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Original Article

Acid Sphingomyelinase Mediates Oxidized-LDL Induced Apoptosis in Macrophage *via* Endoplasmic Reticulum Stress

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***Aim*: Macrophage apoptosis is a vital event in advanced atherosclerosis, and oxidized low-density lipoprotein (ox-LDL) is a major contributor to this process. Acid sphingomyelinase (ASM) and ceramide are also involved in the induction of apoptosis, particularly in macrophages. Our current study focuses on ASM and investigates its role in ox-LDL-induced macrophage apoptosis.**
***Methods*: Human THP-1 and mouse peritoneal macrophages were cultured *in vitro* and treated with ox-LDL. ASM activity and ceramide levels were quantified using ultra performance liquid chromatography. Protein and mRNA levels were analyzed using Western blot analysis and quantitative real-time PCR, respectively. Cell apoptosis was determined using Hoechst staining and flow cytometry.**
***Results*: Ox-LDL-induced macrophage apoptosis was triggered by profound endoplasmic reticulum (ER) stress, leading to an upregulation of ASM activity and ceramide levels at an early stage. ASM was inhibited by siRNA or desipramine (DES), and/or ceramide was degraded by recombinant acid ceramidase (AC). These events attenuated the effect of ox-LDL on ER stress. In contrast, recombinant ASM upregulated ceramide and ER stress. ASM siRNA, DES, recombinant AC, and ER stress inhibitor 4-phenylbutyric acid were blocked by elevated levels of C/EBP homologous protein (CHOP); ox-LDL induced elevated levels of CHOP. These events attenuated macrophage apoptosis.**
***Conclusion*: These results indicate that ASM/ceramide signaling pathway is involved in ox-LDL-induced macrophage apoptosis *via* ER stress pathway.**

Key words: Atherosclerosis, Macrophage apoptosis, Acid sphingomyelinase, Ceramide, ER stress

Introduction

In several previous studies, researchers established that macrophage apoptosis occurs in patients with advanced atherosclerotic (AS) lesions. These lesions lead to the formation of vulnerable plaques, which are characterized by a necrotic core and defective phagocytosis[1, 2].The AS plaques are extremely susceptible to rupture and erosion, causing cardiovascular complications that are difficult to manage. Oxidized low-density lipoprotein (ox-LDL) is a major lipid found in AS lesions, and it is considered a major causative factor in the development of AS[3]. Ox-LDL triggers lipid storage, foam cell formation, and macrophage apoptosis. All these events lead to the development of AS[4, 5]. However, so far, the underlying mechanism(s) through which ox-LDL induces macrophage apoptosis is still unknown.

The accumulated ceramide acts as an apoptosis regulator in various cell types, including macrophages[6]. Ceramide is generated through multiple pathways: catabolism of sphingomyelin *via* the activ-

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ity of acid sphingomyelinases (ASM); *de novo* synthesis from palmitoyl-CoA and serine; and hydrolysis of glucosylceramide and galactosylceramide. However, these multiple pathways of ceramide generation are cell-specific. They do not manifest themselves in all types of cells. Nevertheless, the ASM/ceramide signal transduction pathway plays an important role in apoptosis. A previous study proved that ASM activation and ceramide generation stimulate ox-LDL, inducing the apoptosis of human macrophage[7]. However, they could not clearly elucidate the underlying mechanism through which ox-LDL induces the apoptosis of human macrophage.

Endoplasmic reticulum (ER) stress is a crosspoint through which many cellular processes can be linked to risk factors that cause the development of AS. In particular, ER stress induces macrophage apoptosis, leading to the development of vulnerable plaques[8]. Initially, transmembrane receptors of ER elicited an unfolded protein response (UPR) to increase the chances of cell survival. UPR comprises of three transducers: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1(IRE1). These transducers activate several downstream targets. Glucose-regulated protein 78 (GRP78) is a master regulator, controlling the activation of UPR. However, if the stimuli are severe or prolonged, ER stress causes mitochondrial dysfunction and apoptotic signals. In the latter case, the three UPR transmembrane receptors disassociate from GRP78, while the processed ATF6 translocates to the nucleus and becomes activated.

C/EBP homologous protein (CHOP) is an important transcription factor that mediates ER stress-induced apoptosis[9, 10]. Apoptosis is not induced only by the overexpression of CHOP; however, the overexpressed CHOP stimulates ER stress and induces apoptosis[11]. It has been found CHOP deficiency has a protective effect on macrophages *in vitro*[9, 12]. On the other hand, an increase in ER stress is found to occur when CHOP is induced. Finally, this leads to the development of apoptosis. Recent studies state that an exogenous cell-permeable, short-chain ceramide (C_2-ceramide or C_6-ceramide) is sufficient to induce ER stress[13, 14]. Therefore, we inferred that accumulated ceramide as a stimulus regulates ER stress. The resultant ceramide is generated through *de novo* synthesis pathways[15, 16]. In this study, we provide novel evidences to prove that the ASM/ceramide pathway modulates ER stress, inducing macrophage apoptosis in an experimental model of AS.

Materials and Methods

Chemicals and Reagents

We purchased desipramine (DES) and 4-phenylbutyric acid (PBA) from Sigma–Aldrich (St. Louis, MO, USA). In this case, DES is an ASM inhibitor, while PBA is an ER stress inhibitor. Recombinant acid ceramidase (AC) and recombinant human ASM (rhASM) were purified from a medium containing overexpressed Chinese hamster ovary cells, as previously described[17, 18].

Cell Lines and Culture

THP-1 monocytes were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Then, they were seeded in Roswell Park Memorial Institute 1640 medium (Hyclone, Logan, UT, USA). The reaction medium consisted of the following reagents: 2 mM glutamine, 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/mol penicillin, and 100 U/mol streptomycin. The cell culture was maintained at 37℃ in a humidified atmosphere containing 5% CO_2. The cell density was maintained between 5×10^5 and 1×10^6. Then, this cell culture was treated for 48 h with 130 ng/ml of phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO, USA). Cell differentiation was triggered with this treatment, and the differentiated cells were transformed into macrophages. The cell differentiation status was observed under a light microscope (Olympus Co., Tokyo, Japan).

Mouse peritoneal macrophages (MPMs) were isolated by performing a previously described procedure[19]. Briefly, thioglycolate (Sigma, St. Louis, MO, USA) was injected into the peritoneal cavity of C57BL/6 mice. After 3 days, macrophages were collected and washed in serum-free Dulbecco's Modified Eagle's Medium (DMEM). Then, these cells were plated in DMEM, which contained 10% FBS. Finally, they were incubated at 37℃ for 4 h. Subsequently, we removed non-adherent cells and incubated the remaining cells at 37℃ before subjecting them to treatment. MPMs were identified *via* immunofluorescence using a CD68 antibody (Abcam, Cambridge, MA, USA; **Supplementary Fig. 1**). The procedure was approved by the Local Ethics Committee of Xiangya Hospital of Central South University, Hunan, China.

Isolation and Oxidation of LDL

Native LDL was isolated from the pooled plasma of healthy donors by performing sequential density gradient ultracentrifugation in sodium bromide solutions; the density of sodium bromide solutions was in the range of 1.019–1.063 g/ml. The process was per-

formed according to a previously described procedure[20]. LDL preparations were washed by ultracentrifugation. Then, they were dialyzed with 0.15 mol/l NaCl solution (pH=7.4), containing 1 mmol/l EDTA. Thereafter, LDL preparations were passed through an acrodisc filter (0.22 μm) to remove aggregates. Finally, they were stored under nitrogen in darkness. Thereafter, LDL was oxidized by dialysis for 20 h at 37℃. For this purpose, we used 10 μmol/l $CuSO_4$ solution that was prepared in phosphate buffered saline (PBS). The oxidation reaction was terminated by adding an excess of EDTA-Na_2. To determine the migration of LDL, we performed agarose gel electrophoresis on each lot. Protein concentration was measured using Lowry's method; thereafter, the protein concentration was adjusted to 1–1.5 mg of proteins/ml. The levels of thiobarbituric acid-reactive substances (TBARS), which reflect the extent of LDL oxidation, were determined colorimetrically using malondialdehyde. The levels of TBARS, indicating LDL and ox-LDL, were 0.14±0.03 and 30.25±0.69 nmol/mg protein, respectively.

RNA Interference and siRNA Transfection

The specific anti-ASM siRNA (si-ASM) and negative control siRNA(si-NC) were synthesized and obtained from RiboBio Co. Ltd. (Guangzhou, China). Si-ASM-1 sequences were as follows: 50'-GCCUCAUCUCUCUCAAUAUdTdT-3' (sense) and 3'-dTdTCGGAGUAGAGAGAGUUAUA-5' (antisense); si-ASM-2 sequences were as follows: 5'-CCAGUGCAACUACCUACAUdTdT-3' (sense) and 3'-dTdTGGUCACGUUGAUGGAU GUA-5' (antisense); si-ASM-3 sequences were 5'-GUCUAUUCACCGCCAUCAAdTdT-3' (sense) and 3'-dTdTCAGAUAAGUGGCGGUAGUU-5' (antisense). To knockdown the expression of ASM, we seeded human THP-1 macrophages in 6-well plates. The resultant culture was grown at 50%–70% confluency. Using Ribo FECT^{TM} CP Transfection Kit (RiboBio Co. Ltd. Guangzhou, China) according to the manufacturer's protocol, we added Si-ASM or negative control siRNA (si-NC) into the cell culture. Then, the cells were transfected for either 24 h or 48 h. Thereafter, the cells were incubated with ox-LDL for 24 h.

Detection of Cell Apoptosis

Cells were seeded in a 24-well plate and washed twice with PBS. Then, the cell culture was fixed in 4% formaldehyde for 30 min. Thereafter, the cells were stained with Hoechst 33258 (10 μg/ml, Beyotime, Jiangsu, China) for 5 min at room temperature in darkness. Subsequently, under a fluorescence microscope (Olympus, Japan), we observed Hoechst-stained nuclei at an emission wave length of 521 nm. Under five random high-power fields (200× magnification), we counted and quantified 200 cells in total. The percentage of apoptosis was expressed as a ratio of apoptotic cells to total cells.

The apoptotic cells were double stained with Annexin V and PI using Annexin-V 488-PI apoptosis detection kit (Invitrogen, Carlsbad, CA, USA). Then, they were detected using flow cytometry. Specifically, cells were cultured in a 6-well plate and washed twice with PBS; the cell culture was resuspended in 100 μl of binding buffer. The cell density of this culture was 1×10^6 cells/ml. Next, 5 μl AnnexinV-488 and 1 μl PI (100 μg/ml) were added into 100 μl cell suspension, and the cells were incubated in dark at room temperature for 15 min. Then, 400 μl of binding buffer was added into the cell solution, and the level of cell fluorescence was immediately determined using a flow cytometer (Becton-Dickinson, New Jersey, NJ, USA).

ASM Activity Assay

Total macrophage proteins were extracted using the Cell Lytic^{TM} Cell Lysis Reagent (Sigma, St. Louis, MO, USA). ASM activity assay was performed according to a previously described procedure[21]. Briefly, 3 μl cell lysis was mixed with 3 μl ASM buffer [200 μm BODIPY®-labeled C12-Sphingomyelin (Thermofisher Scientific, Waltham, MA, USA)]. Then, it was diluted in an assay buffer [0.2 M sodium acetate (pH=5.0) containing 0.2 mM $ZnCl_2$ and 0.2% Igepal CA-630]. Finally, it was incubated at 37℃ for 20 h. Furthermore, the reaction was terminated by adding ethanol. The hydrolytic product was detected and quantified using ultra performance liquid chromatography (UPLC) system (Acquity UPLC H-class, Waters, Milford, MA, USA). In this system, we used a reversed phase column (Acquity BEH Amide, 2.1×50 mm, 1.7 μm, Waters, USA).

Lipid Extraction and Ceramide Assay

Lipids were also extracted from cell lysates. Ceramide levels were quantified by a previously described procedure[22]. Briefly, 25 μl of the sample was added to 150 μl of dichloromethane/methanol (1:2; v/v) and vortexed for 1 min. Then, we added 100 μl of 1 M NaCl (in 10 % HCl). An additional amount of dichloromethane was also added. After vigorous vortex and top spin, we obtained 100 μl of the organic phase in the lower layer. This organic phase was dried by evaporation to obtain the lipid in dried form. The dried lipid was dissolved in 20 μl of 2% Igepal CA630. The resultant solution was shaken at 80℃ for 5 min. Finally, it was cooled down to room temperature before performing ceramide assay. To per-

form ceramide assay, recombinant AC was added into lipid extracts, and the ceramide was fully degraded to sphingosine. Then, we mixed 2 μl of each lipid solution and ceramide hydrolysis buffer (0.2 M citrate/phosphate buffer (pH=4.5), which contained 0.3 M NaCl and 0.5 mg/ml recombinant AC. Finally, we incubated the reaction medium at 37℃ for 1 h. Furthermore, this reaction medium was treated with 56 μl of naphthalene-2,2-dicarboxaldehyde (NDA) mixtures (10 μl of 5 mM NDA, 10 μl of 5 mM NaCN, 16 μl of 100% ethanol, 20 μl of 50 mM Borate buffer) at 50℃ for 10 min. After centrifugation, the supernatants were transferred into the UPLC system. In the UPLC system, we used a reversed phase column (Acquity BEH Shield RP18, 2.1×50 mm, 1.7 μm, Waters, USA) to determine total ceramide.

Western Blotting Analysis

Protein quantification was performed using BCA protein assay kit (Beyotime, Jiangsu, China). To perform Western blot analysis, we used the following primary antibodies: A rabbit anti-GRP78 (1:800) or β-actin antibody (1:6000) (Sigma, St. Louis, MO, USA), a rabbit against IRE1(1:1000) antibody (Abcam, Cambridge, MA, USA), and a rabbit phosphorylated PERK (1:100) or mouse anti-CHOP antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A rabbit anti-ASM (smpd1) antibody (1:1000) was kindly provided by Professor Xingxuan He (Icahn School of Medicine at Mount Sinai, New York, NY, USA).

RNA Isolation and qRT-PCR

Total RNA was isolated from cells by performing a previously described procedure[20]. The quality and concentration of RNA were measured using nanodrop method. Of the total RNA sample of each group, 1 μg was reversely transcribed into cDNA using a reverse transcription kit (Fermentas, Waltham, MA, USA); amplification was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA); qPCR results were calculated using the comparative C_t (threshold cycle) method; the results were normalized against glyceraldehyde-3-phosphate dehydrogenase. The primer sequences of amplified genes are enlisted in **Supplementary Table 1**.

Immunofluorescence

Cells were permeabilized in 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) for 5 min at room temperature. Then, they were fixed with 4% formaldehyde in PBS for 15 min at room temperature. These cells were blocked with 0.5% bovine serum albumin for 1 h, and then they were incubated with a primary rabbit anti-ATF6 antibody (Abgent, San Diego, CA, USA) at a dilution of 1:50 and a secondary anti-rabbit IgG, which was conjugated with AlexaFluor-488 (1:1000; Cell Signaling Technology, Danvers, MA, USA). After washing with PBS, the cells were counterstained with 4', 6-diamidino-2-phenylindole (Sigma, St. Louis, MO, USA). The images were captured using a Leica DM5000B microscope (Leica Microsystems, Cambridge, United Kingdom).

Statistical Analysis

Data were expressed in terms of mean ± standard deviation. To compare the variable group, data by the following statistical techniques: Student's *t*-test, one-way analysis of variance, and Newman–Student–Keuls test. The results were considered to be statistically significant if $P<0.05$.

Results

Effect of Ox-LDL on THP-1 Macrophages Apoptosis and the Activation of ER Stress

We first treated macrophages with 75 μg of proteins/ml (μg/ml), which were isolated from ox-LDL. The treatment was performed for 0, 12, 24, and 48 h according to a procedure described in a previous study[23]. The data proved that ox-LDL-induced cell apoptosis in a time-dependent manner (**Fig. 1A**). We stained these cells using Hoechst 33258 and observed them under a microscope. Thus, we detected cell shrinkage, chromatin condensation, and nuclear fragmentation. In addition, when the macrophages were treated with ox-LDL, there was an increase in the expression of ER stress sensor proteins, such as GRP78, IRE1, and p-PERK (**Fig. 1B**, **C**). This indicates that ox-LDL activated ER stress and apoptosis in a concentration- and time-dependent manner. Based on these findings, we used ox-LDL at an optimal concentration and time (75 μg/ml for 24 h) in subsequent experiments. In the nuclear region of cells treated with ox-LDL, we performed immunofluorescence analysis and detected clusters of ATF6 protein. ATF6 protein was found to be diffused in the cytoplasm of untreated control cells, which are an inactive form of ATF6 protein (**Fig. 1D**).

Ox-LDL Stimulates ASM Activity and Enhances Ceramide Levels in THP-1 Macrophages

To investigate the effect of ox-LDL on ASM and ceramide, we treated macrophages with 75 μg/ml of ox-LDL for 24 h. Within 2 h of treatment, ox-LDL induced maximal levels of ASM in macrophages (2-fold); however, ASM concentration dropped to basal levels within 12 h. We observed a slight increase

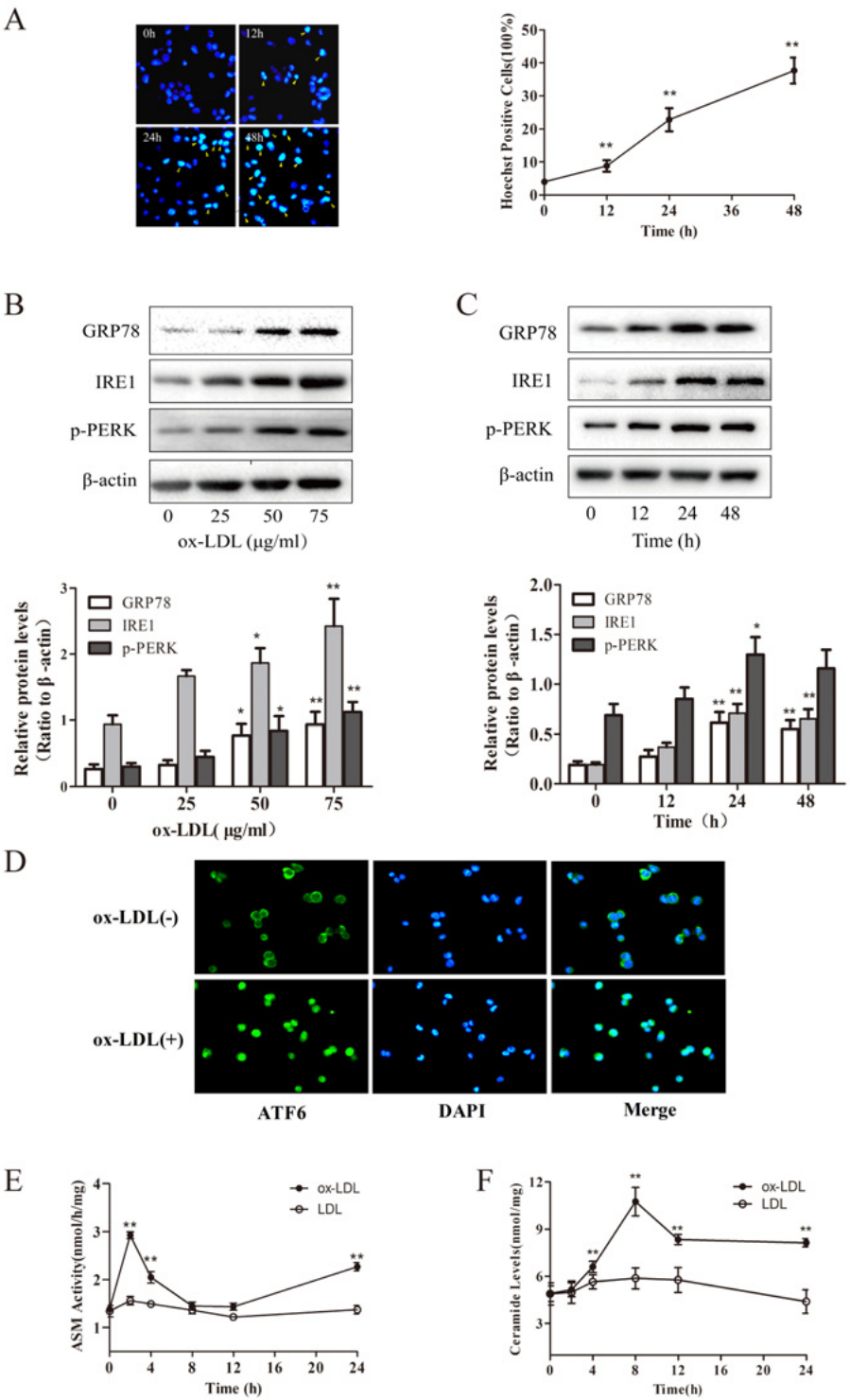

Fig. 1. Ox-LDL induces cell apoptosis, ER stress, and ASM/ceramide activation in THP-1 macrophages

(A) Cells were treated with ox-LDL (75 μg/ml) for 0, 12, 24, and 48 h. Apoptosis was detected by Hoechst 33258 staining. The apoptotic cells (yellow arrows) were observed under a microscope at 200-fold magnification. To perform Western blot analysis of macrophages, they were incubated with ox-LDL at different concentrations (0, 25, 50, or 75 μg/ml) for 24 h (B), or 75 μg/ml of ox-LDL at different time points (0, 12, 24, or 48 h) (C), respectively. (D) Immunofluorescence analysis showed ATF6 nuclear translocation occurred when the cells were treated with ox-LDL (75 μg/ml) for 24 h. The cells were observed at 400-fold magnification with a microscope. (E) ASM activity and (F) ceramide levels were measured after the cells were exposed to 75 μg/ml ox-LDL or 75 μg/ml LDL at the indicated time points (0, 2, 4, 8, 12, or 24 h). All the data are representative of at least three independent experiments. $^{**}p<0.01$, $^{*}p<0.05$ vs. control.

in ASM concentration during the latter half of this experiment (**Fig. 1E**). Moreover, when macrophages were treated with ox-LDL, the levels of ceramide also increased by 2.2-fold within 8 h; however, the ceramide levels also dropped to 1.7-fold at 24 h (**Fig. 1F**). In contrast, there was no significant increase in ASM activity and ceramide levels when the cells were treated with LDL. Thus, the levels of ASM-generated ceramide got elevated before we could induce ER stress and apoptosis.

By Inhibiting ASM, Ox-LDL-Induced Activation of ER Stress is Blocked in THP-1 Macrophages

To further determine the role of ASM in ER stress that is induced by ox-LDL, we transfected ASM siRNA and knocked down ASM expression. Furthermore, ASM siRNA successfully suppressed mRNA, protein expression, and activity of ASM (**Fig. 2A**, **B**, and **C**). Subsequently, we treated macrophages with ox-LDL. Consequently, the elevation in ceramide levels, which was induced by ox-LDL, was significantly attenuated by ASM siRNA transfection (**Fig. 2D**). Next, we examined the effect of ASM knockdown on ER stress via siRNA. Our data indicated that ox-LDL significantly increased the expression of the following ER stress sensors: GRP78, IRE1, and phosphorylated PERK. On the other hand, ASM siRNA blunted the stimulation of ox-LDL (**Fig. 2E**).

In addition, when we treated macrophages with ASM inhibitor DES, there was significant suppression in ER stress. Moreover, the ox-LDL-induced elevated ceramide levels were also significantly attenuated (**Fig. 2F** and **G**).

Exogenous ASM Targets ER Stress in THP-1 Macrophages

In a previous study, it was proved that cultured skin fibroblasts of patients with Niemann–Pick disease (NPD) could rapidly internalize the recombinant ASM in acidic conditions. This is because NPD patients had a deficiency in ASM activity due to genetic and biochemical abnormalities. Furthermore, within these cultured skin fibroblasts, ASM activity was almost 2–3-folds greater than that found in normal cells[24]. To further determine whether ASM directly targets ER stress, we incubated macrophages with recombinant ASM. The ceramide levels increased by 2–3-folds when the macrophages were treated with ASM for 2 h (**Fig. 3A**); this observation agreed well with the findings of a previous study[25]. When these macrophages were exposed to ASM for 24 h, we observed an obvious upregulation of ER stress markers (**Fig. 3C**). In addition, when ASM was added to cells for 24 h, there was significant nuclear translocation of ATF6 protein. This phenomenon was not observed in control cells because they were not treated with ASM (**Fig. 3B**).

AC Attenuates Ox-LDL-Induced ER Stress in THP-1 Macrophages

AC is an enzyme that is mostly located intracellularly, presumably in acidic compartments. Recombinant AC catalyzed the hydrolysis of ceramide in several types of cells[26]. We found that recombinant AC distinctly reduced ceramide levels in THP-1 macrophages (**Fig. 3D**). To confirm that ceramide was involved in ER stress that was induced by ox-LDL, we treated macrophages with recombinant AC in the presence of ox-LDL. The data indicated that AC significantly attenuated the activation of GRP78, IRE1, and phosphorylated PERK, an event which was induced by ox-LDL (**Fig. 3E**). Therefore, these results indicate that when ceramide levels were inhibited, ER stress attenuated in macrophages.

ASM/Ceramide Signaling maybe the Upstream of ER Stress

We wanted to determine whether the ASM/ceramide signaling pathway was absolutely necessary for upregulating ER stress. We treated macrophages for 2 h with tunicamycin, an ER stress inducer. Subsequently, we found that ASM activity and ceramide levels had slightly increased. This increase was not statistically significant (**Supplementary Fig. 2**). This indicates that the ASM/ceramide signaling is not required for activating tunicamycin-induced ER stress. Thus, we deduced that ASM/ceramide signaling directly induces ER stress, regardless of other pathways.

Ox-LDL Induced the Upregulation of CHOP and Apoptosis can be Attenuated by ASM/Ceramide/ER Stress Suppressors in THP-1 Macrophages

To determine whether ox-LDL induces apoptosis in macrophages by ASM/ceramide/ER stress pathway, we treated cells with ASM siRNA. Then, we exposed them to ox-LDL. ASM knockdown remarkably suppressed the expression of CHOP and cell apoptosis, which was induced by ox-LDL (**Fig. 4A**, **B**, and **E**). Furthermore, we treated the cells with DES, recombinant AC, and PBA. Finally, we treated cells with ox-LDL. These suppressors significantly decreased CHOP expression and cell apoptosis (**Fig. 4C**, **D**, and **F**). These data indicate that the ASM/ceramide/ER stress signaling pathway elicits ox-LDL-induced CHOP upregulation and apoptosis of cells.

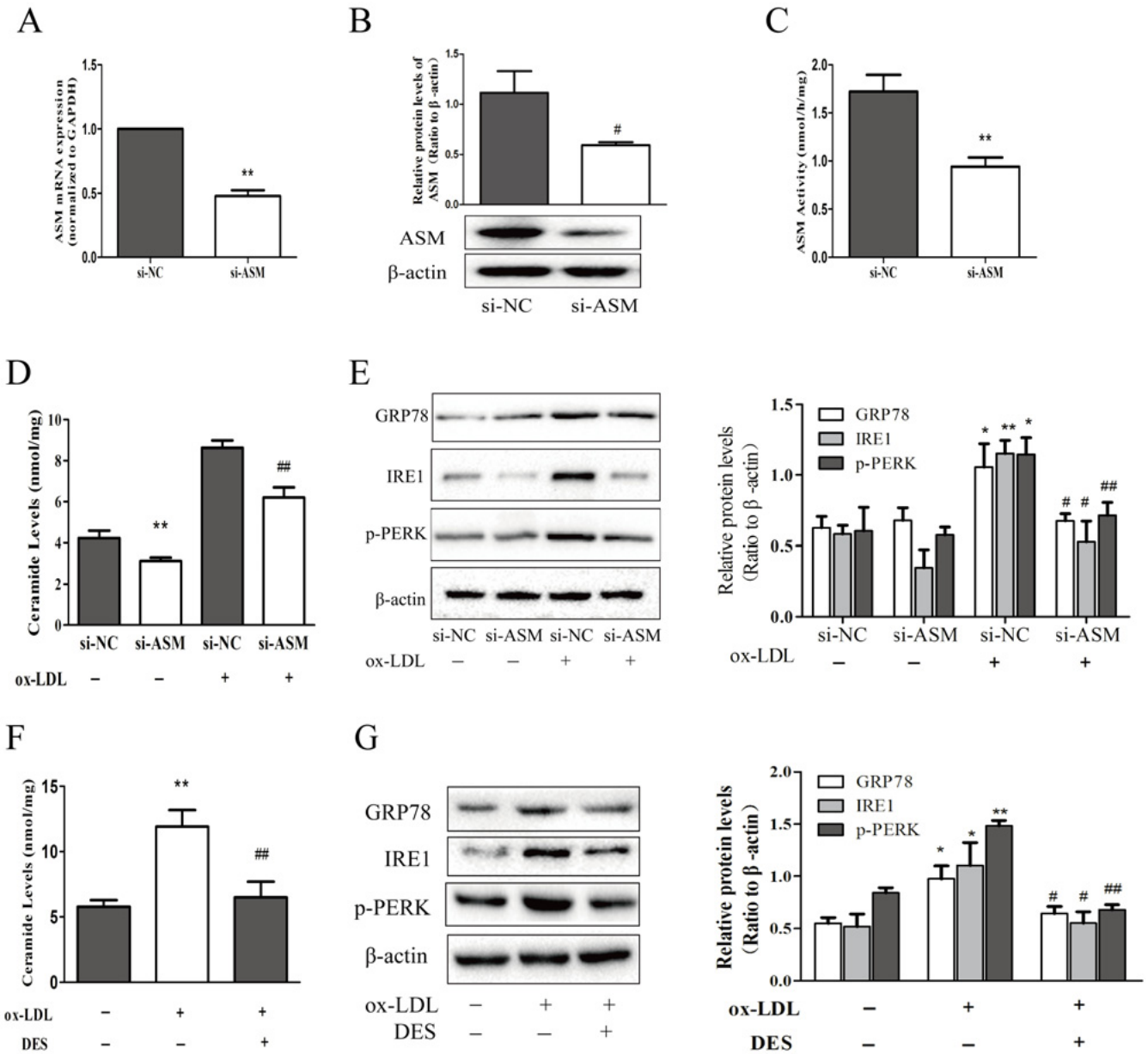

Fig. 2. Inhibition of ASM blocks the upregulation of ox-LDL-induced ceramide and ER stress in THP-1 macrophages

(A) ASM mRNA, (B) ASM protein, and (C) ASM activity were suppressed significantly by transfecting ASM with siRNA ($^{*}p<0.05$, $^{**}p<0.01$ vs. control group transfected with si-NC). Cells were transfected with si-ASM or si-NC, and then they were treated with 75 μg/ml ox-LDL for 24 h: (D) The ceramide levels were validated by UPLC; (E) the expressions of GRP78, IRE1, and phosphorylated PERK proteins were evaluated by Western blot ($^{**}p<0.01$, $^{*}p<0.05$ vs. control group transfected with si-NC; $^{\#\#}p<0.01$, $^{\#}p<0.05$ vs. ox-LDL group transfected with si-NC). DES (20 μM) was added to cultures 2 h before treating them with ox-LDL (75 μg/ml); the ox-LDL treatment was carried out for 24 h. (F) Ceramide levels, and (G) the expression of GRP78 and IRE1 proteins, and phosphorylated PERK were markedly suppressed ($^{**}p<0.01$, $^{*}p<0.05$ vs. control; $^{\#\#}p<0.01$, $^{\#}p<0.05$ vs. ox-LDL). All data are representative of three independent experiments.

Inhibition of ASM/Ceramide Signaling Pathway Attenuates Ox-LDL Induced ER Stress in MPMs

To further determine the effect of the ASM/ceramide signaling pathway on ox-LDL-induced ER stress and to prove that CHOP upregulation was not cell-specific, we used DES and recombinant AC and alleviated the ASM/ceramide signaling pathway. Then, we determined whether there was any mitigation in ox-LDL-induced ER stress. We also determined whether CHOP upregulation was affected in MPMs. As shown in **Supplementary Fig. 3A**, both DES and recombinant AC significantly inhibited ox-LDL-

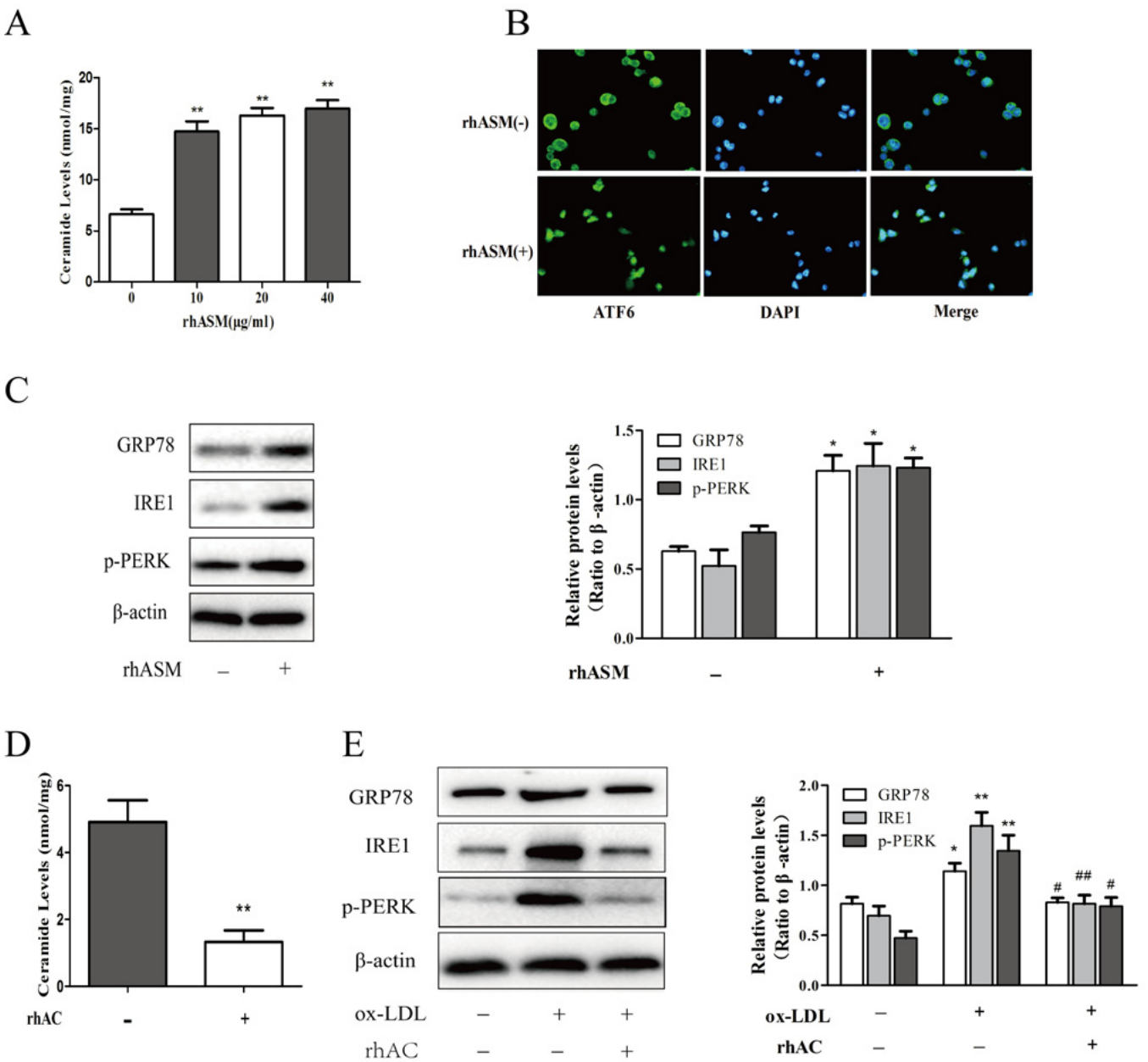

Fig. 3. Exogenous ASM induces ER stress, and recombinant AC attenuates ox-LDL-induced ER stress in THP-1 macrophages

(A) Dose response of rhASM-induced ceramide increase (2 h). (B) ATF6 nuclear translocation was detected by immunofluorescence after treating the cell culture with rhASM (20 μg/ml) for 24 h. The cells were observed at 400-fold magnification under a microscope. (C) Cells that were treated with rhASM(20 μg/ml) for 24 h were analyzed by Western blot to determine the protein expression of GRP78, IRE1, and phosphorylated PERK. (D) Ceramide levels decreased when they were treated with recombinant AC(rhAC) (50 μg/ml) for 24 h. (E) RhAC (50 μg/ml) was added to cultures 2 h before carrying out ox-LDL treatment (75 μg/ml) for 24 h. The expression of GRP78, IRE1 and phosphorylated PERK was also suppressed. All data are representative of three independent experiments. $^{**}p<0.01$, $^{*}p<0.05$ vs. control; $^{\#\#}p<0.01$, $^{\#}p<0.05$ vs. ox-LDL treatment.

induced upregulation of the following proteins: GRP78, IRE1, and phosphorylated PERK. Thus, the activation of ER stress was inhibited by both DES and recombinant AC. Furthermore, DES and recombinant AC also inhibited ox-LDL-induced upregulation of CHOP protein (**Supplementary Fig. 3B**). These results indicated that when the ASM/ceramide signaling pathway is alleviated, there is mitigation in ox-LDL-induced ER stress and CHOP in macrophages. Thus, we concluded that ox-LDL activates the ASM/

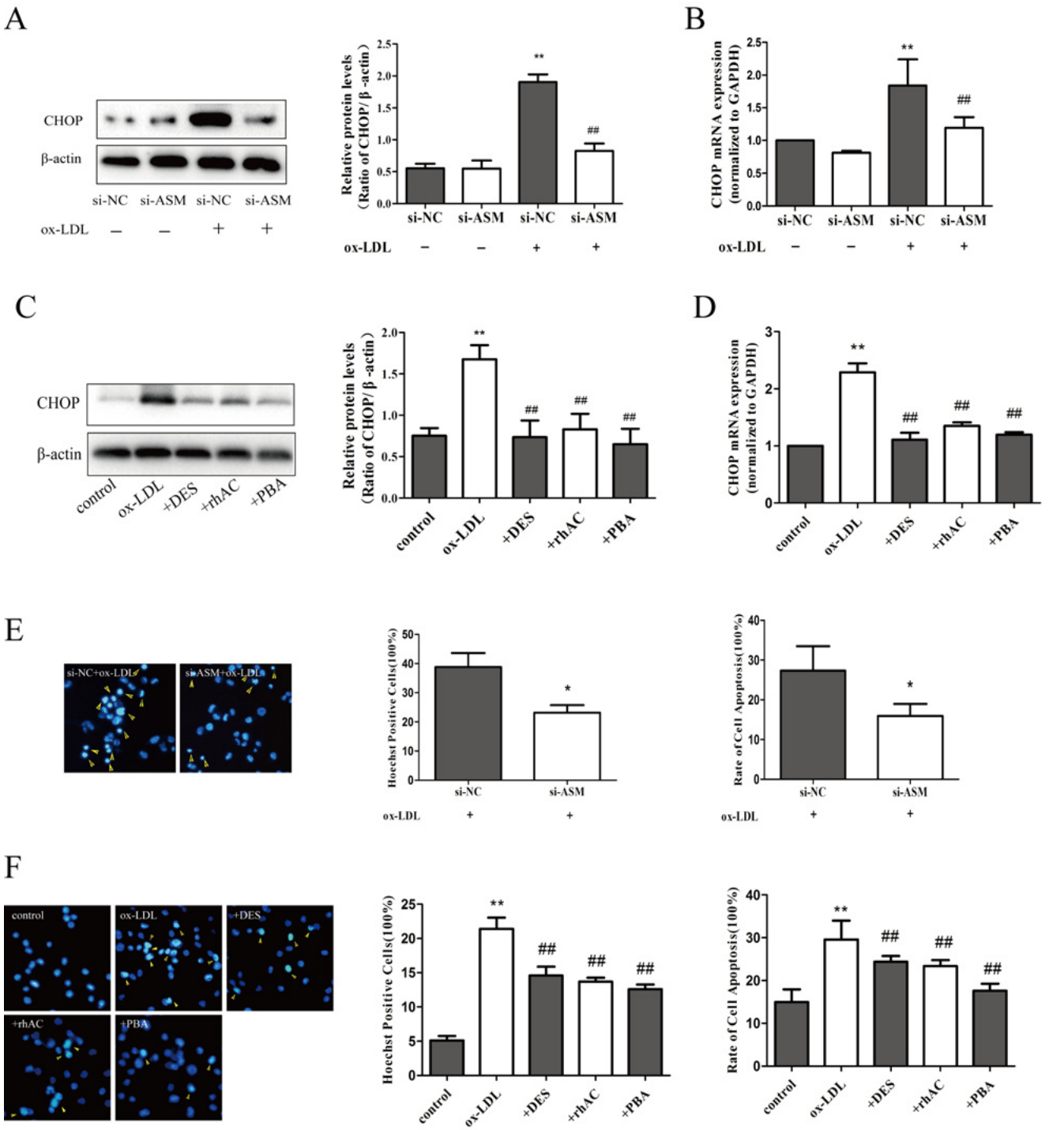

Fig. 4. Ox-LDL-induced upregulation of CHOP and apoptosis can be attenuated by ASM/ceramide/ER stress suppressors in THP-1 macrophages

(A) Protein, and (B) mRNA levels of ox-LDL induced CHOP were blocked by si-ASM transfection. Then, they were compared with si-NC (**$p<0.01$ vs. control group transfected with si-NC; ##$p<0.01$ vs. ox-LDL group transfected with si-NC). (C) Protein, and (D) mRNA levels of ox-LDL induced CHOP were inhibited by treating it with DES (20 μM), rhAC (50 μg/ml), and PBA (10 μM) for 24 h (**$p<0.01$ vs. control; ##$p<0.01$ vs. ox-LDL). (E) Hoechst 33258 and Annexin V-PI double staining coupled along with flow cytometry showed that transfecting si-ASM attenuated ox-LDL induced apoptosis (*$p<0.05$ vs. ox-LDL group transfected with si-NC). (F) DES (20 μM), rhAC (50 μg/ml) and PBA(10 μM) inhibited ox-LDL induced macrophage apoptosis for 48 h; these inhibitory effects were observed by Hoechst 33258 and Annexin V-PI double staining and measured using flow cytometry (**$p<0.01$ vs. control; ##$p<0.01$ vs. ox-LDL). All data are representative of three independent experiments.

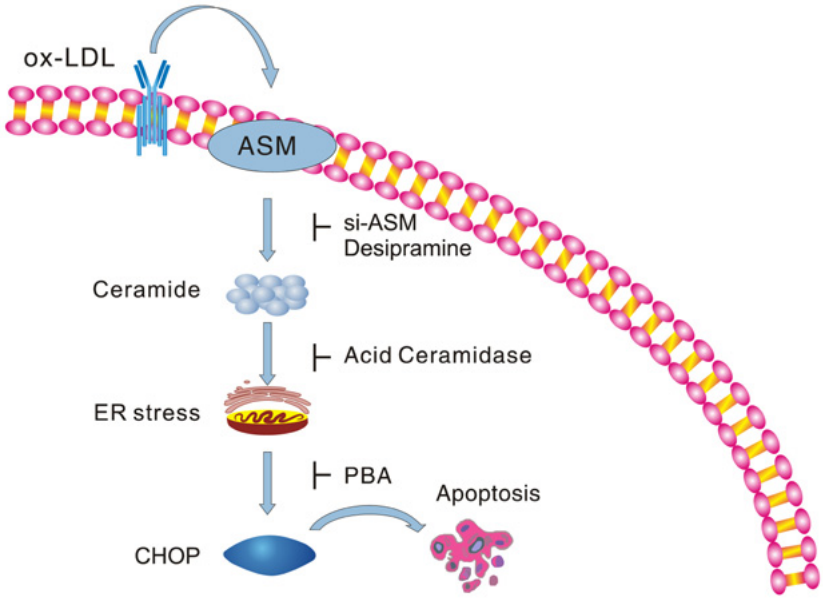

Fig. 5. Schematic representation of ox-LDL, ASM, ceramide, and ER stress in macrophage apoptosis

Ox-LDL induces ASM activation, which increases the generation of ceramide. Then, activated ceramide signaling stimulates ER stress and triggers the expression of pro-apoptotic CHOP, leading to macrophage apoptosis. This proposed signaling axis was suppressed by si-ASM, DES, acid ceramidase, and PBA, leading to the inhibition of ox-LDL induced macrophage apoptosis.

ceramide/ER stress signaling to promote apoptosis of macrophages.

Discussion

In this study, we described for the first time how ER stress was stimulated through the ASM/ceramide signaling pathway. These events lead to ox-LDL-induced apoptosis of macrophages. Owing to ox-LDL-induced cell apoptosis, there was enhancement in ASM activity and elevation in ceramide levels. Furthermore, there was nuclear translocation of ATF6, and upregulation in the expressions of ER stress sensors, such as GRP78, IER1, and phosphorylated PERK proteins: these ER stress sensors activated CHOP. This sequence of events could likely be the underlying mechanism of ox-LDL-induced apoptosis of macrophages. The findings of our present study agree well with those of previous studies[27, 28]. In this study, we also inhibited ASM activity either by using siRNA to knockdown ASM expression or through pharmaceutical manipulation. Thus, we strongly prevented ox-LDL-induced ER stress response, ceramide formation, and apoptosis. Furthermore, exogenous ASM accelerated the expression of ER stress markers in macrophages. Moreover, recombinant AC blocked ceramide, significantly attenuating ox-LDL-induced ER stress and apoptosis. PBA, an ER stress inhibitor, partially alleviated apoptosis of ox-LDL induced macrophages. Interestingly, when ER stress was activated with tunicamycin, the activation of ASM and the production of ceramide remained unaffected. A summary of these changes is shown in **Fig. 5**.

Previous studies prove that ox-LDL is either cytotoxic or proapoptotic to macrophages[29]. However, the pro- (or anti-) apoptotic behavior of ox-LDL depends on numerous factors, such as the nature and extent of LDL oxidation, ox-LDL concentration, and cell types. For example, Asmis *et al.*[30] reported that ox-LDL-induced cytotoxicity (measured as [^{3}H] adenine release) at a threshold concentration of 75 μg/ml. To the best of our knowledge, only a few studies investigated the role of ASM in ox-LDL signaling pathways, but previous studies often established contradictory findings. Some of these previous studies reported that ox-LDL (25 μg/ml) prevents macrophage apoptosis by blocking ASM activity and ceramide expression. Moreover, when macrophages were pre-treated with C2-ceramide, there was reversal in ox-LDL induced upregulation of PKB. This led to a reduction in cell apoptosis[31]. These findings indicate that at low concentrations, ox-LDL promotes the

survival of macrophages by inhibiting the activity of ASM. In another study, researchers reported that ceramide, which was released by ASM, played a positive role in eliciting macrophages. These macrophages responded to ox-LDL treatment: ox-LDL-induced apoptosis in human macrophages by selectively upregulating the transcription of ASM. In a later study, they found that ox-LDL also increased ASM activity in smooth muscle cells[32]. Furthermore, previous studies proved that ox-LDL increases ceramide levels in RAW264 cells, but this ceramide production could be inhibited by a ceramide synthase inhibitor, fumonisin B1. This indicates that in *de-novo* synthesis, ox-LDL induces elevation in the levels of ceramide[33]. In this study, we proved that the release of ASM in human macrophages is promoted and regulated when there is an uptake in the concentrations of ox-LDL (75 μg/ml). We observed that ASM activity increased after 2 h in ox-LDL treated cells; however, it dropped to baseline levels within 12 h. Furthermore, it increased slightly in the latter half of this experiment. This discrepancy in the findings of previous studies and ours may be attributed to different cell types and nature or concentration of ox-LDL.

In a previous study, researchers found that ASM plays a pivotal role in the development of AS. They found that an increase in ASM activity leads to the development of arterial intima and AS plaques[34]. Moreover, due to low-density lipoprotein (LDL) receptor gene and ASM (ldlr-/asm-), lipoproteins are retained to a lesser extent in double-knockout mice. As a result, the development of atheroma is also reduced in these mice[35]. ASM is mainly located intracellularly. Furthermore, ASM is rapidly translocated to the outer level of the plasma membrane when it is subjected to cellular stress. When ceramide is generated under the influence of ASM, there is reorganization of the plasma membrane: there is formation of a ceramide-enriched microdomain that alters fluidity and curvature of the plasma membrane. These lipid microdomains have a higher tendency to fuse together and form larger ceramide-enriched platforms, promoting clustering of membrane receptors and other signaling molecules. These signaling molecules amplify membrane signals and induce apoptosis of cells. Recently, the concept of ASM-induced ER stress was reported in another cell type. For example, Fernandez[36] *et al*. proved exogenous ASM, which was obtained from human placenta, increases mRNA levels of ER stress markers, such as GRP78 and CHOP, in HepG2 cells. Our results agree with this report and prove that activated ASM is involved in ox-LDL-induced ER stress. Thus, activated ASM probably induces ER stress in different cell types.

Recently, a research study proved that *de novo*-generated ceramide plays a pivotal role in the activation of ER stress. However, previous studies did not report that ASM/ceramide signaling pathway is involved in the regulation of ER stress and macrophage apoptosis. In patients with Farber disease, there is an accumulation of ceramide due to AC deficiency. The accumulated ceramide induces abundant morphological changes that are associated with apoptosis[37]. In contrast, an overexpressed AC offers protection from apoptosis[38]. A previous study proved that recombinant AC enzyme rapidly increased AC activity, degrading ceramide levels in primary chondrocytes[39]. In the present study, we assessed the effect of ox-LDL on ceramide levels in macrophages. We found that ceramide levels significantly increased when macrophages were subjected to ox-LDL treatment. In addition, we brought about the degradation of ceramide using recombinant AC. Thus, using fumonisin B1 and myriocin, we discovered a mechanism that was significantly different from the inhibitory mechanism of *de novo* ceramide synthesis. Subsequently, we substantially inhibited the upregulation of ER stress sensors and attenuated ox-LDL-induced apoptosis simply by reducing the accumulation of ceramide. Collectively, these findings support our hypothesis that ox-LDL induced ER stress and apoptosis are involved in the early stages of ceramide generation in macrophages.

To determine whether induced ER stress alters the generation of ASM and ceramide, we treated macrophages with tunicamycin, a classic ER stress inducer. Interestingly, tunicamycin does not upregulate ASM activity and ceramide levels, indicating the ASM/ceramide signaling pathway acts upstream of ER stress.

Several ER stress sensors regulate CHOP expression and promote cell apoptosis[40]. Moreover, CHOP initiates a mitochondrial apoptosis pathway by altering the downstream connection between Bcl-2 family members and Caspase-12. ER stress inhibitors, including chemical chaperones like PBA and tauroursodeoxycholic acid, improve ER folding capacity. Thus, they stabilize the conformation of protein[41]. In our present study, we proved that ox-LDL-induced upregulation of CHOP and apoptosis in macrophages could be prevented when PBA inhibited ER stress. This finding agrees well with that of a previous study[41-42].

In summary, our current study proved that ASM/ceramide signaling pathway regulates ER stress to prevent ox-LDL-induced macrophage apoptosis. Furthermore, ceramide acts as a novel therapeutic target into which ASM inhibitors and/or recombinant AC can be

introduced to treat advanced stages of atherosclerosis, a condition characterized by intense apoptosis of macrophages. Ceramide is considered to be an intermediate rather than an end product. Nevertheless, future studies must further elucidate the relationship between the derivatives of ceramide and ER stress.

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Conflicts of Interest

None.

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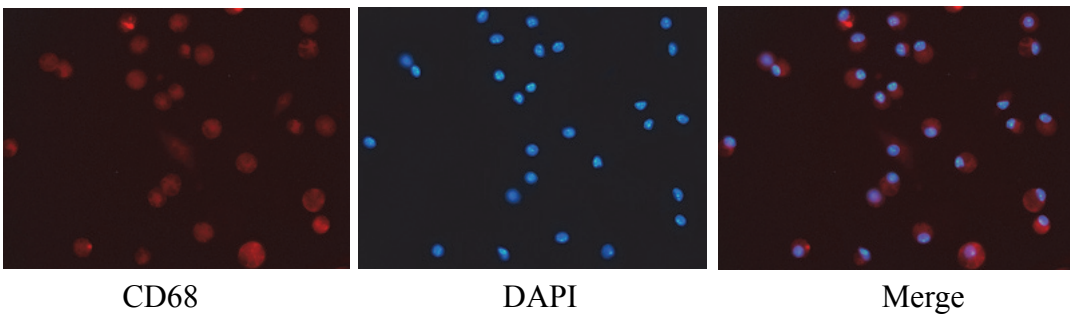
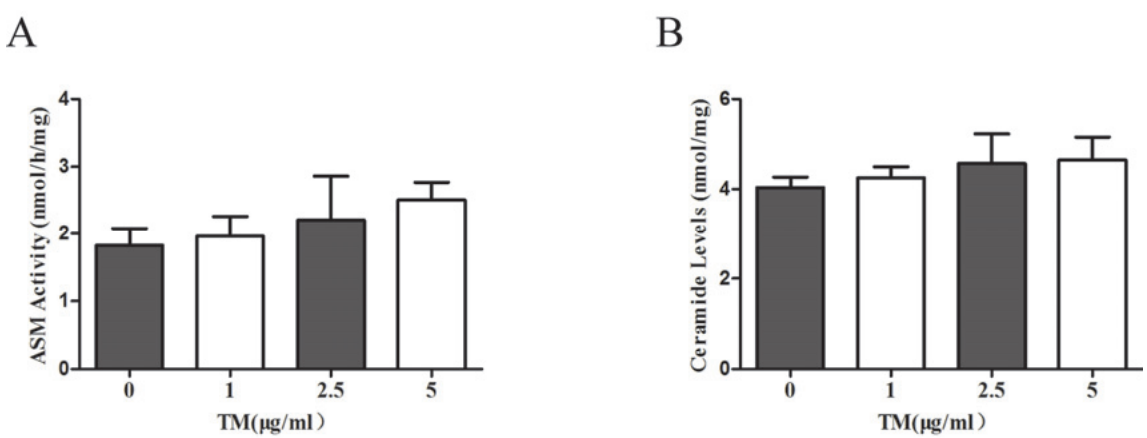


Supplementary Fig. 1.

Primary MPMs were confirmed by immunofluorescence with CD68 antibody

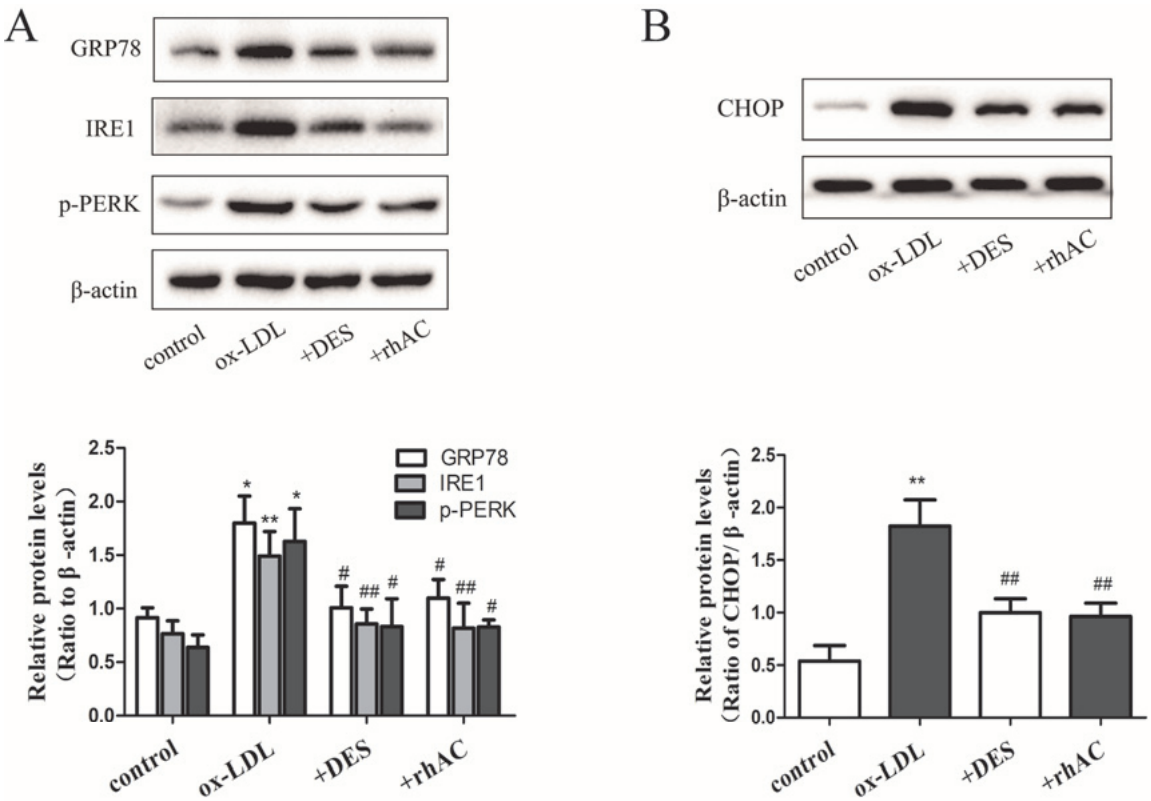
Supplementary Table 1. Primer sequence of specific genes in the present study

RNA		Prime
ASM	Forward	5'-TCTGACTCTCGGGTTCTCT-3'
	Reverse	5'-CACATTGGGTTCCTTCTTCA-3'
CHOP	Forward	5'-CTTCTCTGGCTTGGCTGACT-3'
	Reverse	5'-ACCACTCTGTTTCCGTTTCCT-3'
GAPDH	Forward	5'-CAGGAGGCATTGCTGATGA-3'
	Reverse	5'-GAAGGCTGGGGCTCATTT-3'



Supplementary Fig. 2.

(A) ASM activity and (B) ceramide levels could not increase significantly after treatment with Tunicamycin (TM) at different concentration.



Supplementary Fig. 3. Inhibition of ASM/ceramide attenuates ox-LDL induced ER stress in MPMs.

Desipramine (20 μM) and rhAC (50 μg/ml) were incubated with MPMs for 2 h. Then, MPMs were treated with 75 μg/ml ox-LDL for 24 h. (A) The proteins of ER sensors, including GRP78, IRE1, and phosphorylated PERK, as well as (B) CHOP protein were evaluated by western blot. All data are representative of three independent experiments. $^{**}p<0.01$, $^{*}p<0.05$ vs. control; $^{\#\#}p<0.01$, $^{\#}p<0.05$ vs. ox-LDL treatment.