Hydrogen Sulfide Attenuated Tumor Necrosis Factor-α-Induced Inflammatory Signaling and Dysfunction in Vascular Endothelial Cells

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Abstract

**Background:** Hydrogen sulfide (H₂S), the third physiologically relevant gaseous molecule, is recognized increasingly as an anti-inflammatory mediator in various inflammatory conditions. Herein, we explored the effects and mechanisms of sodium hydrosulfide (NaHS, a H₂S donor) on tumor necrosis factor (TNF-α)-induced human umbilical vein endothelial cells (HUVEC) dysfunction.

**Methodology and Principal Findings:** Application of NaHS concentration-dependently suppressed TNF-α-induced mRNA and proteins expressions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), mRNA expression of P-selectin and E-selectin as well as U937 monocytes adhesion to HUVEC. Western blot analysis revealed that the expression of the cytoprotective enzyme, heme oxygenase-1 (HO-1), was induced and coincident with the anti-inflammatory action of NaHS. Furthermore, TNF-α-induced NF-κB activation assessed by IκBα degradation and p65 phosphorylation and nuclear translocation and ROS production were diminished in cells subjected to treatment with NaHS.

**Significance:** H₂S can exert an anti-inflammatory effect in endothelial cells through a mechanism that involves the up-regulation of HO-1.

Introduction

Endothelial dysfunction elicited by inflammatory cytokines is regarded as a key event in the pathogenesis of cardiovascular disorders [1], [2]. Inflammatory cytokines change the secretory activities of endothelium and cause endothelium to become dysfunctional [3]. Enhanced expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin, is an early marker of endothelial activation and dysfunction in the development of early cardiovascular events [4]. Expression of adhesion molecules on the endothelium facilitates the adherence of leukocytes [2],[5], ultimately leading to the progression of numerous vascular diseases [5].

Several studies indicated expression of adhesion molecules on endothelial cells induced by tumor necrosis factor-α (TNF-α) is associated with activation of multiple signal transduction pathways, including mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB) [6],[7]. In addition, the endothelial generation of reactive oxygen species (ROS) during inflammatory stimuli acts as a triggering mechanism for NF-κB activation and the elevation in adhesion molecules and chemokines expression and could ultimately contribute to endothelial dysfunction [8–10]. Thus, the modulation of these processes, i.e., activation of NF-κB, expression of adhesion molecules, and elimination of ROS, assumes great significance in the prevention and treatment of inflammatory cardiovascular diseases.

Hydrogen sulfide (H₂S), a colorless gas with a characteristic rotten-egg odor, has traditionally been considered to be a toxic environmental pollutant. More recently, H₂S has been identified as the third, physiologically relevant, gaseous signaling molecule with a diverse physiological profile [11]. H₂S production has been attributed to three key enzymes cystathionine γ-lyase [12], cystathionine β-synthase, and the newest one, 3-mercaptopyruvate sulfurtransferase [13], while cystathionine γ-lyase is abundant in heart and smooth muscle and is the most relevant enzyme for the cardiovascular system [12],[14]. As the third gaseotransmitter H₂S appears to confer cytoprotection via multiple mechanisms including anti-oxidant and anti-inflammatory effects [14–16]. For instance, recent reports demonstrated H₂S acts as an endogenous scavenger for ROS and reactive nitrogen species [11],[17–19]. In addition, although H₂S has been implicated to play a pro-inflammatory role in systemic inflammation [20–22], a majority of elegant studies strongly suggest that H₂S is a potent...
anti-inflammatory molecule in various models [16,23–25]. However, to the best of our knowledge, the potent anti-inflammatory mechanism of H$_2$S in endothelial cells has not yet been clarified. Here, our present work investigated if H$_2$S exerts anti-inflammatory and thereby potential anti-atherogenic properties in endothelial cells through inhibition of pro-inflammatory processes, such as the expression of adhesion molecules, intracellular ROS production. In addition, the underlying mechanisms and intracellular signaling pathways affected by H$_2$S in TNF-α-stimulated endothelial cells were investigated.

**Results**

**NaHS is non-toxic to HUVEC**

The cytotoxicity experiments of NaHS in this study were performed at 10–100 μM concentration. Non-cytotoxic effect of NaHS was observed at the dosage used in this study (data not shown).

**NaHS inhibited U937 cells adhesion to TNF-α-stimulated HUVEC**

We first investigated the effect of NaHS on the adhesion of U937 cells to TNF-α-activated endothelial cells, a critical step in vascular inflammation. As shown in Figure 1, control-confluent HUVEC showed minimal binding of U937 cells, while the adhesion of U937 cells was remarkably increased when HUVEC were stimulated with TNF-α (10 ng/ml) for 6 h. This dose of TNF-α has been widely used to investigate the proinflammatory effects of TNF-α in cultured cells. The adhesion of U937 cells to TNF-α-stimulated HUVEC was significantly attenuated by NaHS in a concentration dependent manner. In addition, the adhesion of U937 cells to TNF-α-stimulated HUVEC was inhibited by pretreatment with NAC (5 mM) (Figure 1). Importantly, NaHS (100 μM) and NAC (5 mM), but not Dex (10 μM) have a similar inhibitory effect on U937 cells adhesion to TNF-α-activated endothelial cells (P<0.05). These results suggested that ROS is involved in the binding of U937 cells to TNF-α-stimulated endothelial cells.

**NaHS inhibited TNF-α-induced mRNA levels of adhesion molecules**

The effects of NaHS on TNF-α-induced mRNA levels of adhesion molecules by HUVEC were studied by quantitative real-time RT-PCR. Resting HUVEC showed a low constitutive transcription of adhesion molecules, while TNF-α caused a significant increase in mRNA levels of E-selectin, P-selectin, VCAM-1, and ICAM-1 in HUVEC after 4 h of incubation. Treatment of cells with NaHS (10-100 μM) for 30 min resulted in concentration-dependent decreases in TNF-α-induced mRNA levels of E-selectin (Figure 2A), P-selectin (Figure 2B), VCAM-1 (Figure 2C), and ICAM-1 (Figure 2D). Dex (10 μM) also significantly inhibited TNF-α-induced mRNA levels of adhesion molecules, but not significant decreased E-selectin mRNA level (Figure 2). Importantly, NaHS (100 μM) and Dex (10 μM) have a similar inhibitory effect on TNF-α-induced mRNA levels of P-selectin (Figure 2B), VCAM-1 (Figure 2C), and ICAM-1(Figure 2D) (P>0.05).

**NaHS decreased TNF-α-induced expression of ICAM-1 and VCAM-1**

As the expression of adhesion molecules on endothelial cells is a prerequisite for adhesion of leukocytes, we investigated the effect of NaHS on TNF-α-induced ICAM-1 and VCAM-1 expression. Western blot analysis of cell lysates showed that levels of VCAM-1 and ICAM-1 were very low in unstimulated HUVEC, but were significantly increased by TNF-α treatment. NaHS attenuated TNF-α-induced ICAM-1 expression only at higher concentration (100 μM) (Figure 3B), while NaHS suppressed TNF-α-induced VCAM-1 expression in a dose-concentration manner at concentrations ranging from 10 to 100 μM (Figure 3C). In addition, a similar profile was also observed by pretreatment with Dex (Figure 3), as previously reported [26].

**NaHS up-regulated HO-1 expression in TNF-α-stimulated cells**

The effect of NaHS on HO-1 expression was initially explored in cultured HUVEC, Figure 4A shows upon incubation with NaHS (10–100 μM) for 6 hours, a substantial increase in the expression of HO-1 was observed. MTt cell survival assays failed to demonstrate any cellular cytotoxicity at these concentrations (not shown). Thus, we examined the effects of NaHS on HO-1 expression in TNF-α-stimulated HUVEC. As shown in Figure 4B, treatment with TNF-α didn’t reveal significant effect on HO-1 expression compared with resting cells. Contrary, NaHS concentration-dependently increased HO-1 expression in TNF-α-stimulated cells. Meanwhile, the upregulation of HO-1 was also observed in NAC-treated cells. Taken together, these results suggested that the expression of HO-1 induced by NaHS may functions as a negative regulator of TNF-α-induced inflammatory responses in HUVEC.

**NaHS reduced intracellular ROS production in TNF-α-stimulated HUVEC**

To confirm whether the inhibitory effect of H$_2$S on TNF-α-induced intracellular ROS production, HUVEC were labeled with a cell-permeable fluorescent dye H$_2$DCF-DA and analyzed by spectrophotometer or fluorescence microscope. Stimulation with TNF-α resulted in a great increase in the amount of intracellular ROS generation in HUVEC compared with unstimulated cells (Figure 5). However, pretreatment with NaHS (10–100 μM) significantly decreased TNF-α-induced intracellular ROS production. In addition, coincident with the reports that free radical scavenger NAC (5 mM) also abolished TNF-α-induced intracellular ROS production [27] (Figure 5). Meanwhile, NaHS (100 μM) and NAC (5 mM) also showed similar free radical scavenging capacity (P>0.05). These results strongly suggested that scavenging ROS by H$_2$S may be responsible for inhibition in the binding of U937 cells to TNF-α-stimulated endothelial cells.

**NaHS inhibited TNF-α-induced p38 MAPK activation**

Activation MAPK signaling pathway induced by TNF-α plays an important role in the regulation of adhesion molecules expression. In unstimulated cells, there was almost no detectable phosphorylation of p38, ERK1/2, or JNK1/2 in HUVEC. TNF-α caused a rapid phosphorylation of MAPK within 5 min, with phosphorylation peaking at 15 min and followed by dropping to normal level (Figure 6A). Based on this time course, 15 min was chosen for subsequent experiments.

To examine whether MAPK activation was involved in the regulation of inflammatory response by NaHS in TNF-α-stimulated HUVEC, phosphorylation of MAPK (p38, JNK1/2, and ERK1/2) were analyzed by Western blot. As shown in Figure 6, phosphorylation of p38 (Figure 6B), JNK1/2 (Figure 6C), and ERK1/2 (Figure 6D) in resting cells was significantly increased after 15 minutes treatment with TNF-α. NaHS concentration-dependently abolished the p38 phosphorylation induced by TNF-α (Figure 6B), but had little effect on JNK1/2 and ERK1/2 phosphorylation (Figure 6C, 6D).
NaHS inhibited TNF-α-induced IκBα degradation

Translocation of NF-κB from cytoplasm to the nucleus is preceded by the phosphorylation and subsequent degradation of IκBα. To determine the effect of NaHS on TNF-α-induced IκBα degradation, total cell lysate was prepared from the TNF-α with or without NaHS-treated cells. Using Western blot analysis, we demonstrated that the degradation of IκBα took place in a time dependent manner after the TNF-α induction (Figure 7A). As shown in Figure 7B, upon induction with TNF-α for 15 min the intensity of IκBα was significantly reduced. In contrast, pretreatment with NaHS inhibited TNF-α-induced IκBα degradation in a concentration dependent manner.

NaHS inhibited TNF-α-induced phosphorylation and nuclear translocation of NF-κB p65

The phosphorylation of NF-κB p65 subunit, particular on serine residues 536 in the C-terminal transactivation domain, plays an important role in regulation transcription of adhesion molecules

Figure 1. NaHS inhibited U937 cells adhesion to TNF-α-activated HUVEC. HUVEC were incubated with indicated concentrations of NaHS, Dex (10 μM) or NAC (5 mM) for 30 min, then stimulated with TNF-α (10 ng/ml) for 6 h, U937 cells seed onto HUVEC and co-cultured for 2 h. After removing the non-adherent cells, adherent cells were detected and counted under a light microscope. (A) Pictures are representative optical fields. NaHS, Dex, and NAC concentrations were 100 μM, 10 μM, and 5 mM, respectively. (B) Quantitative analysis of the binding of U937 cells to HUVEC presented by bar graphs was counted under a light microscope. #P<0.05 compared with unstimulated cells, *P<0.05, **P<0.01 compared with TNF-α-stimulated control. Data are the mean ± S.E.M of results from at least three independent experiments, each performed in duplicate.
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following exposure to inflammatory stimuli [9],[28]. To examine whether NaHS might play a role in the regulation of phosphorylation of NF-κB p65<sup>Ser536</sup> in the TNF-α-activated HUVEC, Western blot analysis was performed. As shown in Figure 7C, TNF-α significantly induced phosphorylation of NF-κB p65<sup>Ser536</sup> in HUVEC. NaHS markedly reduced TNF-α-induced phosphorylation of NF-κB p65<sup>Ser536</sup> in a concentration dependent manner.

We next asked whether NaHS might play a role in the regulation of nuclear NF-κB p65 translocation in TNF-α-stimulated HUVEC. The translocation of NF-κB p65 from the cytoplasm to the nucleus was visualized by Western blot and immunofluorescence (Figure 7D, E). TNF-α result in a significantly induction of nuclear NF-κB p65 translocation, while NaHS (100 μM) significantly suppressed TNF-α-induced nuclear NF-κB p65 translocation.

**Discussion**

Inflammation contributes to the pathogenesis of cardiovascular disease and elevated level of pro-inflammatory cytokine TNF-α is
Figure 3. NaHS inhibited TNF-α-induced expression of ICAM-1 and VCAM-1. HUVEC were pre-treated with NaHS (50–100 μM) or Dex (10 μM) for 30 min and then stimulated with TNF-α (10 ng/ml) for 6 h. (A) Representative Western blot showed the expression of ICAM-1 and VCAM-1. Tubulin was used as loading control. Bar graphs represent the quantitative difference in expression of ICAM-1 (B) and VCAM-1 (C), respectively, in arbitrary units. *P<0.05 compared with unstimulated cells, **P<0.01 compared with TNF-α-stimulated cells. Data are the mean ± S.E.M of results from at least three independent experiments, each performed in duplicate.

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Figure 4. NaHS upregulated expression of HO-1 in HUVEC. (A) HUVEC were incubated with indicated concentrations of NaHS for 6 hours. Cells were then lysed, and HO-1 expression was analyzed by Western blot. Tubulin was used as loading control. Data represent mean ± S.E.M from 3
associated with endothelial dysfunction [1]. The outcome of the present study indicated that exogenous H$_2$S, at the dosage used in this study, attenuated TNF-$\alpha$-induced endothelial dysfunction in vitro. Our major findings showed that exogenous H$_2$S blocked the adhesion of U937 cells to TNF-$\alpha$-activated HUVEC by inhibiting expression of adhesion molecules; suppressed the TNF-$\alpha$-induced activation of NF-κB by inhibiting degradation of IκB and activation of p38 signaling pathway; eliminated TNF-$\alpha$-induced intracellular ROS production; and up-regulated HO-1 expression in HUVEC.

As the third gaseous mediator H$_2$S has multiple positive physiological functions, but the role of H$_2$S during systemic inflammatory diseases is still a matter of debate or may be double-edged. NaHS was used as a H$_2$S donor, because it can dissolve into Na$^+$ and HS$^{-}$ in solution, HS$^{-}$ is released and forms H$_2$S with H$^+$. This provides a solution of H$_2$S at a concentration that is about 33% of the original concentration of NaHS [29]. Several reports describe a significant decrease in plasma H$_2$S level in cardiovascular disease [23],[30],[31]. H$_2$S has a protective effect against atherosclerosis in apoE$^{-/-}$ mice and attenuated TNF-$\alpha$-induced ICAM-1 expression in HUVEC [23]. Several reports mention generous plasma basal H$_2$S levels in the 50–150 μM range [31]. So, the present study explored that in atherosclerosis-associated inflammation, H$_2$S may function as a modulator of endothelial function at the relevant physiological concentrations (10–100 μM).

It is well known that adhesion molecules are strong predictors of atherosclerotic lesion development and future cardiovascular events [4]. TNF-$\alpha$ is recognized as a major risk factor in the initiation and progression of atherosclerotic lesion development and future cardiovascular events, which may promote endothelial dysfunction by increasing the production of endothelium-derived ROS and enhancing the expression of adhesion molecules on the endothelial cells [27],[32],[33]. Recent evidence suggested that H$_2$S might exert anti-inflammatory effect via multiple mechanisms such as upregulation of antioxidant defense [17]. Exogenous H$_2$S exert their anti-oxidative effects by inhibiting ROS production induced by cytokines or hydrogen peroxide in mouse pancreatic β-cells [25]. Consistent with the finding, we also demonstrated that NaHS treatment attenuated TNF-$\alpha$-induced intracellular ROS generation in HUVEC. Meanwhile, treatment with NaHS significantly attenuated TNF-$\alpha$-induced increases in the mRNA expression of ICAM-1, VCAM-1, P-selectin, and E-selectin and subsequent proteins expression of ICAM-1 and VCAM-1 as well as U937 cells adhesiveness to endothelial cells. We also demonstrated that the NAC significantly inhibited intracellular ROS production and subsequent U937 cells adhesiveness. These observations are consistent with numerous reports that demonstrate that scavenging intracellular ROS production inhibits monocytes adhesiveness to endothelial cells by reducing the expression of various adhesion molecules [33],[34]. Thus, inhibition of TNF-$\alpha$-induced ROS by NaHS may be responsible for attenuation of TNF-$\alpha$-induced endothelial dysfunction.

Although the effects of Dex or NaHS on adhesion molecules expression and adhesion of U937 cells were similar, their inhibitory effects on adhesion molecules may differ. Dex, a typical steroidal anti-inflammatory drug, attenuates adhesion molecules expression through direct interaction with glucocorticoid receptors [26]. Our results were consistent with the study that H$_2$S inhibited TNF-$\alpha$-induced expression of ICAM-1, as previously reported by Wang [23]. But there are some earlier reports indicated that H$_2$S has been demonstrated to play a proinflammatory role in various disease states [20],[21]. The inconsistency between the present study and earlier studies may be a result of the dose of H$_2$S donor used or a different inflammatory model.

HO-1 is the inducible isoform of the first and rate-controlling enzyme of heme degradation and plays a central role in the regulation of inflammatory reaction via its products bilirubin and carbon monoxide in a variety of experimental systems [33]. We reported that NaHS can dose-dependently induce HO-1 expression in endothelial cells. The persistent HO-1 induction also observed after TNF-$\alpha$ challenge may be due to the ability of H$_2$S to coordinate to other thiol-containing protein, including a number of redox-sensitive transcription factors and kinases, which is a crucial modulator of the expression of antioxidant genes, including HO-1. We also suggested that HO-1 induction by NaHS might contribute to its anti-inflammatory action. Because the expression of HO-1 was induced concomitantly with the attenuation of expression of adhesion molecules and the binding of U937 cells to TNF-$\alpha$-stimulated HUVEC excited by NaHS. The result is consistent with the report that overexpression of HO-1 prevented adhesion molecules expression and leukocytes to activated endothelial cells [34],[35]. Our results suggested that the induction of HO-1 by NaHS may function in a negative feedback manner to down-regulate adhesion molecules expression, as reported by Paine A [33].

MAPK and NF-κB are key players in intracellular signaling pathways in response to inflammatory stimuli and required for adhesion molecules expression [36],[37]. Therefore, to further investigate the molecular mechanism responsible for the inhibitory effect of NaHS on expression of adhesion molecules, we examined the effect of NaHS on NF-κB and MAPK activation. Our results demonstrated that NaHS potently suppressed TNF-$\alpha$-stimulated phosphorylation and nuclear translocation of NF-κB p65 in HUVEC. Consistent with previous report [38], phosphorylation and nuclear translocation of NF-κB p65 were found to be the main components of TNF-$\alpha$-induced NF-κB activation in HUVEC. The results suggested that the induction of NF-κB activaton by NaHS is mediated by modulation of upstream signaling pathway involved in NF-κB activation. Numerous natural components and therapeutic agents have been shown to inhibit NF-κB activation by preventing IκB degradation [39]. Our data indicate that NaHS not only inhibited the IκB degradation, but also attenuated the nuclear translocation of NF-κB. This provides evidence that H$_2$S can attenuate TNF-$\alpha$-induced NF-κB activation, as previously reported [23],[24]. However, the kinase responsible for IκB degradation has not been identified. There are also studies suggesting that MAPK is involved in the regulation of NF-κB activation in TNF-$\alpha$-induced endothelial cells [40]. Here, we demonstrated that NaHS inhibited TNF-$\alpha$-stimulated p38 MAPK signal pathway in HUVEC, but had little effect on ERK1/2 or JNK1/2 phosphorylation. Although ERK1/2 mainly mediates cellular responses to hormones and growth factors, JNK1/2 and p38 are primarily activated by stress-related stimuli [41]. Inhibition of p38 MAPK markedly inhibited the NF-κB activation and subsequent the expression of adhesion molecules in TNF-$\alpha$-stimulated endothelial cells [27]. Meanwhile, inhibition and
Figure 5. NaHS inhibited TNF-α-induced intracellular ROS generation. HUVEC were incubated with NaHS or NAC for 30 min, and then stimulated with TNF-α (10 ng/ml) for 1 h. (A) Pictures are representative fields detected by fluorescence microscope. NaHS and NAC concentration were 100 μM and 5 mM, respectively. (B) Quantitation of intracellular ROS was determined by fluorescence spectrophotometer. NAC concentration was 5 mM. #P<0.05 compared with unstimulated cells, *P<0.05, **P<0.01 compared with TNF-α-stimulated cells. Data are the mean ±S.E.M of results from at least three independent experiments, each performed in duplicate.

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genetic deficiency of p38 MAPK also contribute to induce HO-1 expression [42], which functions in a negative feedback manner to inhibit NF-kB activation [34].

In summary and conclusion, we demonstrated that NaHS attenuated expression of adhesion molecules and monocyte adhesion to endothelial cells, and decreased intracellular ROS production in TNF-α-stimulated endothelial cells. Remarkably, this anti-inflammatory effect is primarily achieved by the inhibition of NF-kB and p38 signaling pathways, and by the upregulation of HO-1 expression. We, thus, concluded that the inhibition of NF-kB and p38 signaling pathways, adhesion molecules, and modulation of cellular redox balance might be one of the important mechanisms of H2S that improved TNF-α-induced endothelial dysfunction. These findings suggested that H2S release agents could represent a promising approach for the treatment of inflammatory vascular diseases.

Materials and Methods

Reagents

DMEM, RPMI-1640, and fetal bovine serum (FBS) were from GIBCO-BRL (USA). Recombinant human TNF-α was purchased
Figure 7. NaHS inhibited TNF-α-induced IκBα degradation and NF-κB activation. (A) IκBα degradation was analyzed by Western blot in HUVEC stimulated with TNF-α (10 ng/ml) for indicated periods. (B) HUVEC were incubated with indicated concentrations of NaHS for 30 min, then
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stimulated with TNF-α (10 ng/ml) for another 15 min. IkB degradation was analyzed by Western blot. (C) HUVEC were incubated with indicated concentrations of NaHS for 30 min, then stimulated with TNF-α (10 ng/ml) for another 15 min. Phosphorylation levels of NF-kB p65 were analyzed by Western blot. (D) HUVEC were incubated with NaHS (100 μM) for 30 min, then stimulated with TNF-α (10 ng/ml) for another 1 h. Cytoplasmic and nuclear levels of NF-kB p65 were analyzed by Western blot. *P<0.05 compared with unstimulated cells, **P<0.05, ***P<0.01 compared with TNF-α stimulated cells. Data are the mean ± S.E.M of results from at least three independent experiments, each performed in duplicate. (E) HUVEC were incubated with NaHS (50, 100 μM) for 30 min, then stimulated with TNF-α (10 ng/ml) for another 1 h. NF-kB p65 translocation was detected by fluorescent microscope.

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from Millipore (Billerica, MA, USA). 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) was from Molecular probes (Eugene, OR, USA). NaHS, N-acetyl-L-cysteine (NAC), and dexamethasone (Dex) were purchased from Sigma-Aldrich (St Louis, MO, USA). NaHS has been well established as a reliable donor of H2S in culture media [15].

Cell culture studies
HUVEC (ATCC, Manassas, VA) were grown in DMEM supplemented with 1800 mg/L NaHCO3, 4500 mg/L glucose, and 110 mg/L sodium pyruvate, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO2. U937 human monocytes (ATCC, Manassas, VA) were maintained in RPMI-1640 containing 1800 mg/L NaHCO3, 4500 mg/L, glucose, and 110 mg/L sodium pyruvate, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO2.

For experiments, HUVEC were grown to confluence in 6-well plates, 24-well plates, or 60 mm dishes (Costar, Cambridge, MA). Cells were serum-starved for 12 h, to assess the effect of TNF-α or NaHS on MAPK phosphorylation, cells were incubated with TNF-α (10 ng/ml) for different periods (5, 15, 30, 45, and 90 min). In some experiments, cells were pre-incubated with or without NAC (5 mM), Dex (10 μM), or NaHS (10–100 μM) for 30 min before stimulating with TNF-α (10 ng/ml) for various periods: 15 min for measurement of MAPK (p38, JNK1/2 and ERK1/2) phosphorylation, IkBα phosphorylation, and NF-kB p65 translocation; 4 h for measurement of intracellular ROS production and NF-kB p65 translocation; 6 h for measurement of proteins expression of adhesion molecules, HO-1, and adhesion assay.

Cell viability assay
The cytotoxicity of NaHS was analyzed by colorimetric MTT assay as previously described [43].

Adhesion assay
Endothelial cells were starved for 12 h with serum-free medium, and then exposed to NaHS, NAC, or Dex for 30 min, TNF-α stimulated for another 6 h. An exact number of U937 monocytes was seeded on TNF-α-activated HUVEC and incubated for 2 h at 37°C in a humidified 5% CO2 atmosphere as described previously [44]. Nonadhering U937 cells were then removed and washed with PBS for 3 times. Finally, the HUVEC were fixed with 4% paraformaldehyde in PBS for 10 min and the number of adhered U937 monocytes to endothelial cells was calculated using a Zeiss optical microscope system. Results are expressed as means and standard deviations of number of cells counted. All experiments were repeated at least three times.

Quantitative real-time RT-PCR analysis
Total RNA was extracted from HUVEC with TRizol Reagent (Takara, TaKaRa Biotechnology, Dalian, China) following the manufacturer’s instructions. Total RNA (2 μg) of each sample was reverse-transcribed into cDNA and amplified using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer’s directions. real-time RT-PCR conditions include denaturation (95°C for 30 s); annealing (60°C for 30 s; number of cycles (40) and were performed using iQ5 real-time RT-PCR detection System (Bio-Rad, Richmond, USA) in a total volume of 25 μl reaction mixture containing 2 μl cDNA, 12.5 μl 2× SYBR Green 1 Master Mix (Takara), and 1 μl of each primer. GAPDH was used as an internal control to compare the amount of total mRNA of each sample. The primers used in this experiment were indicated in Table 1.

Preparation of whole cell extracts and isolation of cell fractions
For whole cell extraction, cells were washed twice with ice-cold PBS and lysed in RIPA buffer with protease & phosphatase inhibitor. After centrifugation (4°C, 10 min, 10,000 g), samples were prepared for Western blot analysis.

For preparation of cytoplasmic and nuclear fraction, HUVEC were pretreated with NaHS (100 μM), NAC (5 mM), or Dex(10 μM) for 30 min, and then stimulated with TNF-α for 1 h. Nuclear and cytoplasmic proteins of HUVEC were extracted using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Inc) according to manufacturer’s instructions.

Western blot analysis
Equal amounts (30 μg) of proteins were separated on 8-10 % sodium dodecyl sulfate-polyacylamide gels and transferred to polyvinyl difluoride membrane (Millipore, USA). After being blocked with 5% nonfat dry milk, membranes were incubated overnight at 4°C with primary antibodies against ERK1/2, phosphorylated [p]ERK1/2 (Thr183/Tyr185), JNK1/2, p-p38, p-Jun/1/2 (Thr180/Tyr182), NF-kB p65, p-NF-kB p65 (Ser326), IkBα (1:1000) [all 1:1000, Cell Signaling Technology, Beverly, MA, USA]; ICAM-1 (1:500), VCAM-1 (1:2000), β-tubulin (1:2000), β-actin (1:500), GAPDH (1:2000), or HO-1 (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Cell Signaling Technology, Beverly, MA, USA). The immunoreactive bands were detected with enhanced chemiluminescence reagents, and the protein bands were visualized on X-ray film.

Table 1. Primers sequences used in the present study.

| Genes      | Sequences                        |
|------------|----------------------------------|
| ICAM-1     | Sense 5′-TCACGGAGCTCCAGTGCTAA-3′ |
| E-selectin | Sense 5′-CAGTGGCAGCACCAGAAGC-3′  |
| VCAM-1     | Sense 5′-CGAAGGGCCAGTGAAGAC-3′   |
| P-selectin | Sense 5′-ACCTTCAGGAGCAGAGCAAGC-3′|
| GAPDH      | Sense 5′-GGACTGAGCAGGACTAC-3′    |

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USA: After incubation with appropriate secondary antibodies for 1 h at room temperature, proteins were visualized by enhanced chemiluminescence with a camera-based imaging system (Alpha Innotech, Santa Clara, CA, USA). The density of the signals was quantified with the AlphaEase software.

Immunofluorescence

The endothelial cells were grown on glass slides in 6-well plates. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Immunostained using rabbit anti-NF-xB p65 antibody (1:250; Cell Signaling) and Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:200; Invitrogen), and counterstained for nuclei with DAPI. Immunofluorescence was visualized using a fluorescent microscope (Carl Zeiss Inc.). The results were based on three independent analyses.

Intracellular ROS production assay

The fluorescent probe, H₂DCF-DA, was used to measure the intracellular generation of ROS by TNF-α [45]. Briefly, confluent HUVEC in 24-well plates were pretreated with NaHS (10–100 μM) and NAC (5 mM) for 30 min. After removing the NaHS and NAC from the wells, the cells were incubated with 20 μM H₂DCF-DA for 30 min. Then stimulated with TNF-α (10 ng/ml) for 1 h, and the fluorescence intensity was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively, using a fluorescence spectrophotometer (M1000, TECAN, Austria GmbH, Austria) or a fluorescence microscope (Carl Zeiss).

Statistical analysis

Data are presented as mean ± S.E.M. Differences between mean values of multiple groups were analyzed by one-way analysis of variance with Dunnett’s test for post hoc comparisons. Statistical significance was considered at P<0.05.

Author Contributions

Conceived and designed the experiments: Y-ZZ L-LP X-HL. Performed the experiments: L-LP X-HL. Analyzed the data: L-LP X-HL Q-HG. Contributed reagents/materials/analysis tools: Q-HG DW. Wrote the paper: L-LP X-HL.

References

1. Ross R (1999) Atherosclerosis—an inflammatory disease. N Engl J Med 340: 115–126.
2. Libby P (2002) Inflammation in atherosclerosis. Nature 420: 868–874.
3. Borel JC, Roux-Lombard P, Tannirier R, Arnaud C, Monneret D, et al. (2009) Endothelial dysfunction and specific inflammation in obesity hyperventilation syndrome. PLoS One 4: e6733.
4. Simikko PE, Yang CH, Weisel RD, Jeffries GA, Anderson TJ, et al. (2003) Biomarkers of vascular disease linking inflammation to endothelial activation: Part II. Circulation 108: 2011–8.
5. Sprague AH, Khalil RA (2009) Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol 78: 539–552.
6. Li JM, Fan LM, Christie MR, Shah AM (2005) Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cell role of p17phox phosphorylation and binding to TRAF4. Mol Cell Biol 25: 2320–2330.
7. Lin CC, Liu XM, Peyton K, Wang H, Yang WC, et al. (2008) Far infrared light therapy inhibits vascular endothelial inflammation via the induction of heme oxygenase-1. Arterioscler Thromb Vasc Biol 28: 739–745.
8. Kumar S, Sharma A, Madan B, Singhal V, Ghosh B (2007) Isoliquiritigenin inhibits iNOS and P-selectin expression in human endothelial cells. Biochem Pharmacol 73: 1602–1612.
9. Viatour P, Merville MP, Bours V, Chariot A (2005) Phosphorylation of NF-κB and iNOS proteins: implications in cancer and inflammation. Trends Biochem Sci 30: 43–52.
10. Sasaki M, Ostanin D, Elrod JW, Oshima T, Jordan P, et al. (2003) TNF-alpha-induced endothelial cell adhesion molecule expression is cytochrome P-450 monooxygenase dependent. Am J Physiol Cell Physiol 284: C422–428.
11. Wang R (2002) Two’s company: three’s a crowd: can H2S be the third endogenous gaseous transmitter? FASEB J 16: 1792–1798.
12. Zhao W, Zhang J, Liu Y, Wang R (2001) The vasorelaxant effect of H2S as a novel gaseous factor. K(ATP) channel opener. EMBO J 20: 6008–6016.
13. Shibuya N, Tanaka M, Yoshida M, Ogawa Y, Togawa T, et al. (2009) Monoacetylated sullurtransferase produces hydrogen sulfide and bound sulfate sulfane in the brain. Antioxid Redox Signal 11: 703–714.
14. Wang R (2003) The gasotransmitter role of hydrogen sulfide. Antioxid Redox Signal 5: 493–501.
15. Liu L, Moore PK (2008) Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air? Trends Pharmacol Sci 29: 84–90.
16. Lefer DJ (2007) A new gaseous signaling molecule emerges: Cardioprotective role of hydrogen sulfide. Proc Natl Acad Sci USA 104: 17907–17908.
17. Kida K, Yamada M, Tokuda K, Marutani E, Kakinohana M, et al. (2010) Hydrogen sulfide prevents neurodegeneration and movement disorder in a mouse model of Parkinson’s disease. Antioxid Redox Signal 11: 25–33.
18. Yang J, Zhang X, Liu J, Wang W, et al. (2007) Endogenous hydrogen sulfide regulates leukocyte trafficking in cecal ligation and puncture-induced sepsis. J Leukoc Biol 82: 594–605.
19. Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, et al. (2005) Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 19: 1196–1198.
20. Wang Y, Zhao X, Jin H, Wei H, Li W, et al. (2009) Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 29: 173–179.
21. Oh GS, Par HO, Lee BS, Kim BN, Kim JM, et al. (2006) Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via homocysteine-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med 41: 106–119.
22. Taniguchi S, Kang I, Kimura T, Niki I (2011) Hydrogen sulfide protects mouse pancreatic β-cells from cell death induced by oxidative stress, but not by endoplasmic reticulum stress. Br J Pharmacol 162: 1711–8.
23. Barnes PJ, Adcock I (1993) Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 14: 436–441.
24. Yang WS, Lee JM, Han SJ, Kim YJ, Chang JW, et al. (2010) Mycophenolic acid attenuates tumor necrosis factor-alpha-induced endothelin-1 production in human aortic endothelial cells. Atherosclerosis 211: 61–65.
25. Reffertmann RJ, Holbert WC, Roth SH (1992) Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 32: 109–134.
26. Yang G, Wu L, Jiang B, Yang W, Qi J, et al. (2008) H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine γ -lyase. Science 322: 597–598.
27. Szabó C (2007) Hydrogen sulfide and its therapeutic potential. Nat Rev Drug Discov 6: 917–935.
28. Mukherjee TK, Mishra AK, Mukhopadhyay S, Hoidal JR (2007) High concentration of antioxidants N-acetylcysteine and mitoquinone-Q induces intercellular adhesion molecule 1 and oxidative stress by increasing intracellular glutathione. J Immunol 178: 1833–1844.
29. Deneke A, Eiz-Vesper B, Blaszczyk R, Immenschuh S (2010) Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 80: 1895–1903.
30. Li J, Shyu SC, Hung YL, Chen YH, Ku HH, et al. (2005) Superoxide dismutase inhibits the expression of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 induced by tumor necrosis factor-alpha in human endothelial cells through the JNK/p38 pathways. Arterioscler Thromb Vasc Biol 25: 334–340.
31. Seldin MP, Silva G, Pejanovic N, Larsen R, Greigioire IP, et al. (2007) Heme oxygenase-1 inhibits the expression of adhesion molecules associated with endothelial cell activation via inhibition of NF-kappaB RelA phosphorylation at serine 276. J Immunol 179: 7840–7851.
32. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, et al. (1995) Role of human endothelial cells in the inflammatory response to bacterial endotoxin. J Exp Med 180: 109–134.
33. Paine A, Eiz-Vesper B, Blaszczyk R, Immenschuh S (2010) Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 80: 1895–1903.
38. Kim EJ, Park WH, Ahn SG, Yoon JH, Kim SW, et al. (2010) 5'-nitrindirubinoxime inhibits inflammatory response in TNF-alpha stimulated human umbilical vein endothelial cells. Atherosclerosis 211: 77–83.

39. Wang TM, Chen CJ, Lee TS, Chao HY, Wu WH, et al. (2011) Docosahexaenoic acid attenuates VCAM-1 expression and NF-kappaB activation in TNF-alpha-treated human aortic endothelial cells. J Nutr Biochem 22: 187–94.

40. Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, et al. (2006) Phosphatidylinositol 3-kinase gamma signaling through protein kinase C zeta induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. J Biol Chem 281: 16128–16138.

41. Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81: 807–869.

42. Naidu S, Vijayan V, Santoso S, Kietzmann T, Immenschuh S (2009) Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2. J Immunol 182: 7048–7057.

43. Liu XS, Pan LL, Gong QH, Zhu YZ (2010) Antiapoptotic effect of novel compound from herba leonuri - leonurine (SCM-198): a mechanism through inhibition of mitochondrial dysfunction in H9c2 cells. Curr Pharm Biotechnol 11: 895–905.

44. Pawlowski NA, Abraham EL, Pontier S, Scott WA, Cohn ZA (1985) Human monocyte-endothelial cell interaction in vitro. Proc Natl Acad Sci USA 82: 8208–8212.

45. Wang H, Joseph JA (1999) Quantifying cellular oxidative stress by dichloro-fluorescein assay using microplate reader. Free Radic Biol Med 27: 612–616.