Hydrogen Sulfide Attenuates Particulate Matter–Induced Human Lung Endothelial Barrier Disruption via Combined Reactive Oxygen Species Scavenging and Akt Activation

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Exposure to particulate air pollution is associated with increased cardiopulmonary morbidity and mortality, although the pathogenic mechanisms are poorly understood. We previously demonstrated that particulate matter (PM) exposure triggers massive oxidative stress in vascular endothelial cells (ECs), resulting in the loss of EC integrity and lung vascular hyperpermeability. We investigated the protective role of hydrogen sulfide (H_2S), an endogenous gaseous molecule present in the circulation, on PM-induced human lung EC barrier disruption and pulmonary inflammation. Alterations in EC monolayer permeability, as reflected by transendothelial electrical resistance (TER), the generation of reactive oxygen species (ROS), and murine pulmonary inflammatory responses, were studied after exposures to PM and NaSH, an H_2S donor. Similar to *N*-acetyl cysteine (5 mM), NaSH (10 μM) significantly scavenged PM-induced EC ROS and inhibited the oxidative activation of p38 mitogen-activated protein kinase. Concurrent with these events, NaSH (10 μM) activated Akt, which helps maintain endothelial integrity. Both of these pathways contribute to the protective effect of H_2S against PM-induced endothelial barrier dysfunction. Furthermore, NaSH (20 mg/kg) reduced vascular protein leakage, leukocyte infiltration, and proinflammatory cytokine release in bronchoalveolar lavage fluids in a murine model of PM-induced lung inflammation. These data suggest a potentially protective role for H_2S in PM-induced inflammatory lung injury and vascular hyperpermeability.

Keywords: particulate matter; hydrogen sulfide; endothelial permeability; Akt

Nitric oxide (NO), formerly referred to as endothelial cell–derived relaxing factor (EDRF), is an established physiologic messenger molecule with an important role in endothelial-dependent vasorelaxation and endothelial protection (1). Recent indirect evidence implies that another endogenous, gaseous, diffusible bioactive agent, hydrogen sulfide (H_2S), also acts as an EDRF, and plays an important role in the functional regulation and protection of the endothelium (2). H_2S is ubiquitously generated *in vivo* at active concentrations (3), and has been studied in models of ischemia/reperfusion (4), endotoxemia (5), bacterial sepsis (6), and nonmicrobial inflammation (7). Studies using inhibitors of its endogenous production or compounds that donate H_2S have yielded conflicting results regarding the inherent proinflammatory or anti-inflammatory properties of H_2S, possibly because of various dose-dependent effects. However, a number of reports have also indicated that at its physiological plasma concentrations (10–50 μM range), H_2S exhibits free radical scavenging properties, and serves as a leukocyte adherence/infiltration suppressor (8), an endothelium-dependent vasodilator (9), and an endothelial cell migration activator (10). Each of these functions suggests that H_2S potentially protects the endothelium from inflammatory injury, although the mechanisms associated with these protective effects remain unclear.

Particulate air pollution is known to be associated with cardiopulmonary morbidity and mortality (11, 12). Exposure to particulate matter (PM) triggers dramatic oxidative stress in endothelial cells, which in turn induces endothelial integrity disruption and lung vascular hyperpermeability (13). The persistent pulmonary inflammation induced by polluted air particulates is considered one of the main factors exacerbating preexisting cardiopulmonary problems, including asthma, chronic obstructive pulmonary disease (COPD), myocardial infarction (MI), and congestive heart failure (CHF) (14).

In this study, we examined whether the H_2S donor, NaSH, protects against PM-induced endothelial barrier disruption and vascular hyperpermeability, both *in vitro* and *in vivo*. NaSH prevented the generation of PM-mediated reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (MAPK) activation, thereby inhibiting PM-induced endothelial cytoskeletal rearrangement and vascular hyperpermeability. In addition, we identified that H_2S strongly mediated the activation of Akt, which was essential to the resulting endothelial integrity protection against PM challenge. These observations provide novel information in regard to how H_2S regulates endothelial cytoskeletal rearrangement after PM challenge, to ensure the protection of pulmonary vasculature from pathological hyperpermeability. Because pulmonary endothelial barrier disruption is central to triggering multiple acute and chronic pulmonary inflammatory diseases, these novel observations may have broad therapeutic applicability in pulmonary inflammatory diseases.

MATERIALS AND METHODS

Reagents and Chemicals

The urban PM sample (SRM1648a) was obtained from National Institute of Standards and Technology (Gaithersburg, MD). Protein molecular mass standards, polyacrylamide gels, and cell lysis buffer were purchased from Bio-Rad (Hercules, CA). Antibodies and the p38 MAPK activity kit were purchased from Cell Signaling (Danvers, MA). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

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Cell Culture

Human lung microvascular endothelial cells (HLMVECs) were obtained from Lonza (Basel, Switzerland) and were cultured in EGM-2MV complete medium (Lonza) with 10% FBS at 37°C in a humidified atmosphere with 5% CO_2, as previously described (13). The culture medium was changed to EGM-2MV with 2% FBS (Lonza), 24 hours before experiments.

Transendothelial Electrical Resistance

HLMVECs were grown to 95% confluence on gold microelectrodes in eight-well polycarbonate wells (Applied Biophysics, Troy, NY). Data regarding transendothelial electrical resistance (TER) were continuously recorded, using an electrical cell–substrate impedance sensing system (Applied Biophysics), as previously described in detail (13).

Detection of Intracellular Reactive Oxygen Species

Reactive oxygen species (ROS) in HLMVECs were quantified by fluorescence microscopy, as described previously (13). HLMVECs grown on 35-mm dishes (~95% confluent) were loaded with 2′,7′–dichlorofluorescein diacetate (DCFDA; 10 μM) in EBM-2 basal medium for 30 minutes. The medium containing DCFDA was aspirated and the cells were rinsed, and then incubated with the designated reagents for the indicated time periods. At the end of incubation, cells were washed and examined.

F-Actin Staining

HLMVECs were grown on glass coverslips to 95% confluence. After the indicated treatment, HLMVECs were washed, fixed in 3.7% formaldehyde, and permeabilized with 0.25% Triton X-100 for 5 minutes. HLMVECs were washed, blocked with 5% BSA for 30 minutes, and then incubated with Texas Red–conjugated phalloidin for 60 minutes. Coverslips were washed, mounted using Slow Fade (Molecular Probes, Inc., Eugene, OR), and analyzed.

Animals

Male A/J mice (10–12 wk of age) from Jackson Laboratories (Bar Harbor, ME) were housed in an environmentally controlled animal facility at the University of Chicago for the duration of the experiments. All animal procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Illinois at Chicago. Fifteen minutes after a bolus intraperitoneal injection of 100 μl NaSH solution (20 mg/kg), PM (10 mg/kg, in 50 μl of saline) was delivered via intratracheal instillation, as previously described (15). Animals were killed 24 hours after PM treatment, and bronchoalveolar lavage fluid (BAL) and lung tissue were harvested for further analysis.

Protein and Cytokine Measurement

Protein concentrations in BAL fluid were measured using the RC DC Protein Assay (Bio-Rad), according to the manufacturer's recommendations. BAL IL-6 and TNF-α concentrations were measured using murine Elisa kits (BD Biosciences, Franklin Lakes, NJ), according to the user's manual.

Statistical Analysis

Data are presented as group means ± SE. We performed statistical comparisons among treatment groups by randomized-design, two-way ANOVA, followed by the Newman-Keuls *post hoc* test for more than two groups, or by an unpaired Student *t* test for the two groups. In all cases, we defined statistical significance as $P < 0.05$.

RESULTS

NaSH Prevents PM-Induced Disruption of Endothelial Cell Integrity

PM triggers pulmonary inflammation with characteristic increases in endothelial cell barrier disruption and vascular hyperpermeability. PM induces a time-dependent disruption of the endothelial barrier (Figure 1A), as indicated by reduced TER measurements. NaSH (10 μM, 15-min pretreatment) significantly attenuated PM-induced endothelial barrier disruption (> 50% inhibition; Figure 1A). The effective dose range is 10–50 μM, and higher concentrations (100–200 μM) of NaSH mediated cellular toxicity and endothelial hyperpermeability (*see* Figure E1 in the online supplement). Alterations in endothelial cell barrier function are intimately linked to alterations in the actin cytoskeleton (16). In endothelial cells, PM induces the phosphorylation of heat-shock protein–27 (HSP27) via activated p38 MAPK, leading to stress fiber and paracellular gap formation (17). Actin organization in control and PM-challenged HLMVEC monolayers was examined. Under basal conditions, actin was arranged in a fine reticular pattern, partly localized at the cell periphery (Figure 1B), whereas PM challenge (100 μg/ml, 1 h) increased F-actin fluorescence, with actin rearranged into axially oriented stress fibers, resulting in the formation of paracellular gaps. NaSH pretreatment (10 μM) prevented EC PM–induced stress fiber and gap formation (Figure 1B), thereby protecting against PM-mediated barrier disruption in HLMVECs.

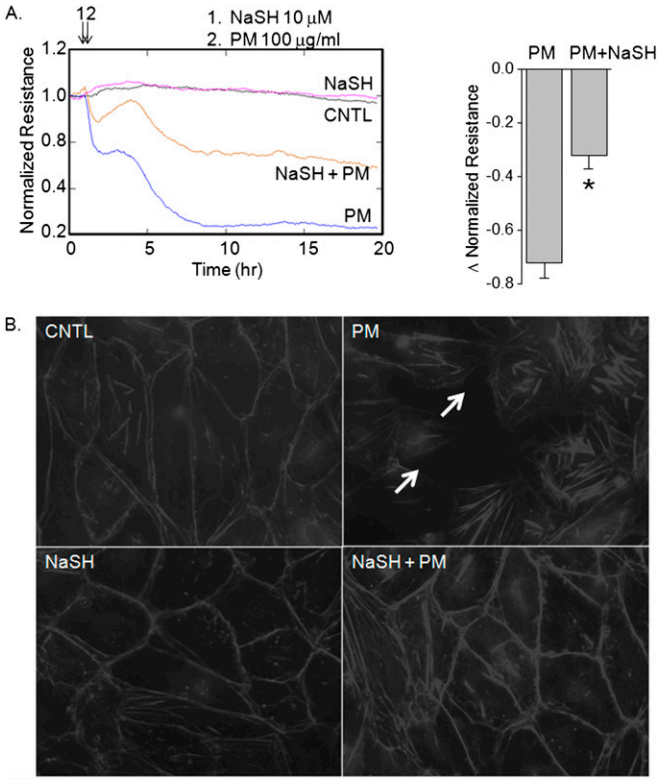

Figure 1. Effects of NaSH on particulate matter (PM)–induced endothelial cell (EC) barrier disruption and cytoskeletal rearrangement. (*A*) Human lung microvascular endothelial cells (HLMVECs; 95% confluence on gold electrodes) were treated with or without NaSH (10 μM) for 15 minutes, and then challenged with or without PM (100 ng/ml). Changes in transendothelial electrical resistance (TER) were measured, and representative TER traces are shown for three independent experiments. Values represent the mean ± SE of three independent experiments. $^{*}P < 0.05$, compared with control samples. $^{**}P < 0.05$, compared with PM challenge. (*B*) HLMVECs (95% confluence on coverslips) were treated with or without NaSH (10 μM) for 15 minutes, and then challenged with or without PM (100 ng/ml). Actin cytoskeletal remodeling was assessed by immunofluorescence staining with Texas Red phalloidin. The formation of paracellular gaps is indicated by *arrows*. Panels are representative of the entire cell monolayer. Results of three independent experiments are shown.

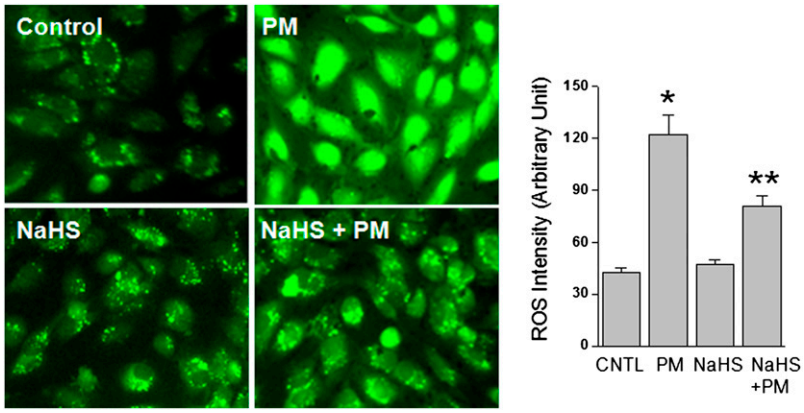

Figure 2. Effects of NaSH on PM-induced generation of reactive oxygen species (ROS). (*A*) HLMVECs (95% confluence on 35-mm dishes) were pretreated with NaSH (10 μM) for 15 minutes before loading with 10 μM 2′,7′–dichlorofluorescein diacetate (DCFDA) for 30 minutes. ECs were rinsed and challenged with vehicle or vehicle plus PM (100 μg/ml) for 1 hour. Formation of ROS was quantified by immunofluorescence microscopy. Values represent the mean ± SE of three independent experiments. *$P < 0.5$, compared with control samples. **$P < 0.05$, compared with PM challenge. CNTL, control.

NaSH Inhibits PM-Stimulated ROS Generation in HLMVECs

We previously reported that massive increases in intracellular oxidative stress via mitochondria dysregulation occur after PM exposure (18). In the present study, NaSH (10 μM, 15-min pretreatment) abolished the PM (100 μg/ml, 1 h)–induced, substantial production of HLMVEC ROS, as measured by DCFDA oxidation (Figure 2). Like the ROS scavenger *N*-acetylcysteine (NAC, 5 mM) (13), NaSH inhibits most of the ROS generated by PM challenge. However, no significant additive effects of ROS scavenging were evident between NAC and NaSH (Figure E2). Thus, NaSH acts as a ROS scavenger in endothelial cells, preventing PM-induced ROS accumulation and significantly inhibiting PM-mediated EC barrier disruption.

NaSH Blocks ROS-Dependent p38 MAPK Activation

We next investigated cell signaling pathways potentially activated by the PM-stimulated generation of ROS. ROS-activated p38 MAPK mediated PM-induced EC hyperpermeability in HLMVECs (Figure E3), similar to human pulmonary artery endothelial cells (13). PM challenge (100 μg/ml, 1 h) increased p38 MAPK phosphorylation, which was inhibited by NaSH (10 μM, 15-min pretreatment) and NAC (5 mM, 1-h pretreatment; Figure 3A). We also confirmed the regulation of p38 MAPK enzymatic activity by the quantification of its ability to phosphorylate recombinant activating transcription factor 2 or ATF-2 (Cell Signaling Technology, Inc.; Figure 3B). PM-activated p38 MAPK activation was inhibited by the pretreatment of NAC or NaSH, indicating that NaSH inhibits the ROS-dependent p38 MAPK activation known to mediate PM-induced EC barrier disruption (13). Interestingly, at higher concentrations (⩾ 50 μM), NaSH inhibited p38 MAPK activity, independent of ROS (Figure E4).

NaSH-Activated Akt Contributes to the Attenuation of PM-Induced Barrier Disruption

H_2S increases cell migration via the activation of Akt (10). In HLMVECs, NaSH (10 μM) significantly induced Akt phosphorylation, which was attenuated by the PI3 kinase inhibitor LY294002 (10 μM; Figure 4A). This Akt phosphorylation persisted despite PM treatment (100 μg/ml, 1 h). The addition of LY294002 (10 μM) to the HLMVECs significantly reduced the protective ability of NaSH against PM-induced EC barrier disruption (Figures 4B and 4C). To define the specific role of NaSH-induced Akt activation, we next knocked down Akt1, the major Akt isotype in endothelium (19). Akt1 small interfering RNA (siRNA) reduced Akt protein expression and significantly prevented the NaSH-mediated protective effects again PM challenge (Figure 4D), independent of ROS-dependent pathways (Figure E5). Taken together, these results indicate that NaSH induces Akt phosphorylation, which contributes to the attenuation of PM-induced HLMVEC barrier disruption.

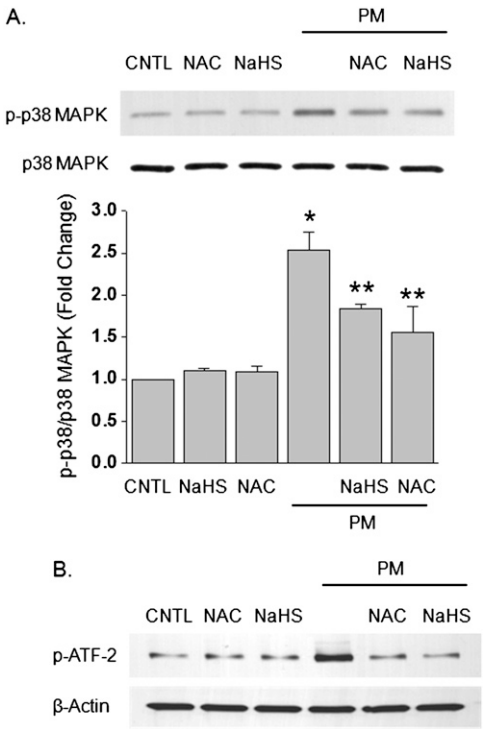

Figure 3. Attenuation of PM-induced p38 mitogen-activated protein kinase (MAPK) activation by NaSH and *N*-acetyl cysteine (NAC). (*A*) HLMVECs (95% confluence on 35-mm dishes) were pretreated with NaSH (10 μM, 15 min) or NAC (5 mM, 30 min), and challenged with PM (100 ng/ml) for 1 hour. Cell lysates were analyzed by Western blotting with phospho-p38 MAPK antibody and total p38 MAPK antibody. Shown are representative blots and quantitative analyses (mean ± SE) from three independent experiments. *$P < 0.05$, compared with control samples. **$P < 0.05$, compared with PM challenge. (*B*) HLMVECs (95% confluence on 35-mm dishes) were pretreated with NaSH (10 μM, 15 min) or NAC (5 mM, 30 min), and challenged with PM (100 ng/ml) for 1 hour. Cell lysates were analyzed with a nonradioactive p38 MAP Kinase Assay Kit (Cell Signaling Technology). Phospho-p38 MAPK was immunoprecipitated with phospho-p38 MAPK antibody, washed, and incubated with recombinant activating transcription factor 2 or ATF-2 protein. The mixture was analyzed by Western blotting with phosphor–ATF-2 (p-ATF-2) antibody to represent the activity of p38 MAPK. Shown are representative blots and quantitative analyses (mean ± SE) from three independent experiments. *$P < 0.05$, compared with control samples. **$P < 0.05$, compared with PM challenge.

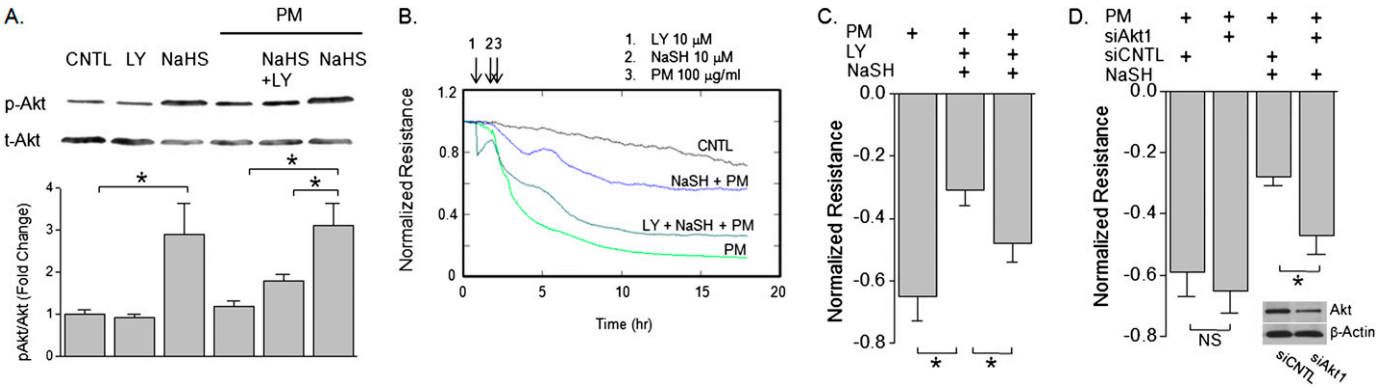

Figure 4. Effects of NaSH on Akt phosphorylation and EC barrier function. (*A*) HLMVECs (95% confluence, 35-mm dishes) were pretreated with NaSH (10 μM, 15 min) or NAC (5 mM, 30 min), and challenged with PM (100 ng/ml) for 1 hour. Cell lysates were analyzed by Western blotting with phospho-Akt MAPK antibody and total Akt MAPK antibody. Shown are representative blots and quantitative analyses (mean ± SE) from three independent experiments. $*P < 0.05$. (*B* and *C*) HLMVECs (95% confluence on gold electrodes) were pretreated with LY294002 (LY; 10 μM) for 45 minutes, followed by treatment with NaSH (10 μM, 15 min). ECs were then challenged with PM (100 ng/ml), and changes in TER were measured. Shown are representative TER traces of three independent experiments. Values represent the mean ± SE of three independent experiments. $*P < 0.05$, between the two groups. (*D*) HLMVECs (60% confluence on gold electrodes) were treated with Akt1 siRNA (small interfering RNA) or control siRNA (100 μM) for 48 hours, followed by TER measurement with treatment with NaSH (10 μM, 15-min pretreatment) and PM (100 ng/ml). Shown are representative TER traces of three independent experiments. Values represent the mean ± SE of three independent experiments. $*P < 0.05$, between the two groups. NS, no significance.

NaSH Attenuates PM-Induced Pulmonary Inflammation *In Vivo*

We next examined *in vivo* vascular permeability in a murine model of PM-induced pulmonary inflammatory injury. PM exposure (10 mg/kg, 24 h, intratracheal instillation) induced significant protein leakage into the bronchoalveolar space (3.5-fold increase; Figure 5A), consistent with our previous report (15). NaSH (20 mg/kg, 30-min intraperitoneal pretreatment) significantly attenuated PM-induced protein leakage (> 50%), thereby reducing endothelial barrier disruption and vascular hyperpermeability. PM mediated the increased infiltration of inflammatory leukocytes and significant leukocyte elevation in BAL, which was partly prevented by NaSH (Figure 5B). Furthermore, NaSH attenuated the PM-stimulated release of inflammatory cytokines IL-6 and TNF-α into BAL fluid (> 50% inhibition; Figures 5C and 5D). Histological studies confirmed that NaSH (20 mg/kg, 30-min pretreatment)–mediated reductions in PM (10 mg/kg, 24 h)–induced dramatic increases in leukocyte infiltration in murine lung tissue (Figure 5E). Higher doses of NaSH (> 100 mg/kg) exhibited deleterious effects, suggesting a small therapeutic window for this molecule (Figure E6). These data indicate that NaSH attenuated PM-induced pulmonary vascular hyperpermeability, inflammatory leukocyte infiltration, and proinflammatory cytokine release, thereby protecting against PM-mediated pulmonary inflammatory injury.

DISCUSSION

Endothelial integrity disruption and vascular hyperpermeability are prominent features of inflammatory syndromes, tumor angiogenesis, and atherosclerosis. The prevention of such endothelial barrier disruption has obvious therapeutic applications. Airborne particulates containing high concentrations of transition metals and polycyclic aromatic hydrocarbons stimulate the excessive generation of ROS in multiple lung tissue types, particularly in the vascular endothelium, which is readily susceptible to PM-induced oxidative stress, and which plays a critical role in the pathophysiology of several vascular disorders (20, 21). The present study demonstrates that NaSH, a donor of active free H_2S, inhibits PM-induced endothelial monolayer disruption and attenuates PM-induced lung vascular leakage via ROS scavenging and Akt activation.

Growing epidemiological evidence supports the deleterious cardiopulmonary health effects and increased cardiopulmonary mortality and morbidity after exposure to ambient pollutant particles (11, 12). Various mechanisms have been proposed to explain the cardiopulmonary health effects of PM, including pulmonary and systemic oxidative stress and inflammation (22), enhanced coagulation pathways (23), and altered cardiac autonomic function (24). Our recent *in vivo* and *in vitro* studies revealed that PM triggered very significant lung inflammation and vascular hyperpermeability, including endothelial barrier disruption, vascular protein leakage, neutrophil infiltration, and proinflammatory cytokine release (13, 15, 18). These pathophysiological alterations in pulmonary cells and tissues lead to systemic inflammation, which is known as one of the primary aggravators of existing cardiopulmonary conditions such as asthma, COPD, cardiac arrhythmias, and CHF.

Inhaled PM, especially the fine and ultrafine portion with diameters of less than 2.5 μm, was reported to travel from the alveolar space into the pulmonary circulation and to trigger amplified endothelial dysfunction (25–27). In addition, PM challenge causes activated neutrophils to migrate into the lung interstitium from the blood circulation and produce significant amounts of ROS, leading to further escalations of inflammation (28). We reported that PM induced significant ROS production in pulmonary ECs, thereby activating p38 MAPK. Activated p38 MAPK may be involved in actin filament reorganization, in response to oxidative stress via the MAPK-dependent phosphorylation of the actin-binding effector HSP27, to facilitate stress actin fiber synthesis and paracellular gap formation, essential markers of EC integrity disruption (13). The deactivation of the activated p38–HSP27 pathway via the inhibition of oxidative stress by the ROS scavenger NAC protects endothelial integrity against PM challenge. Like NAC, H_2S is considered an endogenous ROS scavenger, with the potential function of guarding endothelial integrity against oxidative stress.

Similar to NO and CO, H_2S is a small, gaseous molecule generated in mammalian cells by enzymatic catalysis, and it diffuses freely across the lipid bilayer (2). In mammalian cells, H_2S is produced from L-cysteine, and is catalyzed by one of two pyridoxal 5′-phosphate–dependent enzymes, cystathionine β-synthase or

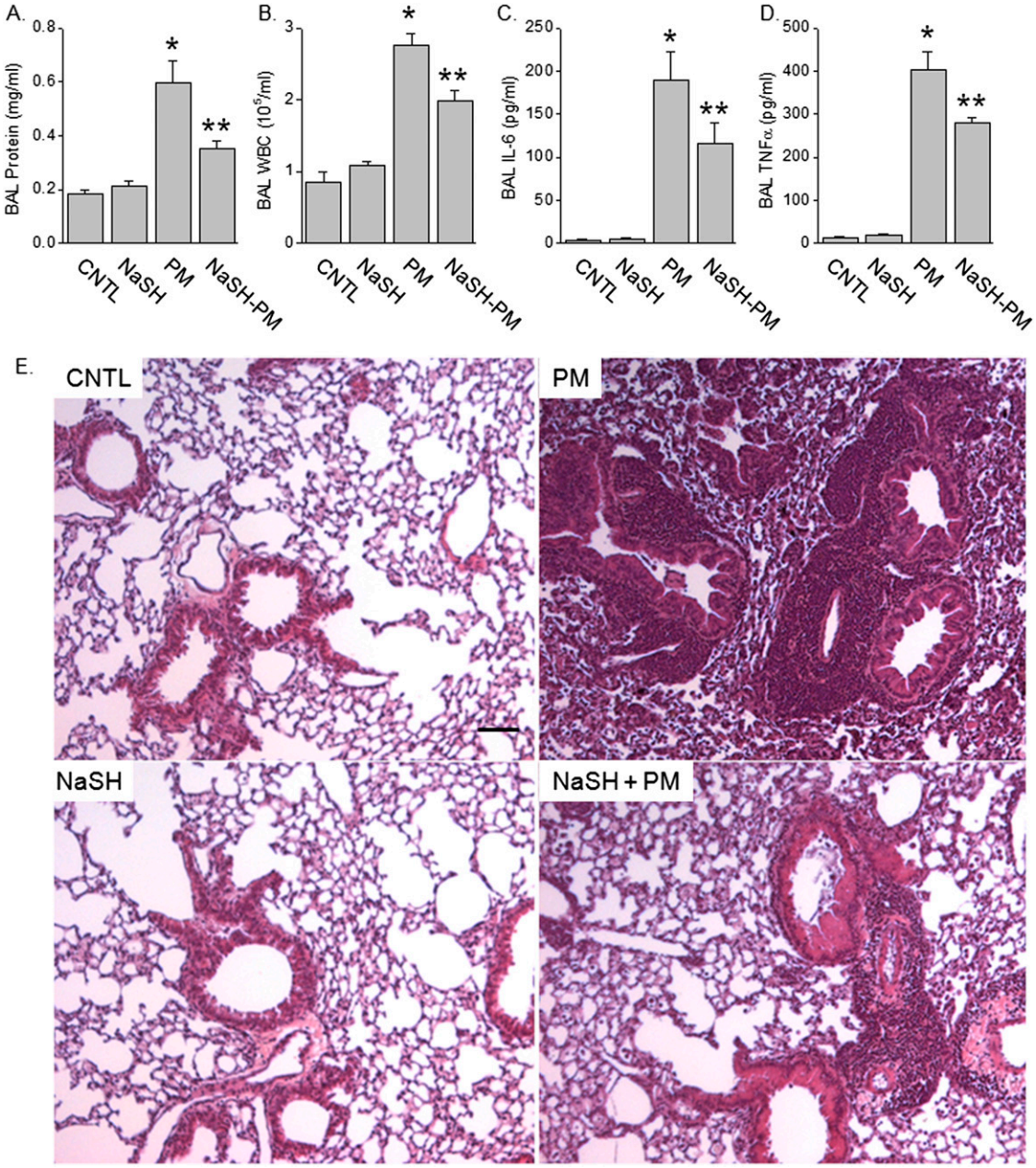

Figure 5. NaSH reduces PM-induced pulmonary inflammation. After intraperitoneal injection of NaSH (20 mg/kg, 30 min), mice ($n = 4$) were intratracheally challenged with PM (10 mg/kg in 50 μl saline). (*A*) After 24 hours, total protein concentration in bronchoalveolar lavage (BAL) fluids was detected. *$P < 0.05$, compared with control samples. **$P < 0.05$, compared with PM challenge. (*B*) Total number of white blood cells in BAL fluids was counted. *$P < 0.05$, compared with control samples. **$P < 0.05$, compared with PM challenge. (*C* and *D*) IL-6 and TNF-α concentrations in BAL fluids were measured. *$P < 0.05$, compared with control samples. **$P < 0.05$, compared with PM challenge. (*E*) Lung tissues were examined by hematoxylin and eosin staining. Shown are representative images from each group of mice ($n = 4$).

cystathionine γ-lyase (CSE) (2). H_2S has been traditionally viewed as a toxic gas, and is infamous for its "rotten egg" smell. Its toxicological effect is mainly manifested as acute intoxication and loss of central respiratory drive (29). Only in the past decade has the biological and physiological importance of this gas molecule been exposed to the limelight. H_2S acts as an endogenous scavenger for ROS and reactive nitrogen species (30). H_2S has been demonstrated to protect cells and proteins from oxidative modifications by peroxynitrite and HOCl (31). H_2O_2 and organic hydroperoxides such as lipid hydroperoxides (LOOHs) are also known to be destroyed by H_2S (32). In endothelial cells, LOOHs are responsible for the activation of heme oxygenase–1, one of the ROS responders that trigger extensive oxidative damage in endothelial cells. Although H_2S is an endogenous metabolic product of methionine-transformed homocysteine (33), it protects against homocysteine-induced endothelial dysfunction and impaired endothelial-dependent vasodilation. NaSH treatment also induces a dose-dependent and time-dependent increase in Akt phosphorylation in endothelial cells (10), which can be inhibited by the PI3K inhibitors LY 294002 and wortmannin, strongly suggesting that H_2S stimulates the activation of Akt to trigger intracellular events. The activation of Akt by various extracellular signals increases endothelial cell proliferation, migration, and tube formation *in vitro* (34), and mediates protective cytoskeletal rearrangement (35). However, the mechanism by which H_2S activates Akt is poorly understood and remains to be investigated.

H_2S exists in the circulation at active concentrations (10–50 μM). However, higher concentrations of H_2S (> 100 μM) are deleterious (Figure E1). The addition of PM slightly reduced H_2S concentrations in the cell culture media (Figure E7), which is presumably attributable to the reduction of cellular s-adenysyl methionine (our unpublished observations), the stimulator of CSE activity. A more important topic for further study is the internal regulation of H_2S concentrations *in vivo*. For instance, a lack of CSE leads to less circulating H_2S, and makes vasodilation less responsive to acetylcholine, leading to vasotone dysregulation and hypertension (9). Thus, the careful analysis of tissue H_2S concentrations, using more elaborate techniques such as mass spectrometry, will be essential for the precise characterization of the composition and amounts of endogenous H_2S generated under different pathological conditions, including endothelial injury–related conditions. These studies will allow for a better understanding of the role of H_2S in cardiopulmonary pathogenesis.

These data demonstrate that NaSH prevents the PM-induced disruption of endothelial integrity and vascular hyperpermeability via the dual actions of ROS scavenging and Akt pathway activation (Figure E8). Our study suggests that the role of H_2S may involve the attenuation of ROS-mediated pulmonary inflammation by PM air pollution.

Author disclosures are available with the text of this article at www.atsjournals.org.

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