Protective effect of a hydrogen sulfide donor on balloon injury-induced restenosis via the Nrf2/HIF-1α signaling pathway

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Abstract. Restenosis is liable to occur following treatment with endovascular interventional therapy. Increasing evidence indicates that hydrogen sulfide (H_2S) exhibits numerous physiological properties, including antioxidative and cardioprotective disease properties. Thus, the present study aimed to investigate the anti-restenosis effects of H_2S and its protective mechanisms. A balloon dilatation restenosis model was used, in which model Sprague-Dawley rats were treated with sodium hydrosulfide (NaHS: A donor of H_2S, 30 μmol/kg) by intraperitoneal injection for 4 weeks. Histological observations of the carotid artery were performed, and H_2S production and the expression of Nuclear factor-E2-related factor 2 (Nrf2)/hypoxia-inducible factor (HIF)-1α signaling pathway proteins were measured. In addition, human umbilical vein endothelial cells (HUVECs) were treated with NaHS following the inhibition of Nrf2 or HIF-1α expression. The expression of Nrf2/HIF-1α signaling pathway proteins, tube formation and cell migration were evaluated thereafter. The results demonstrated that NaHS treatment significantly increased H_2S production in rats with restenosis, and that neointimal thickness decreased significantly in arteries with restenosis. Furthermore, an increase in H_2S production enhanced the nuclear accumulation of Nrf2 and expression of its downstream targets, heme oxygenase-1 and superoxide dismutase, as well as HIF-1α. Similar effects of NaHS on the expression of these proteins were observed in HUVECs. Additionally, these findings indicated that NaHS-induced HIF-1α expression was dependent on Nrf2 expression. NaHS treatment also markedly increased tube formation by upregulating vascular endothelial growth factor expression and cell migration, both of which were mediated by the Nrf2/HIF-1α signaling pathway, and suppressed the migration and proliferation of human vascular smooth muscle cells. Thus, NaHS-mediated H_2S production was observed to prevent neointimal hyperplasia, promote activation of the Nrf2/HIF-1α signal pathway, and enhance HUVEC tube formation and migration, thereby exerting protective effects on balloon injury-induced restenosis.

Introduction

As percutaneous coronary intervention became widely adopted in the 1980s, restenosis was reported in 30-60% of patients, most presenting with recurrent symptoms 1-4 months post-procedure (1,2). In recent years, with the increasing prevalence of vascular stenosis, potential remedial strategies, including medical therapy, surgical bypass and endovascular revascularization, are becoming more widely applied in its clinical treatment (3,4). Endovascular interventional therapy, including percutaneous transluminal angioplasty (PTA) and stent implantation, has demonstrated positive effects in the treatment of vascular stenosis (5,6). However, the recurrence rate of restenosis, a major drawback of PTA, is as high as 19.7 and 60% within the first and fifth years following surgery, respectively (7,8). Restenosis is a pathological outcome of excessive oxidative stress and repair responses in injured local blood vessels in response to interventional therapy (8), leading to a poor prognosis and inferior quality of life in patients with vascular stenosis. The post-operative occurrence of restenosis is one of the major challenges associated with endovascular intervention and represents a worldwide problem.

Vascular endothelial cell damage induced by PTA is an essential factor in the occurrence of restenosis (9). Furthermore, injured endothelia induce the production of reactive intermediates, including reactive nitrogen species and reactive oxygen species (ROS), leading to endothelial dysfunction (10). It has previously been demonstrated that the level of ROS in local blood vessels increases following PTA surgery or stent implantation (11-13). Upon injury, endothelial dysfunction promotes

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the excessive proliferation and migration of vascular smooth muscle cells (VSMCs) and triggers neointimal hyperplasia, resulting in narrowing of the lumen following endovascular intervention (14,15).

Hydrogen sulfide (H$_2$S), one of the important gasotransmitters, is an endogenously produced gas signal molecule that is synthesized and catalyzed *in vivo* through cystathionine β-synthase and cystathionine γ-lyase, respectively (16). H$_2$S is involved in a number of beneficial reactions in cardiovascular tissues, thereby regulating multiple physiological functions, including anti-oxidation, angiogenesis and vasodilation (17). According to the literature, H$_2$S has cardioprotective effects via the inhibition of intimal hyperplasia after peripheral angioplasty (18,19) and antioxidative effects through the elimination of hydrogen peroxide (H$_2$O$_2$) and superoxide anion, thereby suppressing myocardial ischemic injury (20). Nuclear factor-E2-related factor 2 (Nrf2), a transcription factor with a high sensitivity to oxidative stress, exerts antioxidative effects by binding to antioxidant response elements (AREs) in the nucleus and regulating the expression of downstream antioxidant genes, including heme oxygenase (HO)-1 (21). A previous study demonstrated that Nrf2 may be involved in the antioxidative activity of H$_2$S in H$_2$S-mediated cardioprotection (22). In addition, hypoxia-inducible factor (HIF-1), a protein comprising HIF-1α and HIF-1β subunits, has been revealed to serve an important role in regulating angiogenesis, which is beneficial for wound healing during peripheral angioplasty-induced blood vessel injury (23). Thus, it may be hypothesized that the Nrf2 signaling pathway and HIF-1α serve roles in the anti-restenosis effects of H$_2$S.

Although the physiological and cardioprotective effects of H$_2$S have previously been documented, the anti-restenosis effect and molecular mechanisms have not been fully evaluated. Therefore, the purpose of the present study was to investigate the anti-restenosis effect and signaling mechanisms induced by H$_2$S donor (NaHS) treatment using an *in vivo* model of restenosis and *in vitro* cell culture.

Materials and methods

Animals. A total of 24 healthy adult male Sprague-Dawley (SD) rats (8-9 weeks, 250±30 g) were purchased from the Hubei Provincial Center for Disease Control and Prevention (Hubei, China). The rats were housed under controlled conditions of 22±2˚C and 55±5% humidity under a 12-h light/12-h dark cycle and access to food and water *ad libitum*. The rats were acclimatized for at least 1 week prior to being used in the experiments. All experiments involving animal treatment were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Ethics Committee of Union Hospital, affiliated to the Huazhong University of Science and Technology (approval no. TJ-A20161216).

Construction of the balloon dilatation restenosis model and NaHS treatment. All animals were randomly divided into three groups: Normal, model and model+NaHS groups (n=8). The rats that did not receive the balloon injury (sham-operated) were used as normal controls. The rats in the model group (balloon injury) underwent balloon catheter injury to construct the balloon dilatation restenosis model as previously described (8,24) and were allowed to recover for 4 weeks. In brief, the rats were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital and the right external carotid artery in the neck was isolated using a midline incision. The distal end of the right external carotid artery was ligated with a nylon suture, while the proximal part was clamped with a bulldog clamp. Then, a small opening was made in the distal end of the right external carotid artery using eye scissors. A 2F balloon catheter (Edwards Lifesciences, Irvine, CA, USA) was inserted into the right external carotid artery and inflated with 200 µl saline. Next, the balloon catheter was pulled back through the external carotid artery to break the blood vessel endothelium. This manipulation was repeated thrice. Then, the balloon catheter was removed after 20 min; the external carotid artery was ligated and the incisions to the vessel and skin were closed. Penicillin was used topically to prevent infection following the surgery. The rats in the model+NaHS group underwent the same balloon catheter injury and received treatment with H$_2$S in the form of sodium hydrosulfide (NaHS, a donor of H$_2$S; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Treatment with NaHS (30 µmol/kg) was performed by intraperitoneal injection prior to the surgery and continued for 4 consecutive weeks following the surgery, once a day.

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in M200 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). Human vascular smooth muscle cells (HVSMCs) were obtained from American Type Culture Collection and cultured in smooth muscle cell medium (ScienCell Research Laboratories, Inc., San Diego, CA, USA) containing 10% FBS. The two cell types were maintained at 37˚C in a humidified incubator with 5% CO$_2$. Once 75% cell confluence was achieved, they were treated with NaHS (0, 50, 100 or 200 µmol/l) for 48 h. The effect of NaHS on HVSMC proliferation was detected using an MTT cell proliferation and cytotoxicity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The untreated group was considered as the control group. Furthermore, ~2x10^5 HUVECs were cultured in 6-well plates and at 70-80% cell confluence, the cells were treated with NaHS and then transfected with small interfering (si)RNA of target sequences, including siNrf2 (5'-CAUUGAUGUUUCUGAUCUAUU-3'; 100 nM, 48 h), siHIF-1α (5'-CUGAUAACGUGAACAAAUAUU-3'; 100 nM, 48 h) or siRNA negative control (siNC; 5'-GGUCUCACU CCCCAUAGAGUU-3'; 100 nM, 48 h; all Guangzhou RiboBio Co., Ltd., Guangzhou, China) using the Lipofectamine RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). Cultured cells that had not been transfected with siRNA or treated with NaHS were used as controls.

Histological observation. After 4 weeks of treatment, the rats were euthanized by overdose anesthetics (100 mg/kg pentobarbital). Then, the right carotid arteries were isolated, placed in a cold saline solution and the surrounding adipose tissue was dislodged from the carotid artery samples. Subsequently,

the artery samples were cut into vessel segments (3.0 mm) and fixed in 4% paraformaldehyde for 1 h at room temperature. The artery samples were embedded into paraffin blocks using conventional procedures (25), including dehydration, transparency, dipping and embedding. For morphological observation, 5.0-μm thick sections were stained with hematoxylin and eosin (H&E) staining solution (0.2% hematoxylin solution and 1% eosin solution; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. The areas occupied by the external elastic lamina, the internal elastic lamina and the lumen areas were measured by an optical microscope at a magnification of x100. Other areas were calculated as follows: Neointimal area, internal elastic lamina area - lumen area; medial area, external elastic lamina - internal elastic lamina; and % of lumen stenosis/normal, (lumen area in normal group - lumen area in model or treatment group) / lumen area in normal group.

Measurement of endogenous H_2S production. Carotid artery tissue and plasma H_2S production was measured using the H_2S content assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Carotid arteries were ground using liquid nitrogen (-196°C). Total RNA was extracted from carotid arteries using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and dissolved in RNA-free water. Next, cDNA was synthesized using the superscript™ II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and diluted into the concentration of 100 ng/μl for RT-qPCR. The thermal cycling conditions for reverse transcription were as follows: 70°C for 10 min, 42°C for 50 min and 70°C for 15 min. RT-qPCR was performed using the QuantiFast SYBR-Green PCR kit (Qiagen, Inc., Valencia, CA, USA). RT-qPCR was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 45 sec and 72°C for 30 sec; and a last step at 72°C for 10 min. The primers were purchased from Sangon Biotech Co., Ltd., (Shanghai, China). The primer sequences used in present study as follows: Nrf2 forward, 5'-ATTCAAGCCGATTAGAGG-3' and reverse, 5'-ATTGCTCCTTGGACATCA-3'; HO-1 forward, 5'-ATGTCCCAGGATTTGTC-3' and reverse, 5'-CTGCTT GTTTCGCTCT-3'; HIF-1α forward, 5'-GGCTTTGTTATG GTGCTA-3' and reverse, 5'-TTGTTCTTTCCCCTTTCT-3'; vascular endothelial growth factor (VEGF) forward, 5'-AGGGCAGAATCATCACGAAGT-3' and reverse, 5'-AGG GTCTCGATTGGATGGCA-3'; GAPDH forward, 5'-CAA GTTCAACGGCACAG-3' and reverse, 5'-CCAGTAGACTCC ACGACAT-3'. The quantified results were normalized to those of GADPH using the $2^{-\Delta\Delta Cq}$ method (26).

Western blot analysis. Nuclear and cytoplasmic proteins of tissue samples were extracted using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. The total protein samples from HUVECs treated with different concentrations of NaHS (0, 50, 100 or 200 μmol/l), or transfected with siNrf2, siHIF-1α or si-NC were extracted using RIPA Lysis buffer containing a protease inhibitor cocktail (Roche Applied Science, Madison, WI, USA). The concentra-

tion of protein was measured using a bicinchoninic acid protein assay kit. Subsequently, 15 μg of protein/sample was subjected to 12% SDS-PAGE and separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk [dissolved in PBS containing 0.1% Tween-20 (PBST)] for 1 h at room temperature. Then, the membranes were incubated at 4°C overnight with the following primary antibodies: Rabbit monoclonal anti-Nrf2 (1:2,000; cat. no. ab62352); mouse monoclonal anti-HO-1 (1:1,000; cat. no. ab13248); mouse monoclonal anti-HIF-1α (1:2,000; cat. no. ab16066); rabbit polyclonal anti-VEGF (1:2,000; cat. no. ab32152); rabbit polyclonal anti-Histone H3 (1:800; cat. no. ab61251); mouse monoclonal anti-GAPDH (1:2,000; cat. no. ab8245) (all from Abcam, Cambridge, UK). Following washing with PBST thrice, the membranes were incubated with the goat anti-rabbit IgG H&L (1:2,000; cat. no. ab6702) or goat anti-mouse IgG H&L (1:5,000; cat. no. ab6708) secondary antibodies (both from Abcam) for 1.5 h at room temperature. The protein band signals were detected using BeyoECL Plus [ultra-sensitive electrochemical luminescence (ECL) kit; Beyotime Institute of Biotechnology] and an automatic chemiluminescence analyzer (Tanon-5200; Tanon Science and Technology Co., Ltd., Shanghai, China). Gray values of bands were quantified using ImageJ software (version 1.42; National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH or Histone H3.

Immunofluorescence. HUVECs were divided into five groups: Control, NaHS treated, NaHS treated + siRNA-NC, siNrf2 transfected and NaHS treated + siNrf2. After 48 h, ~$1x10^5$ HUVECs cultured on slides were fixed in 4% paraformaldehyde for 10 min at room temperature, treated with 0.5% Triton X-100 at 37°C for 15 min and blocked with 1% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Subsequently, the cells were incubated with the mouse monoclonal anti-HIF-1α (1:200; cat. no. ab16066; Abcam) and rabbit monoclonal anti-Nrf2 (1:300; cat. no. ab62352; Abcam) primary antibodies at room temperature for 1 h. Next, the slides were incubated with the goat anti-mouse IgG H&L (Alexa Fluor 488) (1:1,000; cat. no. ab150113; Abcam) and goat anti-rabbit IgG H&L (Alexa Fluor 647) (1:1,000; cat. no. ab150079; Abcam) fluorescein-conjugated secondary antibodies. The fluorescence signals were detected using an inverted fluorescence microscope at a magnification of x200.

Tube formation assay. HUVECs were divided into five groups: Control, NaHS treated, NaHS treated + siRNA-NC, NaHS treated + siHIF-1α and VEGF treated. Matrigel (BD Biosciences; Becton, Dickinson and Company, San Jose, CA, USA) was diluted with FBS-free and growth factor-free M200 medium at a ratio of 1:1. Then, 50 μl of the mixture was placed into 96-well plates and incubated at 37°C for 30 min. Subsequently, a cell suspension containing $1x10^4$ HUVECs were added into the 96-well plates coated with Matrigel. Next, 50 ng/ml of recombinant human VEGF (Gibco; Thermo Fisher Scientific, Inc.) was added to the medium as the positive control group (the VEGF group). Following culturing for 12 h, tube formation was observed under a light microscope at a magnification x100 and total tube area was evaluated using ImageJ software (v.1.42).
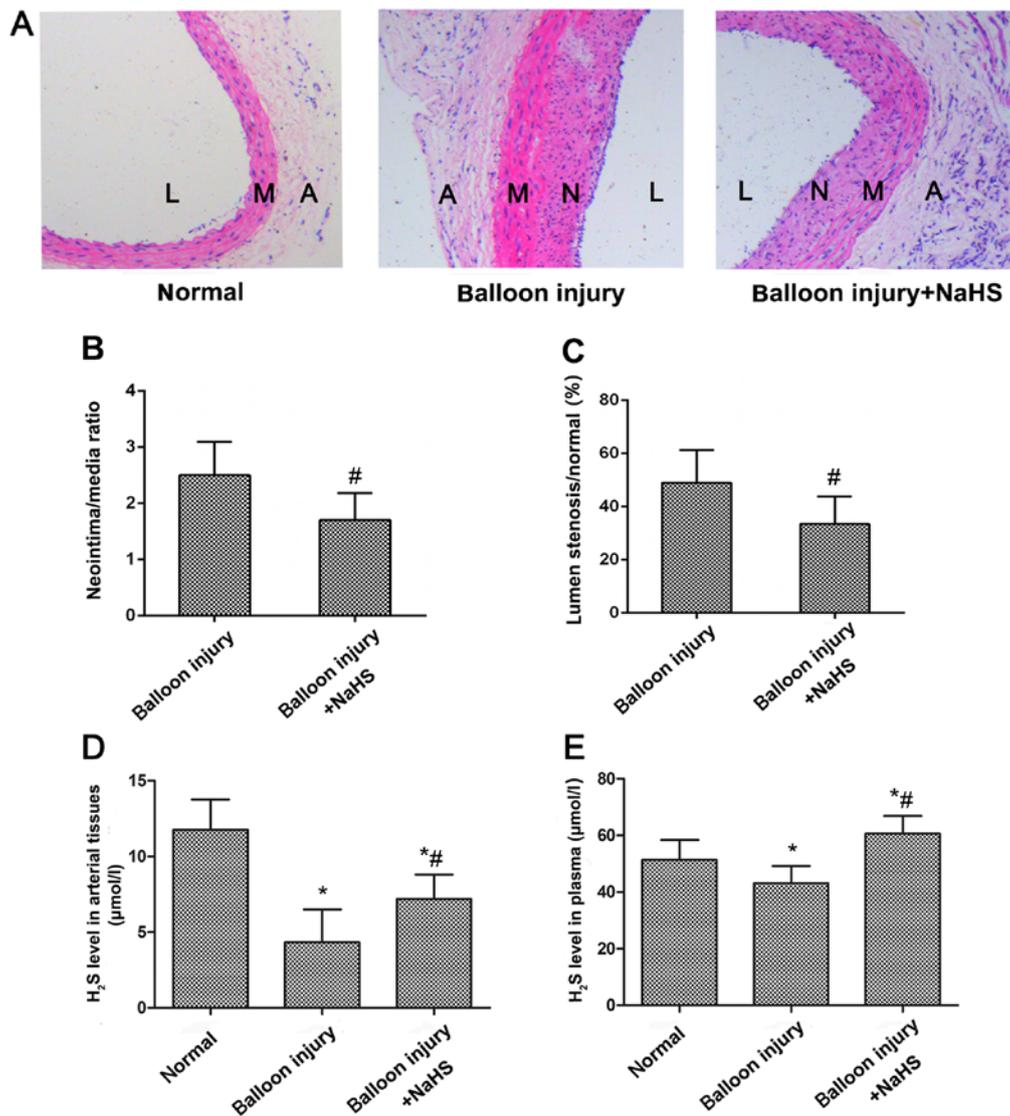
Figure 1. Effects of NaHS treatment on neointimal hyperplasia and H2S production. (A) Histological changes in the carotid artery were observed following balloon injury or NaHS treatment using haematoxylin and eosin staining (magnification, x100). Quantification of (B) neointima/media ratio and (C) % of luminal stenosis. Increase in H2S production in (D) carotid artery tissues and (E) plasma were detected following treatment with NaHS. $^*P<0.05$ vs. the normal group; $^#P<0.05$ vs. the model group. A, adventitia; M, media; N, neointima; L, lumen; NaHS, sodium hydrosulfide H2S, hydrogen sulfide.

Wound scratch assay. HUVECs were divided into six groups: Control, NaHS treated, siNrf2, NaHS treated + siNrf2, siHIF-1α, NaHS treated + siHIF-1α. HVSMCs were divided into four groups: Control, 50 μmol/l NaHS treated, 100 μmol/l NaHS treated and 200 μmol/l NaHS treated. A cell suspension containing $2x10^5$ HUVECs or $2x10^5$ HVSMCs was added into 6-well plates and cultured for 12 h. Subsequently, the medium was replaced with fresh medium containing 2% FBS and 1 μg/ml mitomycin C to suppress cell proliferation for 12 h. Wounds were made to the cell monolayer using 10-μl pipette tips, and the detached cells were removed following washing with PBS heated to 37˚C. The cells were further cultured, and wound closure area was monitored for 8 h. The migrative rate was evaluated using ImageJ software (v.1.42).

Statistical analysis. The data in the present study are expressed as the mean ± standard deviation. Differences among multiple groups were evaluated using one-way analysis of variance followed by the Tukey's post hoc test and differences between two groups were evaluated using the Student's t-test using the statistical software SPSS v.19.0 (IBM, Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Increased H₂S production prevents neointimal hyperplasia in SD rats with carotid artery restenosis. A restenosis model was established to explore whether H₂S was able to inhibit carotid artery restenosis. As shown in Fig. 1A, neointimal hyperplasia was evident in the carotid arteries of the model group rats following balloon catheter injury. However, neointimal hyperplasia was reduced following NaHS treatment compared with the model group, as demonstrated by the significant reduction in the luminal stenosis ratio, and the ratio between the neointima and media areas (Fig. 1B and C). Measurement of H₂S production in the arterial tissues and the plasma indicated that restenosis significantly reduced H₂S expression

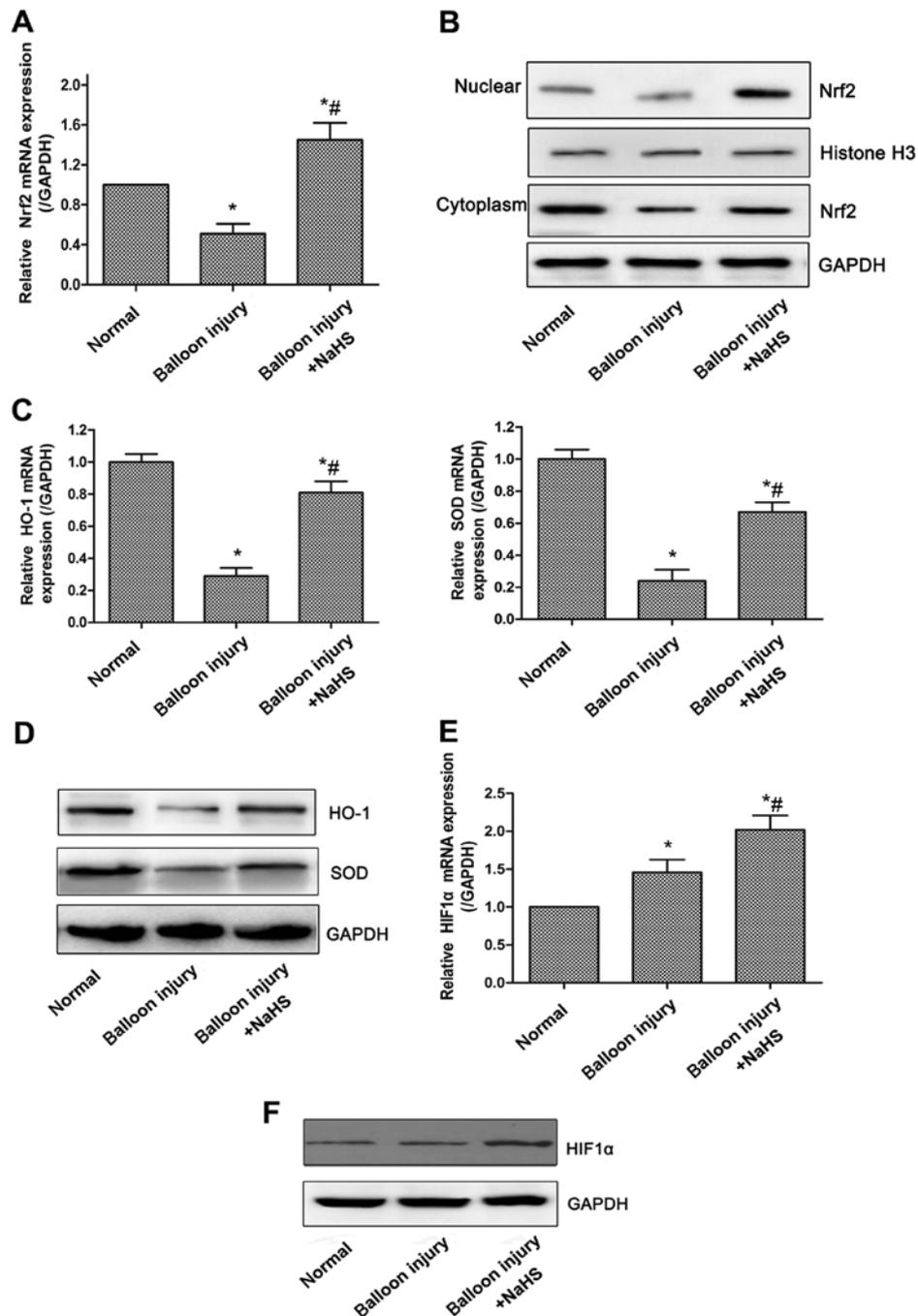Figure 2. Effects of NaHS-induced H2S production on the expression of Nrf2/HIF-1α pathway-associated proteins in carotid artery tissues. The transcriptional and protein levels of proteins were detected by RT-qPCR and western blot analysis, respectively. (A) Upregulation of Nrf2 mRNA expression in carotid artery tissues following NaHS treatment compared with model rats. (B) Nuclear and cytoplasm levels of Nrf2 protein demonstrating nuclear accumulation following treatment with NaHS. NaHS-induced increase (C) mRNA and (D) protein expression of HO-1 and SOD in carotid artery tissues. NaHS-induced increase (E) mRNA and (F) protein expression of HIF-1α. Representative data from three independent experiments are presented. *P<0.05 vs. the normal group; #P<0.05 vs. the model group. NaHS, sodium hydrosulfide; Nrf2, nuclear factor-E2-related factor 2; HIF-1α, hypoxia-inducible factor-1α; HO-1, heme oxygenase-1; SOD, superoxide dismutase; GAPDH, glyceraldehyde-phosphate dehydrogenase.

levels compared with the sham control group (Fig. 1D and E). However, following treatment with NaHS for 4 weeks, H$_2$S levels in the arterial tissues and the plasma in the treatment group were significantly increased compared with the model group.

Increased H$_2$S production enhances antioxidative effects through Nrf2/HIF-1α signaling pathway activation in vivo. Oxidative stress is associated with the progression of reste-

nosis following percutaneous coronary intervention (27), and the Nrf2 signaling system has been regarded as perhaps the most important cellular defense against oxidative stress (28). Initially, the mRNA and protein expression levels of Nrf2 were examined, and it was revealed that the total mRNA expression of Nrf2 was significantly reduced in the model group compared with the sham group, whereas compared with the model group, significantly increased expression levels of Nrf2 were observed in rats treated with NaHS (Fig. 2A). Nrf2
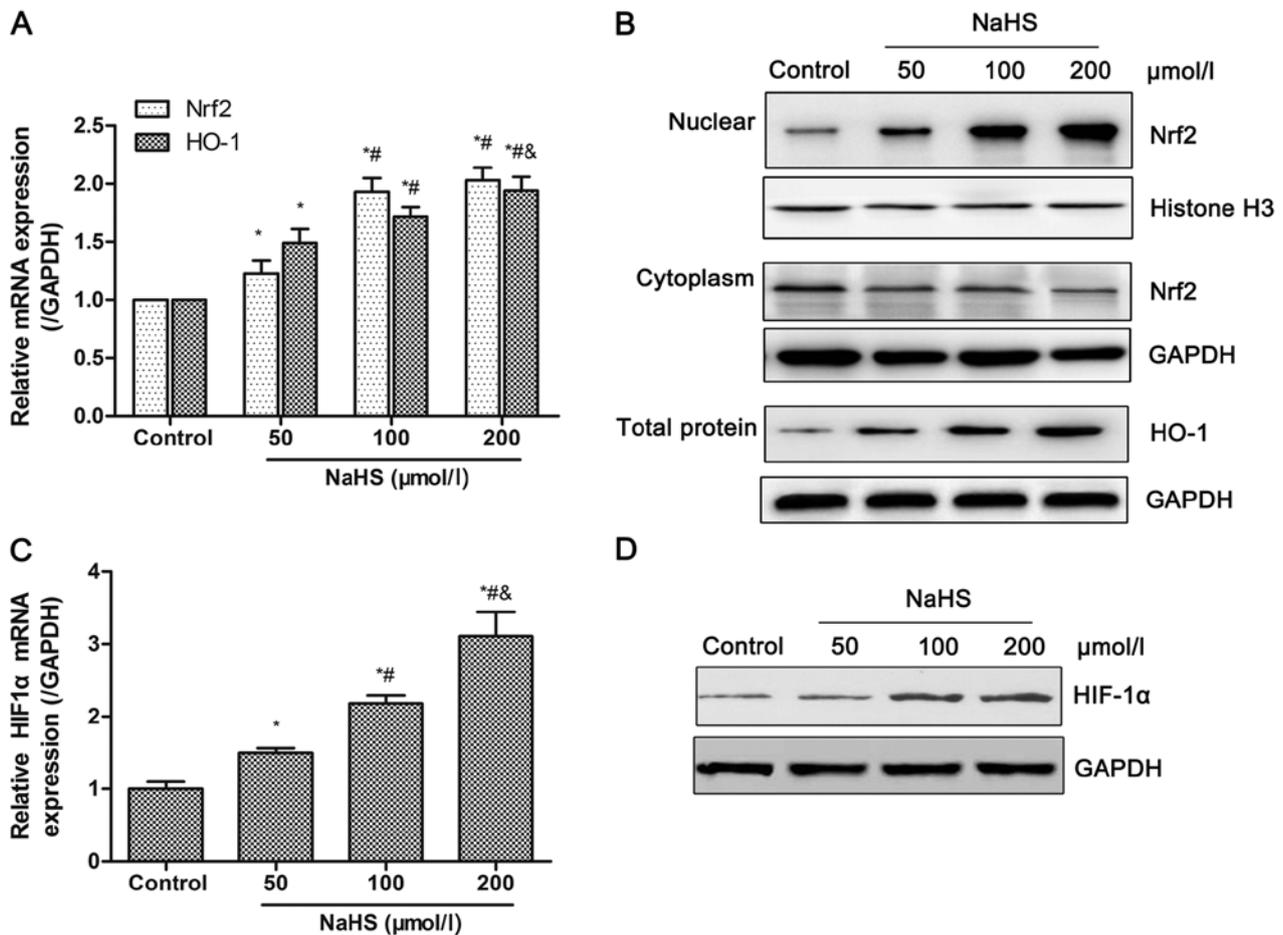
Figure 3. Effects of NaHS on the activation of the Nrf2/HIF-1α signaling pathway in HUVECs. HUVECs were treated with different dosage of NaHS (0, 50, 100, 200 μmol/l) for 48 h. Then, the transcriptional and protein levels of Nrf2, HO-1 and HIF-1α were detected by RT-qPCR and western blot analysis, respectively. (A and B) Treatment with NaHS increased (A) mRNA levels, and (B) nuclear accumulation of Nrf2 and expression of HO-1. Following treatment with NaHS, (C) mRNA and (D) protein expression levels of HIF-1α increased. Representative data from three independent experiments are presented. *P<0.05 vs. the control group; #P<0.05 vs. the 50 μmol/l NaHS treated group; &P<0.05 vs. the 100 μmol/l NaHS treated group. NaHS, sodium hydrosulfide; Nrf2, nuclear factor-E2-related factor 2; HIF-1α, hypoxia-inducible factor-1α; HO-1, heme oxygenase-1; GAPDH, glyceraldehyde-phosphate dehydrogenase.

expression in the nucleoprotein and plasmocin of the arterial tissues demonstrated similar changes (Fig. 2B). The expression levels of HO-1 and the antioxidative enzyme superoxide dismutase (SOD), two of the downstream genes of the Nrf2 signaling pathway, were also significantly decreased in the model group, whereas NaHS significantly upregulated HO-1 and SOD expression (Fig. 2C and D). Furthermore, HIF-1α appeared to be involved in the oxidative stress response, as compared with the model group, the expression of HIF-1α was significantly increased in the carotid artery tissues of rats in the model + NaHS group, whereas the expression of HIF-1α was increased slightly in the model group compared with the sham group (Fig. 2E and F).

H₂S donor promotes the activation of the Nrf2/HIF-1α signaling pathway in HUVECs. Oxidative stress leads to endothelial dysfunctions, which are a key factor for intimal hyperplasia (29). In order to assess the effects and its mechanisms of H₂S on oxidative stress, the expression of Nrf2 and HO-1 was detected in HUVECs following treatment with different dosage of NaHS (0, 50, 100 or 200 μmol/l). As shown in Fig. 3A, the transcriptional levels of total Nrf2 and HO-1 increased in a dose-dependent manner. The nuclear

protein level of Nrf2 and total protein level of HO-1 also demonstrated an increasing trend, while the cytoplasmic protein level of Nrf2 reduced with increased concentrations of NaHS (Fig. 3B). HIF-1α mRNA levels significantly increased in a dose-dependent manner, which was also confirmed at the protein level (Fig. 3C and D). These data indicated that the increased H₂S production, induced by NaHS, promoted activation of the Nrf2/HIF-1α signaling pathway in HUVECs. The comparison among groups demonstrated significant differences between the NaHS and control groups, whereas no significant differences were observed for Nrf2 mRNA expression and HIF-1α protein expression between the 100 and 200 μmol/l NaHS groups. Thus, HUVECs was treated with 100 μmol/l NaHS in the subsequent experiments.

H₂S donor-induced HIF-1α expression is dependent on the Nrf2 signaling pathway in HUVECs. In order to explore the association between HIF-1α and Nrf2, Nrf2 expression was inhibited using siNrf2. As shown in Fig. 4A and B, NaHS treatment significantly increased Nrf2 mRNA expression and markedly increased Nrf2 protein expression compared with the control group. Similarly, the mRNA and protein expression levels of downstream HO-1 and HIF-1α increased
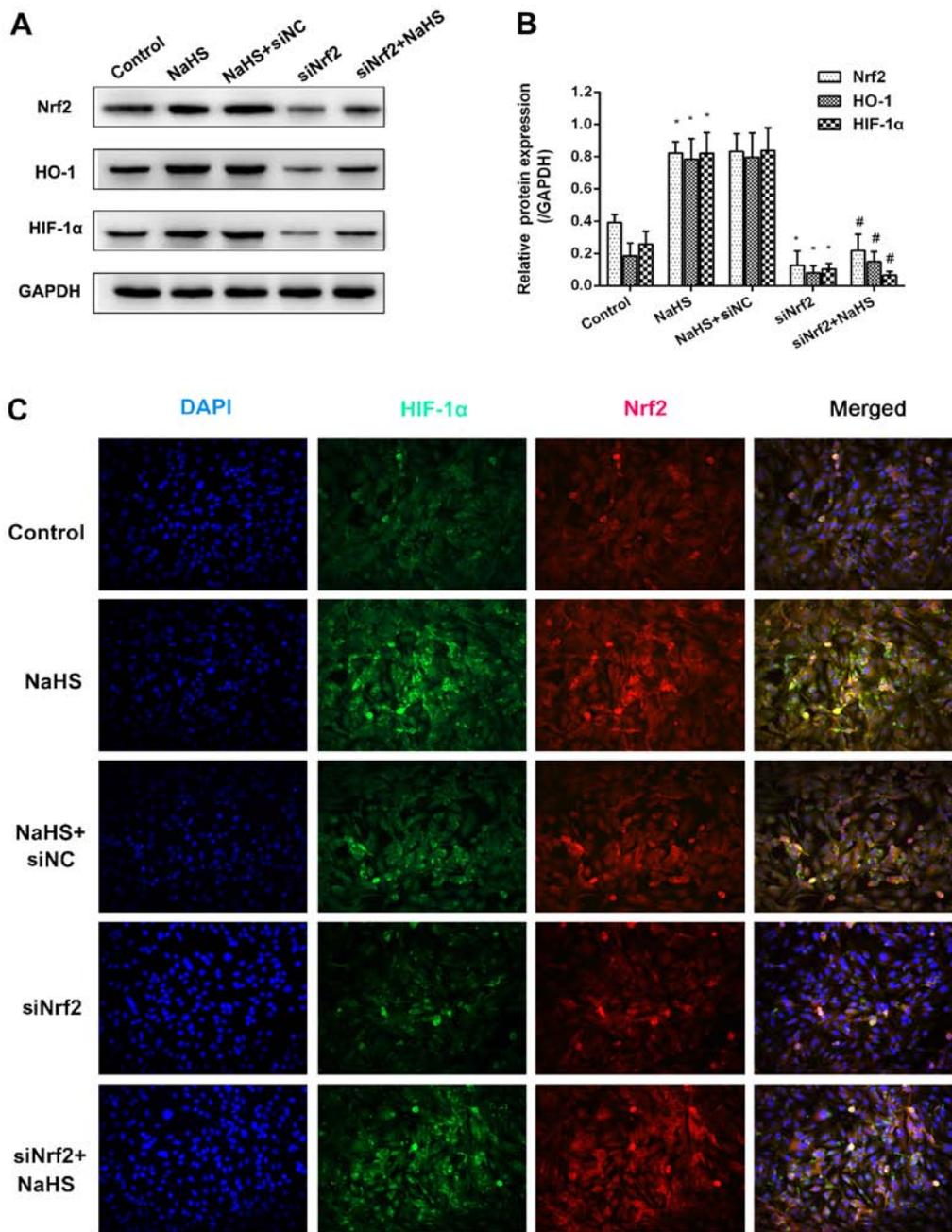
Figure 4. NaHS-induced HIF-1α expression is dependent on the Nrf2 signaling pathway. (A) Representative blot and (B) quantification of Nrf2, HO-1 and HIF-1α protein expression following treatment with NaHS and siNrf2. (C) The co-expression of NrF2 and HIF-1α in HUVECs following treatment with NaHS and siNrf2 using immunofluorescence. Representative data from three independent experiments are presented. *P<0.05 vs. the control group; #P<0.05 vs. the NaHS+siNC treated group. si, small interfering RNA.

following treatment with NaHS. The use of siNrf2 resulted in a significant reduction in all three mRNA levels compared with the control group. However, the effects of NaHS on the expression of HO-1 and HIF-1α were not observed when Nrf2 expression was suppressed using siNrf2 when compared with the siNrf2 alone group. Furthermore, the results of immunofluorescence revealed that the fluorescence signal of Nrf2 and HIF-1α evidently increased in the NaHS group, whereas the fluorescence signals of the two were markedly attenuated in the siNrf2+NaHS group compared with the NaHS group (Fig. 4C). These suggested that the H₂S-induced HIF-1α accumulation was dependent on the expression of Nrf2.

H₂S donor increases VEGF expression and tube formation via Nrf2/HIF-1α activation in HUVECs. VEGF is a well-known pro-angiogenic factor. The mRNA and protein expressions of VEGF in HUVECs were examined by RT-qPCR and western blot analysis. As shown in Fig. 5, the mRNA and protein expression levels of VEGF were significantly increased in the NaHS group. However, inhibition of Nrf2 or HIF-1α expression significantly suppressed the increased VEGF expression induced by NaHS treatment. Thus, NaHS-induced VEGF expression was dependent on the activation of the Nrf2/HIF-1α signaling pathway in HUVECs. Furthermore, the tube formation assay demonstrated that compared with the control group, NaHS or VEGF treatment significantly
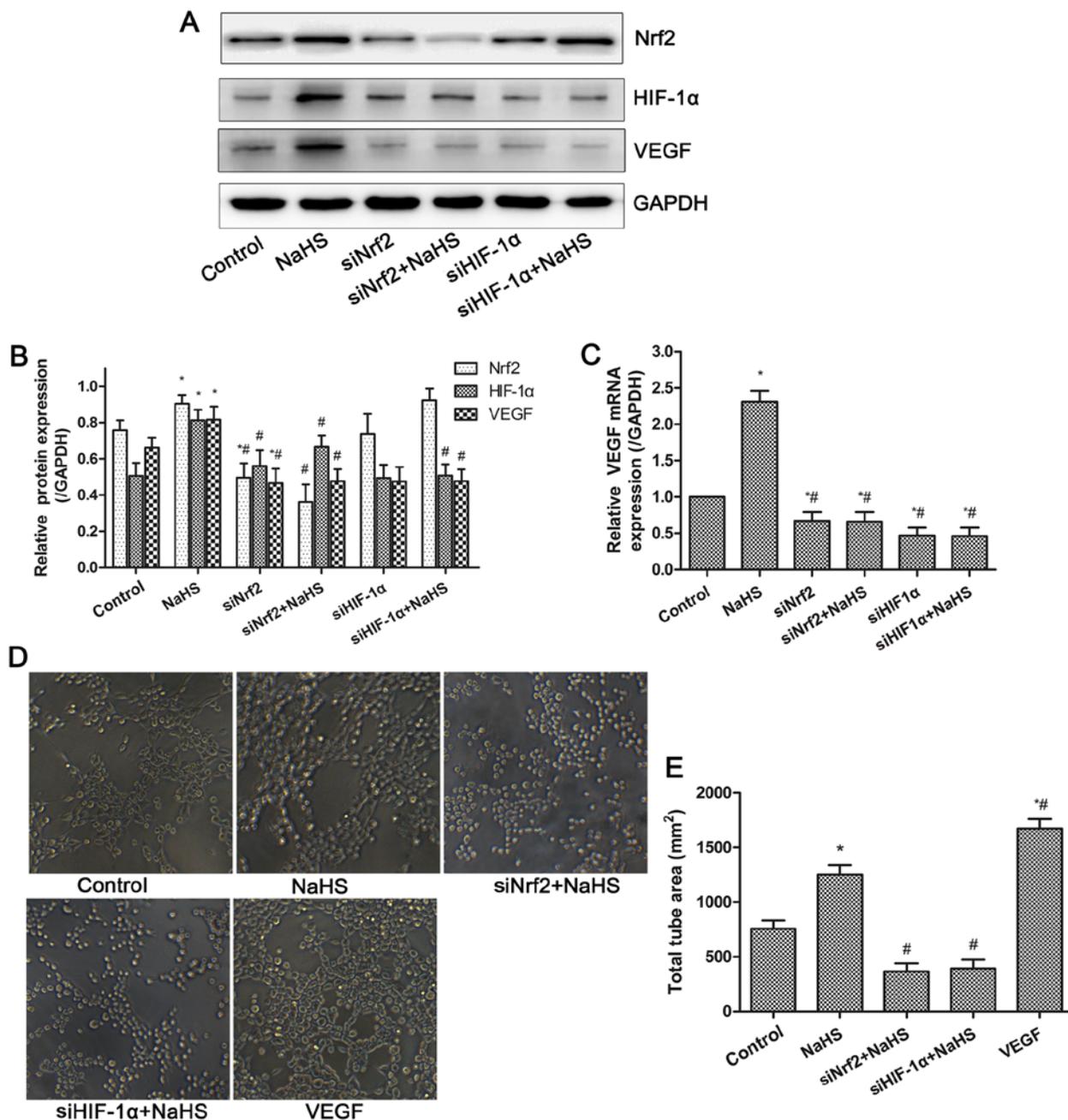
Figure 5. Effects of NaHS on VEGF expression and tube formation in HUVECs. (A) Representative blot and (B) quantification of Nrf2, HIF-1α and VEGF protein expression following treatment with NaHS and siNrf2/siHIF-1α. (C) Decreased mRNA expression of VEGF following the treatment with NaHS and siNrf2/siHIF-1α, demonstrating NaHS-induced VEGF expression was dependent on the Nrf2/HIF-1α signaling pathway. (D) Representative image and (E) quantification of NaHS-induced tube formation, demonstrating the inhibition of tube formation by siNrf2/siHIF-1α. Representative data from three independent experiments are presented. *P<0.05 vs. the control group; #P<0.05 vs. the NaHS treated group.

promoted tube formation of HUVECs, whereas this effect was inhibited following the suppression of Nrf2 and HIF-1α expression. These results indicated that NaHS treatment increased VEGF expression and tube formation via activation of Nrf2/HIF-1α in HUVECs.

H₂S donor induces cell migration via Nrf2/HIF-1α activation in HUVECs but inhibits HVSMC proliferation and migration. Cell migration of vascular endothelial cells to impaired region is an essential process for the repair of damaged blood vessels. The cell migratory abilities of HUVECs and HVSMCs were examined using a wound scratch migration assay. The results

demonstrated that NaHS treatment accelerated cell migration of HUVECs, meanwhile cell migration ability was inhibited when Nrf2 or HIF-1α expression levels were suppressed by siNrf2 and siHIF-1α, respectively (Fig. 6A and B). These results suggested that NaHS treatment induced cell migration by activating the Nrf2/HIF-1α signaling pathway in HUVECs. For the HVSMC proliferation assay, as shown in Fig. 6C, NaHS inhibited HVSMC proliferation in a concentration-dependent manner. Similarly, this phenomenon was observed on the migratory ability of HVSMCs (Fig. 6D and E), suggesting that NaHS treatment suppressed HVSMC migration and proliferation.
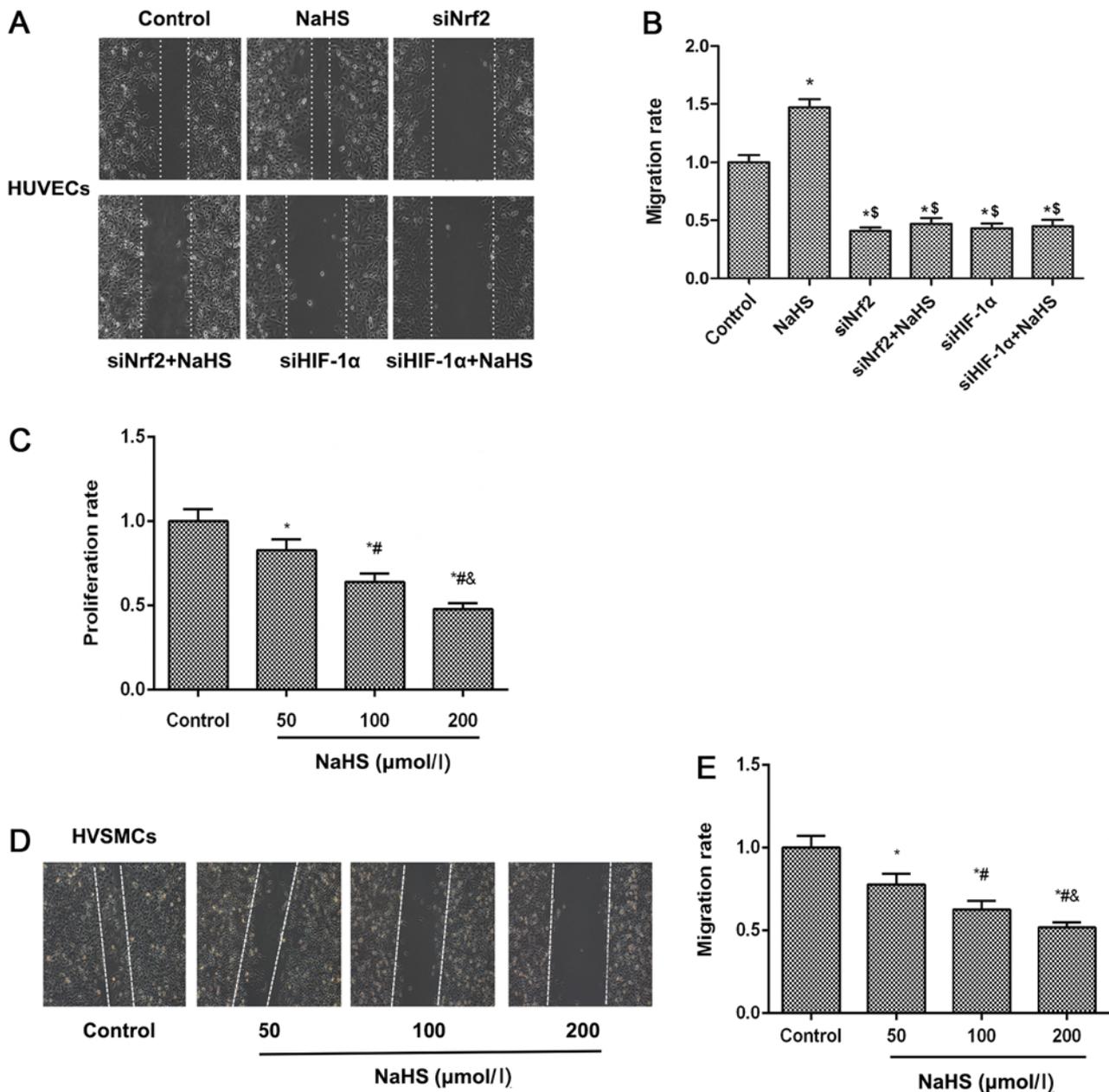
Figure 6. Effects of NaHS on cell migration and proliferation in HUVECs and HVSMCs. HUVEC migration was detected following treatment with NaHS and siNrf2/siHIF-1α using a (A) wound scratch assay and the (B) migratory rate was calculated. (C) The HVSMC proliferation rates were calculated following treatment with different concentrations of NaHS using an MTT assay. (D) Representative image and (E) quantification of HVSMC migration rates as detected using a wound scratch assay. Representative data from three independent experiments are presented. *P<0.05 vs. the control group; $^{\$}$P<0.05 vs. the NaHS treated group; $^{\#}$P<0.05 vs. the 50 μmol/l NaHS treated group; $^{\&}$P<0.05 vs. the 100 μmol/l NaHS treated group. HUEVCs, human umbilical vein endothelial cells; HVSMCs, human vascular smooth muscle cells; si, small interfering; NaHS, sodium hydrosulfide; Nrf2, nuclear factor-E2-related factor 2; HIF-1α, hypoxia-inducible factor-1α.

Discussion

It is well known that intimal hyperplasia induced by endothelial dysfunction and excessive VSMC proliferation is the central feature associated with the development of luminal stenosis diseases. During the treatment of these diseases, endovascular interventional therapies, including balloon catheter angioplasty, may induce exfoliation of endothelial cells, which triggers platelet activation and aggregation on vascular walls, leading to endothelial dysfunction (19). In response to these events, VSMCs switch to a proliferative phenotype. HVSMCs migrate through the endothelium layer and continue to grow

on the inner surface of vessels, resulting in neointimal tissue formation. Accumulating evidence indicates that H$_2$S diminishes the progression of intimal hyperplasia and suppresses restenosis following balloon angioplasty (20,30,31). In present study, a balloon injury model was used to investigate the molecular mechanisms underlying the anti-restenosis effects of H$_2$S. Consistent with the hypothesis, the contents of H$_2$S in the plasma and arterial tissues were increased in the balloon injury model following NaHS (a donor of H$_2$S) treatment, whereas intimal hyperplasia was suppressed. Previously, using a rabbit model with atherosclerotic-like lesions, it was demonstrated that treatment with NaHS significantly inhib-

ited arterial restenosis by reducing the intimal area and the intima/media ratio, accompanied by reduced SMC proliferation and elevated SMC apoptosis in the neointima (32). These results are similar to those obtained in the present study, and thereby indicate that H$_2$S derived from exogenous NaHS is effective in treating experimentally induced vascular stenosis.

Furthermore, with respect to the pathogenesis of restenosis, increased ROS production has been observed to occur at an early stage following angioplasty (33). ROS are considered to serve a role as universal regulators in vascular biology, including in endothelial and VSMC homeostasis, the inflammatory response, vascular remodeling, and endothelial dysfunction (24). Increased ROS production may trigger increased oxidative stress, resulting in vascular damage by accelerating endothelial dysfunction and macrophage activation (33). Oxidative stress may aggravate endothelial dysfunction through a variety of pathways, including oxidation of low-density lipoproteins, upregulation of adhesion molecules and monocyte chemoattractant protein 1 chemokines, activation of matrix metalloproteinases, and inactivation of nitric oxide. In turn, these cytokines and growth factors stimulate VSMC proliferation and extracellular matrix remodeling (33). In addition to direct interaction, increased ROS or oxidative stress promotes VSMC proliferation and migration, eventually leading to the initiation and development of restenosis following PTA (34,35).

Within cells, a variety of physiological antioxidant defense mechanisms have evolved to ameliorate oxidative stress-induced damage. Activation of the Nrf2 signal pathway serves an important role in antioxidant effects through promoting the binding of AREs and upregulation of antioxidant genes. Nrf2, a well-known Cap-N-Collar transcription factor, is essential for ARE-mediated transcription, including that of NADPH quinone oxidoreductase 1 (NQO1) and HO-1 (36). Previous studies have indicated that Nrf2 protects against tissue fibrosis, diabetic nephropathy, and non-alcoholic fatty liver, presumably through an enhancement of cellular antioxidant capacity, such as via increased expression of NQO1 and HO-1 (37-39). *In vitro* experiments have indicated that the transcriptional activity and nuclear localization of Nrf2 are inhibited in various ROS-mediated cell damage models involving HUVECs and human coronary artery endothelial cells, accompanied by increases in cell apoptosis (40). Furthermore, several studies have revealed that overexpression of Nrf2 prevents neointimal hyperplasia by inhibiting the proliferation of VSMCs following vascular injury through HO-1-dependent antioxidant and anti-inflammatory effects (41,42). The results obtained in present study indicate that the mRNA levels of Nrf2 and its nuclear accumulation are markedly decreased in rats with restenosis, and that the mRNA and protein levels of HO-1 and SOD are also reduced. Increasing evidence has indicated that activation of the Nrf2 signal pathway suppresses neointimal hyperplasia by increasing the expression of antioxidant genes, including HO-1 (43,44). Other studies have demonstrated that Nrf2 may be involved in the antioxidant activity of H$_2$S during H$_2$S-mediated cardioprotection (22). As one of the well-known target genes stimulated by Nrf2, the by-products of HO-1 have been reported to inhibit proliferation and induce apoptosis of VSMCs (45). In the present study, it was revealed NaHS treatment significantly prevented neointimal hyperplasia in rats

with restenosis through increasing H$_2$S levels and the nuclear accumulation of Nrf2 protein. Furthermore, on the basis of its effects on HUVEC migration through increasing Nrf2 levels, NaHS treatment is also effective at inhibiting the proliferation and migration of human VSMCs. A previous *in vitro* experiment reported that exogenous H$_2$S inhibits VSMC proliferation in a hyperglycemic state via modulation of mitochondrial fusion-fission (46).

ROS production is involved in the regulation of VEGF and HIF-1α expression, and angiogenesis (47). Abnormal activation of the HIF-1α signaling pathway stimulates the upregulation of VEGF expression, which promotes angiogenesis (48). The results of the current study revealed that NaHS treatment increased the expression of HIF-1α and VEGF, whereas inhibition of Nrf2 or HIF-1α expression significantly suppressed VEGF expression, and decreased the tube formation ability of HUVECs. These results suggest that the Nrf2/HIF-1α signaling pathway is involved in NaHS-induced VEGF expression. In a follicle-stimulating hormone (FSH)-induced ovarian epithelial cancer cell (OEC) model, it was previously reported that FSH induces ROS production and activation of Nrf2 signaling, whereas the elimination of ROS or knockdown of Nrf2 blocks FSH-induced VEGF expression (49). In addition, the knockdown of Nrf2 has been revealed to impair HIF-1α signaling activation, indicating that ROS and the aberrant expression of Nrf2/HIF-1α serve important roles in FSH-induced angiogenesis in OECs (49). The findings of a further study indicated that inhibition of Nrf2 expression restrains angiogenesis in colon cancer through suppression of the HIF-1α signaling pathway (50), indicating that the HIF-1α signaling pathway is regulated by Nrf2. Additionally, it has been reported that H$_2$S induces angiogenesis in endothelial cells and promotes damage repair (18). The proliferation and migration of endothelial cells also contribute to damage repair. The current data also indicated that NaHS treatment promoted the tube formation and migration of HUVECs *in vitro*, whereas the knockdown of Nrf2 or HIF-1α attenuated NaHS-induced tube formation and migration in HUVECs.

In summary, the findings of the current study indicate that NaHS-derived H$_2$S production prevented neointimal hyperplasia in a restenosis rat model, and promoted tube formation and migration of HUVECs to accelerate damage repair. These results also demonstrate that these protective effects may be mediated via the activation of the Nrf2/HIF-1α signaling pathway. Furthermore, it has been suggested that the regulation of H$_2$S production through the administration of H$_2$S donors or targeting of Nrf2/HIF-1α signaling pathway may be potential strategies to prevent the development of cardiovascular narrowing diseases, including restenosis.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

KL and WW conceived of the study and drafted the manuscript together. AX designed the study and performed the statistical analysis. YC participated in the study design and constructed the balloon dilatation restenosis model. XC and YL performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments involving animal treatment were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Ethics Committee of Union Hospital, affiliated to the Huazhong University of Science and Technology (approval no. TJ-A20161216).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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