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RESEARCH ARTICLE | *Cardiovascular Actions of Hydrogen Sulfide and Other Gasotransmitters*

Role of thiosulfate in hydrogen sulfide-dependent redox signaling in endothelial cells

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Leskova A, Pardue S, Glawe JD, Kevil CG, Shen X. Role of thiosulfate in hydrogen sulfide-dependent redox signaling in endothelial cells. *Am J Physiol Heart Circ Physiol* 313: H256–H264, 2017. First published May 26, 2017; doi:10.1152/ajpheart.00723.2016.—Recent reports have revealed that hydrogen sulfide (H_2S) exerts critical actions to promote cardiovascular homeostasis and health. Thiosulfate is one of the products formed during oxidative H_2S metabolism, and thiosulfate has been used extensively and safely to treat calcific uremic arteriopathy in dialysis patients. Yet despite its significance, fundamental questions regarding how thiosulfate and H_2S interact during redox signaling remain unanswered. In the present study, we examined the effect of exogenous thiosulfate on hypoxia-induced H_2S metabolite bioavailability in human umbilical vein endothelial cells (HUVECs). Under hypoxic conditions, we observed a decrease of GSH and GSSG levels in HUVECs at 0.5 and 4 h as well as decreased free H_2S and acid-labile sulfide and increased bound sulfide at all time points. Treatment with exogenous thiosulfate significantly decreased the ratio of GSH/GSSG to total sulfide of HUVECs under 0.5 h of hypoxia but significantly increased this ratio in HUVECs under 4 h of hypoxia. These responses reveal that thiosulfate has different effects at low and high doses and under different O_2 tensions. In addition, treatment with thiosulfate also diminished VEGF-induced cystathionine-γ-lyase expression and reduced VEGF-induced HUVEC proliferation under both normoxic and hypoxic conditions. These results indicate that thiosulfate can modulate H_2S metabolites and signaling under various culture conditions that impact angiogenic activity. Thus, thiosulfate may serve as a unique sulfide donor to modulate endothelial responses under pathophysiological conditions involving angiogenesis.

NEW & NOTEWORTHY This report provides new evidence that different levels of exogenous thiosulfate dynamically change discrete sulfide biochemical metabolite bioavailability in endothelial cells under normoxia or hypoxia, acting in a slow manner to modulate sulfide metabolites. Moreover, our findings also reveal that thiosulfate surprisingly inhibits VEGF-dependent endothelial cell proliferation associated with a reduction in cystathionine-γ-lyase protein levels.

thiosulfate; sulfide; antiangiogenesis; glutathione; donor; endothelial cell; vascular endothelial growth factor

IN MAMMALS, hydrogen sulfide (H_2S), like nitric oxide (NO) and carbon monoxide (CO), is a physiological gaseous signal transmitter (7, 9, 12, 25, 28). Endogenous H_2S is predominantly produced by tissue-specific enzymes, including cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptosulfurtransferase (3-MST). Accumulating evidence strongly shows that H_2S has an important role in the vascular system including vasorelaxation (4, 14, 20, 33), neurotransmission (1, 2, 16, 31, 32), and inflammation (9, 11, 15).

In the biological research of H_2S, inorganic sulfide salts (Na_2S and NaHS), garlic extracts [diallyl sulfide, diallyl disulfide, and diallyl trisulfide (DATS)], Lawesson's reagents, and analogs (GYY4137) have been widely used as H_2S sources. However, these sources have certain disadvantages for studying the physiological function of H_2S. NaHS has been implemented at a concentration of 640 μM as a neuromodulator in brain lysate, and DATS has been used at a concentration of 60 μM for antiangiogenesis in breast cancer cells (24). GYY4137 is a slow-releasing sulfide donor that has been used at a 400 μM concentration in the leukemia cell line HL-60 (21). This concentration is still too high for therapeutic purposes, because H_2S concentrations in tissues range from high nanomolar to low micromolar concentrations (19). In addition, these sulfide donors have variable stability and need to be administered in high doses to achieve various effects (21). Therefore, to benefit from the vasodilatory ability of H_2S in human or animal studies, there is a need for a low-dose, slow-releasing sulfide donor.

Thiosulfate ($S_2O_3^{2-}$) has been used extensively and safely in human clinical trials for vascular calcification studies. It increases the solubility of calcium by up to 100,000-fold and can be used to treat calcific uremic arteriopathy in dialysis patients. In addition, sodium thiosulfate is an Food and Drug Administration-approved medication for the treatment of cyanide poisoning.

Thiosulfate can produce H_2S through a nonenzymatic pathway or by an enzymatic pathway via a glutathione-dependent reduction (shown below in *Eq. 1*). Koj et al. (18) reported that glutathione disulfide (GSSG), H_2S, and labeled sulfite were produced when rat mitochondria were incubated with oxygen, glutathione (GSH), and [S^{35}]thiosulfate. Production of H_2S can be directly compared with the glutathione pathway because high concentrations of H_2S reflect a high rate of consumption (30). Interestingly, Curtis and colleagues (3, 10, 18) reported that when S^{35} was perfused through isolated rat tissue, it was oxidized to thiosulfate. These findings suggest that thiosulfate can involve H_2S metabolism through the glutathione pathway, as follows:

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$$2GSH + S_2O_3^{2-} \rightarrow GSSG + SO_3^{2-} + H_2S \quad (1)$$

H_2S can exist in various forms, including free sulfide, acid-labile sulfide, and bound sulfane sulfur. Free sulfides include S^{2-}, HS^-, and H_2S. Acid-labile sulfides are usually bound to iron in the form of iron-sulfur (Fe-S) clusters but may also include persulfides. Bound sulfides include bound sulfane sulfur, polysulfides, thiosulfate, polythionates, thiosulfonates, bisorganyl-polysulfanes, and elemental sulfur. We have previously developed precise analytical methods to measure sulfide bioavailability in cells and tissues using monobromobimane (MBB) (6, 25). This MBB method for measuring sulfide by reverse-phase (RP)-HPLC with fluorescence detection is a useful and sensitive quantitative method to measure sulfide metabolism in biological samples.

Hypoxia is an effective stimulus of human umbilical vein endothelial cell (HUVEC) activation responses (22). Importantly, these responses to hypoxia can be acute or chronic. Hypoxia activates hypoxia-inducible transcription factors (HIFs) that induce the expression of VEGF (6). Thus, hypoxia can regulate the proliferation and remodeling of endothelial cells. However, little is known about how varying O_2 tensions modulate thiosulfate bioavailability and actions in endothelial cells. Here, we address this topic by the study of thiosulfate on sulfide bioavailability and redox balance in HUVECs under normoxia and hypoxia. Finally, we also investigated the effects of thiosulfate on VEGF-induced proliferation of HUVECs to ascertain its possible role in angiogenic responses.

MATERIALS AND METHODS

Materials. MBB, Tris (2-carboxyethyl)phosphine hydrochloride (TCEP), sulfosalicylic acid (SSA), 1-fluoro-2,4-dinitrobenzene (DNFB), and *N*-ethylmaleimide (NEM) were purchased from Sigma (St. Louis, MO). $VEGF_{164}$ was purchased from Lifeline Cell Technology. Rabbit β-actin polyclonal antibody and rabbit CSE polyclonal antibodies were obtained from Proteintech. The ECL Western Blotting system was acquired from Millipore (Billerica, MA). Vacutainer tubes, 3.5-in. 25-gauge spinal needles, and 0.5-in. 30-gauge needles were purchased from BD Medical Technology. All other reagents were purchased at the analytical grade.

Cell culture and treatment. HUVECs were purchased from Lifeline Cell Technology (catalog no. FC-0044) and cultured in VascuLife Basal Medium (catalog no. LM-0002) supplemented with the appropriate LifeFactors Kit (catalog no. LL-0003). All cells were grown in tissue culture flasks under normoxic conditions at 5% CO_2-21% O_2 at 37°C. Media were changed every 2–3 days. HUVECs were starved 16 h postconfluence in endothelial based media (EBM) supplemented with 0.5% FBS, nonessential amino acids, penicillin-streptomycin, and L-glutamine. HUVECs were the incubated in the hypoxic chamber (5% CO_2-1% O_2 at 37°C) for 0.5 or 4 h.

Sulfide and thiosulfate measurements. Bioavailable sulfide and thiosulfate levels were measured as we have previously reported (6, 25, 29). Levels of free sulfide and thiosulfate in HUVECs were measured by HPLC after derivatization with excess MBB as stable products sulfide-dibimane (SDB) and thiosulfate bimane (TSB), respectively. Briefly, HUVECs were homogenized in Tris·HCl buffer [100 mM Tris·HCl (pH 9.5) and 0.1 mM diethylenetriaminepenta-acetic acid (DTPA)]. Cell lysates were derivatized with MBB and then measured by Shimadzu Prominence 20A equipment with RF-10AXL (excitation wavelength: 390 mm and emission wavelength: 475 mm) and an Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm). Typical retention times of SDB and TSB were around 16.5 and 9.5 min, respectively. Free H_2S levels were calculated according to standard SDB (25). Thiosulfate stock solution was diluted to the desired concentrations in Tris·HCl buffer and derivatized with MBB to make TSB standard solutions. Acid-labile sulfide was released by an acidic solution [100 mM phosphate buffer (pH 2.6) and 0.1 mM DTPA] and then trapped in 100 mM Tris·HCl buffer (pH 9.5, 0.1 mM DTPA). The bound sulfane sulfur was measured by incubating the samples with 1 mM TCEP in 10 mM phosphate buffer (pH 2.6, 0.1 mM DTPA), and sulfide measurements were performed in a manner analogous to that described above. Acid-labile sulfide was determined by subtracting the free H_2S value from the value obtained by the acid-liberation protocol. The bound sulfane sulfur measurement was determined by subtracting the H_2S measurement from the acid-liberation protocol alone from that of the TCEP plus acidic conditions.

Determination of reduced and oxidized glutathione levels by RP-HPLC. Concentrations of GSH and GSSG in HUVECs were measured by HPLC equipped with a ultraviolet detector as previ-

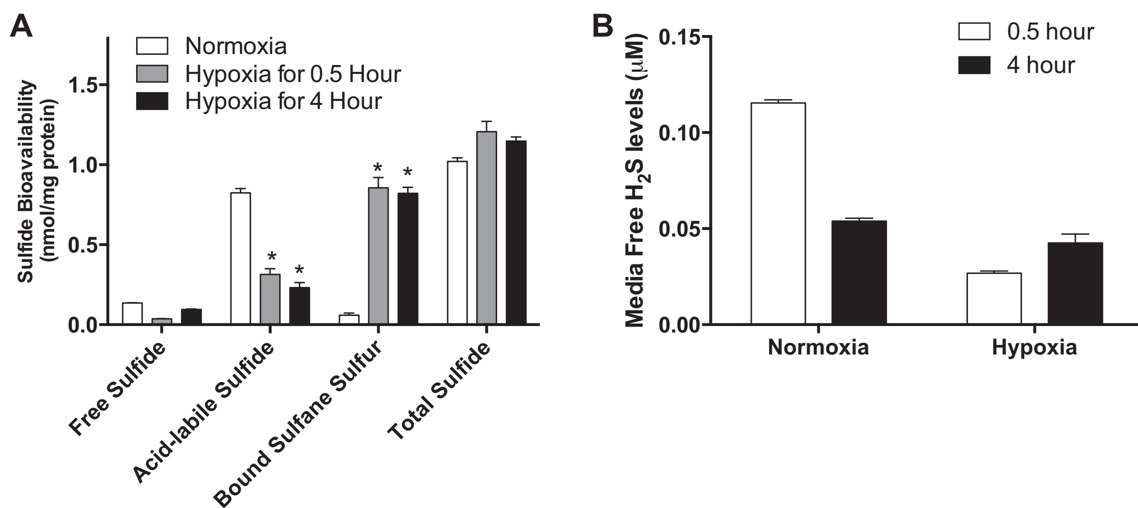

Fig. 1. Sulfide bioavailability of human umbilical vein endothelial cells (HUVECs) under normoxia and hypoxia. HUVECs were incubated under normoxia (21% O_2-5% CO_2 at 37°C) or hypoxia (1.0% O_2-5% CO_2 at 37°C) conditions for 0.5 or 4 h. *A*: cell lysates were collected under hypoxic conditions and used to measure free, acid-labile sulfide and bound sulfane sulfur by HPLC. *$P < 0.05$ vs. normoxia; $n = 8$. *B*: free sulfide levels were measured in endothelial cell conditioned tissue culture media at different time points under normoxia or hypoxia conditions.

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ously described (13). In brief, after homogenates of HUVECs were treated with 100 mM NEM, the mixtures were diluted with an equal volume of trichloroacetic acid (10%) and then centrifuged at 12,000 rpm for 5 min. The excess of NEM in the supernatant was extracted by 5 volumes of dichloromethane. After centrifugation at 12,000 rpm for 5 min, the supernatant was alkalinized by 1 M Tris·HCl (pH 10) and then reacted with an equal volume of DNFB solution (1.5% in ethanol) for 3 h at room temperature in the dark. After acidification with 37% HCl, the samples were loaded onto RP-HPLC with a NH_2 column and an ultraviolet detector at 355 nm. Elution solvents were 80% methanol (*solvent A*) and 0.5 M acetate buffer (pH 4.6) (*solvent B*). Samples were eluted for 0–10 min with an acetate gradient, 70% *solvent A* and 30% *solvent B*, followed by a linear gradient, 40−95% *solvent B*, for 10–35 min. The flow rate was 1 ml/min. The retention times of GSH and GSSG were 7.1 and 23.8 min, respectively.

Cell proliferation measurement and Western blot analysis. HUVECs were seeded in EBM supplemented with 5% FBS, nonessential amino acids, 1× penicillin-streptomycin, and L-glutamine. After cells had settled and attached, the media were replaced with EBM starvation media as described above. After starvation, the bromodeoxyuridine label (Calbiochem) was added, and the assay was completed according to the manufacturer's protocol in the presence or absence 50 ng/ml VEGF. Each treatment was incubated for 4 h in either normoxia or

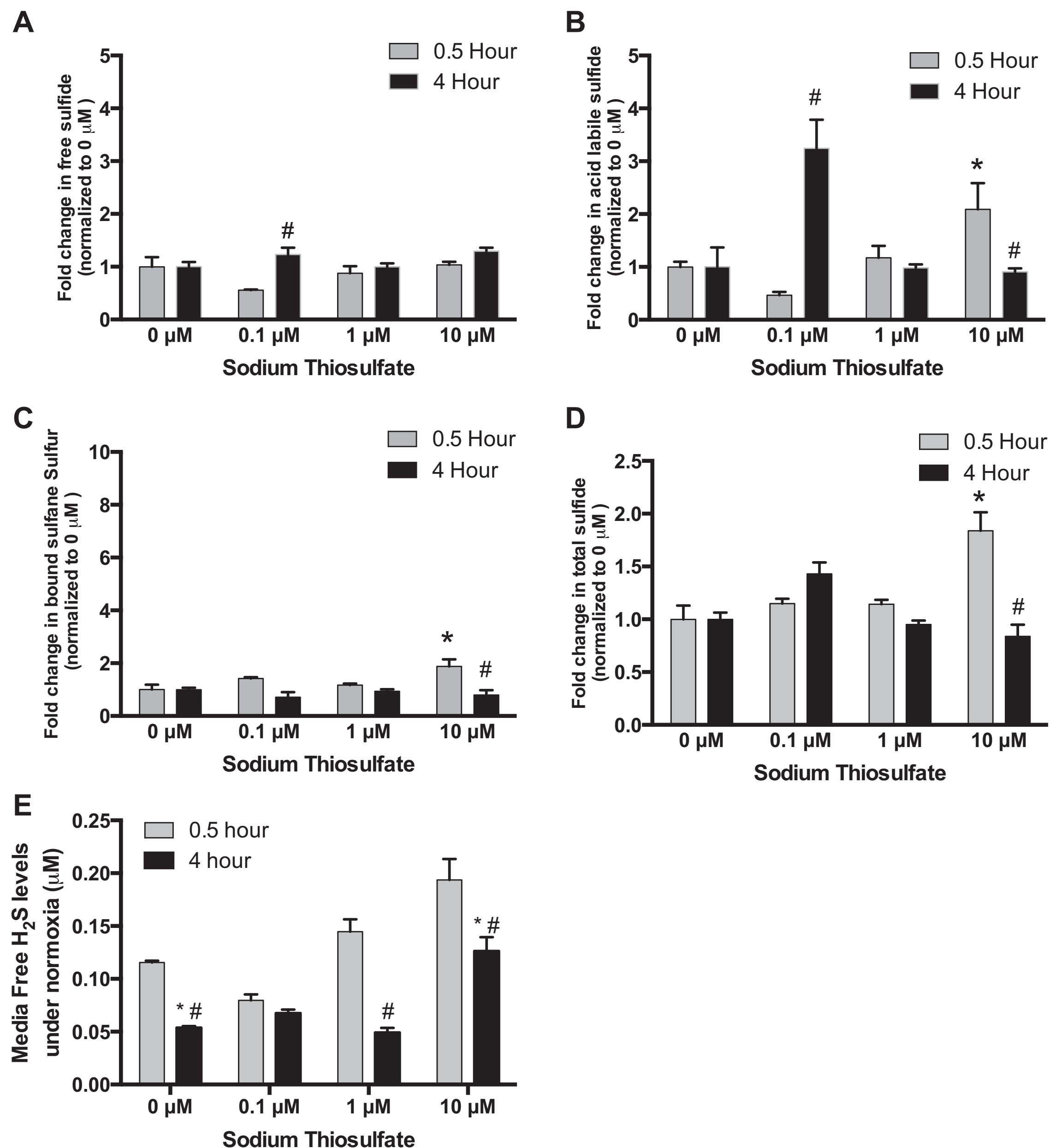

Fig. 2. Effects of thiosulfate on sulfide bioavailability of HUVECs under normoxia. Under normoxia (21% O_2-5% CO_2 at 37°C), HUVECs were treated with 0, 0.1, 1.0, or 10 μM thiosulfate for 0.5 or 4 h, respectively. All data are normalized to respective metabolite levels without thiosulfate treatment (0 μM). *A*: change in free sulfide levels with varying thiosulfate levels at different time points. *B*: changes in acid-labile sulfide levels with varying thiosulfate levels at different time points. *C*: changes in bound sulfane sulfur levels with different thiosulfate doses at different time points. *D*: changes in total sulfide levels in response to different thiosulfate treatments at different time points. *E*: endothelial cell conditioned media free H_2S levels with various thiosulfate treatments over time. *$P < 0.05$ vs. treatment with 0 μM of thiosulfate; #$P < 0.05$ vs. treatment with the same dose of thiosulfate; $n = 8$.

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hypoxia. For Western blot analysis, cells were lysed in 4 × SDS sample buffer and boiled for 5 min. Protein samples were then loaded on a 10% SDS gel and transferred to a polyvinylidene difluoride membrane. Nonspecific proteins were blocked with 5% nonfat milk. Anti-CSE antibody and anti-β-actin antibody were used to detect the CSE expression changes in these HUVECs.

Statistical analysis. All data are presented as means ± SE. Statistical analysis was performed using two-way ANOVA for independent samples with Prism 5.0 (GraphPad Software, La Jolla, CA). The Dunnett posttest method was used to determine statistical significance from the control. A 95% confidence interval ($P < 0.05$) was considered significant.

RESULTS

Sulfide bioavailability of HUVECs under hypoxia. We have previously reported that exogenous H_2S donor treatment augments endothelial cell ischemic proliferation and survival under hypoxia involving complex interactions with other gasotransmitters (6). However, little to no information exists regarding the effect of O_2 tension on endothelial sulfide metabolite levels. Thus, we first examined whether hypoxia influenced sulfide bioavailability of endothelial cells by evaluating the free sulfide, acid-labile sulfide, and bound sulfane

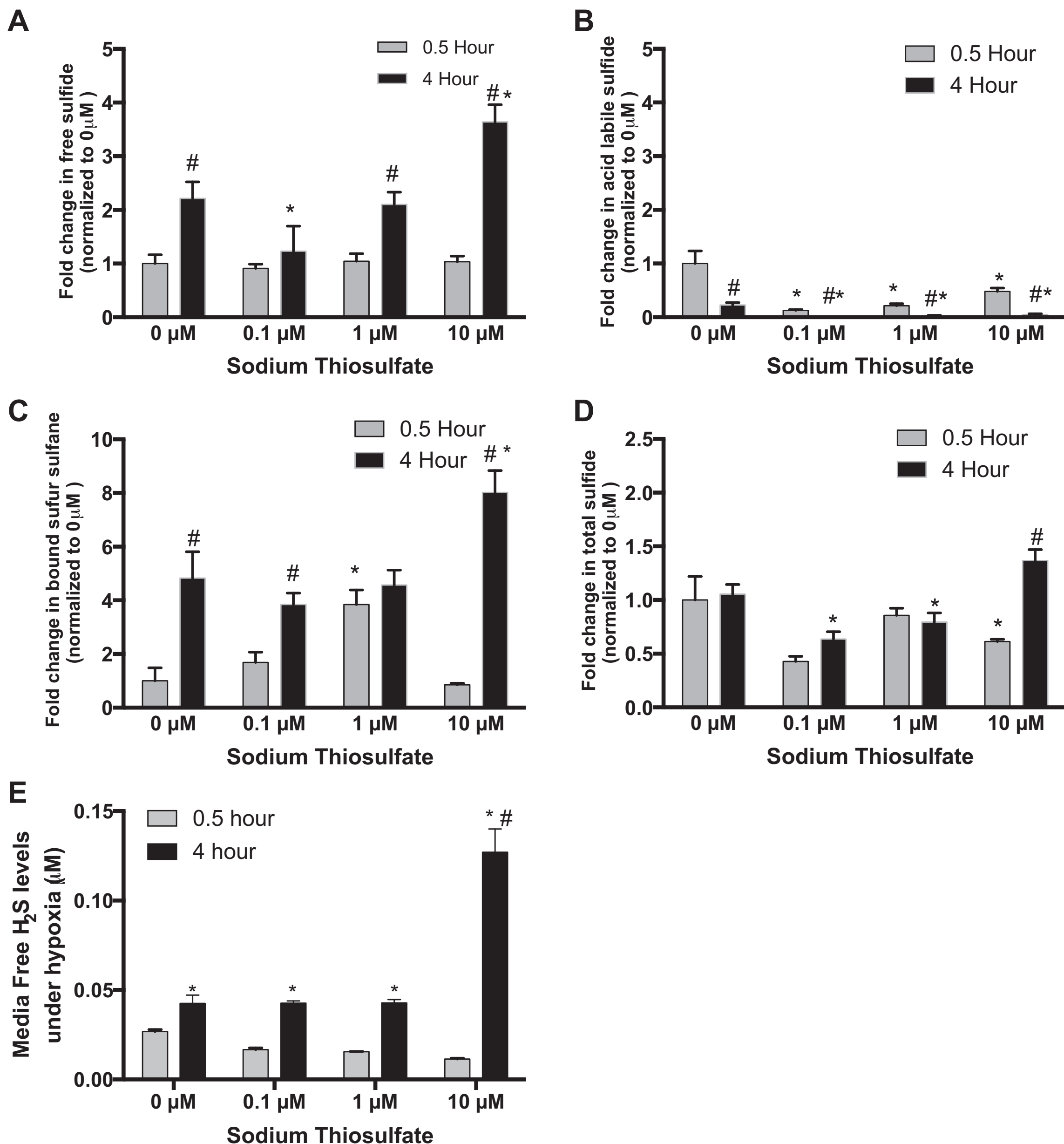

Fig. 3. Effects of thiosulfate on sulfide bioavailability of HUVECs under hypoxia. Under hypoxia (1.0% O_2-5% CO_2 at 37°C), HUVECs were treated with 0, 0.1, 1.0, or 10 μM thiosulfate for 0.5 or 4 h, respectively. All data are normalized to respective metabolite levels without thiosulfate treatment (0 μM). *A*: change in free sulfide levels with varying thiosulfate levels at different time points. *B*: changes in acid-labile sulfide levels with varying thiosulfate levels at different time points *C*: changes in bound sulfane sulfur levels with different thiosulfate doses at different time points. *D*: changes in total sulfide levels in response to different thiosulfate treatments at different time points. *E*: endothelial cell conditioned media free H_2S levels with various thiosulfate treatments over time. *$P < 0.05$ vs. treatment with 0 μM of thiosulfate; #$P < 0.05$ vs. treatment with the same dose of thiosulfate; $n = 8$.

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sulfur levels of HUVECs under normoxia or hypoxia at different time points. As shown as Fig. 1*A*, sulfide bioavailability of HUVECs was significantly altered between normoxia and hypoxia (1% O_2) at different time points. Compared with normoxia, acid-labile sulfide levels of HUVECs under hypoxia were markedly decreased. Conversely, bound sulfane sulfur levels of HUVECs under hypoxia were significantly increased compared with normoxic cells. Finally, no significant changes in total sulfide were observed between the various conditions. Figure 1*B* shows tissue culture media-free H_2S levels under normoxia or hypoxia at different time points, revealing that 4 h of hypoxia significantly increased media-free sulfide levels.

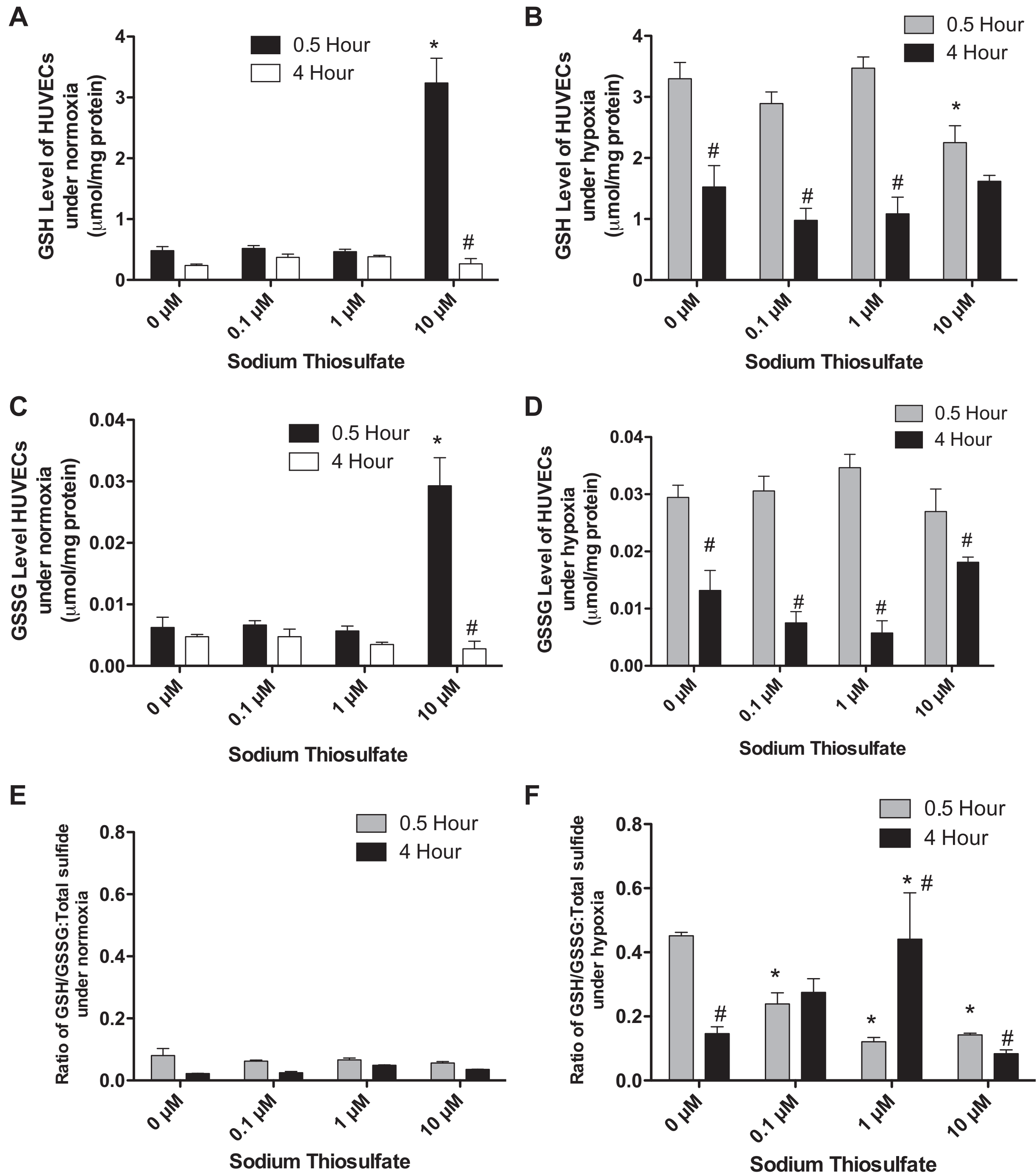

Fig. 4. Effects of thiosulfate on reduced and oxidized glutathione levels of HUVEC cells under normoxia and hypoxia. HUVECs were incubated under normoxia (21% O_2-5% CO_2 at 37°C) or hypoxia (1.0% O_2-5% CO_2 at 37°C) for 0.5 or 4 h along with treatment of 0, 0.1, 1.0, or 10 μM thiosulfate. *A*: reduced GSH levels of HUVECs treated with sodium thiosulfate under normoxia. *B*: HUVECs reduced GSH levels with sodium thiosulfate treatment under hypoxia. *C* and *D*: oxidized GSSG levels in HUVECs with various amounts of sodium thiosulfate under normoxia and hypoxia, respectively. *E* and *F*: ratio of GSH/GSSG to total sulfide levels under normoxia and hypoxia, respectively. $*P < 0.05$ vs. treatment with 0 μM thiosulfate; $\#P < 0.05$ vs. treatment with the same dose of thiosulfate; $n = 8$.

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Together, the effect of hypoxia on sulfide bioavailability is clearly influenced by O_2 tension, as it has been noted that O_2 can be considered a sulfide antagonist (8). However, the significant changes in acid-labile and bound sulfane sulfur forms of sulfide metabolites highlight the dynamic nature of sulfide species bioavailability.

Effects of thiosulfate on sulfide bioavailability. We next studied the effects of thiosulfate on sulfide bioavailability of endothelial cells under normoxia or hypoxia. HUVECs were treated for 0.5 or 4 h with 0, 0.1, 1, or 10 μM thiosulfate. Changes in sulfide metabolite forms were normalized to basal levels of each respective metabolite at 0 μM. Under normoxia, treatment with 0.1 μM thiosulfate significantly increased free and acid-labile sulfide levels of HUVECs at 4 h (Fig. 2, *A* and *B*), and treatment with 10 μM thiosulfate significantly increased acid-labile sulfide and bound sulfane sulfur levels of HUVECs at 0.5 h (Fig. 2, *B* and *D*). However, tissue culture media-free sulfide levels were significantly reduced by thiosulfate treatment at 4 h under normoxia (Fig. 2*E*).

Under hypoxia, free sulfide, acid-labile sulfide, and bound sulfane sulfur levels of HUVECs were significantly changed by treatment with thiosulfate at 4 h (Fig. 3, *A*–*C*). Interestingly, acid-labile sulfide and bound sulfane sulfur levels of HUVECs were significantly reduced and elevated respectively by thiosulfate treatment at both time points (Fig. 3, *B* and *C*). While total intracellular sulfide levels did significantly fluctuate, the overall changes were less dramatic compared with changes in biochemical forms (Fig. 3*D*). Finally, thiosulfate treatment at various doses increased free sulfide levels in the media of hypoxic HUVEC at only the 4-h time point (Fig. 3*E*).

Effects of thiosulfate on redox balance. Due to the complexity of time-dependent redox imbalance effects, we only investigated the changes of GSH and GSSG in HUVECs treated with 0.1, 1, and 10 μM thiosulfate for 0.5 or 4 h. Under normoxia, GSH and GSSG levels were only significantly increased in HUVECs treated with 10 μM thiosulfate for 0.5 h (Fig. 4, *A* and *C*, respectively). There was no difference in the ratio of GSH/GSSG to total sulfide of HUVECs treated with 0, 0.1, 1, or 10 μM thiosulfate for 0.5 or 4 h (Fig. 4*E*).

Under hypoxia, GSH and GSSG levels were not significantly changed except with 10 μM thiosulfate treatment at 0.5 and 4 h but were higher than that of HUVECs under normoxia (Fig. 4, *B* and *D*). From the 0.5- to 4-h time point, GSH and GSSG levels were significantly decreased in HUVECs treated with 0, 0.1, 1, or 10 μM thiosulfate. Under normoxia, there were only significant decreases of the ratio of GSH/GSS to total sulfide in HUVECs treated for 4 h with the same dose of thiosulfate. Under hypoxia, thiosulfate treatment resulted in a significant decrease of the ratio of GSH/GSSG to total sulfide in HUVECs treated for 0.5 h. Interestingly, long-time (4 h) thiosulfate treatment resulted in a significantly different change in the ratio of GSH/GSSG to total sulfide in HUVECs (Fig. 4*F*). These data indicate that thiosulfate preferentially decreased the ratio of GSH/GSSG to total sulfide at lower concentrations of thiosulfate but was increased at higher concentrations at 4 h of hypoxic conditions.

Effects of thiosulfate on VEGF-dependent endothelial proliferation. To determine the role of thiosulfate in angiogenic response, we next investigated the effect of thiosulfate on endothelial proliferation by bromodeoxyuridine assay. As shown in Fig. 5*A*, compared with HUVECs treated with 0 μM thiosulfate, HUVEC proliferation was significantly decreased in HUVECs with treatment of 10 μM sodium thiosulfate under normoxia (0.8639 ± 0.0222, $P < 0.001$) and significantly decreased in HUVECs with treatment of 1.0 or 10 μM sodium thiosulfate under hypoxia (0.7255 ± 0.0284, $P < 0.001$, and 0.7255 ± 0.0358, $P < 0.001$, respectively). In the VEGF induced-proliferation assay (Fig. 5*B*), compared with HUVECs treated with 0 μM thiosulfate, HUVEC proliferation was only significantly decreased in HUVECs treated with 10 μM thiosulfate under normoxia (0.7715 ± 0.0357, $P < 0.001$) and significantly decreased in HUVECs with treatment of 0.1, 1.0, or 10 μM sodium thiosulfate under hypoxia (0.7126 ± 0.0276, $P < 0.001$; 0.6765 ± 0.0306, $P < 0.001$; and 0.5994 ± 0.0247, $P < 0.001$, respectively). In addition, we examined the CSE expression in HUVECs treated with VEGF or 10 μM thiosulfate under normoxia and hypoxia. As shown in Fig. 6, thiosulfate treatment significantly decreased VEGF-regulated CSE expression in HUVECs under either hypoxia or normoxia.

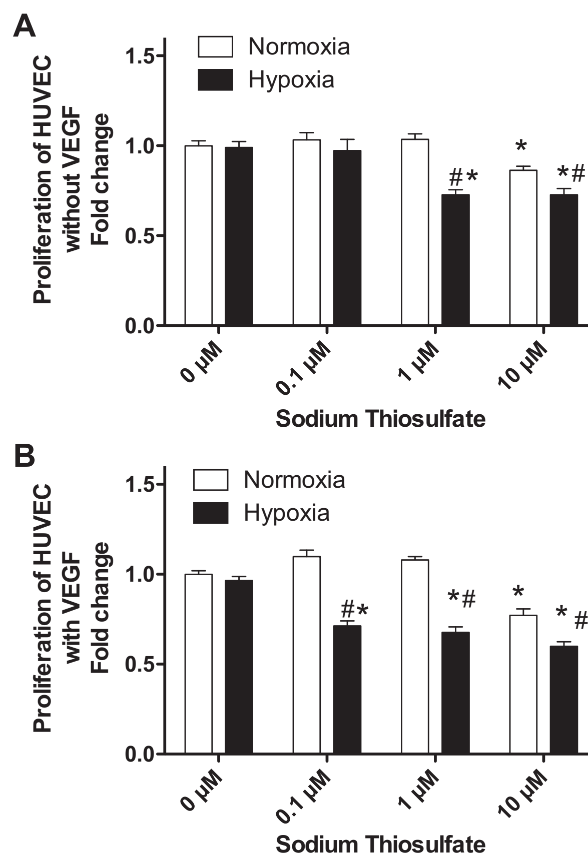

Fig. 5. Effects of thiosulfate on proliferation of endothelial cells. *A*: the effect of thiosulfate treatments on HUVEC proliferation under normoxia or hypoxia without VEGF treatment. *B*: effect of thiosulfate treatment on HUVEC proliferation with VEGF treatment under normoxic and hypoxic conditions. *$P < 0.05$ vs. treatment with 0 μM thiosulfate; #$P < 0.05$ vs. treatment with the same dose of thiosulfate; $n = 8$.

DISCUSSION

Endogenous H_2S is generated in mammalian cells via enzymatic and nonenzymatic pathways. It is well known that CSE, CBS, and 3-MST are key enzymes in the enzymatic pathway of H_2S production. CBS is found predominantly in the brain and

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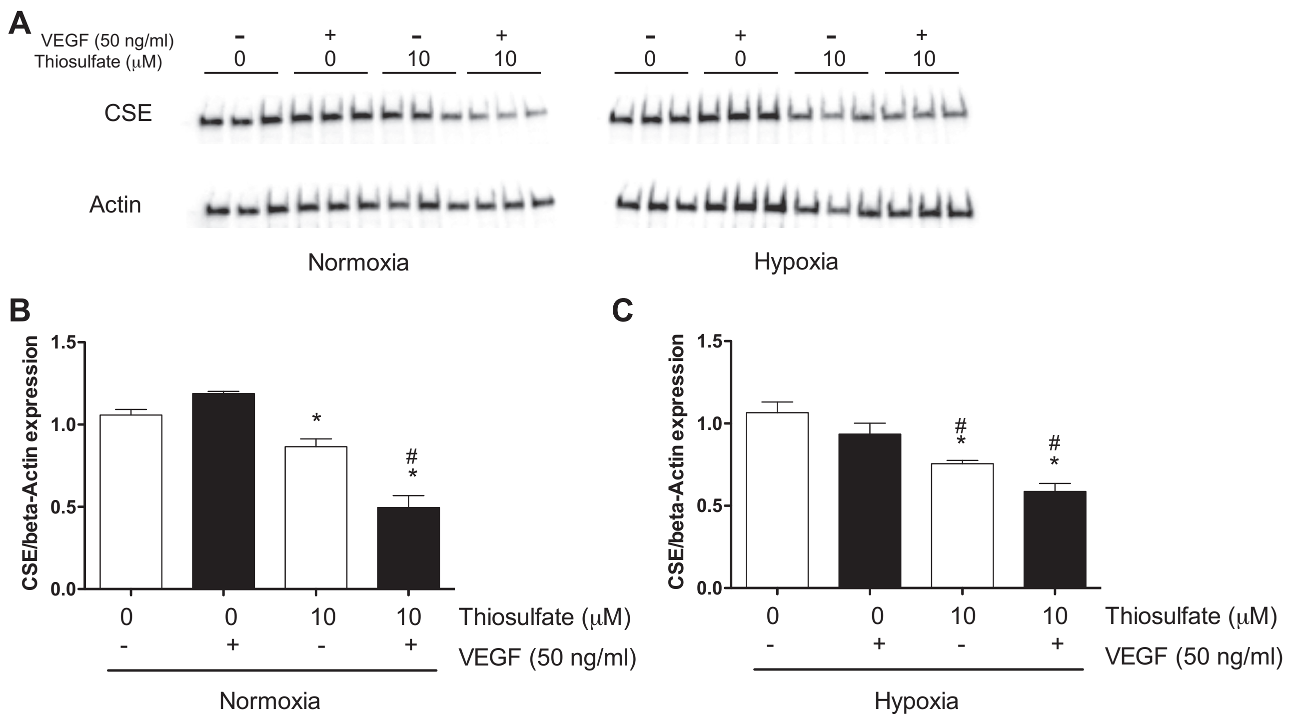

Fig. 6. Effects of hypoxia/thiosulfate treatment on cystathionine-γ-lyase (CSE) expression in HUVECs. *A*: effect of thiosulfate treatments on HUVEC CSE expression under normoxia or hypoxia without VEGF treatment. The expression levels of CSE in HUVECs were examined by Western blot analysis (β-actin was used as an internal control). *B* and *C*: blots were quantified by densitometry and plotted as the ratio of CSE to β-actin. $*P < 0.05$ vs. treatment with 0 μM thiosulfate; $\#P < 0.05$ vs. treatment with the same dose of thiosulfate; $n = 3$.

nervous system, where H_2S is released in the reaction of cysteine and homocysteine to cystathionine. CSE is responsible for H_2S generation in the vascular tissue through a reaction of L-cysteine and cystathionine. 3-MST is mainly localized to the mitochondria. The expression of these enzymes may occur in a preferential tissue manner where they can convert cysteine or cysteine derivatives to H_2S, which contributes to H_2S homeostasis.

However, nonenzymatic processes of H_2S generation are less well understood, including the reaction of glucose and cysteine, direct reduction of glutathione and elemental sulfur, and organic polysulfides (5, 17). Thiosulfate is a metabolite of H_2S and also generates H_2S through a reductive reaction involving pyruvate, which acts as a hydrogen donor (19). In this manner, thiosulfate could serve as a useful recycling pathway to augment or maintain H_2S bioavailability under certain conditions. Use of thiosulfate as a selective H_2S donor for specific disease conditions could be a potent and effective therapeutic modality. Recent work by Ichinose and colleagues (23, 27) has revealed that sodium thiosulfate can indeed confer protection against acute lung injury via LPS or cecal ligation and puncture and against neuronal ischemia. However, it remains unclear how thiosulfate-based therapies alter specific cellular sulfide metabolite levels (e.g., acid-labile and bound sulfane) under hypoxic conditions.

Our goal in the present study was to better understand the impact of exogenous sodium thiosulfate treatment on endothelial sulfide metabolites bioavailability and its subsequent impact on glutathione metabolites in normoxic and hypoxic situations. Importantly, hypoxia alone decreased acid-labile sulfide levels but increased bound sulfane sulfur levels in HUVECs. After treatment with thiosulfate, sulfide bioavailability of HUVECs changed in a temporal manner under both normoxia and hypoxia. Specifically, no significant changes were seen in free, acid-labile, and bound sulfane sulfur levels of HUVECs treated with 0.1 μM sodium thiosulfate for 0.5 h under normoxia; however, all of the levels were increased after 4 h of incubation. This observation may likely involve cellular transportation effects of thiosulfate as it has recently been shown that SCL13A4 serves as a thiosulfate transporter (23). However, the impact of thiosulfate on specific sulfide metabolites was different under hypoxic conditions. A higher amount (10 μM) of thiosulfate was required to significantly increase free sulfide, bound sulfane sulfur, and total sulfide levels. This likely reflects the fact that hypoxia substantially augments sulfide metabolite formation and stability but could also involve changes in thiosulfate transport activity that remain unknown. Together, our findings reveal that thiosulfate is a slow, effective sulfide donor at low concentrations under normoxic conditions, which may provide some insights into its pharmacological behavior for various disease conditions.

In the H_2S field, sulfide donors, including H_2S gas, inorganic sulfide salts (Na_2S and NaHS), garlic and related sulfur compounds (DADS and DATS), Lawesson's reagent, and analogs (GYY4137), have become important (yet sometimes contentious) research tools to study biological functions and mechanisms of H_2S therapy. All of these donors release H_2S through diverse mechanisms and at the same time can form various metabolic byproducts. This likely contributes to the fact that donors often appear to have inconsistent functions potentially due to byproduct interference also showing biological effects. However, the reaction of thiosulfate to produce

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sulfide ($2GSH + S_2O_3^{2-} \rightarrow GSSG + SO_3^- + H_2S$) represents an endogenous metabolic pathway whose end products and their effects are well known. Our results extend this insight revealing the impact of both thiosulfate levels and cellular O_2 tension on endothelial glutathione metabolism, which is useful in understanding how thiosulfate metabolism occurs under relevant cellular pathology conditions.

It is well known that endothelial dysfunction is associated with various cardiovascular diseases, including atherosclerosis, hypertension, and cardiovascular diseases. Numerous studies have shown that H_2S has anti-inflammatory, antiapoptotic, and antioxidant effects (19, 26). In addition, H_2S may prime endothelial cells toward angiogenesis. Hence, H_2S is important in restoring endothelial function. We have previously reported that exogenous sodium sulfide therapy increases VEGF expression in ischemic tissue but not nonischemic tissue (6). Therefore, we examined the effect of thiosulfate on VEGF-induced proliferation of HUVECs under normoxia or hypoxia. To our surprise, it was observed that low concentrations of thiosulfate blunted VEGF-induced proliferation of HUVECs under hypoxia and thiosulfate diminished VEGF-dependent CSE expression. Further investigation is certainly needed to determine how thiosulfate antagonizes VEGF-dependent endothelial proliferation under hypoxia and whether such a response occurs similarly in vivo.

In summary, we report the effects of thiosulfate on sulfide bioavailability in endothelial cells under normoxia and hypoxia. It is clear that exogenous thiosulfate treatment influences sulfide metabolite levels in a dose- and condition-specific manner that impacts endothelial glutathione metabolism. These findings are useful in considering future studies aimed toward the use of thiosulfate as a sulfide donor or investigations targeted toward understanding endogenous recycling of the metabolite.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-113303 (to C. G. Kevil).

DISCLOSURES

C. G. Kevil has intellectual property regarding H_2S measurement and equity in Innolyzer, LLC. X. Shen has intellectual property regarding H_2S measurement and is a consultant for Innolyzer, LLC.

AUTHOR CONTRIBUTIONS

A.L., C.G.K., and X.S. conceived and designed research; A.L., S.P., and X.S. performed experiments; A.L., S.P., J.D.G., C.G.K., and X.S. analyzed data; A.L., C.G.K., and X.S. interpreted results of experiments; A.L., S.P., J.D.G., and C.G.K. prepared figures; A.L., J.D.G., and X.S. drafted manuscript; J.D.G., C.G.K., and X.S. edited and revised manuscript; C.G.K. and X.S. approved final version of manuscript.

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