

RESEARCH PAPER

Hydrogen sulfide inhibits the translational expression of hypoxia-inducible factor-1 α

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BACKGROUND AND PURPOSE

The accumulation of hypoxia-inducible factor-1 α (HIF-1 α) is under the influence of hydrogen sulfide (H₂S), which regulates hypoxia responses. The regulation of HIF-1 α accumulation by H₂S has been shown, but the mechanisms for this effect are largely elusive and controversial. This study aimed at addressing the controversial mechanisms for and the functional importance of the interaction of H₂S and HIF-1 α protein.

EXPERIMENTAL APPROACH

HIF-1 α protein levels and HIF-1 α transcriptional activity were detected by Western blotting and luciferase assay. The mechanisms for H₂S-regulated HIF-1 α protein levels were determined using short interfering RNA transfection, co-immunoprecipitation and 7-methyl-GTP sepharose 4B pull-down assay. Angiogenic activity was evaluated using tube formation assay in EA.hy926 cells.

KEY RESULTS

The accumulation of HIF-1 α protein under hypoxia (1% O₂) or hypoxia-mimetic conditions was reversed by sodium hydrosulfide (NaHS). This effect of NaHS was not altered after blocking the ubiquitin-proteasomal pathway for HIF-1 α degradation; however, blockade of protein translation with cycloheximide abolished the effect of NaHS on the half-life of HIF-1 α protein. Knockdown of eukaryotic translation initiation factor 2 α (eIF2 α) suppressed the effect of NaHS on HIF-1 α protein accumulation under hypoxia. NaHS inhibited the expression of VEGF under hypoxia. It also decreased *in vitro* capillary tube formation and cell proliferation of EA.hy926 cells under hypoxia, but stimulated the tube formation under normoxia.

CONCLUSIONS AND IMPLICATIONS

H₂S suppresses HIF-1 α translation by enhancing eIF2 α phosphorylation under hypoxia. The interaction of H₂S and HIF-1 α inhibits the angiogenic activity of vascular endothelial cells under hypoxia through the down-regulation of VEGF.

Abbreviations

CHX, cycloheximide; CSE, cystathionine γ -lyase; DFX, desferrioxamine; DMOG, dimethylxalyl glycine; eIF2 α , eukaryotic translation initiation factor 2 α ; H₂S, hydrogen sulfide; mTOR, mammalian target of rapamycin; NaHS, sodium hydrosulfide

Introduction

Hydrogen sulfide (H₂S) is a gasotransmitter, endogenously produced by cystathionine γ -lyase (CSE) and cystathionine β -synthase (Wang, 2003; 2010). Endogenous H₂S participates in both physiological regulation and pathophysiological processes of different mammalian systems (Yang *et al.*, 2008; Gil *et al.*, 2011). The therapeutic value of exogenous H₂S has been shown for dealing with different diseases (Esechie *et al.*, 2009; Ekundi-Valentim *et al.*, 2010; Pouokam and Diener, 2011).

A master regulator of cellular responses to changes in oxygen levels is hypoxia-inducible factor-1 (HIF-1), a transcription factor which activates more than 100 genes involved in angiogenesis, glucose metabolism, cell survival and metastasis (Semenza, 2006; Spagnuolo *et al.*, 2011). The dominant form of HIF-1 is a heterodimer consisting of the inducibly regulated HIF-1 α subunit and the constitutively expressed HIF-1 β subunit (Wang *et al.*, 1995; Yang *et al.*, 2004). Under normoxia, the HIF-1 α gene is continuously transcribed and translated, but the level of this protein is very low due to its rapid degradation via the ubiquitin-proteasomal pathway mediated by prolyl hydroxylase (Semenza, 2004). In contrast, hypoxia inhibits prolyl hydroxylase activity and consequently results in the accumulation of HIF-1 α protein (Yee Koh *et al.*, 2008). A wide range of stimuli and cytokines have been shown to affect HIF-1 α protein stability, including two gasotransmitters, NO (Hagen *et al.*, 2003; Metzen *et al.*, 2003) and carbon monoxide (CO) (Choi *et al.*, 2010). One of the two key control elements of HIF-1 α gene translation is the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway responsible for the initiation of cap-dependent HIF-1 α gene translation (Holcik and Sonenberg, 2005; Spriggs *et al.*, 2010). The other element is eukaryotic translation initiation factor 2 α (eIF2 α), a component of the eIF-2 ternary complex. eIF2 α is responsible for the transformation of GDP to GTP, an essential step for translation commencement. Phosphorylation of eIF2 α at Ser⁵¹ prevents the re-formation of the eIF-2 ternary complex and thus suppresses HIF-1 α translation (Rocha, 2007; Yee Koh *et al.*, 2008). Certain anti-tumour compounds decrease HIF-1 α translation due to eIF2 α phosphorylation (Jung *et al.*, 2009; Zhang *et al.*, 2010).

H₂S has been shown to up-regulate HIF-1 protein levels and activity under normoxia in *Caenorhabditis elegans* or down-regulate HIF-1 α protein levels and activity under hypoxia in cultured cells (Budde and Roth, 2009; Kai *et al.*, 2012). The molecular mechanisms through which H₂S mediates HIF-1 α protein level are largely elusive and controversial. Elucidation of the mechanisms for H₂S-induced alteration in HIF-1 α protein level is critical for our understanding of the role and mode of action of H₂S in hypoxia. The present study was undertaken to determine whether and how H₂S regulates HIF-1 α protein level under hypoxia and hypoxia-mimetic conditions in different cell lines. We also investigated the effect of H₂S on HIF-1 α translation and degradation and the relative importance of these two processes. HIF-1 α transcriptional activity and the effect of H₂S on angiogenic activity of endothelial cells were further tested.

Methods

Cell culture and reagents

HEK293T, Hep3B and EA.hy926 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U·mL⁻¹ penicillin and 100 μ g·mL⁻¹ streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 21% O₂. For hypoxic treatment, the cells were incubated in a chamber (STEMCELL Technologies, Vancouver, BC, Canada) at 37°C with a gas mixture (1% O₂, 5% CO₂ and 94% N₂). Sodium hydrosulfide (NaHS), cobalt chloride (CoCl₂), desferrioxamine (DFX) and cycloheximide (CHX) were obtained from Sigma (Oakville, ON, Canada). Dimethylloxalyl glycine (DMOG) was purchased from Cayman (Burlington, ON, Canada). Rapamycin and MG132 were obtained from Cell Signaling (Beverly, MA, USA) and EMD (Philadelphia, PA, USA) respectively.

RNA extraction and real-time quantitative PCR

Total RNA was isolated from HEK293T cells using Trizol reagent (Sigma). First-strand cDNA was synthesized with SuperScript III First-Strand Synthesis System according to the protocol of the manufacturer (Invitrogen, Burlington, ON, Canada). Real-time quantitative PCR was performed as reported previously (Yang *et al.*, 2007). Primers were designed for HIF-1 α (forward) 5'-CTCAAAGTCGGACAGCCT-CA-3', (reverse) 5'-CCCTGCAGTAGGTTCTGCT-3' (Metzen *et al.*, 2003); actin (forward) 5'-GCACAGAGCCTCGCCTT-3', (reverse) 5'-GTTGTCGACGACGAGCG-3'; and VEGF (forward) 5'-CCTTGCTGCTCTACCTCCAC-3', (reverse) 5'-GCAGTAGCTGCGCTGATAGA-3'.

Western blotting

The cultured cells were harvested and solubilized at 4°C with a lysis buffer (40 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.5 mM Na₃VO₄, 20 mM NaF, pH 7.5) containing protease inhibitors (2 μ g·mL⁻¹ aprotinin, 2 μ g·mL⁻¹ leupeptin, 2 μ g·mL⁻¹ pepstatin A and 0.5 mM PMSF), incubated on ice for 20 min and centrifuged at 20817 g for 15 min at 4°C. Equal amounts of protein extracts were resolved on 10–15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). The membranes were probed with appropriate primary antibodies and detected using peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:5000) and visualized by ECL (GE Healthcare, Amersham, UK). Immunoblottings were performed with the following antibodies: mouse monoclonal anti-HIF-1 α (BD Biosciences, Mississauga, ON, Canada); mouse monoclonal anti-ubiquitin (Invitrogen); rabbit polyclonal anti-eIF2 α or anti-phospho-eIF2 α , rabbit monoclonal anti-eIF4G, rabbit monoclonal anti-4E-BP1, mouse monoclonal anti-p53, mouse monoclonal anti-Cyclin D1 (Cell Signaling); rabbit monoclonal anti-CSE (Abnova, Taipei, Taiwan); mouse monoclonal anti-eIF4E, mouse monoclonal anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti- β -actin (Sigma).

Co-immunoprecipitation assay

The cultured cells were harvested and lysed in a lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, pH 7.6) containing protease inhibitors. Protein extracts of 1–2 mg were incubated with 2–3 µg anti-ubiquitin antibody for 4 h at 4°C followed by incubation with protein G (GE Healthcare) for 1 h at 4°C. The beads were washed three times with the lysis buffer, and the bound proteins were eluted by boiling for 5 min with 2 × SDS loading buffer and analysed by Western blotting.

Plasmids and DNA transfection

Plasmid haemagglutinin (HA)-HIF1α-pcDNA3 (#18949) (Kondo *et al.*, 2002) and plasmid HA-Ubiquitin (#18712) (Kamitani *et al.*, 1997) were purchased from Addgene (Cambridge, MA, USA). In brief, HEK293T cells were seeded onto 6-well plates (3×10^5 cells per well) and transfected with 1.6 µg plasmid using Lipofectamine™ 2000 Transfection Reagent (Invitrogen), according to manufacturer's specification.

Luciferase assay

HEK293T cells seeded onto 6-well plates were co-transfected with 600 ng plasmid HRE (hypoxia response element)-luciferase (#26731, Addgene) containing three hypoxia response elements from the *Pgk-1* gene upstream of firefly luciferase (Emerling *et al.*, 2008) and HA-HIF1α-pcDNA3 expressing vector (350 ng) or empty vector (EV) (350 ng) respectively. Fifty nanograms of pRL-TK vector (Promega, Madison, WI, USA) were co-transfected and served as an internal control. After 24 h of transfection, the cells were incubated with NaHS for 4 h under normoxic conditions and then analysed for luciferase activity using Dual-Luciferase® Reporter Assay System (Promega), followed by quantification with a Fluostar Luminometer (BMG Labtech, Offenburg, Germany).

Short interfering RNA (siRNA) transfection

Pre-designed eIF2α-targeted siRNA (eIF2α-siRNA) and control siRNA were purchased from Santa Cruz. Transfections were performed as previously reported (Teng *et al.*, 2009). Briefly, HEK293T cells were seeded onto 6-well plates at a density of 1×10^5 cells per well and transfected using Lipofectamine™ 2000 Transfection Reagent. All transfections were performed according to manufacturer's instructions. The medium was replaced, 48 h after transfection, with normal medium containing NaHS under hypoxic condition for an additional 4 h.

7-Methyl-GTP Sepharose 4B pull-down assay

The cultured cells were lysed in the lysis buffer and centrifuged at 14000 rpm for 15 min at 4°C. The supernatants (500 µg total protein) were incubated with 25 µL of 7-methyl GTP Sepharose (GE Healthcare) on a rotator for 2 h at 4°C. Pelleted beads were washed three times with the lysis buffer and eluted by boiling for 5 min with 2 × SDS loading buffer and analysed by Western blotting.

Endothelial cell tube formation assay

EA.hy926 cells (2×10^4 cells per well) in DMEM supplemented with 0.1% heat-inactivated FBS were seeded in triplicates

onto a 96-well plate coated with Matrigel Basement Membrane Matrix (BD Bioscience) and incubated for 8 h under normoxic or hypoxic conditions. Images of tube formation were taken under an IX71 inverted microscope (Olympus, Center Valley, PA, USA).

Cell viability assay

HEK293T and EA.hy926 cells were seeded at a density of 8×10^3 cells per well in a 96-well plate (Pei *et al.*, 2011). After 24 h of incubation with NaHS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and incubated further for 4 h. The MTT formazan was dissolved in 100 µL of DMSO. The plates were mixed for 30 min on a gyratory shaker, and absorbance was measured at 595 nm using a Fluostar Luminometer.

5-Bromo-2'-deoxyuridine (BrdU) cell proliferation assay

EA.hy926 cells were seeded at a density of 1.5×10^4 cells per well in a 96-well plate. After 24 h of incubation with NaHS, cell proliferation was assessed by measuring the incorporation of BrdU into DNA using BrdU Cell Proliferation Assay Kit (EMD, Philadelphia, PA, USA) according to the manufacturer's instructions. Quantification was performed using a Fluostar Luminometer at a wavelength of 450 nm with reference readings at a wavelength of 570 nm.

Oxygen partial pressure measurement

The cell culture medium was analysed for the partial pressures of O₂ using an electronic blood-gas analyser (GEM Premier 3000, Instrumentation Laboratory, Bedford, MA, USA).

Statistical analysis

All data are expressed as means ± SEM of at least three independent experiments. Statistical analyses were made using Student's *t*-test, and *P* < 0.05 was considered statistically significant.

Results

H₂S lowered HIF-1α protein levels in HEK293T cells challenged by hypoxia or hypoxia-mimetic agents

NaHS (an H₂S donor) lowered hypoxia-elevated protein levels in HEK-293T cells in a dose-dependent manner and nearly eliminated the increase in HIF-1α at concentrations higher than 10 µM (Figure 1A). Overexpression of CSE in HEK293T cells reduced HIF-1α accumulation compared with vector transfected cells under hypoxia (Supporting Information Fig. S1).

To assess whether the suppressive effect of H₂S on HIF-1α protein levels was associated with a decreased activity of HIF-1α, we transfected HEK293T cells with HRE-luciferase expression vector containing three hypoxia response elements from the *Pgk-1* gene upstream of firefly luciferase (Emerling *et al.*, 2008). As shown in Figure 1B, luciferase activity was significantly increased in response to hypoxia. The addition of NaHS substantially attenuated luciferase

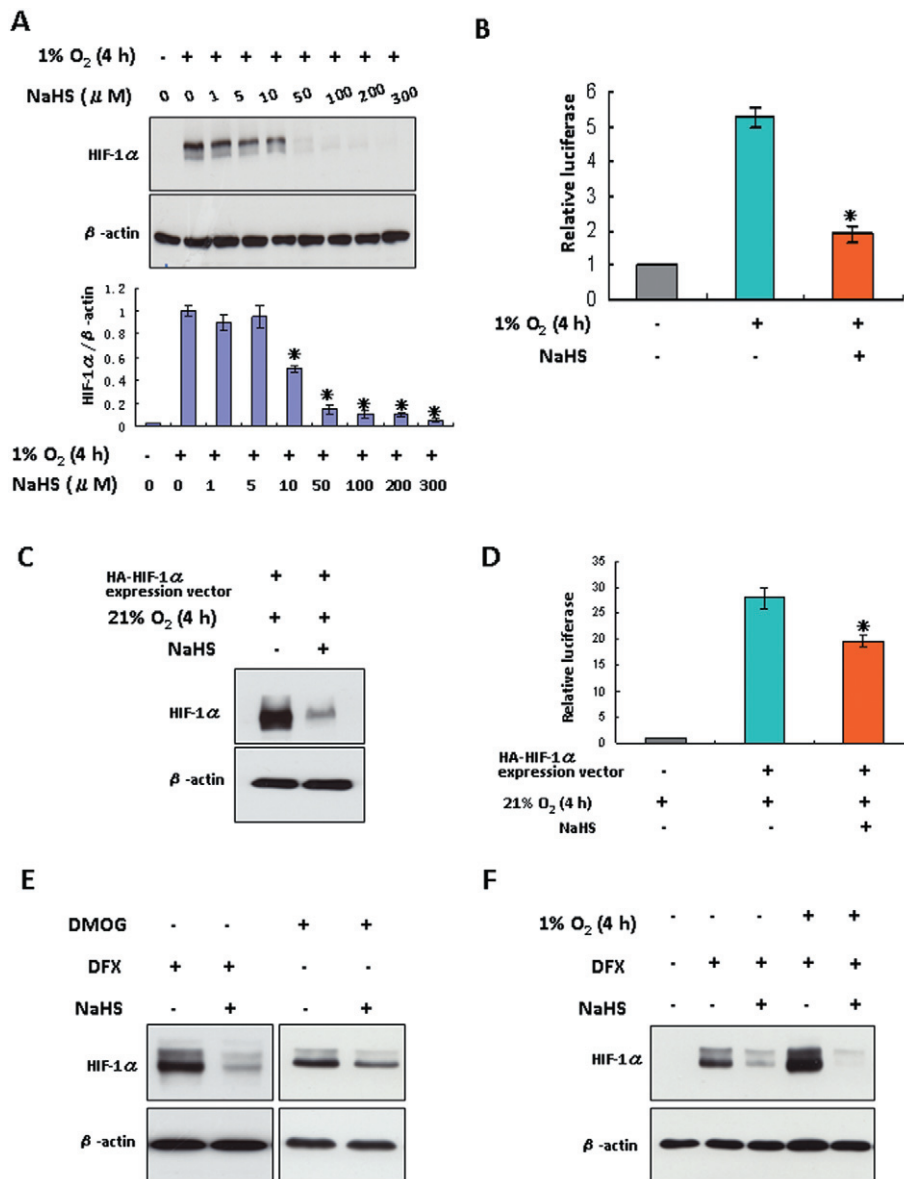


Figure 1

The effects of H₂S on HIF-1 α protein levels in HEK293T cells challenged by hypoxia or hypoxia-mimetic agents. (A) HEK293T cells were treated under hypoxia for 4 h in the presence or absence of increasing concentrations of NaHS. The lower panel shown is the densitometric quantification of HIF-1 α protein normalized to β -actin, and presented as % of hypoxia-alone group. * P < 0.05 versus hypoxia-alone group, n = 5. (B) HEK293T cells were co-transfected with plasmid HRE-luciferase and pRL-TK vector as described in Methods. After 24 h of transfection, cells were exposed to hypoxia for 4 h with or without 100 μ M NaHS. Luciferase activity was measured and normalized to control. Each column represents the mean $\pm$ SEM (n = 3). * P < 0.05 versus group without NaHS. (C) HEK293T cells were transfected with plasmid HA-HIF1 α -pcDNA3 for 24 h, followed by the treatment of 100 μ M NaHS for 4 h under normoxic conditions. (D) HEK293T cells were co-transfected with plasmid HRE-luciferase and pRL-TK vector, and either with plasmid HA-HIF1 α -pcDNA3 or empty vector. After 24 h of transfection, cells were challenged with 100 μ M NaHS under normoxia. Luciferase activity was measured and normalized to control. Each column represents the mean $\pm$ SEM (n = 3). * P < 0.05 versus group without NaHS. (E) HEK293T cells were treated with either 200 μ M DFX or 1 mM DMOG for 2 h, followed by the addition of 100 μ M NaHS for 4 h. (F) HEK293T cells were treated with 200 μ M DFX in either normoxic or hypoxic conditions for 4 h, with or without 100 μ M NaHS. In (A, C, E and F), total protein extracts (40 μ g) were subjected to immunoblot assays with anti-HIF-1 α or anti- β -actin antibodies.

expression under hypoxia, indicating that H₂S decreased HIF-1 α transcriptional activity. We also detected the effect of NaHS on HIF-1 α overexpressed HEK293T cells by using plasmid HA-HIF-1 α -pcDNA3, which contains human wild-

type HIF-1 α cDNA sequences. Heterologous overexpression of HIF-1 α gene in HEK293T cells led to an abundant expression of HIF-1 α proteins under normoxic conditions. Application of NaHS for 4 h significantly lowered the HIF-1 α protein level

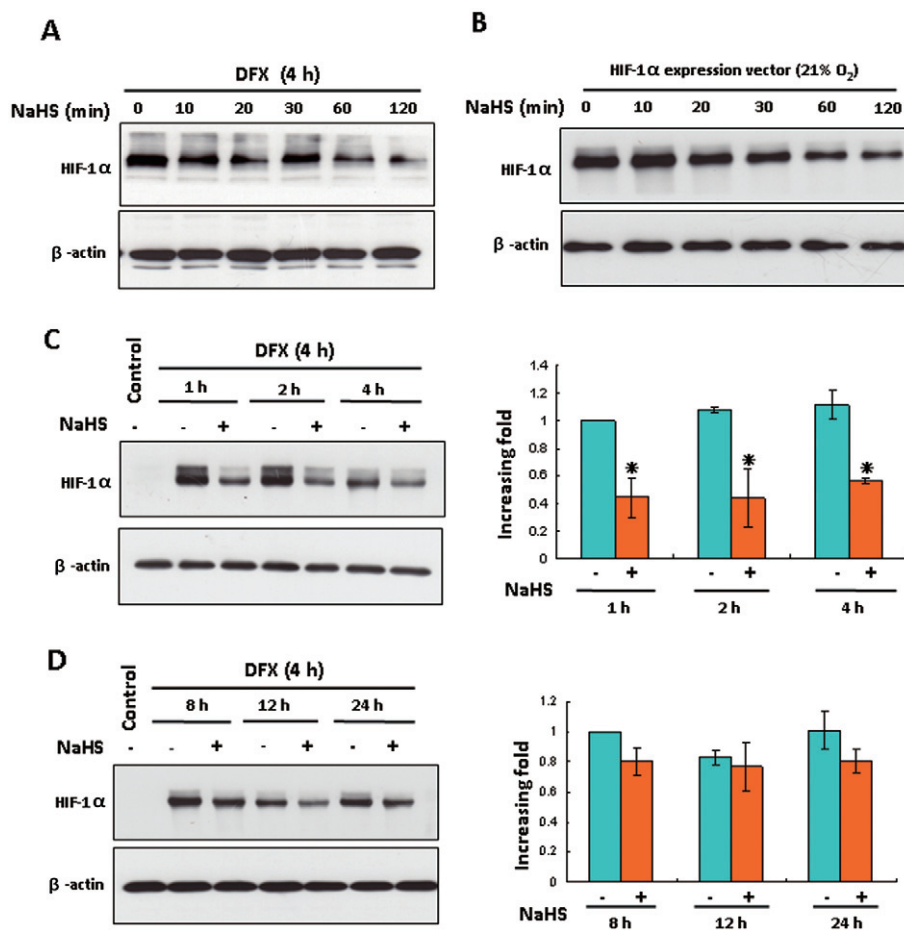


Figure 2

H₂S reduced HIF-1α protein levels in HEK293T cells in a time-dependent manner. (A) HEK293T cells were treated with 200 μM DFX for 4 h followed by 100 μM NaHS treatment for the indicated time. The levels of HIF-1α and β-actin were analysed by Western blotting. (B) HEK293T cells transfected with plasmid HA-HIF1α-pcDNA3 were treated with or without 100 μM NaHS for the indicated time under normoxic conditions. The levels of HIF-1α and β-actin were analysed by Western blotting. (C and D) HEK293T cells were treated with 200 μM DFX for 4 h followed by 100 μM NaHS treatment for the indicated time. The right panel shown is the densitometric quantification of HIF-1α protein normalized to β-actin, and presented as % of cells at 1 h (C) or 8 h (D) without NaHS. In all panels, mean ± SEM is shown, **P* < 0.05 versus group without NaHS at each time point. *n* = 4.

(Figure 1C) and HIF-1α activity (Figure 1D) compared with untreated cells.

Treatment of HEK293T cells with DFX (Figure 1E), DMOG (Figure 1E) or CoCl₂ (Supporting Information Fig. S2) elevated HIF-1α protein levels. These agents have been shown to inhibit hydroxylation of HIF-1α, consequently promoting HIF-1α accumulation (Wang and Semenza, 1993; Keely *et al.*, 2009; Wiley *et al.*, 2010). In the presence of these hypoxia-mimetic agents, NaHS induced HIF-1α down-regulation. Even in the presence of hypoxia combined with DFX, NaHS still potently lowered HIF-1α protein level (Figure 1F). The oxygen partial pressure in the cell culture medium was significantly lower under hypoxic conditions than normoxic conditions (Supporting Information Fig. S3). With hypoxia-mimetic agents, however, the oxygen partial pressure in the cell culture medium was not altered compared with that in the absence of the agents under normoxic culture conditions. NaHS did not alter the oxygen partial pressure of the culture

medium in either hypoxic or hypoxia-mimetic conditions (Supporting Information Fig. S3). Furthermore, NaHS down-regulated HIF-1α in cultured Hep3B cells (a human hepatoma cell line) or EA.hy926 cells (a human umbilical vein endothelial cell line) under both hypoxic and hypoxia-mimetic conditions (Supporting Information Fig. S4 & Figure 6A). Additionally, under normoxic conditions, NaHS treatment of HEK293T cells for either 1 or 4 h had no effect on HIF-1α protein level (Supporting Information Fig. S5). H₂S did not affect HIF-1α expression at the transcription level as real-time quantitative PCR analysis did not reveal any decrease in HIF-1α mRNA level after 4 h of NaHS treatment under hypoxic conditions (Supporting Information Fig. S6).

The down-regulation of HIF-1α protein by NaHS was detected as early as 20 min after 4 h DFX treatment and manifested more as the NaHS treatment time was prolonged (Figure 2A). The NaHS-induced time-dependent suppression of HIF-1α protein levels was also obtained in HEK293T cells

with overexpressed HIF-1 α under normoxic conditions (Figure 2B). As shown in Figure 2C, NaHS treatment for 1–4 h decreased DFX-induced HIF-1 α accumulation by appropriately 50%. This inhibitory effect subsided 8 h after NaHS treatment (Figure 2D).

The inhibitory effect of H₂S on HIF-1 α protein level was independent of the ubiquitin-proteasomal degradation pathway

To investigate whether H₂S-mediated repression of HIF-1 α was due to increased HIF-1 α degradation, we used the proteasome inhibitor MG132 to block the degradation of ubiquitinated HIF-1 α . In the presence of MG132, NaHS down-regulated HIF-1 α under either normoxia or hypoxia (Figure 3A, left). NaHS treatment of plasmid HA-HIF1 α -pcDNA3 transfected HEK293T cells still lowered HIF-1 α level after cells were pretreated with MG132 under normoxia (Figure 3A, right).

Ubiquitination of HIF-1 α is a key step for ubiquitin-proteasome-dependent HIF-1 α degradation. The interaction of H₂S and ubiquitination of HIF-1 α was studied by co-immunoprecipitation assay in HEK293T cells co-transfected with HIF-1 α and ubiquitin expression vectors. After immunoprecipitation with antibodies against ubiquitin and then blotting with antibodies against HIF-1 α , we detected that NaHS decreased the level of ubiquitinated HIF-1 α after the degradation of ubiquitinated HIF-1 α had been blocked with MG132 (Figure 3B, left). Consistent with the immunoprecipitation results, Western blot analysis on cell lysates with equal amounts of that used for immunoprecipitation (input) showed that NaHS decreased the levels of both un-ubiquitinated and ubiquitinated HIF-1 α protein in the presence of MG132 (Figure 3B, right). We also did the same experiment but without MG132 treatment to further evaluate the ubiquitination of HIF-1 α . In this case, NaHS inhibited HIF-1 α accumulation, but did not significantly affect the ubiquitination of HIF-1 α (Supporting Information Fig. S7). These results suggest that NaHS-induced HIF-1 α down-regulation is not due to enhanced ubiquitination of HIF-1 α .

As shown in Figure 3C, 4 h of exposure to hypoxia evoked massive HIF-1 α accumulation in HEK293T cells. Five minutes of re-oxygenation caused a marked decrease in HIF-1 α protein levels, and 15 min after re-oxygenation, HIF-1 α proteins were only barely detectable. Re-oxygenation-induced HIF-1 α reduction was not affected by NaHS.

Next we assessed the effect of NaHS on the rate of HIF-1 α degradation in HEK293T cells. After pretreatment of cells with DMOG for 4 h to induce HIF-1 α accumulation, CHX was added to block new HIF-1 α translation. CHX alone or NaHS alone caused a gradual decrease in HIF-1 α protein level. However, in the presence of CHX, the addition of NaHS did not cause significant changes in HIF-1 α protein levels at each time point compared with that of CHX alone treated cells (Figure 3D). The half-life of HIF-1 α was approximately 40–50 min in either CHX alone or NaHS combined with CHX treated cells. Similar results were obtained in the cells stimulated with DFX (Figure 3E). NaHS did not alter HIF-1 α protein levels at any time point in the presence of CHX. The half-life of HIF-1 α was approximately

60 min in either CHX alone or NaHS combined with CHX treated cells.

H₂S-induced HIF-1 α down-regulation was independent of the mTOR/4E-BP1 pathway

Under hypoxic conditions, H₂S had no significant effect on the expression of p53, p21 and cyclin D1, all of which are short-lived proteins (Figure 4A). These data suggest that the inhibitory effect of H₂S on HIF-1 α expression is relatively specific to hypoxia.

The PI3K-Akt-mTOR pathway has been implicated in the regulation of HIF-1 α gene translation (Hudson *et al.*, 2002). Activation of the PI3K-Akt-mTOR pathway phosphorylates the translational repressor 4E-binding protein 1 (4E-BP1). Phosphorylated 4E-BP1 dissociates from eIF4E, resulting in eIF4E binding to eIF4G, which is the limiting step in the assembly of the translation initiation complex eIF4F (Liu *et al.*, 2006). Pretreatment with rapamycin, an inhibitor of mTOR, did not reverse H₂S-induced reduction in HIF-1 α protein in HEK293T cells exposed to either hypoxia or DFX treatment (Figure 4B), suggesting that mTOR may not be an effector of H₂S for HIF-1 α regulation. Next, we used 7-methyl GTP Sepharose pull-down assays to detect whether H₂S modulates the interaction between 4E-BP1 and eIF4E (Chapuis *et al.*, 2010). As shown in Figure 4C, H₂S had no effect on the dissociation of 4E-BP1 from eIF4E in hypoxic conditions, as detected by pull-down assays. Equal amounts of eIF4G, 4E-BP1 and eIF4E in cell lysates for pull down were measured by Western blotting.

Phosphorylation of eIF2 α was associated with H₂S-induced repressing of HIF-1 α translation

In normal oxygen levels, H₂S reversibly phosphorylated eIF2 α in HEK293T cells with the peak occurring at 30 min (Figure 5A). H₂S treatment also induced a modest phosphorylation of eIF2 α under hypoxic conditions (Figure 5B). eIF2 α knockdown decreased the basal level of HIF-1 α proteins by appropriately 30% in hypoxic conditions compared with the siRNA negative control (Figure 5C). The eIF2 α -depleted cells were highly resistant to H₂S, displaying only a 34% decrease in HIF-1 α expression compared with the 83% decrease in cells transfected with the control siRNA, indicating that eIF2 α is involved in the H₂S-induced repression of HIF-1 α in hypoxic conditions. However, application of the hypoxia-mimetic compound DFX, resulted in a similar H₂S-induced reduction of HIF-1 α in either negative control or eIF2 α knockdown cells, 55 and 48% respectively (Figure 5D). In short, these results show that H₂S-induced eIF2 α phosphorylation contributed to HIF-1 α translational repression under hypoxic but not hypoxia-mimetic conditions.

H₂S inhibited in vitro capillary tube formation under hypoxia

Among all the known genes regulated by HIF-1, VEGF plays a central role in stimulating angiogenesis and oxygen delivery (Forsythe *et al.*, 1996). NaHS, at concentrations higher than 10 μ M, evoked a marked decrease in hypoxia-induced HIF-1 α accumulation in EA.hy926 cells (Figure 6A). Consistent with the result obtained after exogenous NaHS treatment, in recombinant defective adenovirus containing CSE gene

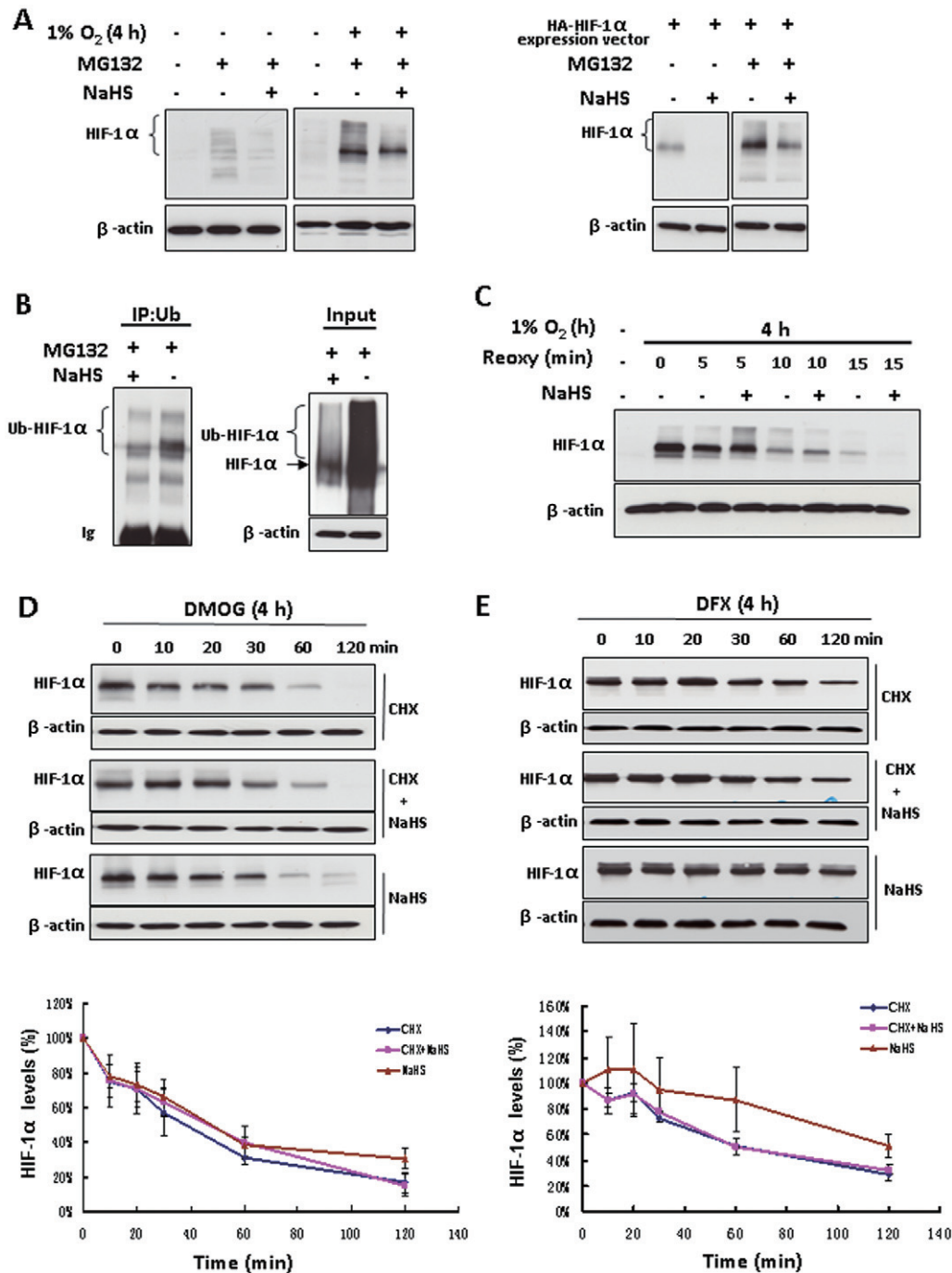


Figure 3

The inhibitory effect of H₂S on HIF-1α protein level was independent of the ubiquitin-proteasomal degradation pathway. (A) Non-transfected HEK293T cells were treated with or without 100 μM NaHS in the presence of 20 μM MG132 for 4 h under either normoxia or hypoxia (left). Plasmid HA-HIF1α-pcDNA3 transfected HEK293T cells were exposed to the same treatments under normoxia (right). Total cell extracts (40 μg) were separated by SDS-PAGE and immunoblotted for HIF-1α protein; β-actin served as a loading control. (B) HEK293T cells co-transfected with plasmid HA-HIF1α-pcDNA3 and plasmid HA-ubiquitin were exposed to 20 μM MG132 with or without 100 μM NaHS for 4 h. Cell extracts were subjected to immunoprecipitation with antibody to ubiquitin (IP) followed by immunoblotting with anti-HIF-1α antibody. An aliquot of cell lysates that was reserved before IP was also analysed (input) by using anti-HIF-1α antibody. Ub, ubiquitin; Ub-HIF-1α, ubiquitinated HIF-1α. (C) HEK293T cells were treated with hypoxia for 4 h followed by re-oxygenation for the indicated time with or without 100 μM NaHS. Total cell extracts (40 μg) were prepared for immunoblotting. HEK293T cells were treated with either 1 mM DMOG (D) or 200 μM DFX (E) for 4 h, followed by the indicated treatments. The concentrations of CHX and NaHS are 25 and 100 μM respectively. Total cell extracts (40 μg) were prepared for immunoblotting with anti-HIF-1α antibody. The lower panels of (D) and (E) are the densitometric quantifications of HIF-1α normalized to β-actin, and presented as % of cells at 0 min. *n* = 3 for each group. Reoxy, re-oxygenation.

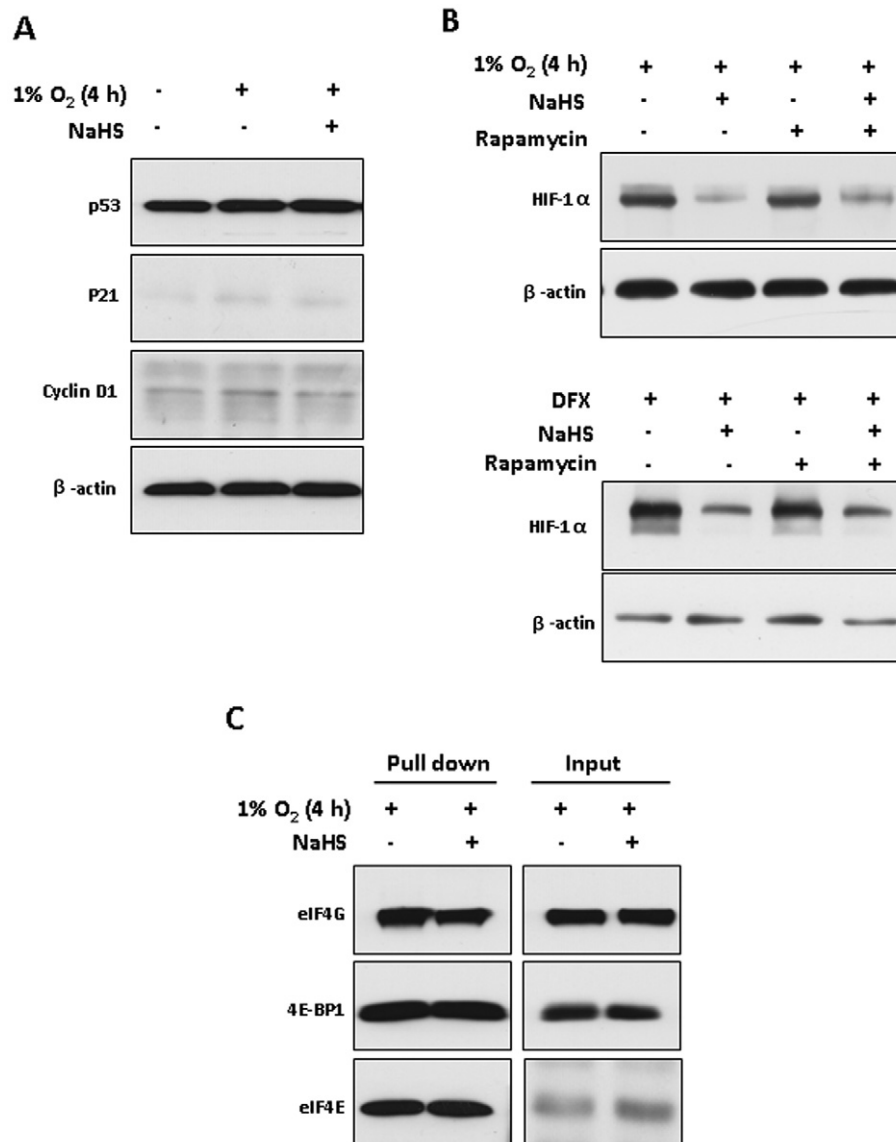


Figure 4

H₂S-induced HIF-1 α down-regulation was independent of the mTOR/4E-BP1 pathway. (A) Western blot analysis of p53, p21 and cyclin D1 in HEK293T cells treated under hypoxia for 4 h with or without 100 μ M NaHS; β -actin served as a loading control. (B) HEK293T cells were pretreated with or without 100 nM rapamycin for 0.5 h followed by exposure to hypoxia (upper) or 200 μ M DFX (lower) for 4 h in the presence or absence of 100 μ M NaHS respectively. Western blot analyses of HIF-1 α and β -actin are shown. (C) HEK293T cells were exposed to hypoxia for 4 h with or without 100 μ M NaHS. Cell lysates (500 μ g) were subjected to 7-methyl GTP Sepharose, followed by immunoblotting with antibodies for eIF4G, 4E-BP1 and eIF4E respectively. Equal amounts of proteins that were reserved before pull down were also analysed (input).

(Ad-CSE) transfected endothelial cells, HIF-1 α accumulation was inhibited under hypoxia compared with that of recombinant adenovirus encoding bacterial β -galactosidase (Ad-lacZ) transfected endothelial cells (Supporting Information Fig. S8). NaHS did not significantly decrease HIF-1 α under hypoxia after eIF2 α knockdown (Figure 6B). In the presence of MG132 to block the degradation of HIF-1 α , NaHS still down-regulated HIF-1 α under hypoxia (Supporting Information Fig. S9A). Additionally, H₂S-induced eIF2 α phosphorylation was also detected (Supporting Information Fig. S9B). The transcriptional expression of VEGF was significantly

increased in hypoxia, and this was down-regulated by NaHS (Figure 6C). Under normoxic conditions, VEGF or NaHS alone stimulated tube formation of EA.hy926 cells. Hypoxia also increased tube formation compared with that under normoxic conditions. However, the application of NaHS reduced capillary tube formation (Figure 6D), and reduced cell viability and inhibited cell proliferation of EA.hy926 cells (Figure 6E,F). Neither eIF2 α phosphorylation (Supporting Information Fig. S9) nor tube formation (Supporting Information Fig. S10) was changed by a lower concentration of NaHS (5 μ M).

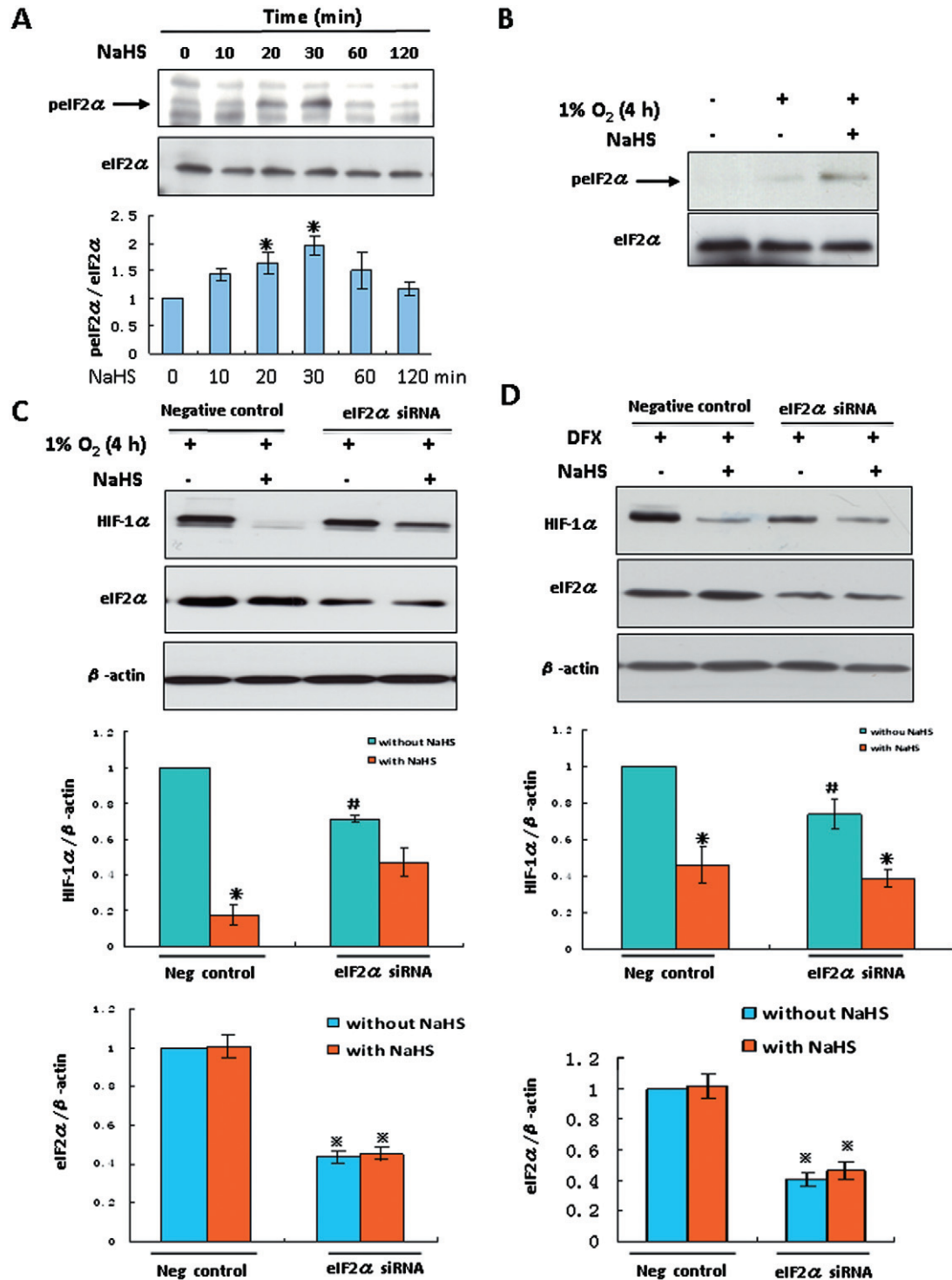


Figure 5

Phosphorylation of eIF2 α was associated with H₂S-induced HIF-1 α translational repression in HEK293T cells. HEK293T cells were treated with 100 μ M NaHS under normoxia (A) or hypoxia (B) for the indicated time. p-eIF2 α were determined by Western blot analyses. The panel shown under (A) is the densitometric quantification of p-eIF2 α normalized to total eIF2 α , and presented as % of value at 0 min. * P < 0.05 versus 0 min, n = 5. Total eIF2 α served as a loading control. HEK293T cells were transfected with a siRNA specific for eIF2 α or a negative control siRNA. Transfected cells were exposed to hypoxia (C) or 200 μ M DFX (D) for 4 h in the presence or absence of 100 μ M NaHS. Levels of HIF-1 α and eIF2 α were examined by immunoblotting and β -actin served as a loading control. The middle and lower panels shown are the densitometric quantification of HIF-1 α normalized to β -actin and eIF2 α normalized to β -actin, respectively, and presented as % of negative control without NaHS from five (C) or seven (D) independent experiments. * P < 0.05 versus the same siRNA transfection group without NaHS. # P < 0.05 versus negative control group without NaHS. * P < 0.05 versus negative control group without NaHS.

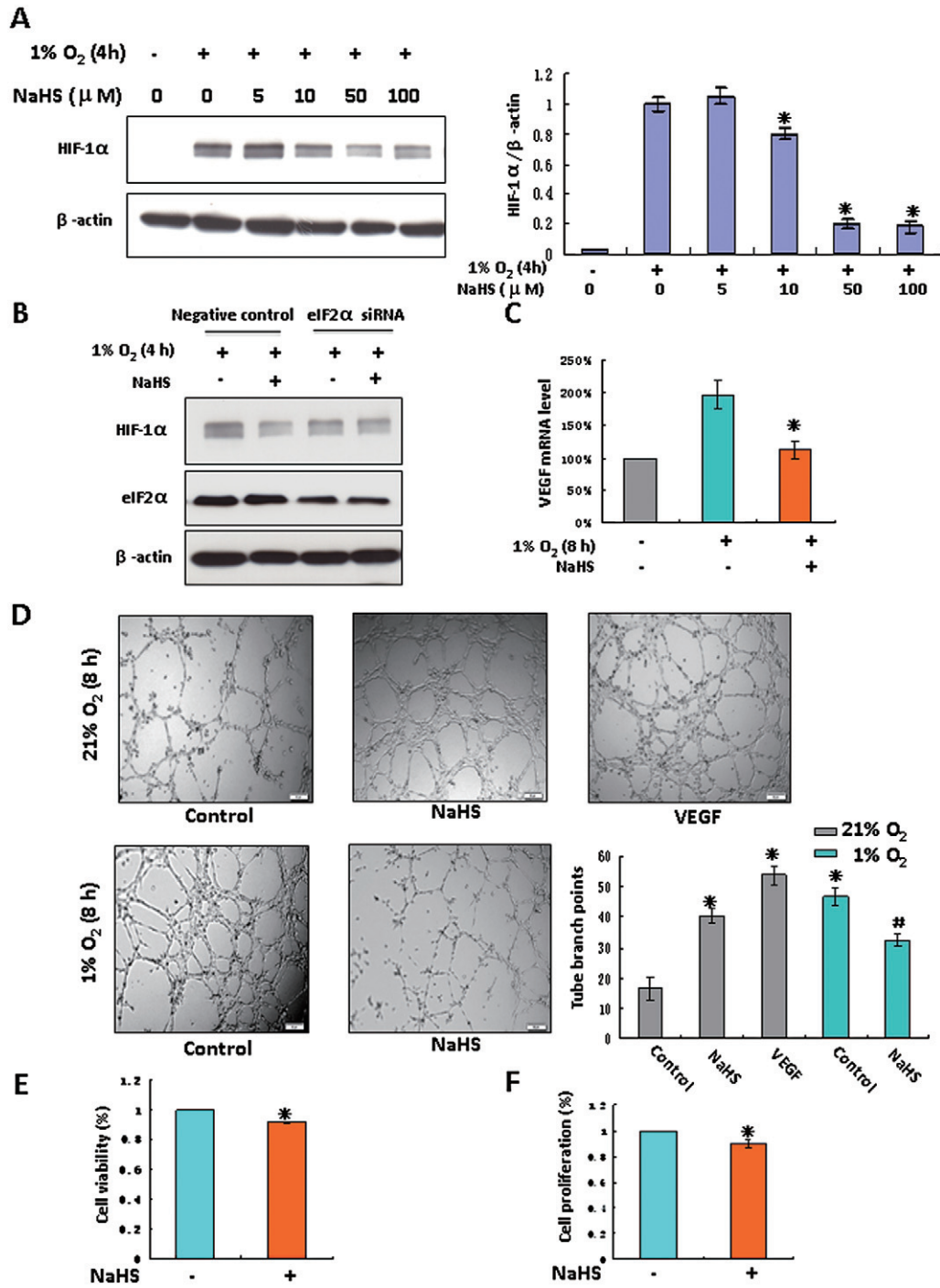


Figure 6

H₂S inhibited *in vitro* angiogenic activity in EA.hy926 cells under hypoxia. (A) EA.hy926 cells were incubated in hypoxic conditions for 4 h in the presence of NaHS at indicated concentrations. The right panel shown is the densitometric quantification of HIF-1 α protein normalized to β -actin, and presented as % of hypoxia alone group. * P < 0.05 versus hypoxia-alone group, n = 4. (B) EA.hy926 cells were transfected with a siRNA specific for eIF2 α or a negative control siRNA. Transfected cells were exposed to hypoxia for 4 h in the presence or absence of 100 μ M NaHS. Levels of HIF-1 α and eIF2 α were examined by immunoblotting and β -actin served as a loading control. (C) EA.hy926 cells were exposed to hypoxia for 8 h with or without 100 μ M NaHS. Total RNA was isolated. VEGF mRNA was analysed by real-time quantitative PCR. Each column represents the mean $\pm$ SEM (n = 3). * P < 0.05 versus hypoxic group without NaHS. (D) EA.hy926 cells were seeded onto 96-well plates coated with Matrigel, followed by incubation with 100 μ M NaHS for 8 h under normoxia or hypoxia. 50 ng·mL⁻¹ VEGF was used as a positive control. Images of tube formation were taken under an inverted light microscope and tube branch points from each of four randomly chosen fields were quantified. Data represent the mean $\pm$ SEM, n = 3. * P < 0.05 versus control under normoxia. # P < 0.05 versus control under hypoxia. Cell viability (E) and cell proliferation (F) in EA.hy926 cells were detected under hypoxia in the presence of 100 μ M NaHS for 24 h. * P < 0.05 versus hypoxia-alone group, n = 5.

Discussion and conclusions

Both NO and CO have been shown to regulate HIF-1 α expression and HIF-1 α transcriptional activity (Hagen *et al.*, 2003; Metzen *et al.*, 2003; Choi *et al.*, 2010). As the third gasotransmitter, H₂S also regulates HIF-1 α activity and expression. An earlier study it was reported that sulforaphane down-regulates the expression of HIF-1 α under hypoxia (Yao *et al.*, 2008). In this regard, sulforaphane has recently been reported to have H₂S-donating properties (Pei *et al.*, 2011). NaHS at 1 mM decreased HIF-1 α protein level under hypoxia in several cell lines, including Hep3B, SH-SY5Y and Hela cells (Kai *et al.*, 2012). Under normoxic conditions, H₂S gas (50 p.p.m.) enhanced HIF-1 activity and HIF-1 protein concentration in *C. elegans* (Budde and Roth, 2009). NaHS (300 μ M) also increases HIF-1 α protein in CoCl₂ (300 μ M) treated vascular smooth muscle cells (Liu *et al.*, 2010). Thus, the regulatory effects of H₂S on HIF-1 α protein levels might be different with different experimental setups, such as animal species and cell types, whether endogenous H₂S levels are manipulated or exogenous H₂S is given in different formulas, and the degree of hypoxia (Jaakkola *et al.*, 2001; Berra *et al.*, 2003).

NaHS at 10–100 μ M has been used in different studies to mimic physiologically relevant concentrations of H₂S *in vivo* (Yang *et al.*, 2012). However, within hours of its administration, the NaHS concentration is decreased (Suzuki *et al.*, 2011; Yang *et al.*, 2012); we previously demonstrated a 55–76% loss of H₂S from NaHS-containing medium during the incubation periods 30 min to 12 h (Zhao *et al.*, 2001; Yang *et al.*, 2012). In addition, the accuracy of conventional methods used in the measurement of H₂S level has been challenged in recent years and the actual level of endogenous H₂S in circulation or tissues *in vivo* has not been unambiguously established. With different H₂S measurement techniques, endogenous levels of H₂S in circulation have been reported from nanomolar to low micromolar range. Therefore, one should be cautious in correlating the concentrations of exogenously applied H₂S to real physiological levels of H₂S *in vivo*. In the present study, we found that NaHS (10–100 μ M) treatment of HEK293T, Hep3B and EA.hy926 cells decreased HIF-1 α protein levels under both hypoxia and hypoxia-mimetic conditions (DFX, DMOG and CoCl₂) in a dose- and time-dependent manner. Down-regulation of HIF-1 α protein was associated with a reduction in HIF-1 α transcriptional activity. In addition, H₂S-induced eIF2 α phosphorylation was shown to contribute partially to the translational suppression of HIF-1 α protein. Finally, H₂S inhibited angiogenesis under hypoxic conditions and this may be involved in the down-regulation of HIF-1 α . NaHS at 5 μ M had no effect on HIF-1 α protein levels, or on eIF2 α phosphorylation or tube formation.

HIF-1 α degradation is mainly mediated by prolyl hydroxylases and von Hippel Lindau protein in different types of cells in an oxygen-dependent manner (Rocha, 2007). The oxygen-independent HIF-1 α degradation involves the receptor of activated PKC and heat-shock protein 90 (Hsp90) (Liu *et al.*, 2007). These mechanisms regulating HIF-1 α stability converge at the ubiquitin-proteasomal system, which ultimately determines HIF-1 α proteolysis. NO and CO have been reported to regulate HIF-1 α accumulation by different mechanisms that affect its degradation. NO-stimulated HIF-1 α

down-regulation under hypoxia results from an increase in its degradation mediated by prolyl hydroxylases (Hagen *et al.*, 2003), whereas CO up-regulates HIF-1 α partially by suppressing its oxygen-independent degradation (Choi *et al.*, 2010). Kai *et al.* showed that H₂S down-regulated HIF-1 α under hypoxic, but not hypoxia-mimetic, conditions in Hep3B cells (Kai *et al.*, 2012). These authors believed that this was due to an H₂S-induced decrease in oxygen consumption under hypoxia. They found that H₂S promoted HIF-1 α degradation under hypoxia after CHX-induced HIF-1 α translational inhibition. Moreover, H₂S did not affect HIF-1 α expression induced by MG132. Their work suggested that H₂S-inhibited mitochondrial oxygen consumption resulted in increased oxygen level in hypoxic cells, which therefore promoted HIF-1 α degradation. On the other hand, H₂S had no effect on DFX- or CoCl₂-induced HIF-1 α accumulation because H₂S could not significantly increase cellular oxygen partial pressure in the presence of hypoxia-mimetic agents (Kai *et al.*, 2012). Unfortunately, most of the key observations that elucidated potential mechanisms for H₂S-inhibited HIF-1 α accumulation were obtained with NaHS at 1 mM, which is clearly not within the physiological range of endogenous H₂S. Kai *et al.* performed a cytotoxicity assay and showed that 4 h treatment with 1 mM NaHS had no effect on cell viability (Kai *et al.*, 2012). However, treatment for 4 h, a relatively short time, may not reflect the potential injury induced by 1 mM NaHS. We found that a 24 h treatment of HEK293T cells with NaHS at 1 mM decreased cell viability (Supporting Information Fig. S11). When we used NaHS at a concentration of 100 μ M, which had no inhibitory effect on cell viability, we reached a different conclusion that H₂S-induced HIF-1 α down-regulation is independent of the ubiquitin-proteasomal degradation pathway. Firstly, in the presence of the proteasome inhibitor MG132 to inhibit HIF-1 α degradation, H₂S still induced HIF-1 α down-regulation. Secondly, co-immunoprecipitation assays showed that H₂S did not induce HIF-1 α ubiquitination which is a key step for HIF-1 α degradation. Thirdly, H₂S had no effect on re-oxygenation-induced reduction in HIF-1 α levels. Fourthly, after HIF-1 α translation had been blocked with CHX, NaHS ceased to affect HIF-1 α protein levels at all of the time points during the observation. Finally, NaHS lowered HIF-1 α protein levels even with hypoxia-mimetic agents, which did not lower oxygen partial pressure in the culture medium. It is worthy of mention that Kai *et al.* performed their experiments by adding hypoxia-mimetic agents and NaHS at the same time (Kai *et al.*, 2012), whereas we pretreated cells with hypoxia-mimetic agents, 2–4 h before the addition of NaHS.

Several lines of evidence have demonstrated that the PI3K-Akt-mTOR pathway, which regulates the formation of translational complex eIF4E, is involved in regulating HIF-1 α protein translation (Rocha, 2007). YC-1 (Sun *et al.*, 2007). Also KC7F2 (Narita *et al.*, 2009) and the promyelocytic leukaemia tumour suppressor (Bernardi *et al.*, 2006), negatively regulate HIF-1 α protein synthesis through inhibition of the PI3K-Akt-mTOR pathway. In our present study, pretreatment with rapamycin did not reverse H₂S-induced HIF-1 α protein reduction under either hypoxic or hypoxia-mimetic conditions, suggesting that the mTOR pathway is not involved in this event. On the other hand, H₂S did not alter the affinity of 4E-BP1 with eIF4E, ruling out the possibility that the eIF4E

complex is involved in H₂S-induced HIF-1 α translational inhibition under hypoxia.

Another critical regulatory molecule for eukaryotic initiation of translation is eIF2 α , of which the phosphorylation at Ser⁵¹ arrests protein synthesis by preventing the re-formation of eIF-2 ternary complex (Rocha, 2007). Previous studies have shown that translational suppression of HIF-1 α by certain anti-tumour compounds is closely correlated with increased eIF2 α phosphorylation (Jung *et al.*, 2009; 2011; Zhang *et al.*, 2010). Our data showed that H₂S led to the phosphorylation of eIF2 α under both normoxic and hypoxic conditions. Restoration of H₂S-induced decrease in HIF-1 α proteins under hypoxia was observed after knockdown of the eIF2 α gene, demonstrating that HIF-1 α translational suppression is associated with H₂S-induced eIF2 α phosphorylation. What is more interesting is that under hypoxia-mimetic conditions, where only the utilization of oxygen is blocked but the actual oxygen partial pressure is not reduced, eIF2 α knockdown did not significantly restore H₂S-induced reduction of HIF-1 α . This phenomenon indicates that eIF2 α phosphorylation may not be a major contributor to H₂S-induced decrease in HIF-1 α expression under hypoxia-mimetic conditions. Although our data showed that the ubiquitin-proteasomal degradation pathway was not responsible for H₂S-induced HIF-1 α down-regulation, we cannot rule out the possibility that the calcium/calpain-mediated HIF-1 α degradation pathway might be involved in this event (Zhou *et al.*, 2006). H₂S has been found to be associated with the regulation of intracellular calcium (Wang, 2011). Thus, under hypoxic-mimetic conditions, whether H₂S inhibits HIF-1 α accumulation by promoting the calcium/calpain pathway needs to be investigated.

EA.hy926 cell line is a useful model for *in vitro* study of angiogenic processes, which has maintained the phenotype of endothelial cells (Bauer *et al.*, 1992; Aranda and Owen, 2009; Schwalm *et al.*, 2010). This cell line has also been reported to produce more consistent responses to specific variables and greater reproducibility of data (Aranda and Owen, 2009). H₂S exhibited an inhibitory effect on the angiogenic activity of EA.hy926 cells under hypoxia, which was associated with H₂S-induced HIF-1 α down-regulation and VEGF inhibition. H₂S stimulated capillary tube formation under normoxia but without an effect on HIF-1 α and VEGF expression, indicating that HIF-1 α is not essential for H₂S-stimulated angiogenic activity under normoxia. In line with this, H₂S has been demonstrated to increase angiogenesis under normoxia through K_{ATP} channels and the MAPK pathway (Papapetropoulos *et al.*, 2009; Szabo and Papapetropoulos, 2011). Thus, our results suggest that H₂S may play a dual role in the regulation of angiogenesis, which is associated with oxygen levels.

In summary, H₂S-induced repression of HIF-1 α protein translation, rather than an effect on the ubiquitin-proteasomal degradation pathway, is the key mechanism for H₂S-mediated HIF-1 α down-regulation under both acute moderate hypoxia and hypoxia-mimetic conditions. This inhibitory effect of H₂S on HIF-1 α translation is partially associated with an increase in eIF2 α phosphorylation. Additionally, H₂S-inhibited HIF-1 α expression is involved in H₂S-induced inhibition of angiogenic activity under hypoxia. These findings elucidate a novel eIF2 α phosphorylation-

related mechanism for the interaction of H₂S and HIF-1 α under hypoxic conditions and its functional consequence in vascular endothelial cells. They also pave the way for further elucidation of the oxygen-sensing role of H₂S in different physiological and pathophysiological situations.

Acknowledgements

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Endogenous H₂S decreased HIF-1 α protein levels in HEK293 cells under hypoxia. HEK293 cell line stably expressing CSE (HEK293 + CSE) and control cell line (HEK293 + vector) were exposed to hypoxia for 4 h. Western blot analyses of HIF-1 α , CSE and β -actin were shown.

Figure S2 H₂S decreased HIF-1 α protein levels in HEK293T cells treated with CoCl₂. HEK293T cells were challenged with 200 μ M CoCl₂ for 4 h followed by 100 μ M NaHS treatment for the indicated time. Western blot analyses of HIF-1 α and β -actin were shown.

Figure S3 Oxygen partial pressure in cell-culture medium in different conditions. HEK293T cells were treated as indicated in the presence or absence of 100 μ M NaHS. Oxygen partial pressure in cell-culture medium was measured by using GEM

Premier 3000 blood gas analyser. $n = 5$ for each independent experiment. $*P < 0.05$ versus control group.

Figure S4 H₂S down-regulated HIF-1 α protein levels in other cell lines. Hep3B cells (A) or EA.hy926 cells (B) were treated with hypoxia or 200 μ M DFX for 4 h in the presence or absence of 100 μ M NaHS. Western blot analyses of HIF-1 α and β -actin were shown.

Figure S5 H₂S did not induce any HIF-1 α accumulation under normoxia in HEK293T cells. HEK293T cells were treated with increasing concentrations of NaHS for 1 h (A) or 4 h (B) under normoxia. Western blot analyses of HIF-1 α and β -actin were shown.

Figure S6 H₂S had no effect on HIF-1 α mRNA levels under hypoxia in HEK293T cells. Total RNA was extracted from HEK293T cells subjected to normoxia or hypoxia for 4 h in the presence or absence of 100 μ M NaHS. Real-time quantitative PCR was performed to analyse HIF-1 α mRNA levels. Results are representative of an average of three independent experiments.

Figure S7 H₂S did not affect the ubiquitination of HIF-1 α in HEK293T cells. HEK293T cells co-transfected with plasmid HA-HIF1 α -pcDNA3 and plasmid HA-Ubiquitin were exposed to 100 μ M NaHS under normoxia for 4 h. Cell extracts were subjected to immunoprecipitation with antibody to ubiquitin (IP) followed by immunoblotting with anti-HIF-1 α antibody (IB). An aliquot of cell lysates that was reserved prior to IP was also analysed (input) using anti-HIF-1 α or anti- β -actin antibody. Ub, ubiquitin; Ub-HIF-1 α , ubiquitinated HIF-1 α .

Figure S8 Endogenous H₂S decreased HIF-1 α protein levels in EA.hy926 cells under hypoxia. EA.hy926 cells infected with recombinant CSE adenovirus (Ad-CSE) or Ad-lacZ were treated under hypoxia for 4 h. Western blot analyses of HIF-1 α , CSE and β -actin were shown.

Figure S9 H₂S-induced HIF-1 α protein translation inhibition was indicated in EA.hy926 cells under hypoxia. (A) EA.hy926 cells were treated with or without 100 μ M NaHS in the presence or absence of 20 μ M MG132 for 4 h under hypoxia. HIF-1 α levels were determined by Western blot analyses. β -actin serves as a loading control. (B) EA.hy926 cells were treated with 5 or 100 μ M NaHS under normoxia for the indicated time. p-eIF2 α was determined by Western blot analyses. Total eIF2 α serves as a loading control.

Figure S10 NaHS at a concentration of 5 μ M did not affect *in vitro* tube formation in EA.hy926 cells. EA.hy926 cells were seeded onto 96-well plates coated with Matrigel, followed by incubation with 5 μ M NaHS for 8 h under normoxia or hypoxia. 50 ng mL⁻¹ VEGF was used as a positive control. Images of tube formation were taken under an inverted light microscope and tube branch points from each of four randomly chosen fields were quantified. Data represent the mean $\pm$ SEM, $n = 3$.

Figure S11 The effects of NaHS at different concentrations on cell viability in HEK293T cells. Cell viability in HEK293T cells were detected in the presence of 100 μ M or 1 mM NaHS for 24 h. $*P < 0.05$ versus control group, $n = 4$.