Original Research

Hydrogen sulfide inhibits macrophage-derived foam cell formation

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Abstract

Recent evidence indicates that hydrogen sulfide (H_2S) exerts an antiatherogenic effect, but the mechanism is unclear. Formation of macrophage-derived foam cells is a crucial event in the development of atherosclerosis. Thus, we explore the effect of H_2S on the formation of macrophage-derived foam cells. Incubation of monocyte-derived macrophages with oxidized LDL (oxLDL) alone caused significant increases both in intracellular lipids revealed by Oil-red O staining and in intracellular total cholesterol (TC) and esterified cholesterol (EC) concentrations assessed by high-performance liquid chromatography. Sodium hydrosulfide (NaHS, an H_2S donor) remarkably abrogated oxLDL-induced intracellular lipid accumulation, and attenuated TC and EC concentrations and EC/TC ratio, whereas DL-propargylglycine (PPG) (a H_2S-generating enzyme cystathionine gamma lyase inhibitor) exacerbated lipid accumulation and augmented TC and EC concentrations and EC/TC ratio. Incubation of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-oxLDL led to lipoprotein binding and uptake of macrophages, which was blunted by NaHS, but enhanced by PPG. Furthermore, OxLDL markedly induced CD36, scavenger receptor A (SR-A) and acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) expressions in macrophages, which was suppressed by NaHS (50–200 μmol/L). Finally, the down-regulations of TC and EC concentrations as well as CD36 and ACAT-1 expressions by NaHS were suppressed by glibenclamide, a K_{ATP} channel blocker, but facilitated by PD98059, an extracellular signal-regulated kinases 1 and 2 (ERK1/2) inhibitor. These results suggested that H_2S inhibits foam cell formation by down-regulating CD36, SR-A and ACAT1 expressions via the K_{ATP}/ERK1/2 pathway in human monocyte-derived macrophages.

Keywords: hydrogen sulfide, foam cells, oxidized low-density lipoprotein, macrophages, scavenger receptors, acyl-coenzyme A:cholesterol acyltransferase-1

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Introduction

Recently, endogenous hydrogen sulfide (H_2S) was recognized as a novel gastrotransmitter, and is synthesized from L-cysteine mainly by cystathionine gamma lyase (CSE) in cardiovascular systems.[1,2] Data from apolipoprotein E-knockout (apoE$^{-/-}$) mice showed that H_2S inhibits atherosclerotic plaque formation,[3] but its mechanisms have not yet been clarified. Some studies have shown several antiatherogenic mechanisms of H_2S, including suppressing the proliferation of rat aortic smooth muscle cells (SMCs),[4] and inhibiting superoxide formation and adhesion molecule expression in arterial endothelial cells.[5] Having known this, it is necessary to further investigate the underlying antiatherogenic mechanism of H_2S because of the complexity of the atherogenic process.

It has been established that macrophage-derived foam cells play a critical role in the initiation and progression of atherosclerosis.[6] Macrophages take up oxidized lipoproteins via scavenger receptors such as CD36 and scavenger receptor A (SR-A) and subsequently esterify free cholesterol to cholesteryl ester (CE) by acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1), which are two key events in the formation of foam cells and the development of atherosclerosis. Furthermore, some studies have suggested that H_2S is endogenously synthesized by monocytes and macrophages, which in turn regulates the functions of these cells.[7,8]

Therefore, in the present study, we aimed to investigate the effects of H_2S on macrophage-derived foam cell formation and its molecular mechanisms.

Materials and methods

Materials

The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Sodium hydrosulfide (NaHS), DL-propargylglycine (PPG), Oil-red O, phorbol 12-myristate 13-acetate (PMA) and glibenclamide were from Sigma (St Louis, MO, USA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Invitrogen (Carlsbad, CA, USA). PD98059 was from Calbiochem (San Diego, CA, USA). The Pierce bicinchoninic acid (BCA) protein assay and mammalian cell extraction kits were from Thermo company (Rockford, IL, USA) and BioVision (Mountain View, CA, USA). CD36 antibody (sc-9154), SR-A (sc-20660), ACAT-1 (sc-20951) and beta-actin antibody (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation.[9] This study was approved by the Ethics Committee of University of South China, and informed consent was obtained from all blood donors. Monocytes purified from mononuclear cells were assessed by flow cytometry using FITC-conjugated anti-human CD14 antibody (AbD Serotec, Oxford, UK) and found to include about 90% $CD14^+$ cells. Both freshly isolated human monocytes and THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine in humidified air, 5% CO_2 at 37°C. Monocytes were differentiated into monocyte-derived macrophages by treatment with 100 ng/mL PMA for 72 h. To evaluate foam cell formation and CD36, SR-A and ACAT-1 expression, macrophages were pretreated with NaHS (25–200 μmol/L) or PPG (3 mmol/L) for one hour, and then incubated for another 48 h in the presence of oxLDL (50 μg/mL). For oxLDL binding experiments, macrophages were pretreated with NaHS (50 μmol/L) and PPG (3 mmol/L) for one hour, and then incubated for another two hours with DiI-labeled oxLDL (DiI-oxLDL; 10 μg/mL) at 4°C. For oxLDL uptake experiments, macrophages were pretreated with NaHS (50 μmol/L) and PPG (3 mmol/L) for one hour, and then incubated for another six hours with DiI-oxLDL (10 μg/mL). To evaluate the intracellular signal transduction pathways, macrophages were pretreated for one hour with the K_{ATP} channel blocker glibenclamide (10 μmol/L), the extracellular signal-regulated kinases 1 and 2 (ERK1/2) inhibitor PD98059 (10 μmol/L) or dimethyl sulfoxide (DMSO) (0.5%) before the addition of NaHS, and then further incubated for 48 h with oxLDL.

Isolation and modification of lipoproteins

LDL was isolated and Cu^{2+}-oxLDL was prepared as described.[10] Briefly, the LDL fraction (1.019–1.063 g/mL) was isolated from human plasma by sequential ultracentrifugation. Then LDL was exposed to 10 μmol/L $CuSO_4$ for 18 h at 37°C, and Cu^{2+} was removed by extensive dialysis. The extent of modification was determined by the measurement of thiobarbituric acid-reactive substances (TBARs). The TBARs content of ox-LDL was 60 nmol/mg LDL protein. OxLDL was sterilized by filtration through a 0.45-μm filter and stored at 4°C.

High-performance liquid chromatography assays

Monocyte-derived macrophages (THP-1 cells, and freshly isolated blood monocytes) were pretreated with NaHS and PPG for one hour, and then incubated for another 48 h in the presence of oxLDL. After incubation, cells were washed thrice with phosphate-buffered saline (PBS) and harvested in PBS. Cells were sonicated in an ice bath using an ultrasonic processor. A portion of the lysate (50 μL) was transferred to microfuge tubes and protein concentration was determined using the BCA assay. One hundred and fifty microliters of Stigmasterol (as a cholesterol internal standard) was added to the rest of the lysate (450 μL) and Vortex-mix. The mix was divided into two equal parts for the total (TC) or free (FC) cholesterol determination. To measure the TC, 100 μL 8.9 mol/L KOH was added to each sample and incubated for two hours in a 50°C water bath. Afterwards, 1 mL of the hexane was added to all tubes and vortexed successively for 15 min. Organic phases were collected and dried in a SpeedVac. The residues were re-suspended in 400 μL acetone and 40 μL 2 mol/L H_2SO4:CrO3 (1:1), and the reaction was allowed to progress for five minutes. After five minutes, 500 μL hexane was added to stop the reaction, followed by the addition of 500 μL water. Organic phases were collected and dried in a SpeedVac. The residues were re-suspended in 400 μL of isopropanol:acetonitrile (20:80,v:v) for further analysis. For the FC determination, the cholesterol in the sample need not be saponified in KOH solution, while the rest of the procedure is the same as that of TC determination. The TC and FC content were analyzed by high-performance liquid chromatography (HPLC) analysis. HPLC was performed on a Hypersil-C18 column, and columns were eluted with the solvent system isopropanol:acetonitrile (20:80, v:v) at a flow rate of 1 mL/min. CE was determined by subtracting the FC values from the TC values. Results were presented as mg lipids/mg cellular proteins.

Enzymatic colorimetric assay

Monocyte-derived macrophages (THP-1 cells) were pretreated for one hour with the K_{ATP} channel blocker glibenclamide (10 μmol/L), the mitogen-activated protein kinase inhibitor D98059 (10 μmol/L) or DMSO (0.5%) before the addition of NaHS, and then further incubated for 48 h with oxLDL. Cells were subjected to hexane–isopropanol (3:2) extraction to isolate cellular lipids and proteins. TC and FC contents present in the lipid extracts were determined by using a cholesterol determination kit from Genmed Scientific Inc. (Shanghai, China) according to the manufacturer's instructions, and CE by subtraction.

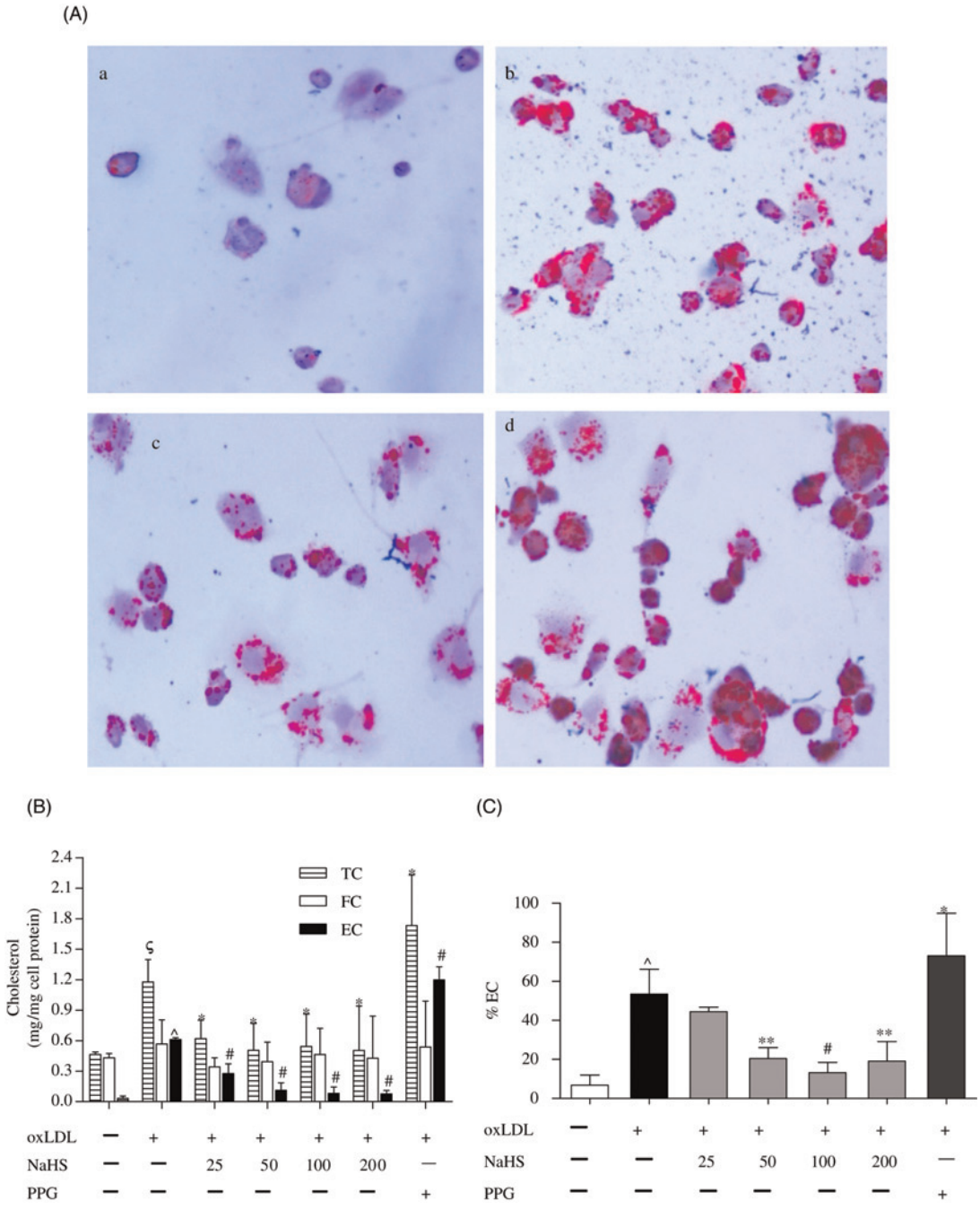

Figure 1 Foam cell formation assay. (A) Light microscopy of monocyte-derived macrophages after staining with Oil-red O. Red particles in the cytoplasm represent lipid droplets. Macrophages were incubated with (a) vehicle (control), (b) oxidized LDL (oxLDL) (50 μg/mL) alone or (c) oxLDL and sodium hydrosulfide (NaHS) (50 μmol/L) and (d) oxLDL and DL-propargylglycine (3 mmol/L) for 48 h. Magnification $\times$400. (B) Intracellular total cholesterol (TC), free cholesterol (FC) and esterified cholesterol (EC) levels in macrophages derived foam cells. TC and FC were determined by high-performance liquid chromatography, and cholesteryl ester was calculated as the difference between TC and FC. $^{\varsigma}P<0.05$, $^{\wedge}P<0.001$ versus control; $^{*}P<0.05$, $^{\#}P<0.001$ versus oxLDL alone. (C) Quantification of percentage of cellular EC. $^{\wedge}P<0.001$ versus control; $^{**}P<0.01$, $^{\#}P<0.001$, $^{*}P<0.05$ versus oxLDL alone. (A color version of this figure is available in the online journal)

Oil-red O staining

Treated macrophages (THP-1 cells) were washed once with PBS and fixed in 4% paraformaldehyde–PBS for 10 min. After rinsing with 60% isopropanol, macrophages were stained with 0.3% Oil-red O in 60% isopropanol for 10 min, washed with 60% isopropanol and then counterstained with hematoxylin for two minutes. After washing with water, macrophages were photographed under a microscope at $\times$400 magnification.

Lipoprotein labeling and oxLDL binding and uptake assay

OxLDL was labeled with the lipophilic tracer, DiI (Molecular Probes), as described.[11] DiI was maintained in the dark at room temperature as a 10 mg/mL stock in DMSO. Lipoprotein labeling was performed by adding 20 μL of DiI stock/1 mL lipoprotein (500 μg/mL) or DMSO. The DiI mixture was vortexed briefly, incubated in the dark at 37°C overnight and centrifuged at 10,000*g* for

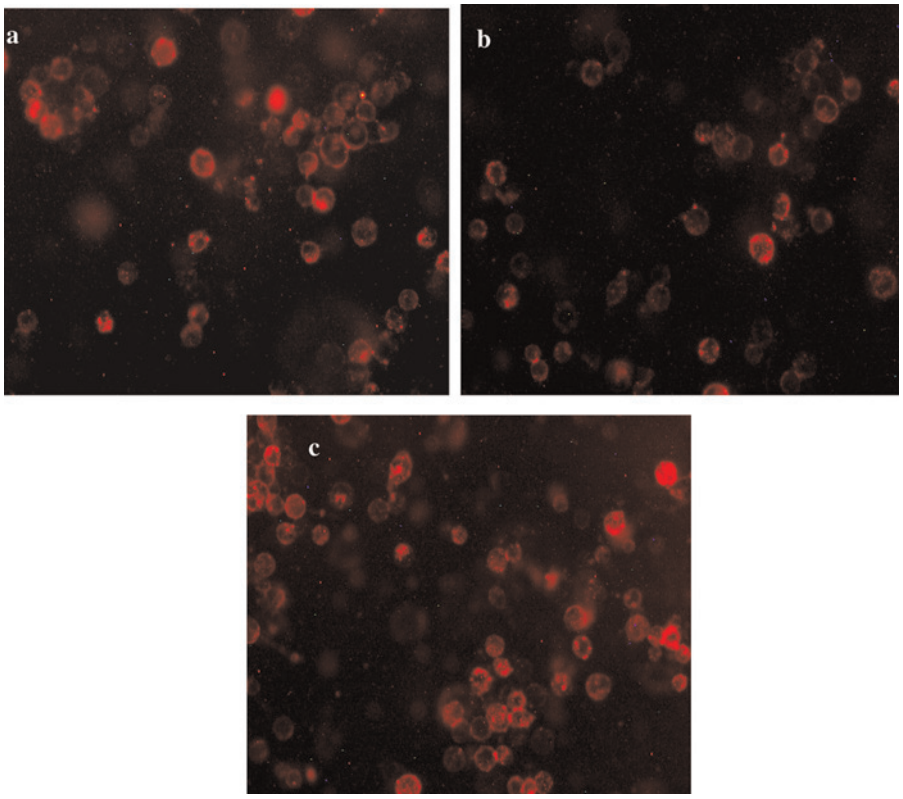

Figure 2 Binding of DiI-oxLDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-oxidized LDL) in monocyte-derived macrophages subjected to DiI-oxLDL for two hours at 4°C. (a) DiI-oxLDL treatment. Macrophages were exposed to DiI-oxLDL in the presence of (b) sodium hydrosulfide (NaHS) (50 μmol/L) and (c) DL-propargylglycine (PPG) (3 mmol/L). Magnification $\times$200. (A color version of this figure is available in the online journal)

30 min to pellet unbound DiI. The supernatant was sterile-filtered with a 0.2-μm filter and kept in the dark.

For oxLDL binding experiments, THP-1 monocyte-derived macrophages were incubated with 10 μg/mL DiI-oxLDL or DMSO-oxLDL for two hours at 4°C in 5% CO_2. DiI-oxLDL binding was assayed by fluorescence microscopy.

For oxLDL uptake experiments, monocyte-derived macrophages (THP-1 cells, and freshly isolated blood monocytes) were incubated with 10 μg/mL DiI-oxLDL or DMSO-oxLDL for six hours at 37°C in 5% CO_2. DiI-oxLDL internalization was assayed by fluorescence microscopy.

Western blot analysis

Protein extracts were prepared by using the mammalian cell extraction kit as per the manufacturer's instructions. All protein concentrations were determined using the Pierce BCA protein assay kit. The protein extracts (50 μg) were treated with 5$\times$ sodium dodecyl sulfate (SDS)–PAGE sample buffer (0.35 mol/L Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, 0.0075% bromophenol blue), then heated at 100°C for five minutes and separated by electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were then transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in TBS-Tween buffer (0.1% Tween 20, pH 7.5) for two hours and incubated overnight with the primary antibodies anti-human CD36 (1:650), anti-human SR-A (1:600), anti-human ACAT-1 (1:600) or anti-human β-actin (1:500). They were washed three times with TBST and incubated for one hour with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000). The protein bands in the blots were detected by using an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL, USA).

Statistical analysis

Results are expressed as mean $\pm$ SD. Differences between groups were evaluated by analysis of variance, Dunnett's test or least significant difference t-test. A $P < 0.05$ was considered statistically significant.

Results

Role of H_2S in oxLDL-induced foam cell formation

Treating macrophages with oxLDL alone caused intracellular lipid accumulation in macrophages as revealed by Oil-red O staining (Figure 1A-b). This oxLDL-induced lipid accumulation was significantly exacerbated by an additional treatment of PPG (a CSE inhibitor) (Figure 1A-d), but suppressed by NaHS treatment (Figure 1A-c). Accordingly, intracellular total and esterified cholesterol (EC) levels, as assessed by HPLC, were markedly higher in macrophages incubated with oxLDL as compared with control cells. However, macrophages treated

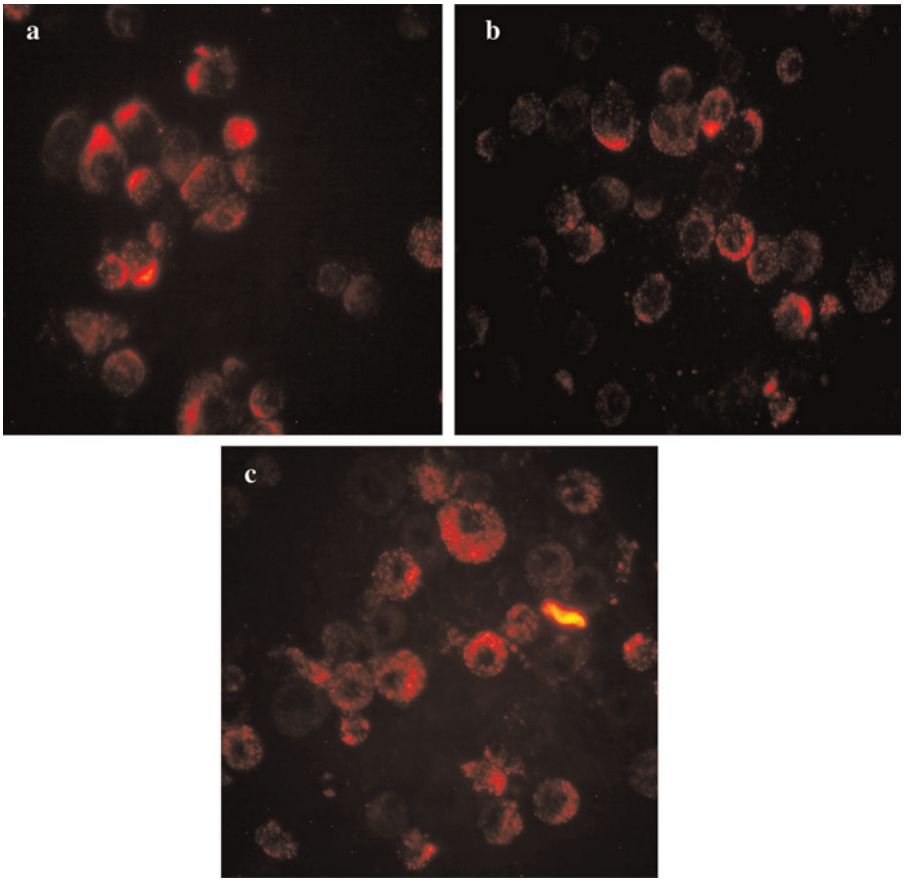

Figure 3 Uptake of DiI-oxLDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-oxidized LDL) in monocyte-derived macrophages on fluorescent microsopy. Red particles in the cytoplasm represent internalized DiI-oxLDL. (a) DiI-oxLDL treatment for six hours at 37°C. (b) Macrophages were incubated with DiI-oxLDL in the presence of (b) sodium hydrosulfide (NaHS) (50 μmol/L) and (c) DL-propargylglycine (PPG) (3 mmol/L). Magnification $\times$400. (A color version of this figure is available in the online journal)

with NaHS (25–200 μmol/L) showed a considerable decrease in intracellular TC and EC concentrations and the percentage of EC, with a maximal decrease of about 58, 88 and 75%, respectively, as compared with oxLDL alone. However, when oxLDL was present in addition to PPG, TC and EC concentrations as well as the percentage of EC were increased to a level higher than that observed with oxLDL alone (Figures 1B and C).

H_2S inhibits oxLDL binding and uptake

We investigated whether decreased binding or uptake of oxidized lipoproteins could account for inhibiting lipid accumulation on treatment with NaHS. Incubation of macrophages with DiI-oxLDL (10 μg/mL) led to the binding of oxLDL to the plasma membrane (Figure 2a) and accumulation of lipoproteins in the cytoplasm (Figure 3a). As expected, NaHS significantly blunted oxLDL binding and uptake in macrophages (Figures 2b and 3b). In contrast, PPG treatment markedly increased the oxLDL binding and uptake in macrophages (Figures 2c and 3c).

H_2S inhibits CD36, SR-A and ACAT-1 expressions

To elucidate the molecular mechanism by which H_2S decreased oxLDL uptake and EC accumulation, the effects of NaHS on the expression of CD36, SR-A and ACAT-1 in macrophages were examined. Macrophages that were untreated showed a relatively low CD36, SR-A and ACAT-1 protein levels; treatment with oxLDL increased CD36, SR-A and ACAT-1 expressions. However, the high levels of CD36, SR-A and ACAT-1 expressions induced by oxLDL were significantly attenuated in the presence of 50–200 μmol/L NaHS. As presented in Figure 4, CD36 expression reached a minimum and a plateau at 50 μmol/L. However, SR-A expression increased gradually after it reached a minimum at 50 μmol/L. ACAT-1 expression was reduced in a dose-dependent manner, with the strongest decrease at 200 μmol/L (Figure 4).

Signaling pathways of H_2S inhibiting foam cell formation

To identify the signaling pathways involved in the inhibitory effect of H_2S on foam cell formation, macrophages were pretreated for one hour with glibenclamide and PD98059 before the addition of NaHS. Results showed that the decrease in cellular TC and EC accumulation by NaHS (50 μmol/L) was reversed by K_{ATP} channel blocker glibenclamide, but was facilitated by ERK1/2 inhibitor PD98059 (Figure 5). Similar results were obtained for CD36 and ACAT-1 expressions (Figure 6).

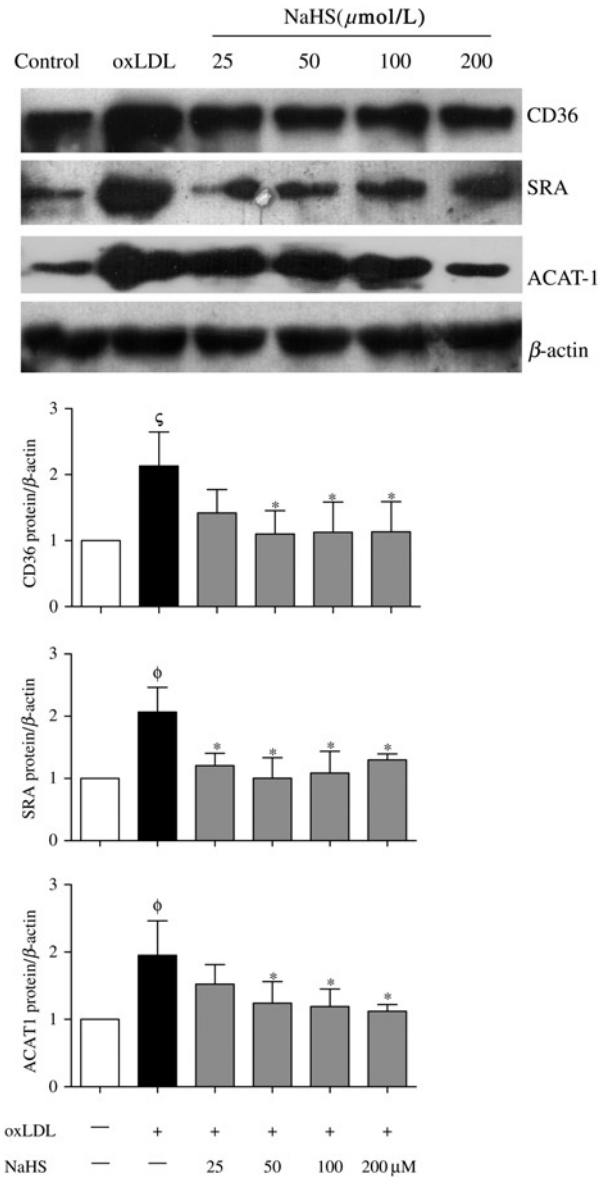

Figure 4 Representative Western blot analysis and densitometric quantification of CD36, scavenger receptor A (SR-A) and acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) protein expression in monocyte-derived macrophages and after incubation with oxidized LDL (oxLDL) with and without sodium hydrosulfide (NaHS) (25–200 μmol/L). Results are mean $\pm$ SD; $n = 3$ in each group. $^{\varsigma}P < 0.05$, $^{\phi}P < 0.01$ versus control; $^{*}P < 0.05$ versus oxLDL alone

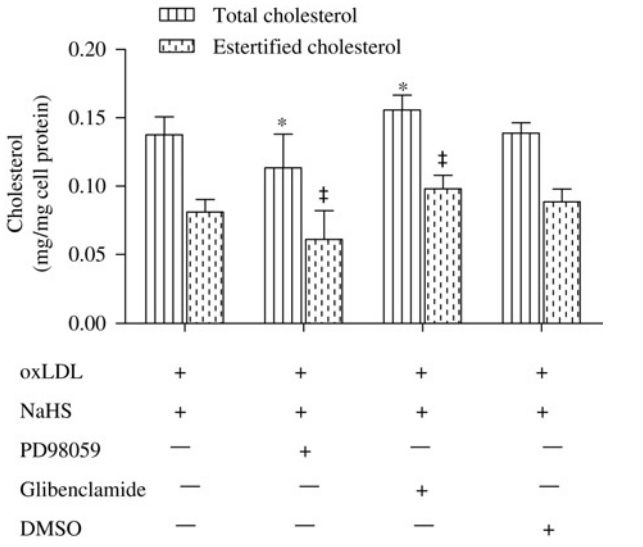

Figure 5 Effect of K_{ATP} channel blocker and extracellular signal-regulated kinases 1 and 2 (ERK1/2) inhibitors on hydrogen sulfide-induced decrease of total (TC) and estertified cholesterol (EC) levels. Macrophages were pretreated for one hour with the K_{ATP} channel blocker glibenclamide (10 μmol/L), the ERK1/2 inhibitor PD98059 (10 μmol/L) or dimethyl sulfoxide (DMSO) (0.5%) before the addition of sodium hydrosulfide (NaHS), and then further incubated for 48 h with oxLDL. At the end of the incubation period, intracellular TC and EC levels were analyzed. Results are mean $\pm$ SD; $n = 5$ (PD98059, DMSO group) or 6 (NaHS, glibenclamide group). $^{*}P<0.05$, $^{\ddagger}P<0.05$ versus oxLDL plus NaHS

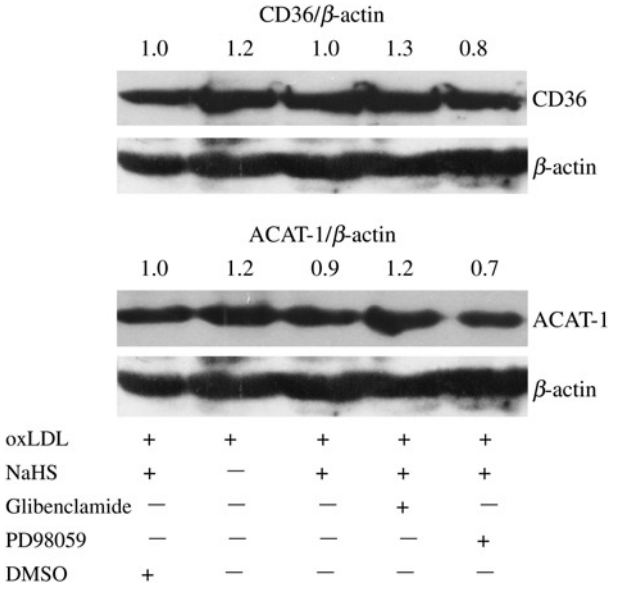

Figure 6 Effect of K_{ATP} channel blocker and extracellular signal-regulated kinases 1 and 2 (ERK1/2) inhibitors on hydrogen sulfide-induced decrease of CD36 and acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) expressions. Macrophages were pretreated for one hour with the K_{ATP} channel blocker glibenclamide (10 μmol/L), the ERK1/2 inhibitor PD98059 (10 μmol/L) or dimethyl sulfoxide (DMSO) (0.5%) before the addition of sodium hydrosulfide (NaHS), and then further incubated for 48 h with oxidized LDL (oxLDL). At the end of the incubation period, cells were lysed and CD36 and ACAT-1 protein expressions were determined by Western blot analysis

Discussion

The accumulation of lipid-laden foam cells in the arterial intima is a critical event in atherogenesis.[12] Despite the recent evidence linking prevention of experimental atherosclerosis with increased H_2S level,[3] no study has examined the role of H_2S in foam cell formation. The present study demonstrates that H_2S inhibits formation of macrophage-derived foam cells. Two lines of evidence support this conclusion. First, both Oil-red O staining and HPLC analysis revealed that the elevation of extracellular H_2S level by NaHS attenuated intracellular lipid and cholesterol (TC, CE) accumulation, and also reduced the percentage of CE. Second, inhibition of endogenous H_2S production by PPG (an inhibitor of CSE) aggravated the above parameters towards foam cell formation.

On exposure to modified lipoproteins such as oxLDL, macrophages take up oxLDL and become foam cells. We

found that NaHS blunted oxLDL binding and uptake of macrophages. In contrast, PPG enhanced oxLDL binding and uptake of macrophages. The binding and uptake of oxLDL is mediated by scavenger receptors.[12]

In multiple scavenger receptors, CD36 and SR-A account for 75–90% of the uptake of modified LDL. CE accumulation was significantly reduced in macrophages from SR-A and CD36 double null mice compared with macrophages from wild-type mice.[13] Additionally, the significance of CD36 and SR-A in atherogenesis is well established. Febbraio *et al.*[14] demonstrated that deletion of CD36 gene slowed lesion development in atherogenic apoE$^{-/-}$ mice. Likewise, a deficiency of the SR-A gene developed less atherosclerosis in apoE$^{-/-}$ or LDLrec$^{-/-}$ mice.[15] The present results showed that NaHS abrogated macrophage CD36 and SR-A expression, and especially reduced CD36 expression within the concentration of 50–200 μmol/L. We conclude that NaHS-induced CD36 and SR-A down-regulation is one way to inhibit foam cell formation.

ACAT-1 is now considered to be a key enzyme promoting intracellular CE accumulation within macrophages. Evidence[16] that overexpression of ACAT-1 in macrophages leads to increased intracellular accumulation of CE supports this role for ACAT-1 in foam cell formation. Furthermore, ACAT-1 is highly expressed in macrophage foam cells in human atherosclerotic lesions,[17] suggesting a crucial role for ACAT-1 in the pathogenesis of atherosclerosis. Notably, partial inhibition of ACAT showed an antiatherogenic effect in apoE-deficient mice.[18] In this study, we demonstrated for the first time that NaHS reduced macrophage ACAT-1 expression, thus suggesting a new role for H_2S, that is, inhibiting foam cell formation.

It has been previously shown that signal transduction pathways of action of H_2S are related to K_{ATP} channel opening.[1,19,20] Spiller *et al.*[19] reported that H_2S improves neutrophil migration and survival in severe sepsis by a K_{ATP}-dependent mechanism. Medeiros *et al.*[20] proved that H_2S prevents ethanol-induced gastric damage in mice by activating K_{ATP} channels. In the present study, we examined the signal transduction pathways by which H_2S inhibits foam cell formation. H_2S-induced inhibitions of EC accumulation and of CD36 and ACAT-1 expressions were abolished by a K_{ATP} channel blocker. The result suggested that the K_{ATP} channel opening is involved in regulation of foam cell formation by H_2S.

In addition to the K_{ATP} channel, we also examined the ERK1/2 pathway. H_2S was previously shown to reduce the activity of ERK1/2. Lu *et al.*[21] reported that H_2S protects astrocytes against H_2O_2-induced neural injury by suppressing ERK1/2 activation. Gobbi *et al.*[22] found that H_2S impairs keratinocyte cell growth and adhesion by inhibiting ERK1/2. Additionally, the inhibitory effect of H_2S on vascular SMC proliferation was due to down-regulation of ERK activation levels by H_2S.[4]

These findings imply that inhibition of ERK1/2 may contribute to the protective effects of H_2S. Inactivation of ERK1/2 prevented cholesterol esterification in human monocyte-derived macrophages treated with acetylated LDL.[23] Our data showed that PD98059 combined with NaHS synergistically lessened accumulation of intracellular EC and protein levels of CD36 and ACAT-1,which suggests that an ERK1/2 signal may be relevant to a pathway of H_2S in inhibiting macrophage foam cell formation. Tay *et al.*[24] recently reported that H_2S antagonizes cerebral hypoxia-induced neuronal cell death via the K_{ATP}/PKC/ERK1/2/Hsp90 pathway. It is tempting to postulate that H_2S inhibits macrophage foam cell formation by the K_{ATP}/ERK1/2 pathway, but the signal transduction pathways between K_{ATP} and ERK1/2 or ERK1/2 and CD36 and ACAT-1 expressions need to be addressed.

In conclusion, our observations identify an antiatherogenic role of H_2S in suppressing human macrophage foam cell formation. The mechanism is via down-regulating expressions of CD36, SR-A and ACAT-1, and inhibiting oxLDL binding and uptake of macrophages, thereby leading to decreased accumulation of EC in macrophages.

Author contributions: Z-ZZ designed and conducted experiments and wrote the manuscript; ZW, G-HL, RW, J-MT, XC and RS performed part of the experiments; and Z-SJ directed the designation of the experiments, evaluated the results and supervised this study.

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