Sodium Thiosulfate Attenuates Acute Lung Injury in Mice

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ABSTRACT

Background: Acute lung injury is characterized by neutrophilic inflammation and increased lung permeability. Thiosulfate is a stable metabolite of hydrogen sulfide, a gaseous mediator that exerts antiinflammatory effects. Although sodium thiosulfate (STS) has been used as an antidote, the effect of STS on acute lung injury is unknown. The authors assessed the effects of STS on mice lung and vascular endothelial cells subjected to acute inflammation.

Methods: Lung injury was assessed in mice challenged with intratracheal lipopolysaccharide or subjected to cecal ligation and puncture with or without STS. Effects of STS on endothelial permeability and the production of inflammatory cytokines and reactive oxygen species were examined in cultured endothelial cells incubated with lipopolysaccharide or tumor necrosis factor-α. Levels of sulfide and sulfane sulfur were measured using novel fluorescence probes.

Results: STS inhibited lipopolysaccharide-induced production of cytokines (interleukin-6 [pg/ml]; 313 ± 164, lipopolysaccharide; 79 ± 27, lipopolysaccharide + STS [n = 10]), lung permeability, histologic lung injury, and nuclear factor-κB activation in the lung. STS also prevented up-regulation of interleukin-6 in the mouse lung subjected to cecal ligation and puncture. In endothelial cells, STS increased intracellular levels of sulfide and sulfane sulfur and inhibited lipopolysaccharide or tumor necrosis factor-α–induced production of cytokines and reactive oxygen species. The beneficial effects of STS were associated with attenuation of the lipopolysaccharide-induced nuclear factor-κB activation through the inhibition of tumor necrosis factor receptor–associated factor 6 ubiquitination.

Conclusions: STS exerts robust antiinflammatory effects in mice lung and vascular endothelium. The results suggest a therapeutic potential of STS in acute lung injury. **(Anesthesiology 2014; 121:1248-57)**

ACUTE lung injury (ALI) is characterized by lung inflammation and increased pulmonary vascular permeability.[1] Sepsis is a major cause of ALI,[2] and lipopolysaccharide, a cell wall component of Gram-negative bacteria, can reproduce the features of human ALI in mice.[3] Studies have revealed that vascular endothelium plays a crucial role in mediating inflammatory response in the lung.[4,5] Therefore, the pulmonary vascular endothelium represents one of the major targets of therapy.[6]

Hydrogen sulfide (H_2S) is a reactive gaseous mediator. In mammalian tissues, H_2S is serially oxidized to persulfide, sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), and sulfate (SO_4^{2-}).[7] In addition, H_2S may be stored as sulfane sulfur–containing polysulfides in cells.[8] Although H_2S can exert a host of biological effects on various targets,[9] it is currently unknown whether the biological effects of H_2S are mediated directly by H_2S itself or its metabolites.[8] In circulation, reaction with plasma proteins or oxidation maintains free plasma H_2S levels very low.[10] Free H_2S levels only transiently increase and quickly return to its baseline after systemic administration of H_2S donor compounds (*e.g.*, Na_2S [sodium sulfide] or NaHS [sodium hydrosulfide]).

We recently reported that the protective effects of inhaled H_2S on mice subjected to lethal lipopolysaccharide challenge are associated with an increased plasma thiosulfate levels.[11]

What We Already Know about This Topic

- Acute lung injury is characterized by neutrophilic inflammation and increased lung permeability. Thiosulfate is a stable metabolite of hydrogen sulfide, a gaseous mediator that exerts antiinflammatory effects, but its role in acute lung injury is unknown.

What This Article Tells Us That Is New

- Using an experimental model of acute lung injury in mice challenged with intratracheal lipopolysaccharide or subjected to cecal ligation and puncture with or without sodium thiosulfate, it was shown that sodium thiosulfate exerts robust antiinflammatory effects in mice lung and vascular endothelium.

Furthermore, intraperitoneal administration of sodium thiosulfate (STS) improved survival after endoxemia[11] and acute liver failure.[12] These observations suggest that thiosulfate may be one of the "carrier" molecules that mediate antiinflammatory effects of H_2S. STS has been used for decades as an antidote against cyanide poisoning.[13] STS has also been used for the treatment of calciphylaxis,[14] vascular calcifications,[15,16] and cisplatin-induced cytotoxicity.[17] Therefore, if antiinflammatory effects of STS are confirmed, then it is highly clinically relevant and readily translatable. Although H_2S has been shown to mitigate lung injury[18,19] and vascular endothelial dysfunction in a variety of animal models,[20,21]

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effects of STS against ALI remain to be determined. Furthermore, mechanisms responsible for the antiinflammatory effects of STS were not investigated in our previous studies.[11,12] It is possible that thiosulfate protects vascular endothelium from inflammatory insults.

H_2S appears to exert antiinflammatory effects at least in part *via* inhibition of nuclear factor-κB (NFκB)–dependent signaling pathway.[11,22] Upon binding of lipopolysaccharide to the toll-like receptor 4, tumor necrosis factor receptor–associated factor 6 (TRAF6) is recruited to the receptor complex, which facilitates lysine 63 (K63)–linked polyubiquitination of TRAF6.[23,24] Polyubiquitinated TRAF6 induces phosphorylation and activation of transforming growth factor-β–activated kinase 1 (TAK1). TAK1 then activates inhibitor of NFκB kinase (IKK), resulting in NFκB activation.[24] Thus, inhibition of TRAF6 ubiquitination has been suggested as a target to modulate NFκB signaling pathway.[25,26]

The objective of the current study is to examine the effects of STS in ALI. We hypothesized that STS prevents ALI *via* inhibition of NFκB signaling in pulmonary vascular endothelium. We observed that STS inhibited ALI and inflammation after intratracheal lipopolysaccharide challenge and cecal ligation and puncture (CLP). These results highlight the therapeutic potential of STS in the treatment of ALI.

Materials and Methods

Animals

Male C57BL6J mice (Jackson Laboratories, Bar Harbor, ME), 8 to 10 weeks old, 19 to 27 g body weight, were used in this study. Protocols for animal use were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (Boston, MA). All animal experiments were performed in accordance with the guidelines of the National Institutes of Health (Bethesda, Maryland).

Lipopolysaccharide-induced Lung Injury

Mice were challenged with 2 mg/kg of lipopolysaccharide (O111: B4; Sigma, St. Louis, MO) in 50-μl saline *via* intratracheal route as an aerosol using a microsprayer (Penn-Century, Philadelphia, PA) with or without intraperitoneal administration of 2 g/kg STS at 0 and 12 h after intratracheal lipopolysaccharide. Control mice received 50 μl intratracheal saline. Mice breathed spontaneously in ambient air, and bronchoalveolar lavage fluid (BALF) was collected 24 h after saline or lipopolysaccharide challenge. We chose this time point because we found that inflammatory reaction peaks at 24 h after lipopolysaccharide challenge at this dose in pilot studies. Total number of leukocytes in BALF was counted using a hemocytometer. The BALF samples were subsequently centrifuged and cytospin samples were prepared from the cell pellet. Cytoslides were stained with Kwik-Diff stain kit (Thermo Shandon, Pittsburgh, PA). The number of polymorphonuclear neutrophils (PMNs) was determined by the cell counting of neutrophil fraction. Supernatant samples were used to quantify the protein levels using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) or stored at −80°C for cytokine and myeloperoxidase activity analysis.

Cecal Ligation and Puncture

Mice were subjected to CLP as previously described.[27] Mice were anesthetized and a midline abdominal incision was made to expose cecum. The cecum was ligated at 1.0 cm from the tip then punctured twice with an 18-gauge needle. A small amount of its contents were extruded before the cecum was returned into the abdominal cavity, and then the abdominal incision was closed in layers. Sham-operated controls underwent laparotomy without CLP. All mice were given fluid resuscitation with prewarmed sterile saline (50 ml/kg) subcutaneously. STS (0.5 g/kg) or saline was intravenously injected *via* tail vein 10 min after CLP. Lungs were harvested 8 h after CLP, homogenized with 0.5% Triton X-100/phosphate-buffered saline, and centrifuged, and then the supernatants were subjected to cytokine measurement after equalization by the protein concentration.

Measurement of BALF Cytokines and Myeloperoxidase

Enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) were used for the measurement of mouse tumor necrosis factor-α (TNFα), interleukin (IL)-6, and myeloperoxidase levels in BALF according to the manufacturer's instruction.

Lung Wet Dry Ratio

Mouse lungs were harvested 24 h after lipopolysaccharide challenge, blotted dry, and weighed immediately to obtain the wet lung weight. Lungs were dried in a 70°C incubator for 3 days to obtain dry lung weight, and then wet dry lung ratio was calculated.

Lung Histology

Mouse lungs were inflated under a pressure of 23 cm H_2O with 4% paraformaldehyde (Boston Bio Products, Ashland, MA) for histologic evaluation by hematoxylin and eosin staining as previously described.[28] Twenty high-power fields (×400 magnification) were taken per mouse, and lung injury was graded using a modified ALI score in each high-power field. Each of three categories, such as (1) thickness of the alveolar walls, (2) infiltration of inflammatory cells, and (3) hemorrhage, was graded in a blinded manner according to the following scale: 0 = minimal damage; 1 = mild damage; 2 = moderate damage; 3 = severe damage; and 4 = maximal damage. The degree of lung damage was assessed by the total of scores ranging from 0 to 12.

Quantitative Polymerase Chain Reaction

Mouse lungs were harvested 24 h after lipopolysaccharide or saline challenge, and total RNA was extracted using the RNAspin mini kit (GE Healthcare, Piscataway, NJ). Complementary DNA was synthesized with Moloney Murine

Leukemia Virus Reverse Transcriptase (Promega, Madison, WI), and RNA transcript levels were measured using a Mastercycler Realplex system (Eppendorf North America, Westbury, NY). The primer sequences are listed in table 1. Gene expression was normalized to 18S ribosomal RNA level. The mean value of control mice was set as 1.

High-performance Liquid Chromatography

To determine the impact of intraperitoneal administration of STS, concentrations of hydrogen sulfide and thiosulfate in lung and plasma were measured by high-performance liquid chromatography as previously described.[11] At 2 h after lipopolysaccharide challenge, blood was drawn and lung was dissected. Plasma and lung homogenates were added to 70 μl of 10 mM Tris-HCl buffer (pH 9.5, 0.1 mM diethylenetriamine pentaacetic acid), followed by addition of 50 μl of 200 mM 5-sulfosalicylic acid after 30 min. The mixture was centrifuged and supernatant was analyzed by high-performance liquid chromatography with a fluorescence detector (Waters, Milford, MA).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) and human lung microvascular endothelial cells (HMVEC-L) were purchased from Lonza (Walkersville, MD). The cells were cultured in EGM-2 or EGM-2 MV (Lonza) supplemented with 2 or 5% fetal bovine serum and endothelial cell growth factors and used between passages 3 to 5 for all experiments.

Measurement of Cytokine Production in Cell Culture Supernatants

Human umbilical vein endothelial cells or HMVEC-L were seeded in 96-well plates at 1×10^4 cells per well and incubated with lipopolysaccharide (10 μg/ml) or recombinant human TNFα (10 ng/ml) (R&D Systems) with or without STS. Cell culture supernatants were collected after incubation for 20 h with lipopolysaccharide or TNFα. Human IL-6 and IL-8 levels were measured by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instruction.

Measurement of Reactive Oxygen Species Production in HUVEC

Chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen, Eugene, OR) was used to measure intracellular production of general reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), hydroxy-radical, and peroxides. HUVEC were seeded at 1×10^4 cells per well in 96-well plate and cultured to confluent. Cells were loaded with 10 μM of CM-H_2DCFDA for 30 min. Then cells were washed twice, lipopolysaccharide (10 μg/ml) or TNFα (10 ng/ml) was added with or without STS for 30 min, followed by fluorescence measurement at excitation and emission wavelengths of 480 and 530 nm. The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was also used to measure H_2O_2 and peroxide production. After treatment with lipopolysaccharide or TNFα, 100 μM of Amplex Red reagent solution (Invitrogen) was added to each well. The cells were treated for 30 min, and the fluorescence intensity was measured at excitation and emission wavelengths of 560 and 590 nm.

Table 1. List of Primer Sequences for Quantitative Polymerase Chain Reaction (5'–3')

TNFα	Forward	CAG CCT CTT CTC ATT CCT GC
	Reverse	GGT CTG GGC CAT AGA ACT GA
IL-6	Forward	CCG GAG AGG AGA CTT CAC AGA
	Reverse	CAG AAT TGC CAT TGC ACA AC
ICAM-1	Forward	TCC GCT GTG CTT TGA GAA CT
	Reverse	AGG GTG AGG TCC TTG CCT AC
KC	Forward	CTG GGA TTC ACC TCA AGA ACA TC
	Reverse	CAG GGT CAA GGC AAG CCT C
MCP-1	Forward	TGT TCA CAG TTG CCG GCT GGA G
	Reverse	AGC TTC TTT GGG ACA CCT GCT GC
NOS2	Forward	GTT CTC AGC CCA ACA ATA CAA GA
	Reverse	GTG GAC GGG TCG ATG TCA C
MMP9	Forward	GCC GAC TTT TGT GGT CTT CC
	Reverse	CGG CCG TAG AGA CTG CTT CT
MMP2	Forward	CAA GTT CCC CGG CGA TGT C
	Reverse	TTC TGG TCA AGG TCA CCT GTC
18S rRNA	Forward	CGG CTA CCA CAT CCA AGG AA
	Reverse	GCT GGA ATT ACC GCG GCT
IL-1β	TaqMan Mm01336189_m1 (Applied Biosystems, Foster City, CA)	

ICAM-1 = intercellular adhesion molecule-1; IL = interleukin; KC = keratinocyte-derived chemokine; MCP-1 = monocyte chemoattractant protein-1; MMP = matrix metalloproteinase; NOS2 = nitric oxide synthase 2; rRNA = ribosomal RNA; TNFα = tumor necrosis factor-α.

Western Blotting

Protein extracts were prepared by lysing cells or homogenizing lung tissue in radio-immunoprecipitation assay buffer (Boston BioProducts) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma). The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA), and immunoblotted. Antibodies used were as follows: TRAF6 (H274), TAK1 (M579) from Santa Cruz (Dallas, TX), K63-linkage–specific ubiquitin (HWA4C4) from Millipore, phospho-TAK1 (4531), phospho-IKKα/β (2697), IKKβ (2678), phospho-NFκB p65 (3033), NFκB p65 (8242), inhibitor of NF-κBα (IκBα) (4812), phospho-IκBα (2859), glyceraldehyde-3-phosphate dehydrogenase (5174), and β-tubulin (2146) from Cell Signaling (Danvers, MA). In densitometric analysis, the mean value of control group was set as 1.

Immunoprecipitation

Cell lysates were incubated with TAK1 or TRAF6 antibodies with Protein G Mag Sepharose (GE Healthcare) at 4°C for overnight. The complexes were washed four times with Tris buffered saline with Tween20 and eluted in sodium

dodecyl sulfate–sample buffer (Boston Bioproducts) at 90°C for 2 min. For TRAF6 immunoprecipitation, cells were pretreated with 10 μM MG132 (Sigma) for 4 h before treatment with lipopolysaccharide and STS. Cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Roche) and 10 mM *N*-ethylmaleimide.

Measurement of Hydrogen Sulfide and Sulfane Sulfur Levels

Two novel fluorescent probes, HSip-1DA[29] and SSP4, an improved version of SSP2,[30] were used for the detection of H_2S and sulfane sulfur, respectively. SSP4 was prepared using the same method reported previously.[30] ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.49 (s, 2H), 6.97 (d, J = 9.0 Hz, 2H), 7.12 (d, J = 9.0 Hz, 2H), 7.32 (t, J = 6.0 Hz, 2H), 7.44 to 7.55 (m, 5H), 7.66 (d, J = 9.0 Hz, 2H), 7.76 to 7.88 (m, 2H), 8.09 (d, J = 9.0 Hz, 1H), 8.19 (d, J = 6.0 Hz, 2H). ^{13}C NMR (75 MHz, CD_3Cl) δ 81.9, 110.9, 116.9, 118.2, 124.3, 124.7, 125.2, 125.5, 129.3, 131.4, 132.5, 133.7, 140.1, 151.8, 152.2, 153.2, 164.9, 169.4; MS (ESI$^+$) m/z 627.6 (M+Na$^+$). HUVEC were seeded at 1×10^4 cells per well in 96-well plate and cultured to confluent. Cells were loaded with 30 μM of HSip-1DA, a cell membrane-permeable derivative of HSip-1, or 50 μM of SSP4 for 30 min. Then cells were washed twice and treated with or without lipopolysaccharide and STS for 20 h, followed by fluorescence measurement at excitation and emission wavelengths of 490 and 515 nm. Intracellular H_2S and sulfane sulfur levels were determined by relative fluorescence intensities normalized to the levels of untreated control at 1 h after treatment.

Statistical Analysis

All data are presented as means ± SD. We did not conduct an *a priori* statistical power calculation. We estimated our sample size based on our previous studies in which the effects of STS were examined in sepsis.[11] No randomization methods were used to assign animals or cells to each treatment. Treatment conditions were alternated between samples in an effort to maintain experimental conditions as constant as possible among groups. Two-tailed hypothesis testing method was used throughout this study. Data were analyzed by one-way or two-way ANOVA followed by Bonferroni *post hoc* comparisons tests as required using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Thiosulfate and sulfide levels of plasma and lung were analyzed by Mann–Whitney U test because the values were not normally distributed. *P* values of less than 0.05 were considered significant.

Results

STS Attenuates Lipopolysaccharide-induced Lung Injury and Enhanced Permeability

The number of cells in BALF was evaluated to examine the effects of STS on lipopolysaccharide-induced lung inflammation and permeability. The recovery rate of BALF was more than 90% in all groups. In lipopolysaccharide-challenged mice, a marked increase in the number of total cells and PMNs in BALF was observed (fig. 1, A and B). However, STS significantly decreased the influx of cells into the alveolar space after lipopolysaccharide challenge. STS treatment alone did not affect the number of cells in BALF. Myeloperoxidase levels, a marker of PMN infiltration, were also measured by enzyme-linked immunosorbent assay. In accordance with the result of PMNs influx into the lung, myeloperoxidase levels were significantly increased in lipopolysaccharide-challenged mice, whereas it was attenuated by STS administration (fig. 1C). We also evaluated the effect of STS on lung vascular leak by measuring BALF protein and lung wet dry weight ratio. Lipopolysaccharide challenge induced a significant increase in BALF protein concentrations and lung wet dry ratio (fig. 1, D and E). STS attenuated the pulmonary vascular leakage and lung edema in lipopolysaccharide-challenged mice. To evaluate inflammatory mediators recruiting PMNs to the lung, we measured cytokine levels in BALF by enzyme-linked immunosorbent assay. STS significantly decreased the lipopolysaccharide-induced IL-6 and TNFα increase in the BALF (IL-6, TNFα [pg/ml]; 49 ± 12, 57 ± 6, control [n = 8]; 313 ± 164, 336 ± 156, lipopolysaccharide [n = 10]; 79 ± 27, 76 ± 88, lipopolysaccharide + STS [n = 10]; fig. 1, F and G). Furthermore, intravenous STS attenuated IL-6 induction in the lung 8 h after CLP (pg ml^{-1} g^{-1} protein; 95 ± 33, control [n = 8]; 424 ± 256, CLP [n = 12]; 241 ± 128, CLP + STS [n = 12]; fig. 1H). These results suggest that STS exerts potent antiinflammatory effects and prevents the increase in lung permeability after lipopolysaccharide challenge or polymicrobial sepsis.

STS Prevents Lipopolysaccharide-induced Lung Injury

Histologic assessment revealed that lipopolysaccharide stimulated a marked influx of PMNs to the alveolar space. Administration of STS attenuated the PMN infiltration into the lung (fig. 2, A and B). Semiquantitative analysis of lung sections by lung injury score demonstrated that lipopolysaccharide-induced lung injury was attenuated by STS treatment (fig. 2C).

STS Attenuates Up-regulation of Proinflammatory Mediators in the Lung after Lipopolysaccharide Challenge

We measured the messenger RNA expression levels of proinflammatory mediators, including cytokines, chemokines, and adhesion molecules, in the whole lung of mice after lipopolysaccharide challenge (fig. 3). STS attenuated lipopolysaccharide-induced up-regulation of cytokines (TNFα, IL-6, and IL-1β), intracellular adhesion molecule-1, chemokines, and nitric oxide synthase 2. STS also attenuated the lipopolysaccharide-induced up-regulation of matrix metalloproteinase 9, a family of proteinases that remodel extracellular matrix components, but not matrix metalloproteinase 2.

STS Inhibits Lipopolysaccharide-induced Activation of IκB/NFκB Signaling Pathway in Mice Lung

Activation of IKK by lipopolysaccharide induces phosphorylation and degradation of IκB, leading to the

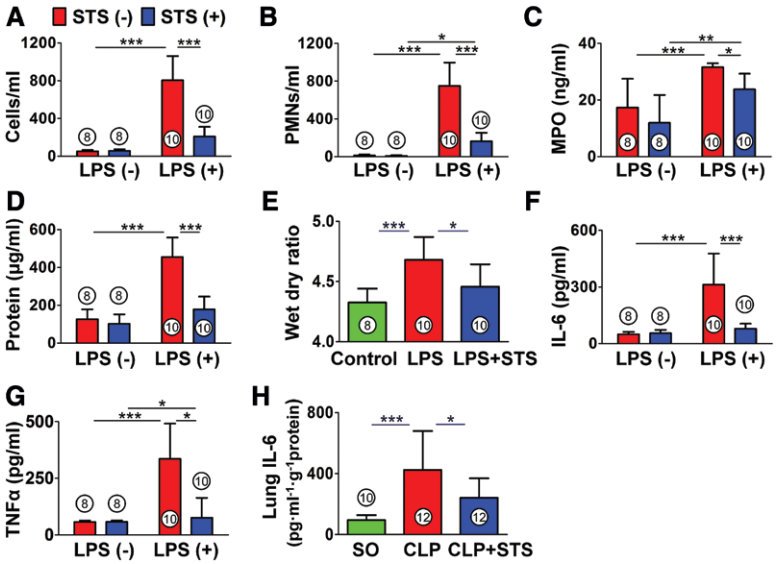

Fig. 1. Effects of sodium thiosulfate (STS) on lipopolysaccharide (LPS)-induced or polymicrobial sepsis-induced lung injury. (*A*) Total number of leukocytes and (*B*) polymorphonuclear neutrophils (PMNs) in bronchoalveolar lavage fluid. (*C*) Myeloperoxidase (MPO) levels and (*D*) total protein concentration in bronchoalveolar lavage fluid. (*E*) Mouse lung wet dry ratio. (*F*, *G*) Inflammatory cytokine levels in bronchoalveolar lavage fluid. (*H*) Interleukin (IL)-6 levels in mouse lung subjected to cecal ligation and puncture (CLP). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; (*A–D*, *F*, *G*) two-way ANOVA Bonferroni posttest, (*E*, *H*) one-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size. SO = sham operation; TNFα = tumor necrosis factor-α.

nuclear translocation of NFκB and transcriptional activation.[23] To elucidate the molecular mechanisms involved in the attenuated inflammatory responses by STS, we examined the effects of STS on IκB/NFκB p65 pathway. STS inhibited lipopolysaccharide-induced IκB phosphorylation and p65 nuclear translocation in mice lung 24 h after lipopolysaccharide challenge (fig. 4). These results suggest that STS inhibits lipopolysaccharide-induced activation of NFκB signaling by inhibiting IκB phosphorylation.

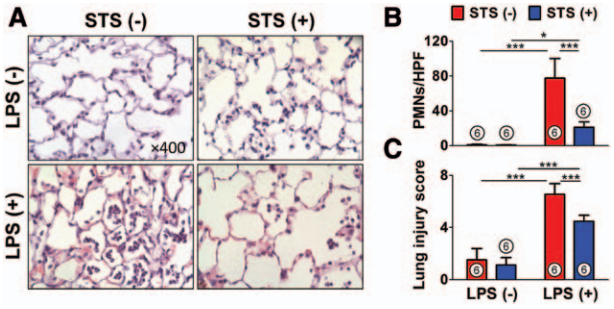

Fig. 2. Histologic evaluation of the effects of sodium thiosulfate (STS) on lipopolysaccharide (LPS)-induced lung injury. (*A*) Representative microscopic images of hematoxylin and eosin–stained lung sections from each group (magnification ×400). (*B*) The number of polymorphonuclear neutrophils (PMNs) in each high-power field (HPF). (*C*) Semiquantitative analysis of lung sections by lung injury score. ***$P < 0.001$, *$P < 0.05$; two-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size.

STS Augments Thiosulfate and Sulfide Levels of Plasma and Lung in Mice

Administration of STS markedly increased thiosulfate levels in plasma and lung with or without lipopolysaccharide challenge (fig. 5). Plasma thiosulfate concentrations reached 1.8 ± 0.3 mM and 1.8 ± 0.9 mM in mice challenged with saline or lipopolysaccharide, respectively, at 2 h after STS administration. Plasma sulfide levels were also augmented to 56 ± 16 μM and 46 ± 22 μM by STS administration in mice challenged with saline or lipopolysaccharide, respectively. Administration of STS markedly increased the levels of thiosulfate in the lung with or without lipopolysaccharide challenge. Similarly, sulfide levels doubled in the lungs of saline-challenged mice and tended to increase in lipopolysaccharide-challenged mice lungs after STS administration.

STS Attenuates Lipopolysaccharide or TNFα-induced Proinflammatory Mediator Production in HUVEC and HMVEC-L

To examine the effects of STS on endothelium, we next evaluated the effects of STS on lipopolysaccharide or TNFα-induced cytokine production in HUVEC and HMVEC-L. STS (20 mM) *per se* had no significant cytotoxicity on HUVEC (data not shown). STS inhibited the lipopolysaccharide or TNFα-induced IL-6 production in HUVEC (fig. 6A) and HMVEC-L (fig. 6B) in a dose-dependent manner. Similarly, STS attenuated lipopolysaccharide or TNFα-induced IL-8 production in HUVEC (fig. 6C).

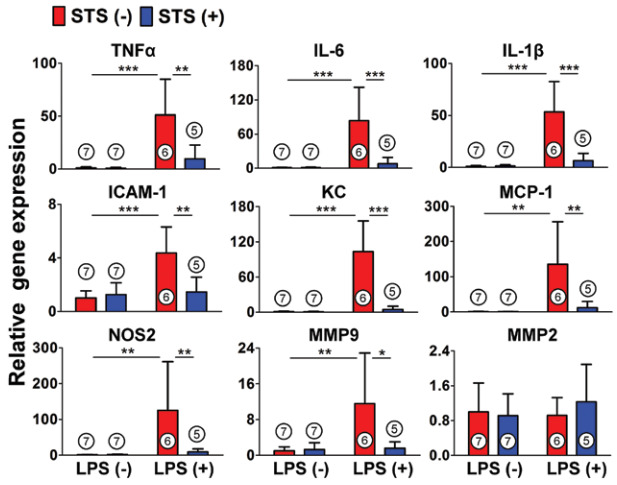

Fig. 3. Relative messenger RNA expression of proinflammatory mediators in lipopolysaccharide (LPS)-induced lung injury. Relative gene expression levels of inflammatory mediators in the lung 24 h after challenge with saline or LPS. Gene expression was normalized to 18S ribosomal RNA expression level, and the mean value for control mice challenged with saline was set to 1. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; two-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size. ICAM-1 = intracellular adhesion molecule-1; IL = interleukin; KC = keratinocyte-derived chemokine; MCP-1 = monocyte chemotactic protein-1; MMP = matrix metalloproteinase; NOS2 = nitric oxide synthase 2; STS = sodium thiosulfate; TNFα = tumor necrosis factor-α.

STS Inhibits Lipopolysaccharide or TNFα-stimulated ROS Production in HUVEC

We assessed whether or not STS affects lipopolysaccharide or TNFα-induced production of ROS, which can lead further inflammatory response in endothelium.[23] Lipopolysaccharide (10 μg/ml) or TNFα (10 ng/ml) significantly increased the fluorescence intensity of CM-H_2DCFDA, an intracellular probe of ROS in HUVEC (fig. 7A). STS inhibited the intracellular ROS production induced by lipopolysaccharide or TNFα. The H_2O_2/peroxidase levels in HUVEC measured by fluorescent intensity of Amplex red (Invitrogen) were significantly increased with lipopolysaccharide or TNFα, which were markedly inhibited by STS (fig. 7B).

STS Inhibits Lipopolysaccharide-induced IKK/NFκB Activation in HUVEC

To determine whether or not STS attenuates lipopolysaccharide-induced activation of IKK/NFκB pathway in endothelial cells, we analyzed IKKα/β, IκB, and p65 in HUVEC. STS inhibited lipopolysaccharide-induced phosphorylation of IKKα/β, IκB, and p65 in a dose-dependent manner (fig. 8, A–C).

STS Inhibits Lipopolysaccharide-induced TAK1 Activation and TRAF6 Polyubiquitination in HUVEC

We examined the effect of STS on the activation of TAK1 and polyubiquitination of TRAF6 as upstream modulators

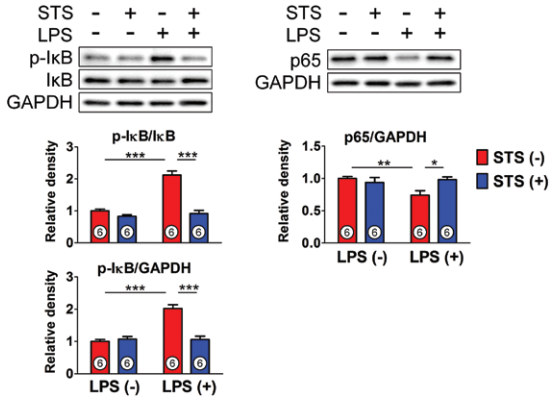

Fig. 4. Effects of sodium thiosulfate (STS) on IκB/nuclear factor-κB (NFκB) signaling pathway. Levels of total and phosphorylated inhibitor of NFκB (p-IκB) and p65 subunit of NFκB normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in lung tissue homogenates 24 h after challenge with saline or lipopolysaccharide (LPS). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; two-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size. p65 = p65 subunit of NFκB.

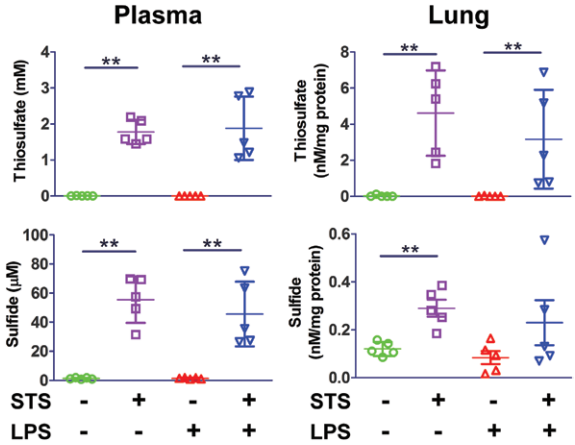

Fig. 5. Levels of thiosulfate and sulfide in plasma and lung 2 h after intratracheal lipopolysaccharide (LPS) challenge. Levels of thiosulfate and sulfide in plasma and lung were measured with high-performance liquid chromatography at 2 h after intratracheal LPS challenge with or without sodium thiosulfate (STS). **$P < 0.01$; Mann–Whitney U test, n = 5 in each group.

of NFκB.[24] Cell lysates were immunoprecipitated with TAK1 antibody and phosphorylated TAK1 was detected by immunoblot. STS inhibited lipopolysaccharide-induced TAK1 phosphorylation (fig. 9A). Next, TRAF6 was immunoprecipitated and probed with anti-K63–specific ubiquitin antibody. STS inhibited lipopolysaccharide-induced K63-linked polyubiquitination of TRAF6 (fig. 9B). These results suggest that STS attenuates lipopolysaccharide-induced IKK/NFκB activation through inhibiting the polyubiquitination of TRAF6 and activation of TAK1.

STS Augments Intracellular H_2S and Sulfane Sulfur Levels

To identify the sulfide metabolites increased by STS in the cells challenged with lipopolysaccharide, intracellular levels of free H_2S/HS^- and sulfane sulfur were evaluated using fluorescent probes HSip-1DA and SSP4, respectively. We performed kinetic measurement of fluorescence up to 20 h after treatment with lipopolysaccharide and STS. HSip-1 and SSP4 fluorescent intensity gradually increased over 20 h in control cells that are loaded with HSip-1DA or SSP4 but without lipopolysaccharide and STS. Lipopolysaccharide alone or lipopolysaccharide with STS at concentrations less than 5 mM did not affect fluorescence intensity of HSip-1 and SSP4 compared with control at all times. Intracellular sulfide levels were augmented by 20 mM of STS between 2 and 20 h after treatment in cells incubated with lipopolysaccharide (fig. 10A). STS at 10 mM augmented intracellular sulfide levels at 6 and 20 h after treatment in lipopolysaccharide-treated cells. Intracellular sulfane sulfur levels were augmented by 20 mM of STS between 6 and 20 h after treatment (fig. 10B). These results suggest that STS increases intracellular levels of sulfide and sulfane sulfur in lipopolysaccharide-treated cells, with the former increasing faster than the latter.

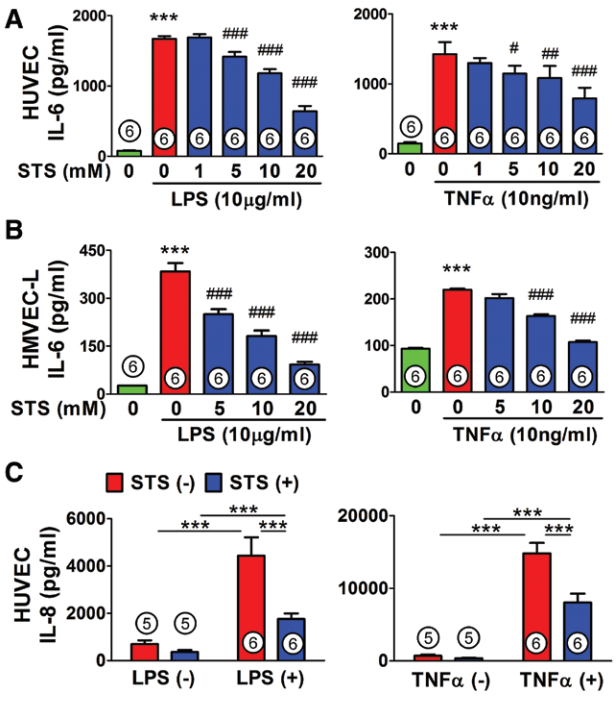

Fig. 6. Effects of sodium thiosulfate (STS) on lipopolysaccharide (LPS) or tumor necrosis factor-α (TNFα)–induced cytokine production in human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HMVEC-L). Interleukin (IL)-6 levels in HUVEC (*A*) and HMVEC-L (*B*) culture medium incubated with LPS or TNFα for 20 h with or without varying concentrations of STS. ***$P < 0.001$ *versus* control. ###$P < 0.001$, ##$P < 0.01$, #$P < 0.05$ *versus* LPS or TNFα; one-way ANOVA Bonferroni posttest, mean ± SD. (*C*) IL-8 levels in HUVEC culture medium incubated with LPS or TNFα for 20 h with or without 20 mM STS. ***$P < 0.001$; two-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size.

Discussion

In the current study, we demonstrated that intraperitoneal administration of STS attenuated ALI in mice. STS inhibited the increase of lung permeability, influx of PMN, and expression of proinflammatory mediators in mice lung subjected to intratracheal lipopolysaccharide challenge. STS also attenuated lung tissue inflammation after polymicrobial sepsis. Administration of STS markedly increased the levels of sulfide and thiosulfate in lung and plasma of mice. We also observed that STS markedly attenuated lipopolysaccharide or TNFα-induced cytokine/ROS production in cultured endothelial cells and prevented the increase in endothelial permeability in vascular endothelial monolayer (data not shown). The beneficial effects of STS were associated with down-regulation of IKK/NFκB signaling pathways. Our results also revealed that STS markedly inhibited the lipopolysaccharide-induced activation of TAK1 and TRAF6 polyubiquitination, suggesting a novel regulatory mechanism responsible for the inhibitory effects of sulfide on NFκB signaling. Last, the current results suggest that STS exerts its beneficial effects at least in part by increasing intracellular sulfide and sulfane sulfur levels in vascular endothelium. Taken together, these observations suggest a therapeutic potential of STS against ALI.

Role of sulfide and sulfide metabolites in inflammatory organ injury remains incompletely defined. Although acute administration of high doses of H_2S donor compounds appears to be invariably toxic, lower and steady levels of H_2S may be cytoprotective against systemic inflammation.[11,22] Along these lines, we have recently reported that breathing low concentration of H_2S prevents lethal endotoxemia and lipopolysaccharide-induced lung and liver injury in mice at least in part by increasing thiosulfate.[11] We also observed that administration of STS dose dependently prevents death from endotoxin shock in the previous study.[11] These studies prompted us to further examine the lung-protective effects of STS in the current study.

Thiosulfate is a potent antioxidant and STS has been used for the treatment of cyanide poisoning and calciphylaxis with a remarkable safety track record.[13,14] In the current study, we observed that STS markedly inhibited ROS production induced by lipopolysaccharide in endothelial cells. Although we used relatively high doses of STS, previous clinical studies have shown extremely low cytotoxicity and effectiveness of STS at similar doses in patients. For example, intravenous administration of STS at dose of 4 or 12 g/m^2 showed no evidence of neuro- or nephrotoxicity in humans.[31,32] Furthermore, Neuwelt *et al.*[17] used as much as 16 and 20 g/m^2 doses of intravenous STS against carboplatin-induced ototoxicity in human. In their study, after intravenous administration of 16 or 20 g/m^2 of STS, serum thiosulfate levels reached 308 mg/dl (12.3 mM) and 330.8 mg/dl (13.2 mM), respectively,

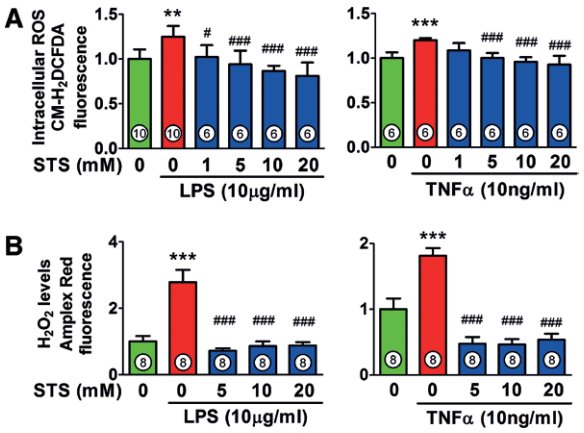

Fig. 7. Effect of sodium thiosulfate (STS) on lipopolysaccharide (LPS) or tumor necrosis factor-α (TNFα)–stimulated reactive oxygen species (ROS) production in human umbilical vein endothelial cells. (*A*) Intracellular levels of ROS measured by chloromethyl-2'7'-dichlorofluorescein diacetate (CM-H_2DCF-DA) in human umbilical vein endothelial cells stimulated with LPS or TNFα for 30 min with or without varying concentrations of STS. (*B*) Levels of H_2O_2 measured by Amplex Red (Invitrogen, Eugene, OR) in human umbilical vein endothelial cells stimulated with LPS or TNFα for 30 min with or without varying concentrations of STS. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control, ###$P < 0.001$, #$P < 0.05$ *versus* LPS or TNFα treatment; one-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size.

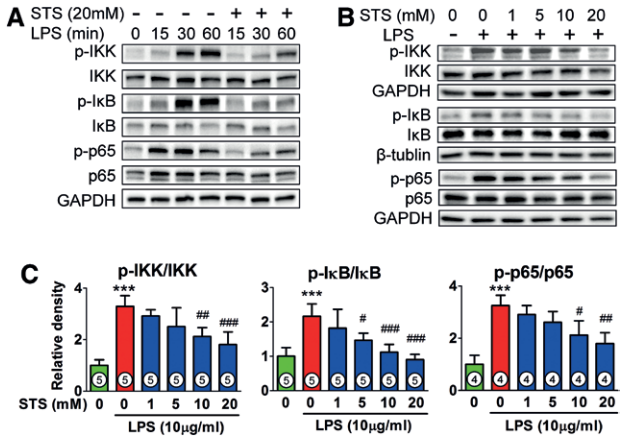

Fig. 8. Effects of sodium thiosulfate (STS) on lipopolysaccharide (LPS)-induced IκB/nuclear factor-κB (NFκB) signaling in human umbilical vein endothelial cells. (*A*) Representative immunoblots of total and phosphorylated IκB kinase (IKK), p65 subunit of NFκB (p65), and inhibitor of NFκB (IκB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human umbilical vein endothelial cells incubated with LPS (10 μg/ml) with or without STS (20 mM) for the indicated times. Representative immunoblots (*B*) and densitometric analyses (*C*) of phosphorylated/total IKK, p65, and IκB in human umbilical vein endothelial cells stimulated with LPS for 30 min with or without varying concentration of STS. $^{***}P < 0.001$ *versus* control, ###$P < 0.001$, ##$P < 0.01$, #$P < 0.05$ *versus* LPS treatment; one-way ANOVA Bonferroni posttest, mean ± SD. *Numbers in bars* represent the sample size.

immediately after bolus infusion, with no signs of toxicity.[17] Given the weight and height of the patient are 50 kg and 160 cm (1.5 m^2 surface area), the doses of 16 to 20 g/m^2 STS are assumed to be 0.48 to 0.6 g/kg, which correspond to the effective intravenous STS dose in mouse subjected to CLP in the current study. Therefore, the doses of STS used in the current study and the resultant plasma concentrations of thiosulfate fall within the doses of STS and plasma levels of thiosulfate that have been observed in patients. These observations suggest clinical relevance of our findings.

It is well established that lipopolysaccharide activates toll-like receptor 4–dependent signaling cascade. Binding of lipopolysaccharide to toll-like receptor 4 in endothelial cells up-regulates production of proinflammatory cytokines, chemokines, and adhesion molecules, predominantly *via* the transcription factor NFκB pathway.[23] These actions lead to cell adhesion or increased vascular permeability that causes neutrophil migration and edema in the lung.[23] STS markedly inhibited lipopolysaccharide-induced phosphorylation of IκBα and nuclear translocation of NFκB p65 in mice lung. STS inhibited the rapid phosphorylation of IKK, IκBα, and p65 in HUVEC that occurred within 1 h after exposure to lipopolysaccharide. Although these observations are consistent with the hypothesis that sulfide exerts antiinflammatory effects *via* inhibition of NFκB,[11,22] impact of sulfide or thiosulfate on NFκB-dependent signaling is incompletely defined. For example, although the majority of studies reported that H_2S inhibits NFκB,[22,33] some studies suggested that NFκB can be activated by sulfide.[34] To further characterize the molecular mechanisms responsible for the inhibitory effects of STS on toll-like receptor 4-NFκB signaling, we examined the impact of STS on lipopolysaccharide-induced TRAF6 ubiquitination and TAK1 activation that are upstream of NFκB.

The protein ubiquitination is carried out through a three stepwise enzymatic reactions, involving E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase).[24] TRAF6 functions as an E3, which catalyzes K63 polyubiquitination. K63-linked autoubiquitination of TRAF6 is required for the activation of TAK1 and subsequent NFκB activation.[24] A crucial role of ubiquitinated TRAF6 on NFkB signaling has been demonstrated by inhibiting TRAF6 polyubiquitination.[25,26] The inhibitory effects of STS on lipopolysaccharide-induced TRAF6 ubiquitination revealed in this study shed light on the novel mechanisms responsible for the antiinflammatory effects of STS and sulfide. In addition, STS inhibited TNFα-induced cytokine production in HUVEC. Of the six TRAF family members, TRAF6 is the only TRAF that mediates both of the TNF receptor and toll-like receptor signaling.[35] Although this unique property of TRAF6 may explain the inhibitory effects of STS on TNFα-induced cytokine production, the effects of STS on TNFα-induced signaling remain to be further elucidated in future studies.

It has been proposed that some of the effects of H_2S are mediated *via* the properties of sulfide metabolites containing reactive sulfane sulfur (S^0), a labile, highly reactive sulfur

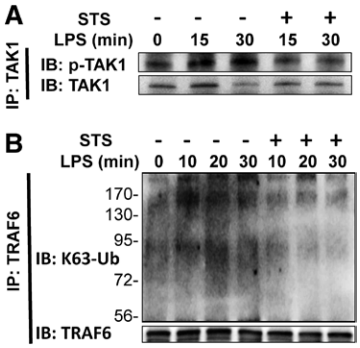

Fig. 9. Effects of sodium thiosulfate (STS) on lipopolysaccharide (LPS)-induced transforming growth factor-β–activated kinase 1 (TAK1) activation and tumor necrosis factor receptor–associated factor 6 (TRAF6) ubiquitination. (*A*) Representative immunoblots of total and phosphorylated TAK1 in human umbilical vein endothelial cells incubated with or without LPS (10 μg/ml) and STS (20 mM) for indicated times. Cell lysates were immunoprecipitated (IP) with anti-TAK1 antibody and then immunoblotted (IB) with anti phospho-TAK1 (p-TAK1) antibody. (*B*) Representative immunoblots of lysine 63 (K63)-polyubiquitinated TRAF6 of the cell lysates of human umbilical vein endothelial cells incubated with LPS with or without STS for indicated times. Cell lysates were immunoprecipitated with anti-TRAF6 antibody and then immunoblotted with anti K63-specific ubiquitin (K63-Ub) antibody.

atom.[8,10] Although thiosulfate is one of the sulfide metabolites that contain sulfane sulfur,[8] thiosulfate itself appears to have limited reactivity. We therefore hypothesized that STS is converted to other sulfide metabolites that exert beneficial effects after lung injury. To determine the sulfide metabolites that are responsible for the beneficial effects of STS, we measured the levels of sulfide and reactive sulfane sulfur in HUVEC using novel fluorescent probes HSip-1[29] and SSP4,[30] respectively. Gradual increase of the HSip-1 and SSP4 fluorescence intensity in control cells may reflect endogenous production of sulfide or sulfane sulfur or leakage of the fluorescence probes to extracellular spaces where the probes can react with sulfide metabolites in culture media. We observed that STS increased intracellular H_2S levels within 2 h after the start of incubation of HUVEC with lipopolysaccharide and STS. In contrast, SSP4-reactive sulfane sulfur levels did not increase in HUVEC treated with lipopolysaccharide and STS until 6 h after the treatment. Because lipopolysaccharide triggered NFκB activation within 1 h and STS markedly inhibited NFκB activation, our data suggest that the antiinflammatory effects of STS are primarily mediated by intracellular sulfide that is converted from STS. It has been reported that thiosulfate can be converted to H_2S *via* 3-mercaptopyruvate sulfurtransferase which is expressed in vascular endothelium.[36,37] However, it has been suggested that H_2S converted from thiosulfate is stored as sulfane sulfur.[37] It is likely that levels of sulfide and sulfane sulfur are dynamically regulated in cells.

In summary, our results revealed that STS exhibits robust antiinflammatory effects on the lung. Taken together with our recent studies,[11,12] results of this study suggest a role of STS in inflammatory ALI in addition to its established role as a therapeutic agent for cyanide toxicity, calciphylaxis, and chemotoxicity. Our results also revealed for the first time that the beneficial effects of STS are associated with the inhibition of TRAF6 ubiquitination, suggesting a novel regulatory mechanism of NFkB signaling by sulfide metabolites. Considering the clinical availability and established safety track record of STS and the critical role of NFkB signaling in cellular survival, further studies examining the beneficial effects of STS in other diverse forms of organ injury are warranted.

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Competing Interests

The authors declare no competing interests.

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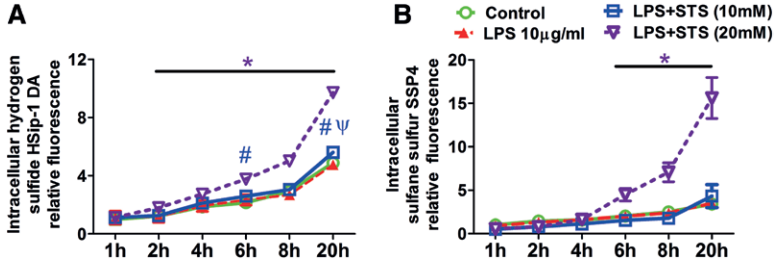

Fig. 10. Effect of sodium thiosulfate (STS) on hydrogen sulfide and sulfane sulfur levels in human umbilical vein endothelial cells challenged with lipopolysaccharide (LPS). Kinetic measurement of HSip-1DA (*A*) and SSP4 (*B*) fluorescence was performed to evaluate the impact of LPS and STS on sulfide metabolism up to 20 h after treatment. The relative fluorescence at each time point was normalized to the values of untreated control at baseline (1 h). **P* < 0.05, LPS + STS (20 mM) *versus* control, LPS and LPS + STS (10 mM); #*P* < 0.05, LPS + STS (10 mM) *versus* control; ψ*P* < 0.05, LPS + STS (10 mM) *versus* LPS; two-way ANOVA Bonferroni posttest, n = 6 in each group.

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