formation and activation in cultured ECs**

Next, we aimed to confirm the contribution of Smpd1 gene overexpression to NLRP3 inflammasome activation and formation in primary cultured ECs from carotid arteries of WT/WT and Smpd1trg/ECcre mice as previously described (41, 42). First, we confirmed that Ami, an ASM inhibitor, dose-dependently decreased ASM protein by Western blot and ASM activity detected by ELISA kit (supplemental Fig. S2A–C). Next, it was found that 7-Ket-induced colocalization of NLRP3 with ASC or caspase-1 was more significantly increased in Smpd1trg/ECcre ECs compared with that in WT/WT ECs, whereas pretreatment of both ECs with Ami, an ASM inhibitor, abolished such 7-Ket-induced increases (Fig. 6A–D).

The NLRP3 inflammasome activation in cultured ECs was further assessed by Western blot analysis of the caspase-1 cleavage, caspase-1 activity assay, and ELISA analysis of IL-1β production. As shown in Figure 7A, B, 7-Ket remarkably increased cleaved caspase-1 expression in WT/WT ECs, which was further enhanced in Smpd1trg/ECcre ECs. Such enhancement in caspase-1 cleavage in Smpd1trg/ECcre ECs was correlated with increased caspase-1 activity (Fig. 7C) and IL-1β production (Fig. 7D). Moreover, 7-Ket-induced increases in caspase-1 cleavage, caspase-1 activity, and IL-1β production were prevented by Ami in both WT/WT and Smpd1trg/ECcre ECs (Fig. 7A–D). It should be noticed that under basal conditions (vehicle), Smpd1trg/ECcre ECs did not have significantly higher levels of NLRP3 inflammasome formation (Fig. 6A–D) and activation (Fig. 7A–D) compared with WT/WT ECs. Therefore, Smpd1 gene overexpression alone seems to be insufficient to trigger NLRP3 inflammasome formation and activation. However, our data indicate that Smpd1 gene overexpression in ECs could enhance 7-Ket-induced NLRP3 inflammasome formation and activation in vitro, which is large because of increased ASM activity.
**Smpd1** gene overexpression enhances the formation and activation of MR-redox signaling platforms in cultured ECs

As shown in Figure 8A–D, untreated Smpd1^{Tg/ECc} ECs had a similar level of colocalization of ceramide or ASM with flotillin compared with that of WT/WT ECs, suggesting that Smpd1 gene overexpression does not trigger ceramide-enriched MR clustering under basal condition (vehicle groups). Interestingly, 7-Ket stimulated the colocalization of ceramide or ASM expression with flotillin in WT/WT ECs, which were enhanced in Smpd1^{Tg/ECc} ECs and inhibited by Ami. Therefore, our data indicate that Smpd1 gene overexpression could enhance 7-Ket-induced ceramide-enriched MR clustering in ECs.

We then examined the effects of Smpd1 gene overexpression on the formation of MR-redox signaling platforms in ECs. MR was labeled with antibodies against CTXB (an MR marker). It was found that the expression of NOX subunits gp91 or gp47 in CTXB-labeled MR clusters was similar between WT/WT ECs and Smpd1^{Tg/ECc} ECs under ND conditions (control groups). 7-Ket increased the levels of colocalization of gp91^{phox} or gp47^{phox} with CTXB in WT/WT ECs, which were enhanced in Smpd1^{Tg/ECc} ECs and inhibited by Ami. Using ESR spectrometry, we measured the \( \text{O}_2^- \) production in cultured ECs. As shown in Figure 9E, 7-Ket increased \( \text{O}_2^- \) production in WT/WT ECs, an effect enhanced in Smpd1^{Tg/ECc} ECs. Treating both types of ECs with Ami significantly attenuated 7-Ket-induced \( \text{O}_2^- \) production. Together, these data suggest that Smpd1 gene overexpression enhances the 7-Ket-induced formation of MR-redox signaling platforms in ECs.

**Txnip** gene silencing abrogates 7-ket-increased NLRP3 inflammasome formation and activation in ECs with Smpd1 gene overexpression

Previous studies demonstrated that TXNIP binds NLRP3 protein and triggers NLRP3 inflammasome formation and activation (43). Here, we examined the
The role of TXNIP in 7-Ket-induced NLRP3 inflammasome formation and activation in Smpd1^trg^ECcre ECs. As shown in Figure 10A–D, Txnip gene silencing by Txnip siRNA transfection markedly attenuated 7-Ket-induced increases in the colocalization of NLRP3 with ASC or caspase-1 in both WT/WT ECs and Smpd1^trg^ECcre ECs. 7-Ket-induced increases in caspase-1 cleavage, caspase-1 activity, and IL-1β production were significantly abolished by Txnip gene silencing in WT/WT ECs or Smpd1^trg^ECcre ECs (Fig. 11A–D).

Together, these results demonstrate that targeting the Txnip gene abrogated 7-Ket-induced NLRP3 inflammasome activation and formation in ECs with Smpd1 gene overexpression.

**DISCUSSION**

The present study revealed the critical contribution of endothelial ASM-ceramide MR redox signaling pathway to hypercholesterolemia-induced NLRP3
inflammasome activation and neointimal hyperplasia. Our results demonstrated that EC-specific overexpression of the Smpd1 gene enhanced hypercholesterolemia-induced neointimal formation, which was accompanied by augmented ASM-ceramide-MR redox signaling and NLRP3 inflammasome formation and activation in the arterial endothelium. Moreover, we demonstrated that TXNIP is a critical mediator that links ASM-ceramide-MR redox signaling to NLRP3 inflammasome formation and activation in ECs. By using Smpd1Trg/ECcre mice and the primary cultures of their ECs, our findings provide the first direct evidence that ASM-ceramide-MR redox signaling and downstream redox sensor TXNIP are involved in the formation and activation of NLRP3 inflammasome activation and subsequent neointimal lesion formation and how ASM regulates NLRP3 inflammasome activation in response to high cholesterol stimulation in vitro and in vivo. In the present study, we generated EC-specific Smpd1 transgenic mice (Smpd1Trg/ECcre), in which the Smpd1 gene was specifically overexpressed in ECs. Their littermate controls (WT/WT or Smpd1Trg/WT) were used for the comparison of the studies. Our data first confirmed that Smpd1 gene overexpression in ECs significantly enhanced the WD-induced neointima formation in the carotid artery wall (Fig. 1). Then, confocal microscopic analysis revealed that EC-specific Smpd1 gene
overexpression markedly enhanced WD-induced increases in NLRP3 inflammasome formation and activation as characterized by increased colocalization of NLRP3 inflammasome components (Figs. 2 and 3). Consistently, we observed that in \textit{Smpd1} \textsuperscript{trg/ECcre} mice, the enhanced WD-induced NLRP3 inflammasome activation and neointimal formation were accompanied by higher levels of ASM protein expression and ceramides in the endothelium (Fig. 4). Similarly, using isolated ECs from \textit{Smpd1} \textsuperscript{trg/ECcre} mice and WT controls, we confirmed that \textit{Smpd1} gene overexpression enhanced NLRP3 inflammasome activation (Figs. 6 and 7) and ASM-ceramide signaling (Fig. 8) in cultured ECs stimulated by 7-Ket. Taken together, these results suggest that endothelial ASM-ceramide signaling promotes endothelial NLRP3 inflammasome activation during hypercholesterolemic stimulation, which contributes to the development of neointima formation or atherogenic pathology. To our knowledge, this current study provides the first evidence that endothelium-specific overexpression of ASM exaggerates endothelial NLRP3 inflammasome formation and activation upon pathological stimuli such as hypercholesterolemia.

It is known that ROS generation is one of the first identified triggers of NLRP3 inflammasome activation (52–54). ROS may be derived from endoplasmic reticulum stress, damaged mitochondria, and NOX, which is an intermediate factor involved in multiple signaling pathways and can trigger the activation of NLRP3 inflammasome (55). Our previous studies have for the first time defined a mechanism mediating NOX activation in response to many different stimuli, termed MR redox signalosomes. These MR signalosomes use MRs as a platform to transduce and amplify the redox signaling and are centered on the enzymatic NOX subunit clustering and activating to produce O\textsubscript{2}\textsuperscript{•−} (56). NOX-derived ROS can act downstream to transduce transmembrane or intracellular signaling, leading to the redox regulation of cell and organ function (16). We reported that different stimuli such as homocysteine, visfatin, or ATP act on the cell membrane to stimulate ASM to produce and form ceramide-enriched MR platforms and thereby increase NOX-dependent O\textsubscript{2}\textsuperscript{•−} production in different types of cells, such as podocytes, ECs, and hepatic stellate cells (12, 17, 57–62). So far, it remains poorly understood whether ASM-ceramide-MR redox signaling platforms are critical in the activation of NLRP3 inflammasomes and atherosclerotic lesions. Using ASM global knockout mice, we recently demonstrated that ASM/ceramide-associated MR clustering with NOX subunits contributes to

Fig. 7. Effects of ASM blockade on NLRP3 inflammasome activation in the primary cultures of ECs from EC-specific \textit{Smpd1} transgenic mice. A: Representative Western blot gel documents showing the expression of cleaved caspase-1 in the primary cultures of carotid ECs from endothelium-specific \textit{Smpd1} transgenic mice. B: Summarized data showing the expression of cleaved caspase-1 in the primary cultures of carotid ECs from endothelium-specific \textit{Smpd1} transgenic mice. C: Summary data showing caspase-1 activity in the primary cultures of carotid ECs from endothelium-specific \textit{Smpd1} transgenic mice. D: Summarized data showing IL-1β production in the primary cultures of carotid ECs from endothelium-specific \textit{Smpd1} transgenic mice. Data are expressed as means ± SEM, \textit{n} = 5. *P < 0.05 is defined as significant.
hypercholesteremia-induced endothelial NLPR3 inflammasome activation and formation (24). In the present study, we further used Smpd1tg/ECcre mice to study the specific role of ASM in the formation and activation of NLRP3 inflammasome and explore related molecular mechanisms. It was found that Smpd1 gene overexpression enhanced WD-induced formation of ceramide-enriched MR redox signaling as shown by increased formation of flotillin-1-positive MR clusters aggregated with ASM/ceramide (Fig. 4) or NOX subunits gp91 and p47 (Fig. 5) and the consequent production of O2•− levels as measured by in situ DHE staining. DHE is well known as a fluorescent probe for superoxide and hydrogen peroxide and is commonly used for the detection of ROS generation in tissues. Our data demonstrated that WD increased arterial fluorescence in the arterial wall including in ECs and SMCs of the media layer, which was further increased when the Smpd1 gene is overexpressed. It is not surprising to observe DHE-ROS fluorescence in the media layer as hydrogen peroxide is cell permeable and could be released into the medial layer. The ROS production in the media layer could also be originated from redox pathways in SMCs that are activated following endothelial activation or injury. Nonetheless, our data indicate that EC-specific Smpd1 gene overexpression enhanced the oxidative stress in the arterial wall. In addition, we confirmed that the Smpd1 gene overexpression enhanced 7-Ket-induced activation of ASM-MR redox signaling in cultured ECs, which were abolished by the ASM inhibitor (Figs. 8 and 9). Together, these results from the ASM overexpression studies provide direct evidence that hypercholesterolemia or cholesterol stimulation instigates the ASM-ceramide pathway to induce MR redox signaling platform formation in ECs and thereby trigger endothelial NLRP3 inflammasomes.

To further study how ASM-mediated activation of MR redox signaling platforms induces NLRP3 inflammasome activation, we determine the role of a redox sensor TXNIP in the process of NLRP3 inflammasome activation. TXNIP is the endogenous inhibitor and regulator of thioredoxin, a major cellular antioxidant, and antiapoptotic system (63). It has been demonstrated that NLRP3 inflammasome activators induce the dissociation of TXNIP from thioredoxin in a ROS-sensitive manner, which allows thioredoxin to bind NLRP3, thereby activating this inflammasome in different cells, such as macrophages (64), ECs (65–68), and podocytes (69, 70). However, the role of TXNIP in ASM-ceramide-mediated NLRP3 inflammasome activation remains largely unexplored.
In the present study, we examined the effects of \( \text{Txnip} \) gene silencing on the NLRP3 inflammasomes in cultured ECs isolated from WT and \( \text{Smpd1}^{trg/ECcre} \) mice. Our results demonstrated that \( \text{Txnip} \) gene silencing abrogated 7-Ket-induced NLRP3 inflammasome formation and activation in ECs from both WT and \( \text{Smpd1}^{trg/ECcre} \) (Figs. 10 and 11). These data demonstrate that TXNIP is a critical downstream mediator of ASM-ceramide-MR redox signaling that triggers endothelial NLRP3 inflammasome formation and activation. Consistent with our finding, Jiang et al. (71) recently reported that lipopolysaccharide/ATP-induced increase of ASM activity and accumulation of ceramide is attributed to TXNIP/NLRP3 inflammasome activation in J774A.1 cell and THP-1 macrophages. Koka et al. (24) have reported that TXNIP inhibitor verapamil attenuated 7-Ket or cholesterol crystal-induced increases in caspase-1 activity in ECs. In addition, metformin and many other compounds were used to lower aortic TXNIP levels in vivo or endothelial levels in vitro to block NLRP3 inflammasome activation and protect from endothelial dysfunction and cardiovascular risk factors (72–76). Nrf2 and AMPK are also believed to control the regulation of
the NLRP3 inflammasome by the TRX-TXNIP complex (77–79). Concerning the mechanism responsible for the action of TXNIP, there is evidence that it activates the TLR4-NFκB-NLRP3 inflammasome signaling pathway with increased MyD88, NLPR3 inflammasome, and ASC expression, as well as the increased phosphorylation of IκBα and p65, thus promoting downstream NF-κB activation (80). The overexpression of TXNIP also led to an increased expression of inflammation genes via chromatin modifications and by promoting nuclear translocation of NF-κB (81). These previous studies have indicated that TXNIP may perform its functions through multiple binding partners and thereby directly or indirectly regulate NLRP3 inflammasome formation and activation. The precise mechanism by which TXNIP links ASM-ceramide-MR redox signaling and its downstream effector, NLRP3 inflammasome, deserves future investigation.

Finally, the present study had several limitations, which should be noted. First, our lipid panel analysis demonstrated that Smpd1Tg/ECcre mice had increased lipid levels induced by the WD. The mechanism by which serum lipid is increased by endothelial overexpression of ASM remains an intriguing question. Recent evidence identifies that liver sinusoidal EC dysfunction is the main characteristic or early event in the pathogenesis of the nonalcohol fatty liver disease, which contributes to impaired hepatic lipid uptake and metabolism (82). Thus, endothelial ASM overexpression in the liver may cause liver sinusoidal EC dysfunction and hepatic dysregulation of lipid metabolism leading to higher serum lipids, which indirectly contribute to the development of neointimal injury. Second, the EC-specific Smpd1 transgene (Smpd1Tg/ECcre) mice were generated by crossing Smpd1Tg/WT mice with EC-specific Tie2-Cre mice as described (40). Tie2 is a receptor tyrosine kinase that binds angiopoietin-1 and angiopoietin-2. Tie2 is not only expressed in ECs (83) but also found in hematopoietic cells in fetal liver and adult bone marrow and several differentiated hematopoietic cells (84, 85). The formation and activation of NLRP3 inflammasome in hematopoietic cells are implicated in cardiovascular diseases (86, 87). In this regard, some of the effects may be mediated by ASM overexpression in the hematopoietic cells on cardiovascular diseases.

In summary, the present study demonstrated that EC-specific Smpd1 gene overexpression enhanced the production of ceramide and the formation of MR redox signaling platforms, promoted the activation of endothelial NLRP3 inflammasomes, and thereby resulted in

Fig. 10. TXNIP gene silencing abrogated NLRP3 inflammasome formation in the primary cultures of ECs from EC-specific Smpd1 transgenic mice. A: Representative fluorescent confocal microscope images displaying the yellow dots or patches showing the colocalization of NLRP3 (green) with ASC (red). B: The summarized data show the colocalization coefficient of NLRP3 with ASC. C: Representative fluorescent confocal microscope images showing the colocalization of NLRP3 (green) with caspase-1 (red). D: The summarized data show the colocalization coefficient of NLRP3 with caspase-1. Data are expressed as means ± SEM, n = 6. *P < 0.05 is defined as significant. The scale bar represents 10 μm.
endothelial dysfunction and atherogenesis. These findings provide the first evidence that endothelial ASM-ceramide-MR redox signaling is directly linked with endothelial NLRP3 inflammasomes and neo-intimal hyperplasia. Moreover, we identified that ASM-ceramide MR redox signaling is coupled with endothelial NLRP3 inflammasome activation via a redox sensor, TXNIP. Our findings may offer novel therapeutic insights into targeting ASM-ceramide-MR redox signaling or TXNIP to suppress the activation of endothelial NLRP3 inflammasomes, thereby preventing and treating vasculopathy associated with hypercholesterolemia.

Data Availability
The data that support the findings of this study are available from the corresponding author on realistic request.

Supplemental Data
This article contains supplemental data.

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Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations
Ami, amitriptyline; ASC, the adaptor molecule apoptosis-associated speck-like protein containing a CARD; ASM, acid sphingomyelinase; CTXB, cholera toxin B; DHE,
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