Hydrogen sulfide attenuates TMAO-induced macrophage inflammation through increased SIRT1 sulfhydration

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Abstract. Chronic inflammation is a key factor that accelerates the progression of inflammatory vascular disease. Hydrogen sulfide (H_2S) has potent anti-inflammatory effects; however, its underlying mechanism of action has not been fully elucidated. The present study aimed to investigate the potential effect of H_2S on sirtuin 1 (SIRT1) sulfhydration in trimethylamine N-oxide (TMAO)-induced macrophage inflammation, and its underlying mechanism. Pro-inflammatory M1 cytokines (MCP-1, IL-1β, and IL-6) and anti-inflammatory M2 cytokines (IL-4 and IL-10) were detected by RT-qPCR. CSE, p65 NF-κB, p-p65 NF-κB, IL-1β, IL-6 and TNF-α levels were measured by Western blot. The results revealed that cystathionine γ-lyase protein expression was negatively associated with TMAO-induced inflammation. Sodium hydrosulfide (a donor of H_2S) increased SIRT1 expression and inhibited the expression of inflammatory cytokines in TMAO-stimulated macrophages. Furthermore, nicotinamide, a SIRT1 inhibitor, antagonized the protective effect of H_2S, which contributed to P65 NF-κB phosphorylation and upregulated the expression of inflammatory factors in macrophages. H_2S ameliorated TMAO-induced activation of the NF-κB signaling pathway via SIRT1 sulfhydration. Moreover, the antagonistic effect of H_2S on inflammatory activation was largely eliminated by the desulfhydration reagent dithiothreitol. These results indicated that H_2S may prevent TMAO-induced macrophage inflammation by reducing P65 NF-κB phosphorylation via the upregulation and sulfhydration of SIRT1, suggesting that H_2S may be used to treat inflammatory vascular diseases.

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Introduction

An increasing amount of evidence has indicated that macrophage inflammation is an important pathological manifestation of inflammatory vascular diseases (1,2). The gut microbiota metabolite trimethylamine N-oxide (TMAO) has been shown to increase the incidence of major cardiovascular diseases (3). TMAO has been reported to promote activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome signaling pathway in endothelial cells (4) and vascular smooth muscle cells (VSMCs) (5), which can promote vascular inflammation and atherosclerosis. Notably, inhibition of TMAO production can attenuate atherosclerosis development in mice (6). However, the specific mechanistic link between TMAO and macrophage inflammation has not yet been elucidated. Therefore, investigating the specific mechanisms underlying TMAO-induced inflammation in macrophages is essential for the development of new therapeutics.

In mammalian cells, endogenous hydrogen sulfide (H_2S) is synthesized by enzymatic catalysis. Cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) are the major enzymes involved in catalyzing H_2S synthesis (7). These enzymes that catalyze H_2S synthesis are distributed in different tissues: CSE is predominantly expressed in the cardiovascular system, CBS is mainly expressed in the central nervous system, and 3-MST is expressed in human renal tissue, cardiomyocytes, neurons and the gastrointestinal tract (7,8). Previous studies have shown that H_2S has a wide range of cardiovascular protective effects, including the inhibition of foam cell formation, endothelial inflammation, VSMC proliferation and platelet aggregation (9). CSE knockdown has been reported to result in decreased endogenous H_2S production and increased atherosclerotic plaque formation (10). Furthermore, in a previous study, CSE expression significantly reduced plaque size in $ApoE^{-/-}$ mice (11). Whether H_2S is involved in TMAO-induced macrophage inflammation remains unclear, despite significant progress in understanding the role of H_2S in cardioprotection (12).

Sirtuin 1 (SIRT1) is an NAD^+-dependent class III histone deacetylase that is widely expressed in various human organs. SIRT1 has numerous biological activities, including anti-inflammatory, anti-aging and antioxidative activities (13,14). Our previous studies indicated that SIRT1 is involved in H_2S-mediated cardiovascular protection (15-17). SIRT1 activators E123 and levogliptin has been shown to protect against experimental atherosclerosis by lowering plasma cholesterol and triglycerides (18), and inhibiting the vascular inflammatory response (19). By contrast, SIRT1 inhibitor sirtinol has been shown to block the protective effects of H_2S in foam cell formation and atherosclerosis (20,21). H_2S has also been reported to act as a novel SIRT1 activator that can directly induce SIRT1 sulfhydration (22). Therefore, H_2S may exert a broad protective effect against atherosclerosis by inducing SIRT1 sulfhydration. Based on the aforementioned studies, the present study investigated the modifying effects of H_2S on SIRT1 sulfhydration and its regulatory role in TMAO-induced macrophage inflammation.

Materials and methods

Cell culture. RAW264.7 macrophages (American Type Culture Collection) were cultured in RPMI 1640 medium (HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Inc.), 100 μg/ml streptomycin and 100 U/ml penicillin (both Beyotime Institute of Biotechnology) in an incubator at 37˚C with 5% CO_2. Before treatment, the macrophages were seeded in 100-mm dishes at a density of $2x10^6$ cells/dish and were cultured with medium containing 10% FBS for 24 h, followed by starvation in 0.5% FBS-containing RPMI 1640 medium for 24 h at 37˚C. RAW264.7 macrophages was used as the control. Subsequently, the macrophages were pretreated with 25 μM resveratrol (Sigma-Aldrich; Merck KGaA) for 24 h before TMAO (100 mM, Sigma-Aldrich) stimulation for 24 h. For certain experiments, the macrophages were pretreated with 40 mM SIRT1 inhibitor nicotinamide (Calbiochem; Merck KGaA) or DL-dithiothreitol (DTT; 1 mM, Phygene, China) for 60 min before sodium hydrosulfide (NaHS, 100 μmol/l, Sigma-Aldrich, USA) stimulation for 24 h at 37˚C. To stimulate SIRT1 expression, macrophages were incubated with NaHS for 12, 24 or 48 h.

Modified biotin switch assay. The SIRT1 S-sulfhydration assay was performed as previously reported (22). The macrophages were lysed in HENS buffer [250 mM HEPES (pH 7.7), 1 mM EDTA, 0.1 mM Neocuproine, and 1% SDS] supplemented with protease inhibitors. The cell lysates were blocked with HEN blocking buffer [250 mM HEPES (pH 7.7), 1 mM EDTA, 0.1 mM Neocuproine] containing 20 mM methyl methanethiosulfonate (MMTS, Sigma-Aldrich) for 20 min at 50˚C. Subsequently, MMTS was eliminated by precipitating with acetone for 20 min at -20˚C. Thereafter, leaving a small part of the proteins as control (input SIRT1), the remaining proteins were resuspended in HENS buffer, and biotin-HPDP (4 mM, Thermo Scientific) was added for 4 h at 37˚C. Subsequently, the remaining proteins were pulled down by streptavidin agarose (MilliporeSigma) at 4˚C overnight under gentle shaking conditions. After centrifugation, biotinylated proteins (SHY-SIRT1) were resuspended in a protein loading buffer. Finally, the samples were analyzed by western blotting. Biotinylated proteins (SHY-SIRT1) were blotted together with total lysates (input SIRT1) and subjected to SIRT1 immunoblotting to assess SIRT1 sulfhydration.

H_2S concentration measurement. As described previously (23), the supernatant of macrophages in control and TMAO group was collected and centrifuged at 12,000 g for 10 min at 4˚C. H_2S concentration measurement was performed according to the methylene blue method. Briefly, 800 μl supernatant was mixed sequentially with 8 μl NaOH (1 M), 80 μl 20 mM N, N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl, and 80 μl 30 mM $FeCl_3$ in 1.2 M HCl. Subsequently, the mixtures were incubated at 37˚C for 20 min. Finally, the formed methylene blue was detected at 668 nm using a microplate reader (ELx800; BioTek Instruments, Inc.). H_2S concentration was quantified using a standard curve of NaHS.

Western blot analysis. The macrophages were lysed using ice-cold RIPA Lysis Buffer (cat. no. P0013B; Beyotime Institute of Biotechnology), containing a protease and phosphatase inhibitor cocktail (cat. no. P1045; Beyotime Institute of Biotechnology). Protein concentrations were measured using a BCA protein assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology). Equal amounts of protein (80 μg) were separated by SDS-PAGE on 10 or 12% gels and were transferred onto PVDF membranes. The membranes were then blocked with 5% non-fat dry milk containing Tris-buffered saline -0.1% Tween 20 at 37˚C for 2 h and were incubated with the following primary antibodies at 4˚C overnight: CSE (1:1,000; cat. 60234-1-AP; Proteintech Group, Inc.), SIRT1 (1;750; cat. no. 13161-1-AP; Proteintech Group, Inc.), p65 NF-κB (1:800; cat. no. 8242; Cell Signaling Technology, Inc.), phosphorylated (p)-p65 NF-κB (1:750; cat. no. 3033; Cell Signaling Technology, Inc.), IL-1β (1:500; cat. no. WL00891; Wanleibio Co., Ltd.), IL-6 (1:600; cat. no. 21865-1-AP; Proteintech Group, Inc.), TNF-α (1:750; cat. no. 17590-1-AP; Proteintech Group, Inc.) and β-actin (1:5,000; cat. no. 20536-1-AP; Proteintech Group, Inc.). Subsequently, the membranes were incubated with a horseradish peroxidase-linked secondary antibody (1:5,000; cat. no. A0208 or A0216; Beyotime Institute of Biotechnology) for 2 h at 37˚C. Eventually, images were acquired using a chemiluminescence reagent kit (WBKlS0100, MilliporeSigma) and the densities of the bands were analyzed using ImageJ 1.47i software (National Institutes of Health) for semi-quantification.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) and RT was performed with a high-capacity cDNA synthesis kit (cat. no. RR037A; Takara Bio, Inc.). The RT protocol was as follows: 37˚C for 15 min, followed by 85˚C for 5 sec, and finished at 4˚C. qPCR analysis was performed using the SYBR Green PCR Mix kit (cat. no. DRR041A; Takara Bio, Inc.) and the ABI7500 System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: One cycle at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. The PCR finished

Table I. Quantitative PCR primer sequences used in the present study.

Mouse gene name	Forward (5'-3')	Reverse (5'-3')
β-actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
MCP-1	GTCTGTGCTGACCCCAAGAAG	TGGTTCCGATCCAGGTTTTTA
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-6	TTCCATCCAGTTGCCTTCTTG	TTGGGAGTGGTATCCTCTGTGA
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
TNF-α	GCGACGTGGAACTGGCAGAAG	GCCACAAGCAGGAATGAGAAGAGG
CSE	CTTGCTGCCACCA TTACG	TTCAGATGCCACCCTCCT

CSE, cystathionine γ-lyase; MCP-1, monocyte chemoattractant protein-1.

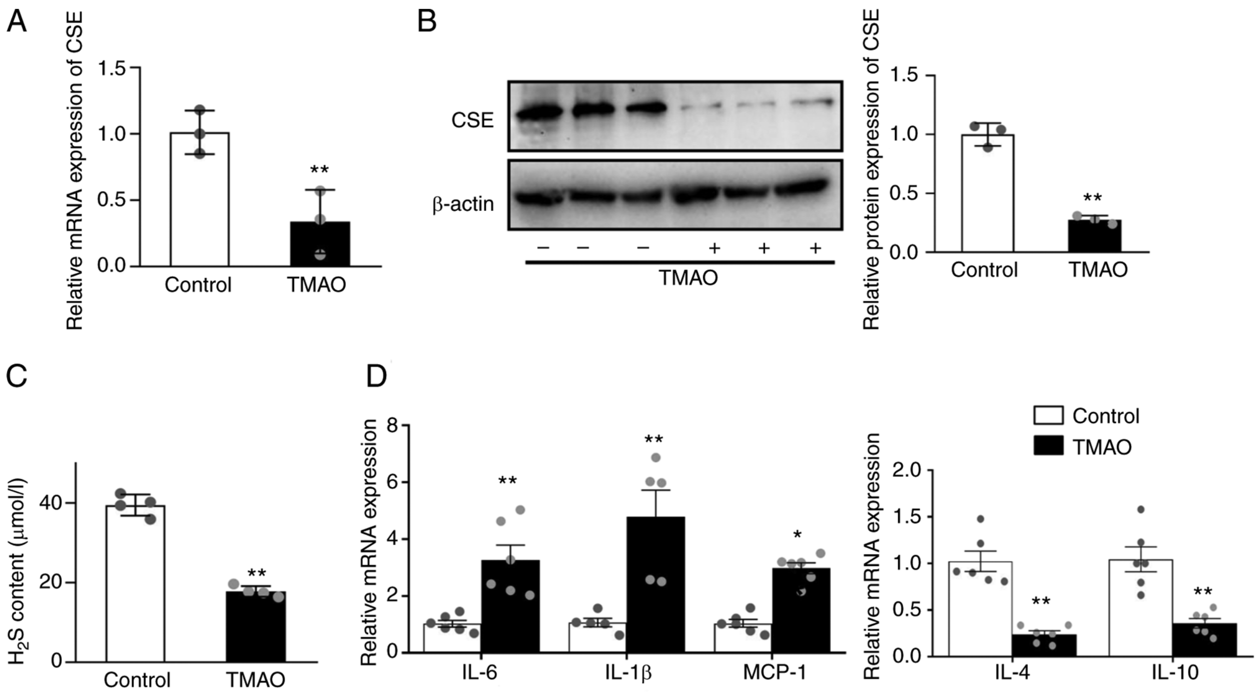

Figure 1. TMAO treatment decreases the expression of CSE and causes inflammation. (A) mRNA expression levels of CSE in the macrophages after TMAO treatment, as measured by RT-qPCR. (B) Representative western blots of CSE in the macrophages after TMAO treatment. (C) H_2S concentration was detected in the supernatant of macrophages after TMAO treatment. (D) mRNA expression levels of IL-1β, IL-6, MCP-1, IL-4 and IL-10 in the macrophages after TMAO treatment, as measured by RT-qPCR (n≥4). $^{*}P<0.05$, $^{**}P<0.01$ vs. control group. CSE, cystathionine γ-lyase; H2S, hydrogen sulfide; MCP-1, monocyte chemoattractant protein-1; RT-qPCR, reverse transcription-quantitative PCR; TMAO, trimethylamine N-oxide.

with extension at 60˚C for 5 min and holding at 4˚C. The relative fold changes in gene expression were normalized to the mRNA expression levels of β-actin and were analyzed using the $2^{-\Delta\Delta Cq}$ method (24). The specific primer sequences are listed in Table I.

Statistical analysis. The data are presented as the mean ± SD. The significant differences between two groups were evaluated using an unpaired Student's t-test. Differences among multiple groups were analyzed by one-way analysis of variance with Tukey's post hoc test. All statistical analyses were performed using SPSS software (version 18; IBM Corp.). All experiments were performed with at least three independent biological samples. P<0.05 was considered to indicate a statistically significant difference.

Results

TMAO downregulates CSE and increases inflammatory cytokine levels. Macrophages were treated for 24 h with 100 mM TMAO to evaluate the effects of TMAO on CSE expression. In the present model, TMAO significantly decreased the mRNA and protein expression levels of CSE in macrophages (Fig. 1A and B) compared with the control group. In addition, TMAO treatment resulted in significantly lower levels of H_2S measured in cell supernatants (Fig. 1C). TMAO upregulated the mRNA

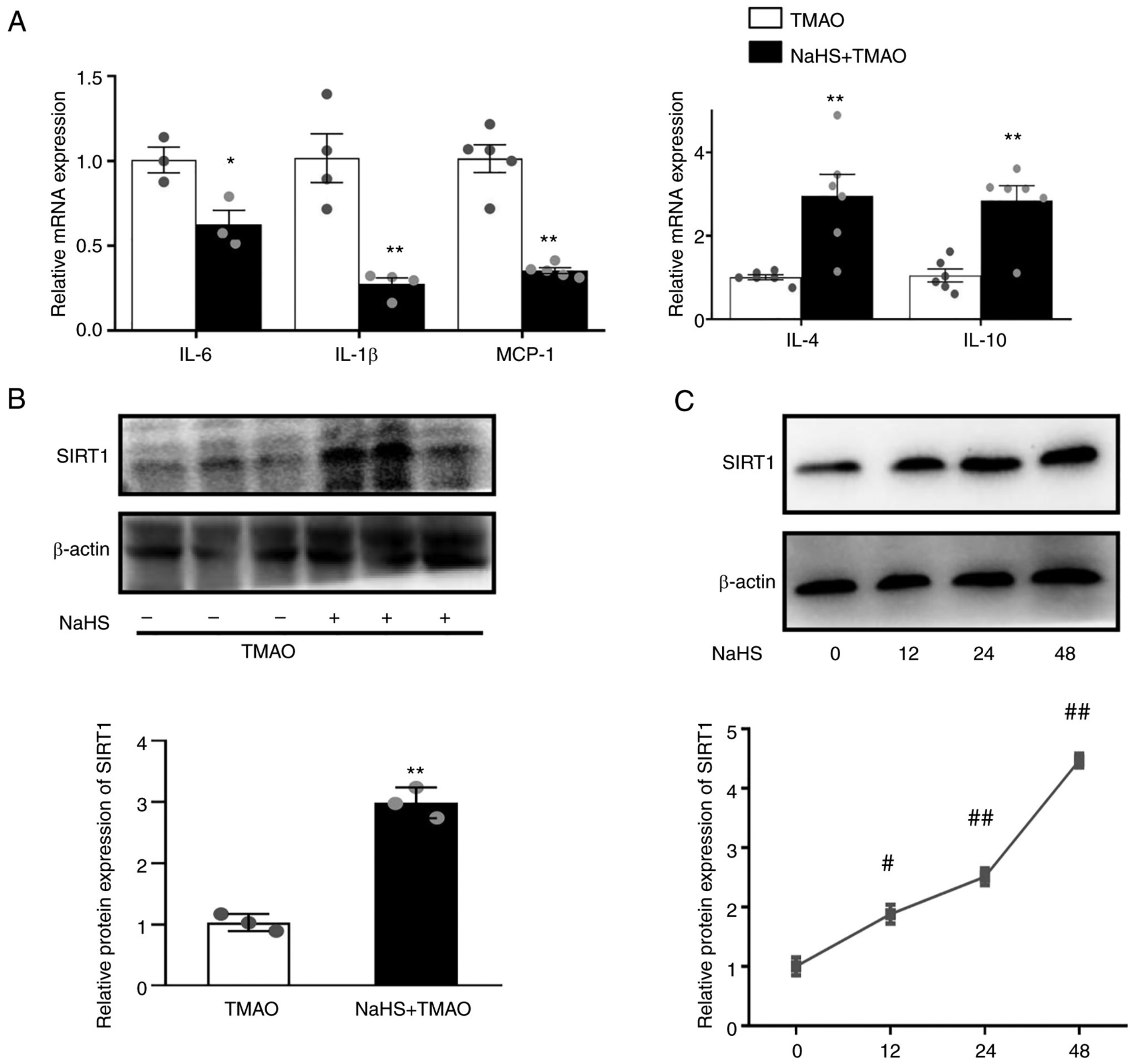

Figure 2. Hydrogen sulfide ameliorates TMAO-induced inflammation and SIRT1 expression. (A) mRNA expression levels of IL-1β, IL-6, MCP-1, IL-4 and IL-10 in the macrophages after NaHS treatment, as measured by reverse transcription-quantitative PCR. (B) Representative western blots of SIRT1 in the macrophages after NaHS and TMAO treatment. (C) Representative western blots of SIRT1 in the macrophages after NaHS treatment for different durations (n=3). *P<0.05, **P<0.01 vs. TMAO group; #P<0.05, ##P<0.01 vs. control group. MCP-1, monocyte chemoattractant protein-1; NaHS, sodium hydrosulfide; SIRT1, sirtuin 1; TMAO, trimethylamine N-oxide.

expression levels of IL-6, IL-1β and monocyte chemoattractant protein-1 (MCP-1), and downregulated IL-4 and IL-10 (Fig. 1D).

NaHS upregulates SIRT1 levels and reduces inflammation in TMAO-stimulated macrophages. H_2S-induced SIRT1 has been reported to confer a protective effect on atherosclerosis (22). NaHS is a donor of H_2S. NaHS downregulated the mRNA expression levels of IL-6, IL-1β and MCP-1, and upregulated the expression levels of IL-4 and IL-10 (Fig. 2A) compared with that in the TMAO group. In addition, NaHS significantly increased the protein expression levels of SIRT1 when RAW264.7 macrophages were challenged with TMAO (Fig. 2B). Furthermore, NaHS induced SIRT1 expression in a time-dependent manner (Fig. 2C) after 12, 24 and 48 h of incubation.

NaHS ameliorates TMAO-induced NF-κB activation. Recent studies have shown that TMAO activates NF-κB signals, resulting in the release of inflammatory cytokines (5,25). The present study examined the effects of H_2S on TMAO-induced NF-κB activation. As shown in Fig. 3A, the TMAO-induced phosphorylation of p65 NF-κB was reversed by NaHS. In addition, NaHS decreased the expression levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in TMAO-stimulated macrophages (Fig. 3B).

NaHS reduces inflammation via enhanced SIRT1 activity. Macrophages were treated with the SIRT1 inhibitor nicotinamide to investigate whether SIRT1 mediated the protective effect of H_2S on TMAO-induced NF-κB activation. The results showed that phosphorylation of p65 NF-κB was significantly

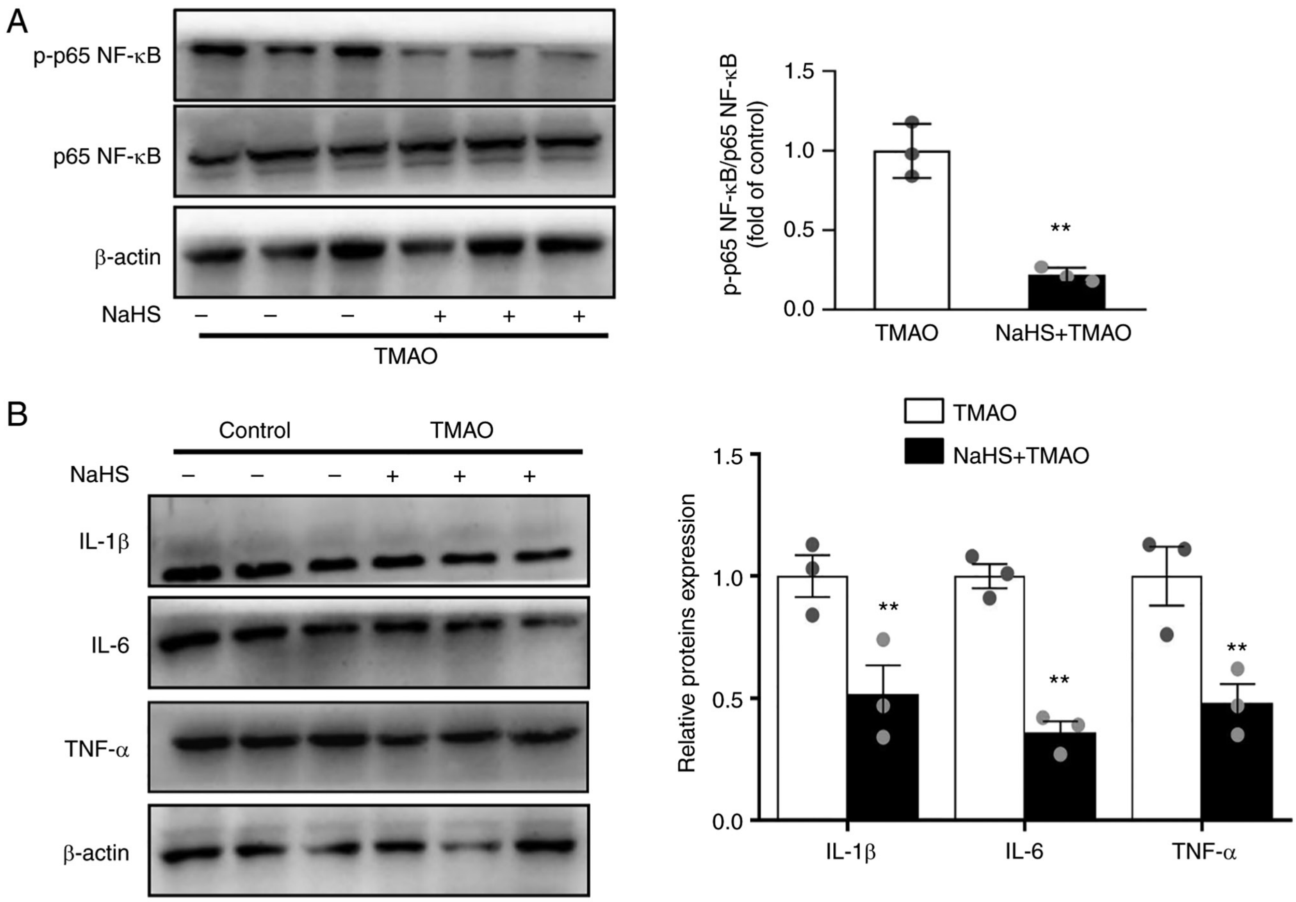

Figure 3. Hydrogen sulfide decreases the phosphorylation of p65 NF-κB and inflammation. (A) Representative western blots of p-p65 NF-κB and p65 NF-κB in the macrophages after NaHS and TMAO treatment. (B) Representative western blots of IL-1β, IL-6 and TNF-α in the macrophages after NaHS and TMAO treatment (n=3). **P<0.01 vs. TMAO. NaHS, sodium hydrosulfide; p-, phosphorylated; TMAO, trimethylamine N-oxide.

increased by nicotinamide compared with that in the NaHS+ TMAO group (Fig. 4A). Furthermore, the protein expression levels of IL-1β, IL-6, and TNF-α were also increased by nicotinamide (Fig. 4B).

Resveratrol counteracts TMAO-induced NF-κB activation via SIRT1. Resveratrol has been identified as a SIRT1 activator, which can modulate the inflammatory response in endothelial cells by inhibiting NF-κB activation (26). Resveratrol reversed the TMAO-induced phosphorylation of p65 NF-κB (Fig. 5A). In addition, resveratrol significantly ameliorated the TMAO-induced expression of IL-1β, IL-6 and TNF-α (Fig. 5B).

H_2S inhibits NF-κB activation via S-sulfhydrated SIRT1. S-sulfhydration refers to the formation of hydropersulfide (-SSH) from H_2S by attaching sulfur to thiol (-SH) groups of cysteines (27). The results revealed that TMAO significantly decreased SIRT1 sulfhydration (Fig. 6A). By contrast, SIRT1 sulfhydration was induced after NaHS treatment in TMAO-stimulated macrophages (Fig. 6B). Given that S-sulfhydrated SIRT1 can reduce inflammation (28), the present study evaluated its effects on NF-κB activation. Following pre-treatment with the thiol-reducing agent DTT in macrophages, the inhibitory effect of NaHS on NF-κB activation was significantly reversed (Fig. 6C). DTT also upregulated the mRNA expression levels of IL-1β, IL-6 and MCP-1, and downregulated IL-4 and IL-10 (Fig. 6D) compared with that in the NaHS +TMAO group. These data indicated that H_2S may attenuate TMAO-induced inflammatory signaling in macrophages by inducing SIRT1 sulfhydration.

Discussion

It has been reported that increased TMAO levels are associated with the development of metabolic diseases, such as diabetes and atherosclerosis (29). To the best of our knowledge, the present study demonstrated for the first time that H_2S was negatively associated with TMAO-induced macrophage inflammation. Mechanistically, it was revealed that H_2S promoted SIRT1 sulfhydration and decreased SIRT1 proteasomal degradation, reducing the phosphorylation of P65 NF-κB, and inhibiting inflammation (Fig. 7). Thus, these results indicated that NaHS attenuated TMAO-induced macrophage inflammation, suggesting that targeting H_2S may be useful in developing novel therapeutic strategies for TMAO-induced cardiovascular events.

TMAO, an intestinal microbial metabolite, has been associated with cardiovascular disease (30). Clinical studies have shown that high TMAO levels are significantly associated with an increased risk of major adverse cardiovascular events (31). TMAO exposure has also been shown to promote endothelial

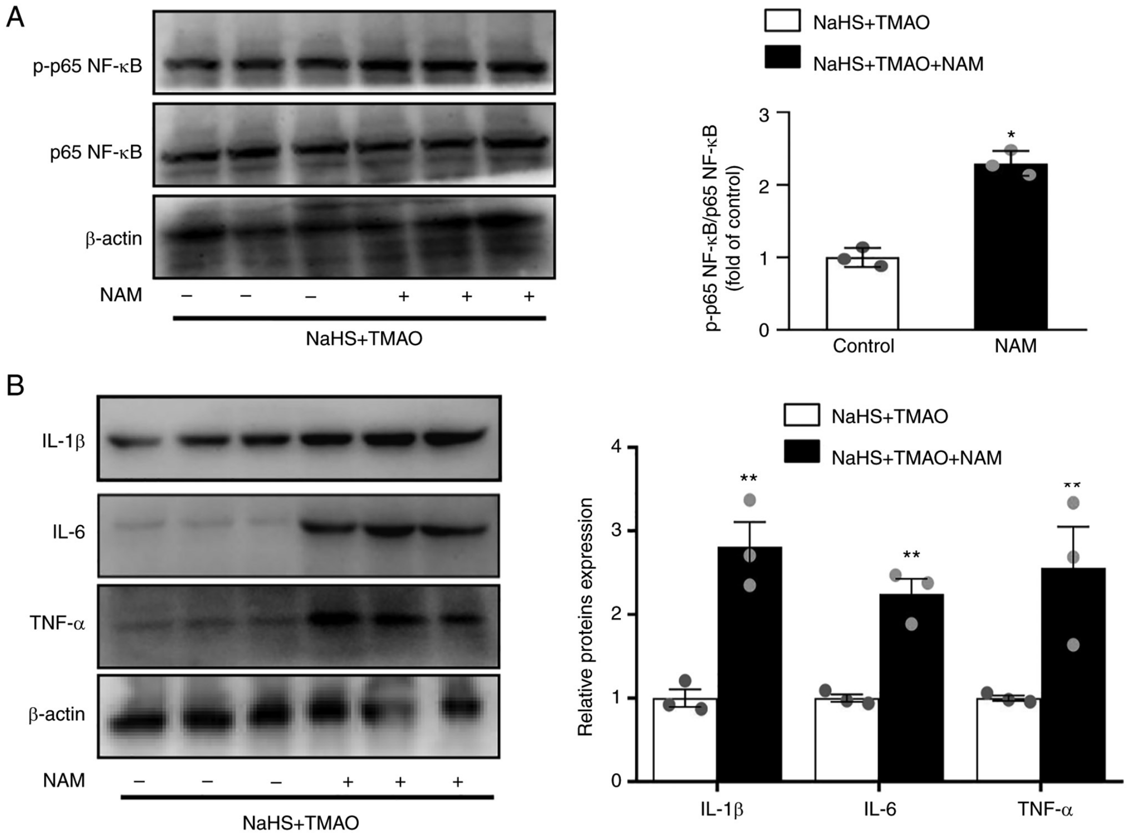

Figure 4. Inhibition of SIRT1 by NAM reverses the protective effects of H_2S. (A) Representative western blots of p-p65 NF-κB and p65 NF-κB in the macrophages after NAM treatment. (B) Representative western blots of IL-1β, IL-6 and TNF-α in the macrophages after NAM pretreatment (n=3). *P<0.05, **P<0.01 vs. NaHS + TMAO group. NAM, nicotinamide; NaHS, sodium hydrosulfide; p-, phosphorylated; TMAO, trimethylamine N-oxide.

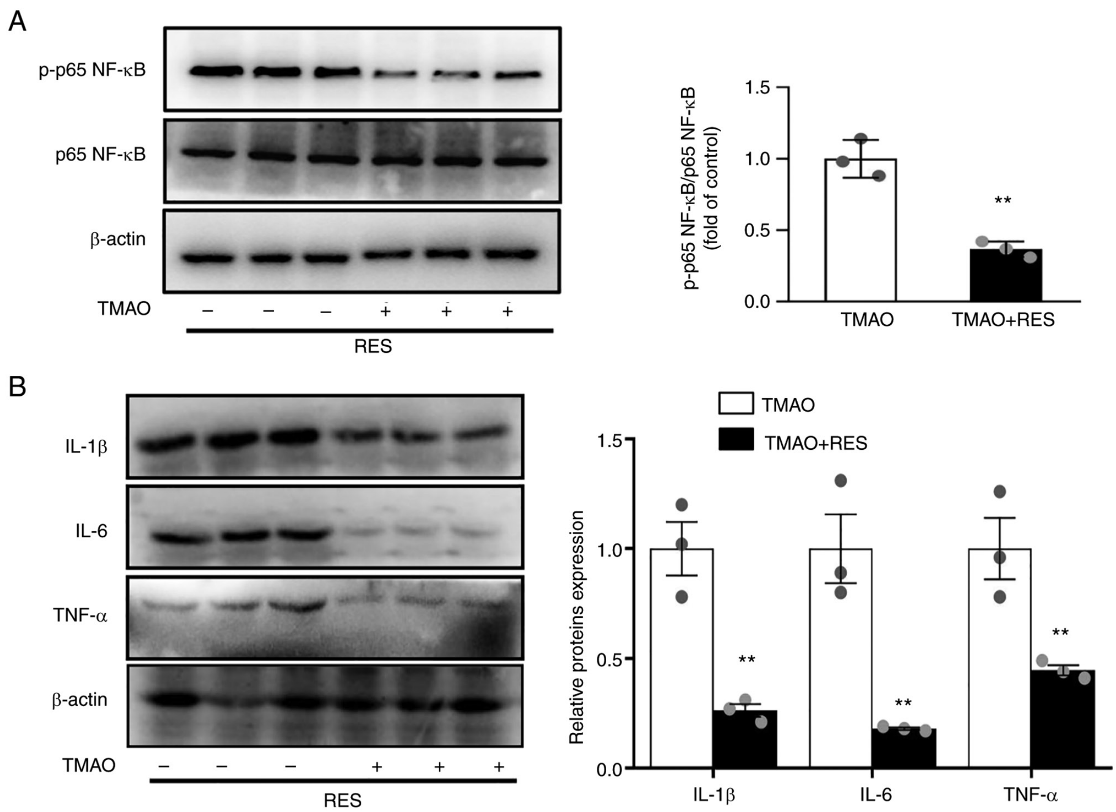

Figure 5. RES, a SIRT1 activator, blocks TMAO-induced macrophage inflammation. (A) Representative western blots of p-p65 NF-κB and p65 NF-κB in the macrophages after treatment. (B) Representative western blots of IL-1β, IL-6, and TNF-α in the macrophages after RES stimulation (n=3). **P<0.01 vs. TMAO group. p-, phosphorylated; RES, resveratrol; TMAO, trimethylamine N-oxide.

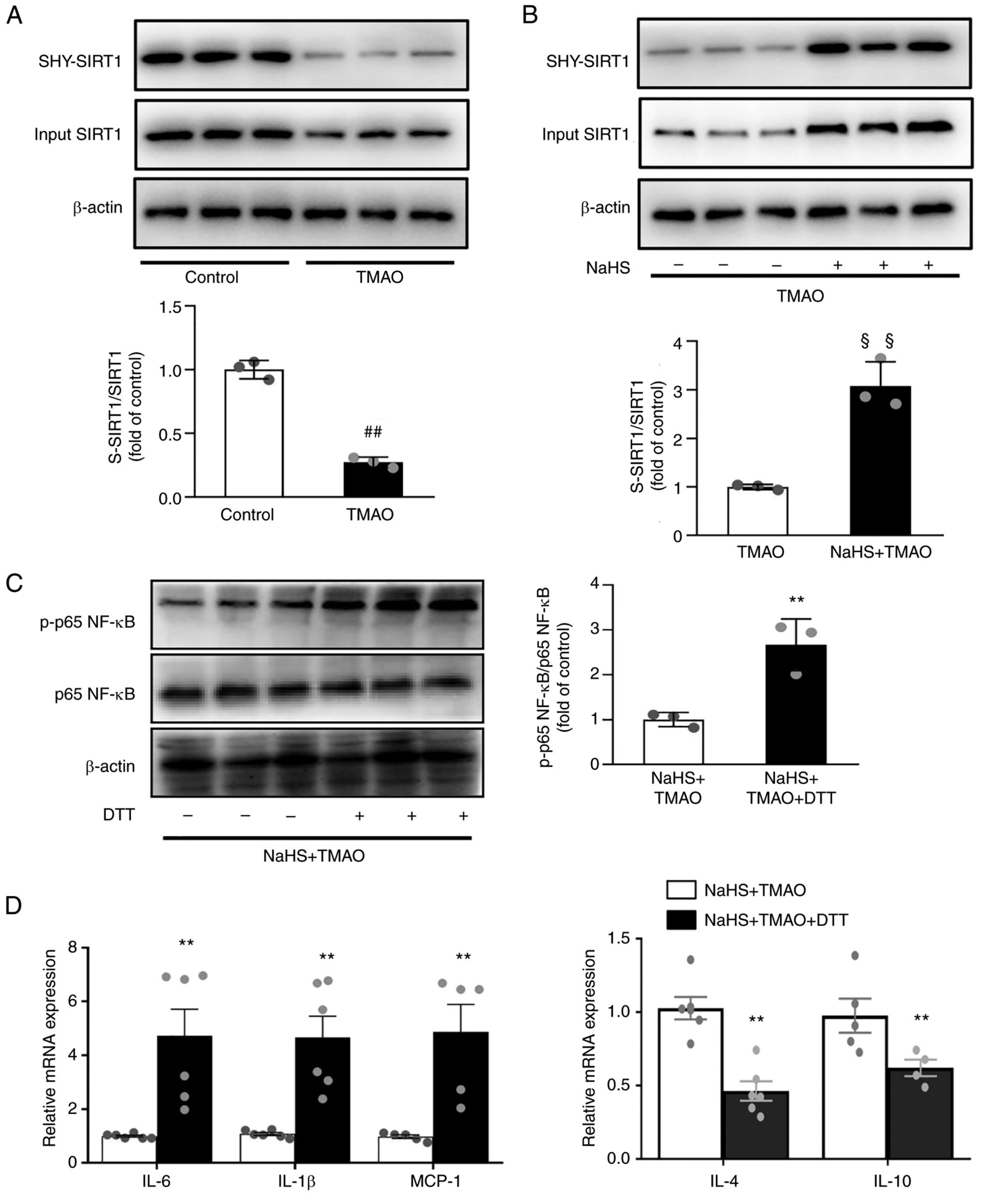

Figure 6. Hydrogen sulfide sulfhydrates SIRT1 and reduces macrophage inflammation. (A) Representative western blots of SHY-SIRT1 in the macrophages after TMAO treatment. (B) Representative western blots of SHY-SIRT1 in the macrophages after NaHS and TMAO treatment. (C) Representative western blots of p-p65 NF-κB and p65 NF-κB in the macrophages after DTT treatment. (D) Reverse transcription-quantitative PCR analysis of the mRNA expression levels of IL-1β, IL-6 TNF-α, IL-4 and IL-10 in the macrophages after DTT treatment (n=4). ##P<0.01 vs. control; §§P<0.01 vs. TMAO group; **P<0.01 vs. NaHS+TMAO group. DTT, DL-dithiothreitol; MCP-1, monocyte chemoattractant protein-1; NaHS, sodium hydrosulfide; p-, phosphorylated; SHY-; sulfhydrated; SIRT1, sirtuin 1; TMAO, trimethylamine N-oxide.

cell inflammation via activation of the NLRP3 inflammasome (32). The present study showed that TMAO induced the expression of inflammatory cytokines in macrophages.

Our previous studies indicated that H_2S has powerful antioxidative (33) and cytoprotective effects (16,23,34) on cardiovascular systems. In addition, H_2S exhibits a significant

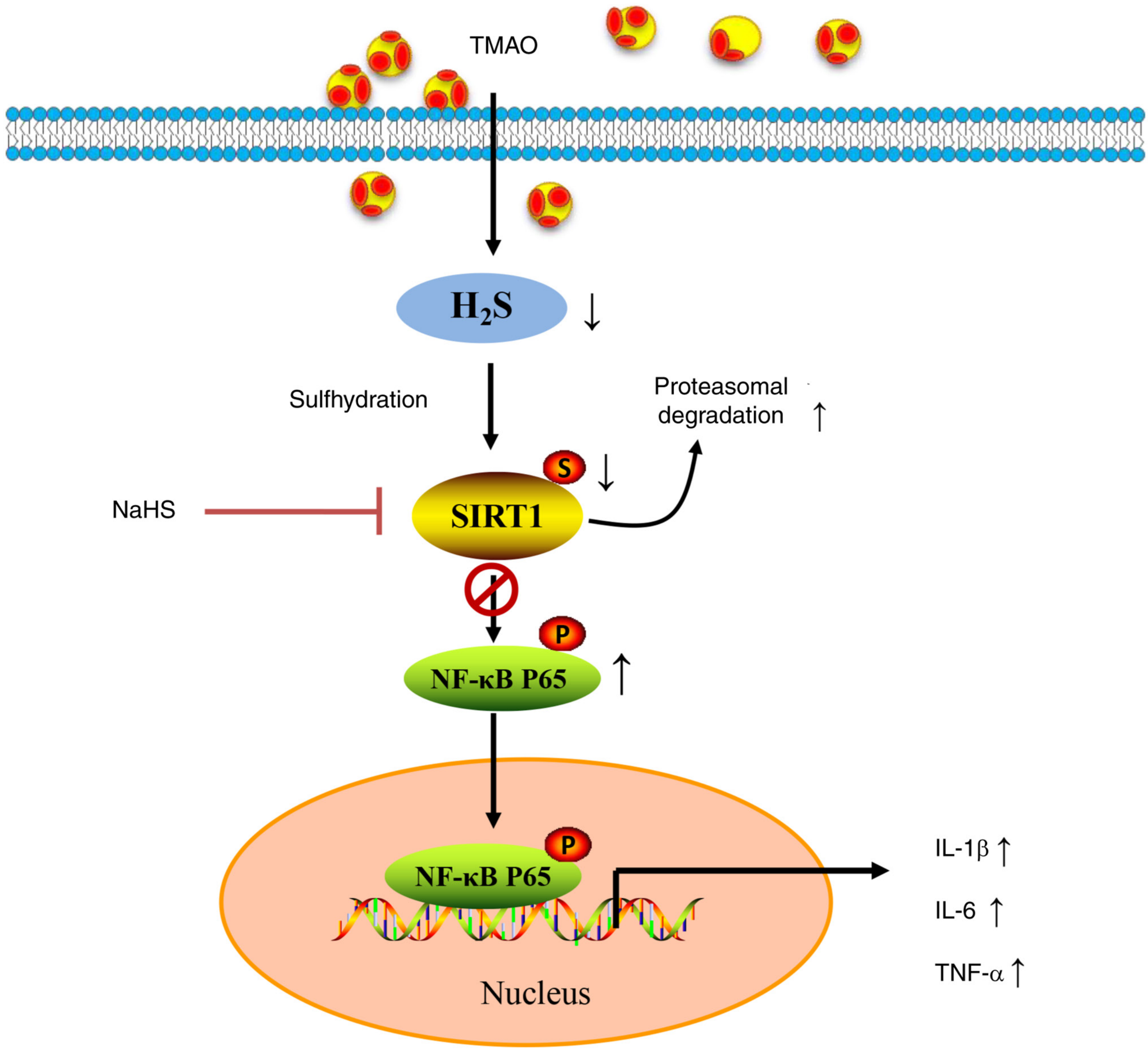

Figure 7. H2S-induced SIRT1 sulfhydration attenuates TMAO-induced inflammatory signaling in macrophages. TMAO inhibited the H2S-induced SIRT1 sulfhydration and caused the proteasomal degradation of SIRT1, resulting in NF-κB activation and inflammation. Endogenous H_2S promoted SIRT1 sulfhydration and decreased SIRT1 proteasomal degradation, reducing the phosphorylation of P65 NF-κB and alleviating inflammation. H_2S, hydrogen sulfide; NaHS, sodium hydrosulfide; SIRT1, sirtuin 1; TMAO, trimethylamine N-oxide.

ability to protect against inflammation (1,35). H_2S can inhibit the TLR4/NF-κB pathway (35,36), scavenge excess reactive oxygen species (37,38) and suppress HMGB1 (39), which are related to NLRP3 activation (40,41) and ferroptosis (42,43). Recently, H_2S has been proven to relieve myocardial infarction by promoting M2 macrophage polarization, macrophage migration and infiltration (1). CSE is the main enzyme for H_2S production in the cardiovascular system. In the present study, it was revealed that TMAO significantly decreased CSE expression and increased expression of pro-inflammatory M1 cytokines, resulting in increased M1 polarization and inflammatory response in macrophages. Notably, the endogenous production of H_2S is largely dependent on the activity of CSE and is less dependent on the activity of CBS or 3-MST in macrophages (44). Previous studies have identified that TMAO significantly decreases plasma H_2S levels (40,45). However, the present study did not investigate whether TMAO influenced the expression levels of CBS and 3-MST in macrophages; therefore, this study has some limitations, which require further study in the future. Moreover, the present data revealed that H_2S promotes a shift from the M1 to M2 macrophage phenotype by decreasing the expression of pro-inflammatory M1 cytokines and by increasing anti-inflammatory M2 cytokines in TMAO-stimulated macrophages, which is consistent with an earlier report (46). Thus, H_2S may be considered a potentially promising therapeutic approach to the treatment of TMAO-induced macrophage inflammation.

SIRT1 is important in the regulation of inflammation and atherosclerosis (47). SIRT1 activation has been shown to ameliorate endothelial function, reduce foam cell formation (48) and attenuate atherosclerotic plaque formation (49). Specific knockdown of SIRT1 in endothelial cells (50) or

VSMCs (51) can significantly increase atherosclerotic plaque area. Our previous study indicated that H_2S induced SIRT1 activation in human umbilical vein endothelial cells (17). The present study demonstrated that NaHS increased SIRT1 expression in TMAO-exposed macrophages, indicating the importance of H_2S in SIRT1 signaling activation. A number of studies have revealed that SIRT1 serves as a potential mediator of NLRP3 inflammasome activation and ferroptosis in chronic inflammation (52,53). NF-κB activity is regulated by post-translational modifications, such as acetylation and phosphorylation (54,55). Notably, SIRT1 signaling has been shown to alleviate NF-κB activity in macrophages after stimulation of the cells with lipopolysaccharide (56). Activating SIRT1 can also deacetylate NF-κB p65 (52) or p53 (53) to alleviate NLRP3 inflammasome activation and ferroptosis in macrophages. Furthermore, SIRT1 defects may lead to NLRP3 activation in hepatocytes (57) or ferroptosis in H9c2 cardiomyocyte cells (58), and thereby SIRT1 signaling relieves inflammation. The present study further verified that TMAO induced activation of the NF-κB signaling pathway by increasing the phosphorylation of p65 NF-κB. Moreover, the SIRT1 inhibitor nicotinamide significantly increased the phosphorylation of p65 NF-κB and impaired the anti-inflammatory actions of H_2S. Conversely, the SIRT1 activator resveratrol attenuated the phosphorylation of p65 NF-κB and inflammation in macrophages. Accordingly, SIRT1 mediates the protective effect of H_2S on TMAO-induced NF-κB activation in macrophages. A previous study also revealed that SIRT1 directly acetylates P65 and promotes inflammation (59). However, the present study did not exclude the effect of SIRT1-induced P65 acetylation on inflammation. Nevertheless, inhibition of P65 phosphorylation by SIRT1 is involved in the protective effects of H_2S against TMAO-induced inflammation in macrophages. The present findings suggested that H_2S inhibited NF-κB p65 mediated inflammatory activation via enhanced SIRT1 activation.

S-sulfhydration is a post-translational modification that increases SIRT1 protein stability and activity (22). NaHS promotes SIRT1 expression and activity by sulfhydrating SIRT1, thereby lowering its ubiquitin-dependent degradation (22). In addition, SIRT1 sulfhydration reduces acetylation and phosphorylation of P65 NF-κB, ultimately inhibiting inflammation (22,28). The present study discovered that NaHS induced SIRT1 sulfhydration to enhance SIRT1 stability and protein levels, which is similar to the findings of a previous study (22). Furthermore, SIRT1 sulfhydration by H_2S decreases the phosphorylation of p65 NF-κB, which downregulates pro-inflammatory cytokine expression in TMAO-exposed macrophages. However, DTT blocked its action, suggesting that SIRT1 sulfhydration mediated the effects of H_2S. Therefore, the present study indicated that SIRT1 sulfhydration mediated the protective effects of H_2S on TMAO-induced M1 polarization and inflammation in macrophages.

In conclusion, the present study demonstrated that H_2S can attenuate TMAO-induced inflammatory signaling in macrophages via the upregulation and sulfhydration of SIRT1. The present study not only provided information on the mechanisms underlying H_2S-mediated anti-inflammatory effects, but also provided evidence that SIRT1 is involved in the protective effects of H_2S on TMAO-associated macrophage inflammation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MHL collected and analyzed the data and wrote the manuscript. MHL and LLX performed the experiments. XLL contributed to the study design and data analyses and revised the manuscript. All authors read and approved the final manuscript. MHL and LLX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing interests.

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