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The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation

Wen-Jie Cai [a], Ming-Jie Wang [a], Philip Keith Moore [b], Hui-Ming Jin [a], Tai Yao [a], Yi-Chun Zhu [a,*]

[a] *Department of Physiology and Pathophysiology, Fudan University Shanghai Medical College, Shanghai, China*
[b] *Cardiovascular Biology Research Group, Department of Pharmacology, National University of Singapore, Singapore*

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Abstract

Objective: Hydrogen sulfide (H_2S) has been reported to be a gasotransmitter which regulates cardiovascular homeostasis. The present study aims to examine the hypothesis that hydrogen sulfide is able to promote angiogenesis.
Methods: Angiogenesis was assessed using *in vitro* parameters (*i.e.* endothelial cell proliferation, adhesion, transwell migration assay, scratched wound healing and formation of tube-like structure) and *in vivo* by assessing neovascularization in mice. Phosphorylation of Akt was measured using Western blot analysis.
Results: Exogenously administered NaHS (H_2S donor) concentration-dependently (10–20 μmol/l) increased cell growth, migration, scratched wound healing and tube-like structure formation in cultured endothelial cells. These effects of NaHS on endothelial wound healing and tube-like structure formation were prevented by either the phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 (5 μmol/l) or transfection of a dominant-negative mutant of Akt. NaHS increased Akt phosphorylation and this effect was also blocked by either LY 294002 or wortmannin (25 nmol/l). NaHS did not significantly alter the levels of vascular endothelial growth factor, mRNA expression of fibroblast growth factor and angiopoietin-1, or nitric oxide metabolites. NaHS treatment (10 and 50 μmol kg^{-1} day^{-1}) significantly promoted neovascularization *in vivo* in mice.
Conclusion: The present study reports a novel proangiogenic role of H_2S which is dependent on activation of Akt.
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Keywords: Angiogenesis; Endothelial cells; Migration

This article is referred to in the Editorial by I.E. Hoefer (pages 1–2) in this issue.

1. Introduction

Hydrogen sulfide (H_2S) is endogenously generated from cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) [1]. CBS is highly expressed in the brain [2], whilst CSE is most concentrated in the vasculature [3]. In recent years, accumulating evidence has suggested that H_2S plays a pivotal role in cardiovascular regulation [4,5]. Intravenous bolus injection of H_2S (in the form of NaHS — a water soluble H_2S donor) transiently decreased blood pressure in rats by 12–30 mm Hg [6,7]. H_2S has been further shown to dilate rat aortic tissues by opening K_{ATP} channels in vascular smooth muscle cells [6]. In addition to the regulation of vascular tone, H_2S has been reported to induce apoptosis of cultured human aortic smooth muscle cells [8]. In spontaneously hypertensive rats, there was a decrease in CSE mRNA

* Corresponding author. Department of Physiology and Pathophysiology Fudan University Shanghai Medical College 138 Yi Xue Yuan Road Shanghai, 200032, China. Tel.: +86 21 5423 7098; fax: +86 21 5423 7098.
E-mail address: yczhu@shmu.edu.cn (Y.-C. Zhu).

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expression, CSE activity and plasma H_2S levels, while exogenous administration of NaHS attenuated the development of hypertension [9]. In isolated perfused rat hearts, exogenous administration of NaHS significantly decreased the duration and severity of ischemia/reperfusion-induced arrhythmias and increased the viability of cardiomyocytes [10].

On the other hand, chronic ischemia may induce angiogenesis which may in turn ameliorate blood supply of the ischemic tissue [11]. For example, an acute, permanent occlusion of the coronary artery usually results in myocardial infarction, however, in some cases suffering from a slow progress of coronary artery occlusion, chronic ischemia has been reported to stimulate angiogenesis around the ischemic region and in certain cases the ischemic tissues can even survive when the supplying coronary artery has been completely occluded. Therefore, exploration of novel approaches to stimulate angiogenesis may potentially lead to better treatment for ischemic disease.

To date, there is no information about the potential role of H_2S in angiogenesis. That H_2S is able to protect against cardiac ischemia [10] raises the possibility that H_2S may be able to regulate the process of angiogenesis.

Angiogenesis involves several sequential phases during which endothelial cells play a major role. Sprout formation is initiated with the release of proteolytic enzymes from endothelial cells to degrade surrounding basement membrane, followed by endothelial cell proliferation and migration. Finally, the migrating cells form tube-like structures [12]. Therefore, the present study aimed to investigate the role of H_2S on endothelial cell proliferation, migration and tube formation in a series of *in vitro* and *in vivo* experiments. Additionally, the intracellular signaling pathways involved in the proangiogenic effect of H_2S were also examined.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and lipofectamine 2000 were from GIBCO-BRL (USA). Antibodies against ERK, p38, Akt were purchased from Cell Signaling Technology (USA). Antibodies against survivin, CD31, and integrin α1, α2, αv, β1, β3 and β5 were from Santa Cruz Biotechnology (CA, USA). LY 294002 and wortmannin were obtained from Calbiochem (USA). Growth factor reduced Matrigel and cell culture insert system were from BD Biosciences (Bedford, MA, USA). Collagen I, hydroxyurea and NaHS were from Sigma (St Louis, MO, USA). H_2S was administered in the form of NaHS which has been well established as a reliable donor of H_2S [6,13,14]. When NaHS was dissolved in saline, about one-third of H_2S exists as undissociated gas, and the remaining two-thirds as HS anion [1]. The concentrations of NaHS selected in the present study did not affect the pH values of the culture medium and the sodium ion content in NaHS is negligible. We also used H_2S solution in scratch wound healing and tube formation assays. The H_2S stock solution was freshly prepared by bubbling distilled water with pure H_2S gas (Summit Specialty Gases, Tianjin, China) to acquire saturated H_2S solution. However precise amount of H_2S generated under these conditions is not clear as highlighted by others [15] and accordingly H_2S was administered in the form of NaHS in the present experiments.

2.2. Cell culture and transfection of the dominant-negative mutant of Akt

RF/6A endothelial cells were maintained in DMEM containing 10% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator. The hemagglutinin (HA)-tagged dominant-negative (DN) (kinase-inactive mutant Myr-Akt-K179M) Akt [16] is a kind gift from Dr. Jin Q. Cheng (Department of Pathology and Interdisciplinary Oncology, University of South Florida College of Medicine, H. Lee Moffitt Cancer Center, Tampa, Florida). pcDNA3 vector containing the DN-Akt cDNA or its control vector was transfected into RF/6A cells using lipofectamine 2000 and incubated for 24 h in DMEM with 10% FBS.

2.3. Cell proliferation assay

RF/6A cells were cultured in 96-well tissue culture plates (1×10^4 cells/well) with 10% FBS for 24 h. Then the serum-free medium was used and cells were exposed to different concentrations of NaHS for another 24 h. Cell viability and proliferation were measured respectively by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [17] and 5-bromo-2′-deoxyuridine (BrdU) (Roche Diagnostics, USA) incorporation assays.

2.4. Cell adhesion assay

Twelve-well tissue culture plates were coated with collagen I (2 mg/ml). Serum-starved endothelial cells were plated at 5×10^4 cells/well with test substances or vehicle and incubated at 37 °C for 30 min. The culture medium was subsequently removed and the cells were gently washed twice using warm PBS. Adherent cells were fixed with 4% paraformaldehyde in PBS and stained with hematoxylin. Five random fields from each of quadruple wells were counted for each experimental condition.

2.5. Cell migration assay

Two types of migration assays were used. The transwell migration assay was performed as described before [18] with some modifications. Briefly, RF/6A cells were seeded at a density of 4.5×10^4 cells/well into the 12-well insert, both upper and lower reservoirs containing serum-free growth media. Test substances or vehicle was added to the lower reservoirs. Cells were subsequently allowed to migrate

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across a collagen I-coated polycarbonate filter (8 μm pore size) for 6 h at 37 °C. Non-migrated cells were removed from the top side of the filter by scraping gently and washing twice in PBS. Migrated cells on the bottom side of the filter were subsequently fixed with 4% paraformaldehyde in PBS for 20 min. The filter was then washed with dH_2O and stained with Harris Hematoxylin solution for 4 min, followed by two further washes in dH_2O. Migrated cells were manually counted using a light microscope. Cells in five random fields for each migration well were counted to determine the average number of migrated cells.

For the scratch wound migration assay, confluent RF/6A cell sheets were starved for 24 h before starting the experiments. Hydroxyurea (5mmol/l) was used to prevent cell proliferation [19]. Confluent cell monolayer was then scraped with a yellow pipette tip to generate scratch wounds and rinsed twice with growth medium. Cells were photographed immediately and 24 h after the scratch with a Nikon digital camera. The wound area was then measured to determine cell migration.

2.6. Angiogenesis in vitro: tube formation on Matrigel

Twenty-four-well plates were coated with 300 μl Matrigel and incubated at 37 °C for 30 min to allow the Matrigel to solidify. RF/6A cells which had been pretreated for 1 h with either vehicle or inhibitors were plated at a density of 5×10^4 cells/well with test substances or vehicle and incubated at 37 °C for 16 h. The cells were then photographed using a Nikon digital camera. Tube formation was quantified by measuring the length of capillary structures using the software NIH ImageJ. Tube length was assessed by drawing a line along each tube and measuring the length of the line in pixels. Branching points were manually counted. Five randomly selected fields of view were photographed in each well. The average of five fields was taken as the value for each sample [20].

2.7. Angiogenesis in vivo: Matrigel plug assay

C57 BL/6 female mice were anesthetized by isoflurane inhalation. Mice were injected subcutaneously with 500 μl Matrigel with Matrigel containing basic fibroblast growth factor (bFGF) (100 ng/ml) acting as a positive control. Different concentrations of NaHS were injected intraperitoneally every day for 7 days. Mice were euthanized after 7 days. The Matrigel plugs were recovered by dissection. Angiogenesis was assessed by hemoglobin measurement or morphological analysis. The hemoglobin concentrations were determined by the tetramethylbenzidine (TMB) method [21], and the values were normalized by the weight of the plugs. Five plugs in each group were paraffin embedded for histological examination. Sections (5 μm) were stained with hematoxylin–eosin. For immunostaining, sections were incubated with rabbit polyclonal anti-CD31 antibody overnight at 4 °C, visualized by using ABC kits (Santa Cruz Biotechnology, CA, USA) with diaminobenzidine as substrate. The investigation conformed to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH) of the United States and was approved by the Ethic Committee of Experimental Research, Fudan University Shanghai Medical College.

2.8. Western immunoblotting

The cells were starved for 24 h and then treated for 30 min with LY 294002 (5 μmol/l), wortmannin (25 nmol/l) or vehicle (DMSO), followed by stimulation with NaHS at 10 μmol/l for 30 min. In another set of experiments, cells were treated either with 10 μmol/l NaHS for different duration (0–120 min) or with increasing dose of NaHS (0–200 μmol/l) for 30 min. Cells were then lysed with 1× SDS sample buffer (62.5mmol/l Tris–HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50mmol/l DTT). Protein concentration was determined by BCA reagent. 30 μg protein was separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride (PVDF) membrane. After blocking with TBST containing 5% milk for 1 h, the membrane was incubated with antibodies against ERK, p38 MAPK, Akt, survivin, integrins or β-actin overnight at 4 °C. After incubation in horseradish peroxidase-conjugated secondary antibody for 1 h, SuperSignal West Pico Chemiluminescent Substrate was used for detection.

2.9. Measurement of plasma H_2S concentration

Plasma H_2S concentrations were measured in C57 BL/6 female mice before or at 5 min, 30 min, 1 h, 3 h, 6 h or 24 h after intraperitoneal injection of NaHS as described elsewhere [9] with some modifications. Briefly, 0.1 ml plasma was added into a test tube containing 0.125 ml 1% zinc acetate and 0.15 ml distilled water. Then 0.067 ml 20mM *N*,*N*-dimethyl-phenylenediamine dihydrochloride in 7.2M HCl was added. This was followed by addition of 0.067 ml 30mM $FeCl_3$ in 1.2M HCl. After the protein in plasma was removed by adding 0.125 ml 10% trichloroacetic acid, the absorbance of the resulting solution was measured with a spectrometer at a wave length of 670nm. The H_2S concentration in the solution was calculated according to the calibration curve of the standard H_2S solution.

2.10. Real-time PCR analysis for bFGF and Ang-1 mRNA expression

The cells were starved for 24 h and then treated with 10 μmol/l NaHS for 6 h. Total RNA was prepared using RNArose Reagent (Watson Biotech, Shanghai, China) according to manufacturer's instructions. cDNA was generated from 2 μg total RNA using a cDNA synthesis kit (Biocolor Biotech, Shanghai, China). Real-time PCR was performed using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Richmond, USA) in a total volume of 25 μl reaction mixture containing 2 μl cDNA, 12.5 μl 2× SYBR Green PCR Master Mix (Toyobo, Japan), and 2 μl of each

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primer (5 μM). To minimize and control the sample variations, mRNA expression of the target gene was normalized relative to the expression of the housekeeping gene GAPDH. Three-step real-time PCR of denaturing, annealing and extension reactions was performed for 40 cycles of 20s at 95 °C, 30s at 58 °C and 30s at 72 °C (for bFGF, angiopoietin-1 (Ang-1) and GAPDH). For the bFGF gene, the forward primer was 5′-AGAAGAGAGAG-GAGTTGTGT-3′ and the reverse primer was 5′-TTGCCCAGTTCGTTTCAGTG-3′. For the Ang-1 gene, the forward primer was 5′-GAGGTCAGAAGAAAGGAG-CAAG-3′ the reverse primer was 5′-GAGTCAGAAT-GGCAGCGAGG-3′. For the GAPDH gene, the forward primer was 5′-ACGGATTTGGTCGTATTGGG-3′ and the reverse primer was 5′-CTCGCTCCTGGAAGATGGTG-3′.

2.11. Measurement of VEGF and NO synthesis

RF/6A cells were stimulated with different concentrations of NaHS for 24 h and then the supernatant was collected. Vascular endothelial growth factor (VEGF) levels were measured by ELISA using a commercially available kit (R&D Systems, MN, USA) according to the manufacturer's instruction. The generation of NO was determined by measuring the stable NO metabolites, *i.e.* total nitrites, in culture medium with a nitrite detection kit (Beyotime Biotech Inc, Jiangsu, China) as described elsewhere [22]. Briefly, 100 μl of medium was mixed with 100 μl of Griess reagent in a 96-well plate. Nitrite concentration was determined by spectrophotometry (540nm) from a standard curve (0–100 μmol/l) derived from $NaNO_2$.

2.12. Measurement of cGMP and cAMP levels

RF/6A cells were stimulated with different concentrations of NaHS for 15 min and then the cells were treated with 0.1mol/l HCl for 20 min to be lysed. cGMP and cAMP measurements were performed with enzyme immunoassay kits (Biomol, PA, USA) according to the manufacturer's instruction. The values were normalized by the protein concentration of the cell lysate.

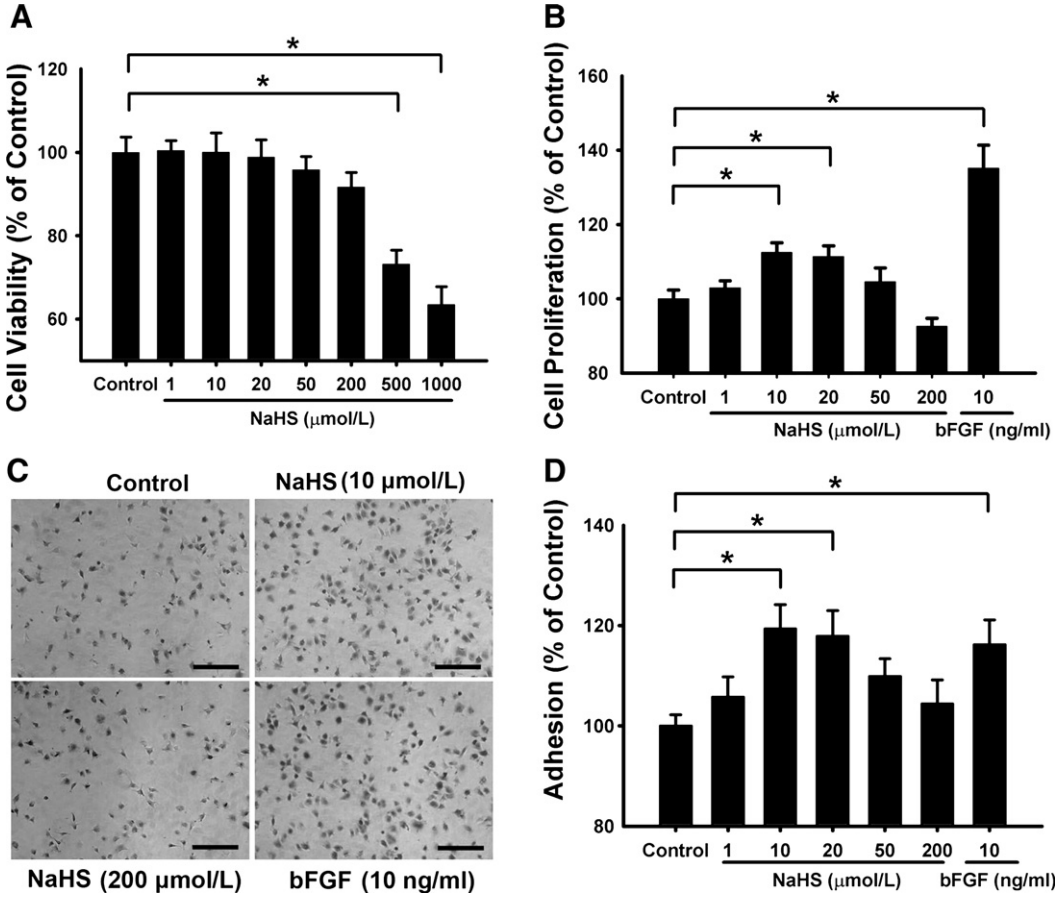

Fig. 1. Effect of H_2S on endothelial cell viability, proliferation and adhesion. Exogenous administration of H_2S was applied by giving the H_2S donor NaHS. A, Cell viability was assessed using MTT method. RF/6A endothelial cells were treated without or with various concentrations of NaHS (1–1000 μmol/l) for 24 h. Only treatment with high concentrations of NaHS (500 and 1000 μmol/l) induced significant reduction in cell viability by 26.8±3.3% and 36.5±4.3%, respectively. B, NaHS treatment promoted RF/6A endothelial cell proliferation as determined by BrdU assay. bFGF (10 ng/ml) treatment significantly promoted cell proliferation by 35.2±6.1%. C and D, At concentration of 10 and 20 μmol/l, NaHS treatment significantly increased adhesion of the endothelial cells to the culture dish. The effect of NaHS (10 and 20 μmol/l) on cell adhesion was comparable to that of bFGF (10 ng/ml). Shown are representative microscopic fields (C) and the values (D) of the endothelial cells treated without or with NaHS (1–200 μmol/l) with the bFGF (10 ng/ml)-treated group acting as a positive control. Bar=200 μm. Data represent the mean±SE of six independent experiments. *$P<0.05$.

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2.13. Statistical analysis

Results are expressed as mean ± SE. Differences between groups were analyzed by one-way ANOVA followed by *post hoc* Tukey's test where applicable. Significance was established at the $P < 0.05$ level.

3. Results

3.1. H_2S increased endothelial cell proliferation

RF/6A endothelial cells were treated without or with increasing concentrations of NaHS (1–1000 μmol/l) for

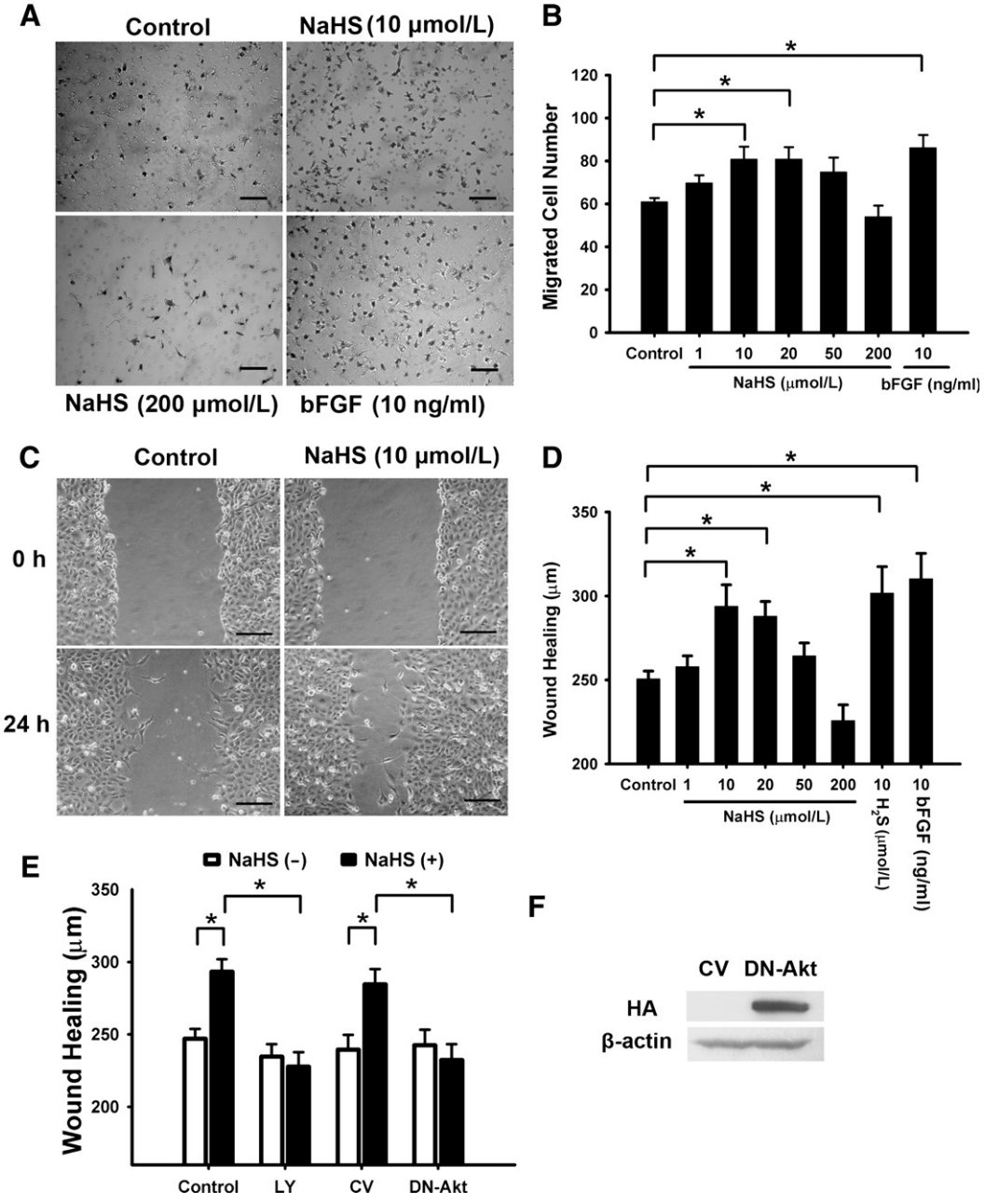

Fig. 2. H_2S promotes endothelial cell migration. A and B, Cell migration was assessed by transwell migration assay. Shown are the representative micrographs (A) and the values (B) of the cells treated with NaHS (1–200 μmol/l), bFGF (10 ng/ml) and vehicle. Treatment with NaHS (10 and 20 μmol/l) and bFGF (10 ng/ml) both increased the number of migrated cells. C, Representative micrographs of scratch wound healing assay of RF/6A endothelial cells treated with or without NaHS (10 μmol/l) at 0 and 24 h after treatment. D, Statistical analysis of scratch wound healing assay of RF/6A endothelial cells treated with various concentrations of NaHS (1–200 μmol/l), H_2S (10 μmol/l) and bFGF (10 ng/ml). E, NaHS-induced promotion of wound healing of RF/6A endothelial cells was prevented by either LY 294002 (5 μmol/l) or transfection of DN-Akt. F, Significant HA expression was detected in the cells transfected with DN-Akt after 24 h suggesting a successful transfection and expression of DN-Akt. Data represent the mean ± SE of six independent experiments. Bar = 200 μm. *$P < 0.05$. LY, LY 294002; CV, control vector; DN-Akt, the dominant-negative mutant of Akt.

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24 h. Only treatment with high concentrations of NaHS (500 and 1000 μmol/l) resulted in a significant reduction in cell viability (26.8±3.3% and 36.5±4.3%, respectively) as assessed using the MTT method (Fig. 1A). Therefore, NaHS was applied as a H_2S donor at concentrations lower than 500 μmol/l in all subsequent experiments. NaHS (10 and 20 μmol/l) stimulated RF/6A endothelial cell growth by 12.5±2.5% and 11.4±2.9% as determined with BrdU assay ($P < 0.05$; Fig. 1B). bFGF induced a more pronounced growth-stimulating effect by 35.2±6.1% (Fig. 1B). These

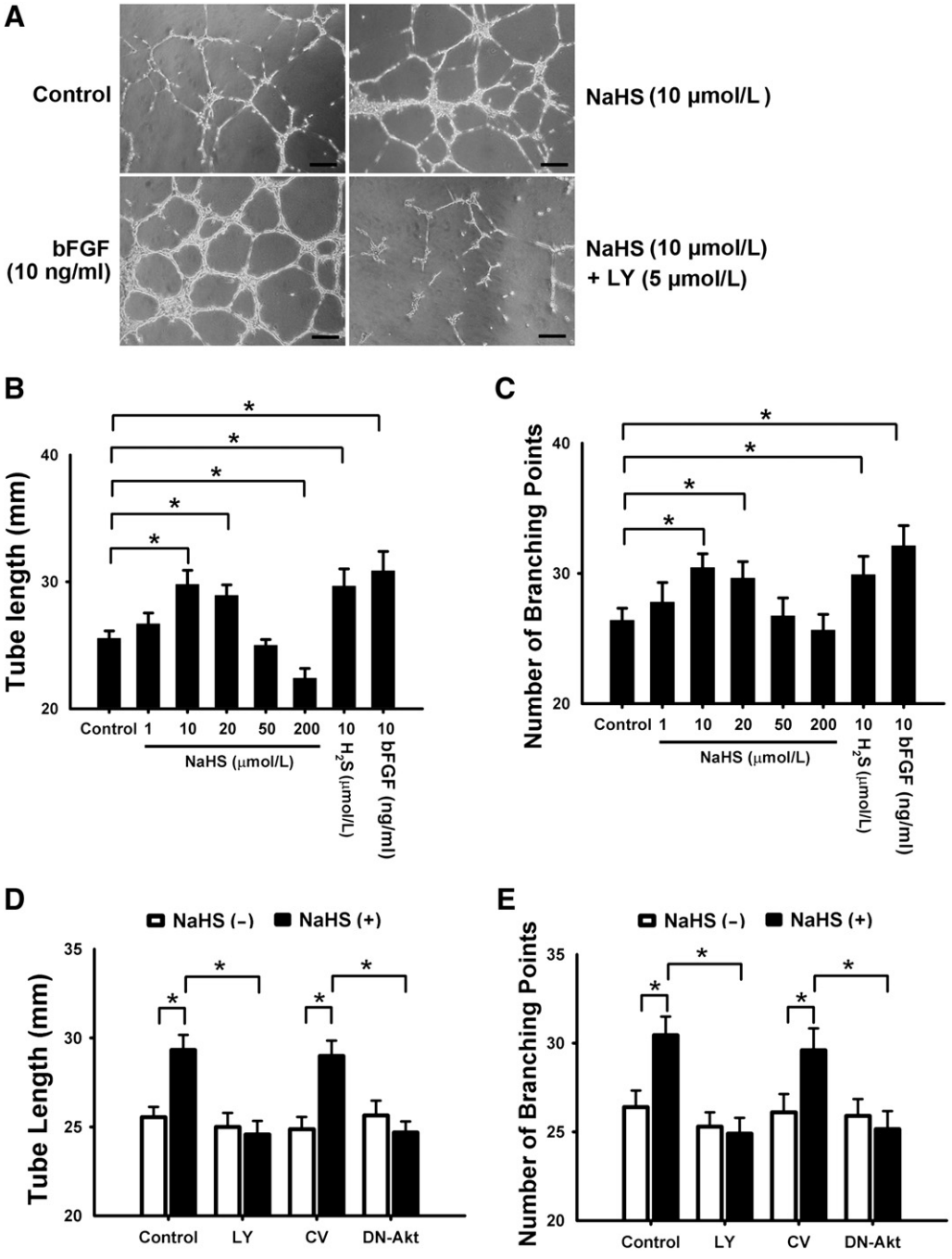

Fig. 3. H_2S promotes microvessel tube formation in three-dimensional culture. RF/6A endothelial cells were starved for 24 h and then plated on Matrigel. Tube formation was determined 16 h after plating. A, Representative micrographs of tube formation of RF/6A endothelial cells treated with NaHS (10 μmol/l) in the presence or absence of LY 294002 (5 μmol/l). B and C, Statistical analysis of tube length (B) and branching points (C) of the RF/6A endothelial cells treated with various concentrations of NaHS (1–200 μmol/l), H_2S (10 μmol/l) and bFGF (10 ng/ml). D and E, NaHS-induced increase in tube length (D) and branching points (E) was prevented by either LY 2940002 (5 μmol/l) or transfection of DN-Akt. Data represent the mean±SE of five independent experiments. Each experiment was performed in duplicate. Bar=200 μm. *$P<0.05$. LY, LY 2940002; CV, control vector; DN-Akt, the dominant-negative mutant of Akt.

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data suggest that NaHS treatment exert a direct growth-stimulating effect on endothelial cells. In addition to cell proliferation, adhesion and migration are also essential events for endothelial cells to form vessel lumen during angiogenesis. Therefore, the effects of NaHS on endothelial cell adhesion and migration were further assessed.

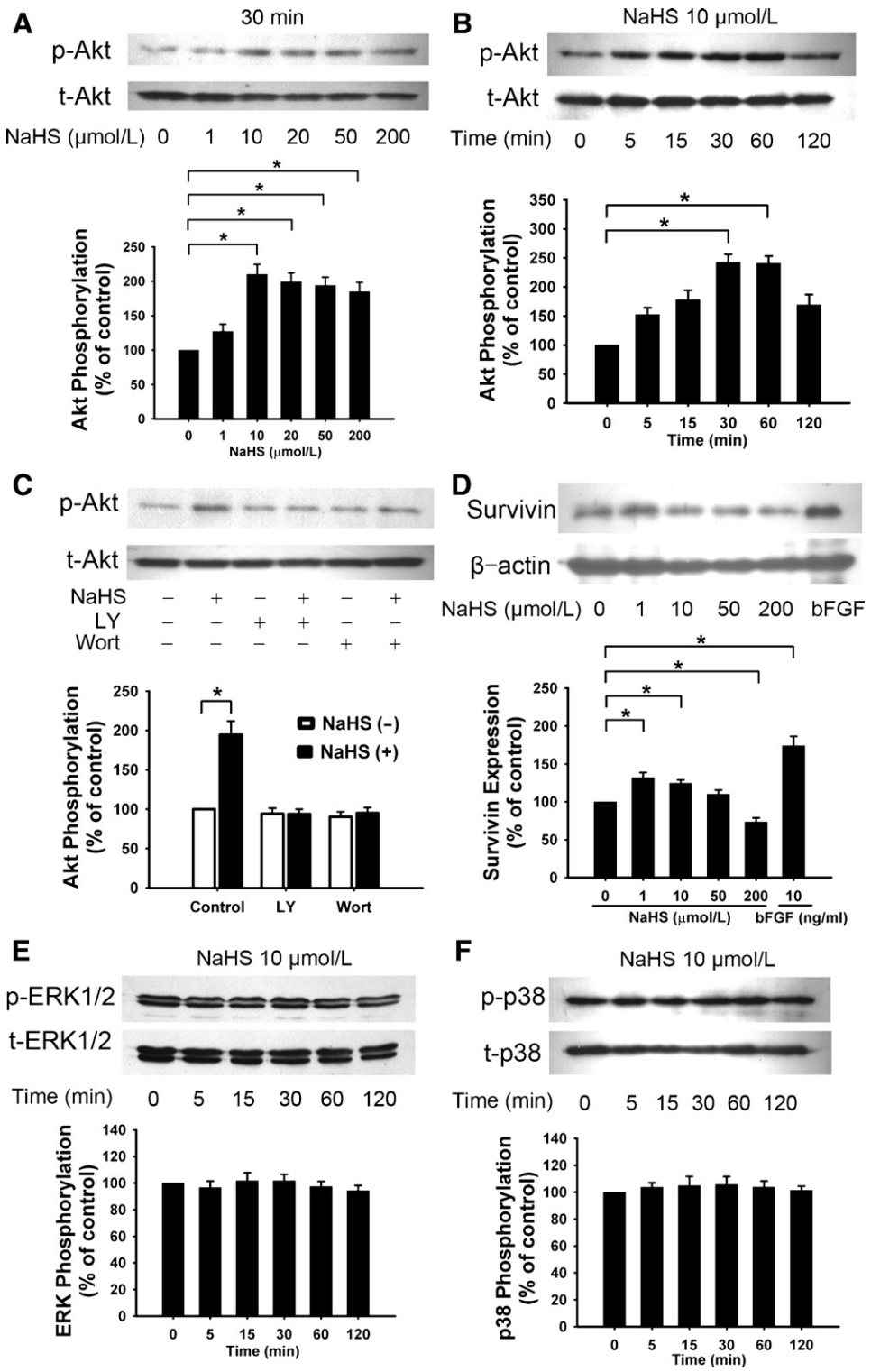

Fig. 4. H_2S increases Akt phosphorylation and survivin levels without inducing phosphorylation of ERK and p38 in endothelial cells. A, Effects of 30 min treatment with various concentrations of NaHS (1–200 μmol/l) on Akt phosphorylation. B, Time course of Akt phosphorylation induced by NaHS (10 μmol/l). C, NaHS-induced Akt phosphorylation was prevented by either wortmannin (25 nmol/l) or LY 294002 (5 μmol/l). D, Effects of various concentrations of NaHS (1–200 μmol/l) and bFGF (10 ng/ml) on survivin expression 24 h after stimulation. E and F, NaHS (10 μmol/l) did not induce phosphorylation of ERK1/2 (E) and p38 (F) within 2 h after treatment. Data represent the mean±SE of six independent experiments. *$P<0.05$.

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3.2. H_2S increased endothelial cell adhesion

RF/6A endothelial cells were treated with or without increasing concentrations of NaHS (1–200 μmol/l) for 30 min. Cell adhesion was increased by 19.4±4.8% and 17.9±5.1% in response to NaHS (10 and 20 μmol/l) treatment, while at higher concentrations this effect was reduced. bFGF (10 ng/ml) induced a similar adhesion promoting effect (Fig. 1C and D).

3.3. H_2S promoted endothelial cell migration

For the transwell migration assay, RF/6A cells were treated with or without increasing concentrations of NaHS (1–200 μmol/l) for 6 h. NaHS (10 and 20 μmol/l) treatment significantly increased cell migration compared with vehicle-treated cells (80.8±5.9 *vs.* 61.0±1.6 and 80.9±5.5 *vs.* 61.0±1.6, respectively; $P < 0.05$; Fig. 2A and B). bFGF showed a comparable migration-promoting effect (86.1±6.0 *vs.* 61.0±1.6, $P < 0.05$; Fig. 2A and B). The effect of NaHS on endothelial cell migration was also assessed using the scratch wound healing assay. As shown in Fig. 2C and D, NaHS (10 and 20 μmol/l) accelerated wound healing of RF/6A endothelial cells compared with vehicle-treated cells (294±13 μm *vs.* 251±5 μm and 288±9 μm *vs.* 251±5 μm, respectively; $P < 0.05$). Similar promoting effect on cell migration was observed in cells treated with H_2S solution (10 μmol/l) (302±15 μm *vs.* 251±5 μm, $P < 0.05$). bFGF (10 ng/ml) treatment elicited wound healing-accelerating effect (310±15 μm *vs.* 251±5 μm, $P < 0.05$; Fig. 2D). Interestingly, the wound healing-accelerating effect of NaHS treatment was blocked by either pretreatment with LY 294002 or transfection of DN-Akt, suggesting a role of PI3K in mediating the H_2S effects (Fig. 2E). Successful transfection of DN-Akt was confirmed by western blot analysis for the HA-tag conjugated with the mutant (Fig. 2F).

3.4. H_2S promoted microvessel tube formation on Matrigel

The initial phase of angiogenesis involves organization of individual endothelial cells into a three-dimensional tube-like structure. Therefore, the effect of H_2S on tube formation was examined using RF/6A endothelial cells cultured on Matrigel. RF/6A endothelial cells were treated without or with increasing concentrations of NaHS (1–200 μmol/l). Tube-like structures appeared on Matrigel after 16 h of culture. NaHS (10 and 20 μmol/l) treatment increased microvessel tube length compared with vehicle treatment (29.8±1.1 mm *vs.* 25.5±0.6 mm and 28.9±0.8 mm *vs.* 25.5 ±0.6 mm, respectively; $P < 0.05$; Fig. 3A and B). NaHS (10 and 20 μmol/l) treatment also increased branching points (30.5±1.1 *vs.* 26.4±0.9 and 29.7±1.2 *vs.* 26.4±0.9, respectively; $P < 0.05$; Fig. 3A and C). Similar promoting effect on an increase in tube length and branching points was observed in cells treated with H_2S solution (10 μmol/l) (Fig. 3B and C). The effect of NaHS in increasing tube length and branching points was prevented by either LY 294002 (5 μmol/l) or transfection of DN-Akt, suggesting a role of PI3K and Akt in this process (Fig. 3D and E).

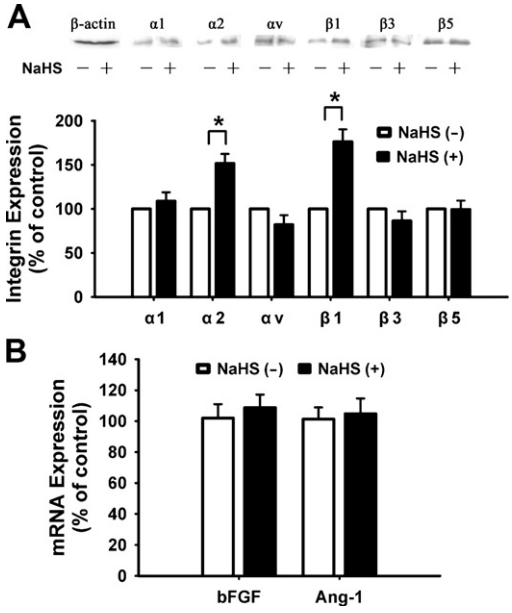

Fig. 5. Effects of H_2S on the expression of integrins, bFGF and Ang-1. A, Integrin α2 and β1 but not α1, αv, β3 or β5 were increased after NaHS treatment (10 μmol/l) for 24 h. B, NaHS (10 μmol/l) treatment for 6 h did not increase bFGF and Ang-1 mRNA expression as assessed by real-time PCR. Data represent the mean±SE of six independent experiments. *$P<0.05$.

3.5. H_2S increased Akt phosphorylation

Since the PI3K inhibitor LY 294002 blocked the pro-angiogenic effects of H_2S, the PI3K downstream effector, Akt, was examined by Western blot analysis in RF/6A endothelial cells upon H_2S stimulation. RF/6A endothelial cells were treated without or with increasing concentrations of NaHS (1–200 μmol/l) for 30 min. Akt phosphorylation was significantly increased by 100.2±9.9% and 84.3±9.3% following administration of NaHS at 10–200 μmol/l, respectively (Fig. 4A). A single dose of NaHS (10 μmol/l) induced

Table 1
H_2S had no effect on VEGF and NO metabolite levels in the culture medium of endothelial cells

	Control	NaHS (μmol/l)				
		1	10	20	50	200
VEGF (pg/ml)	10.58±1.98	10.69±2.42	10.2±1.81	10.24±0.96	9.92±0.51	9.58±1.05
NO metabolites (μmol/l)	0.38±0.05	0.38±0.03	0.35±0.04	0.37±0.07	0.33±0.05	0.35±0.05

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Table 2
H_2S had no effect on cGMP and cAMP levels in cultured endothelial cells

	Control	NaHS (μmol/l)		
		10	50	200
cGMP (pmol/mg protein)	1.21±0.46	0.85±0.35	1.17±0.45	1.17±0.54
cAMP (pmol/mg protein)	2.89±1.00	2.13±0.52	3.06±1.36	2.69±0.65

a time-dependent increase in Akt phosphorylation which peaked at 30 min and lasted till 1 h (Fig. 4B). NaHS-induced Akt phosphorylation was abolished by either wortmannin or LY 294002 suggesting that PI3K is the upstream regulator of Akt upon H_2S stimulation (FS Fig. 4C).

3.6. H_2S increased survivin and integrin α2 and β1 levels without inducing an increase in phosphorylation of ERK and p38

NaHS (1 and 10 μmol/l) treatment significantly increased survivin expression (Fig. 4D). However, administration of NaHS at a high concentration (200 μmol/l) significantly reduced survivin expression (Fig. 4D). bFGF (10 ng/ml) also induced an increase in survivin levels (Fig. 4D). In contrast, NaHS treatment (10 μmol/l) had no effect on ERK (Fig. 4E)

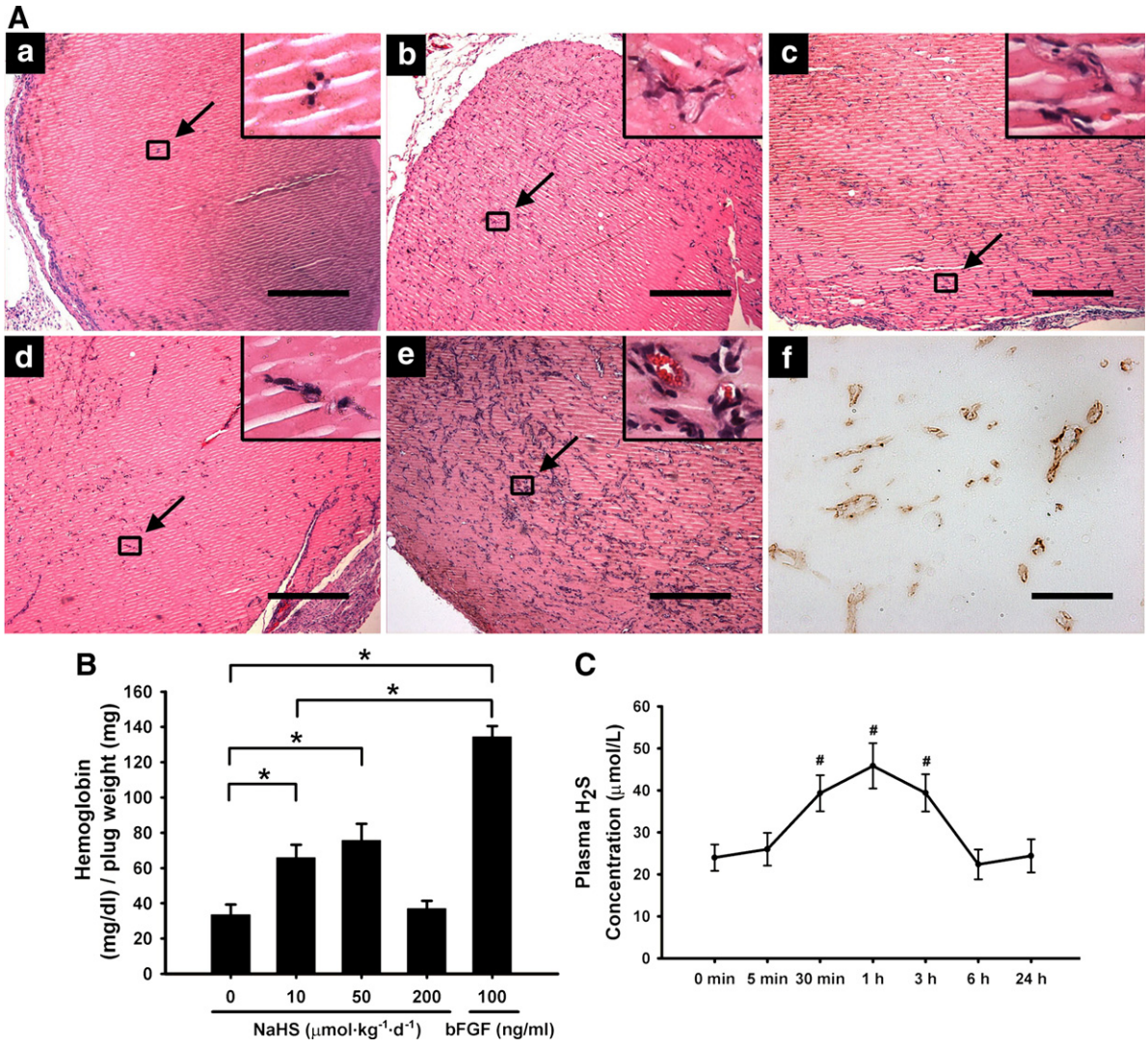

Fig. 6. H_2S treatment promotes angiogenesis *in vivo*. The effects of H_2S on *in vivo* angiogenesis were assessed using Matrigel plug assay in mice. A, Representative photomicrographs of hematoxylin–eosin stained Matrigel sections of mice treated with vehicle (a), various doses of NaHS (b, c and d for 10, 50 and 200 μmol kg^{-1} day^{-1} NaHS, respectively) and bFGF (e, 100 ng/ml in Matrigel). Inserts are higher magnifications of the areas marked in squares (arrow). Capillaries were defined as tubular structures (brown signals) stained with rabbit polyclonal anti-CD31 antibodies in Matrigel sections from the mice treated with 50 μmol kg^{-1} day^{-1} NaHS (f). B, Neovascularization in the Matrigel plugs was quantified by measuring hemoglobin content using the tetramethylbenzidine method. NaHS treatment (10 and 50 μmol kg^{-1} day^{-1}) significantly promoted neovascularization in the Matrigel plugs in mice. This effect of NaHS was less potent than that of bFGF. C, Time course of plasma H_2S concentrations in mice after an intraperitoneal injection of NaHS (100 μmol/kg). Data represent the mean±SE of five mice in each group. Bar=400 μm in Aa–e (for higher magnifications of the areas marked in squares (arrow), bar=50 μm). Bar=50 μm in Af. *$P<0.05$ in B; #$P<0.05$ *vs.* 0 min in C.

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and p38 (Fig. 4F) phosphorylation. While integrin α2 and β1 but not α1, αv, β3 or β5 were increased by NaHS treatment (10 μmol/l) (Fig. 5A).

3.7. H_2S had no effect on VEGF, NO metabolites, cGMP and cAMP levels nor on the mRNA expression of bFGF and Ang-1

Stimulation of RF/6A endothelial cells with increasing concentrations of NaHS (1–200 μmol/l) did not change the levels of VEGF and NO metabolites nitrites in the culture medium (Table 1). Neither cGMP nor cAMP levels were altered by NaHS treatment (10–200 μmol/l) (Table 2). NaHS treatment (10 μmol/l) had no effect on bFGF and Ang-1 mRNA expression in the endothelial cells (Fig. 5B).

3.8. H_2S promoted angiogenesis in vivo

The effect of H_2S on angiogenesis was assessed using an *in vivo* Matrigel plug assay in mice. The mice were treated with vehicle or various doses (10–200 μmol kg^{-1} day^{-1}) of NaHS for 7 days. Intraperitoneal injection of NaHS (100 μmol kg^{-1} day^{-1}) caused a sustained increase in plasma H_2S levels from 0.5 to 3 h after injection (Fig. 6C). There was a significant increase in cellular infiltration and neovascularization in Matrigel following administration of NaHS (10 and 50 μmol kg^{-1} day^{-1}) respectively, suggesting a proangiogenic effect of H_2S *in vivo* (Fig. 6A). This effect was not present following administration of NaHS at a high dose (200 μmol kg^{-1} day^{-1}). In Matrigel containing bFGF (100 ng/ml), there was also a significant increase in cellular infiltration and neovascularization (Fig. 6A). Neovascularization was further quantified by measuring hemoglobin content in the Matrigel plugs. Compared with vehicle treatment, hemoglobin contents were significantly increased in the mice treated with NaHS at doses of 10 and 50 μmol kg^{-1} day^{-1} (66.0±7.2 mg/dl *vs.* 33.6±5.7 mg/dl and 75.7±9.3 mg/dl *vs.* 33.6±5.7 mg/dl, respectively; $P<0.05$; Fig. 6B). Again, there was no change in hemoglobin content following administration of NaHS at a high dose (200 μmol kg^{-1} day^{-1}). bFGF (10 ng/ml) significantly increased hemoglobin contents (Fig. 6B).

4. Discussion

In addition to its effect on regulating vascular tone [6], H_2S has been shown to be involved in gene-expressing-related biological processes such as the pro-apoptotic effect on human aortic vascular smooth muscle cells [15] and human neutrophils [23], anti-proliferative effects on HEK-293 cells [24] and induction of serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells [13]. However, to date, there is no information in the literature concerning the potential role of H_2S in angiogenesis. In the present study, we show that H_2S promotes proliferation, adhesion, migration and tube-like structure formation of endothelial cells *in vitro*, as well as stimulates angiogenesis in a Matrigel plug assay *in vivo* at physiologically relevant concentrations/doses. When H_2S was given at concentrations less than 500 μmol/l (in the form of NaHS) in cultured endothelial cells, cell viability was not affected. Worthy of notice is that high concentration/dose of H_2S, *e.g.* 200 μmol/l *in vitro* or 200 μmol kg^{-1} day^{-1} *in vivo*, did not show any proangiogenic effect. The mechanisms underlying this phenomenon remain to be further investigated.

Since H_2S is endogenously generated from cysteine metabolism and its production has been shown to be decreased in ischemic myocardium [10], decreased H_2S generation may play a negative part in angiogenesis during ischemia. Therefore, identification of the proangiogenic effect of H_2S sheds some light on understanding the mechanisms of angiogenesis and indicates that exogenous administration of H_2S may be explored as a potential novel therapeutic approach in treating chronic ischemic diseases. Plasma H_2S levels have been reported to be ~50 μmol/l in rats [6], ~34 μmol/l in mice [25] and ~44 μmol/l in human [25]. The doses of NaHS (10–50 μmol kg^{-1} day^{-1}) employed *in vivo* in the present study are therefore physiologically relevant.

In the present study, NaHS treatment induced a dose and time-dependent increase in Akt phosphorylation in endothelial cells suggesting a role for Akt in H_2S-induced effects. Both the PI3K inhibitors LY 294002 and wortmannin prevented H_2S-induced Akt phosphorylation. Moreover, H_2S-induced endothelial cell migration and tube formation were also blocked by either LY 294002 or transfection of DN-Akt. These data suggest that H_2S stimulates angiogenesis by activating Akt.

Akt is well established as a pivotal intracellular signaling element of angiogenesis. Activation of the Akt by various extracellular signals has been reported to increase endothelial cell proliferation [26], migration [27] and tube formation [28] *in vitro*, and promote neovascularization [29] *in vivo*. Herein, we showed that this typical proangiogenic pathway might be upregulated by a new gas mediator H_2S. However, how does H_2S activate Akt remains to be further investigated.

The present study showed that expression of integrin α2 and β1 was upregulated by NaHS treatment suggesting a role for these adhesion molecules in H_2S-induded angiogenesis. In line with this hypothesis, integrin α1β1, α2β1, αvβ3 and αvβ5 have been shown to play a role in angiogenesis [30]. Integrin α2 and β1 are required for collagen-driven angiogenesis [31]. While integrin αvβ3 and αvβ5 mediate angiogenesis induced by bFGF and VEGF, respectively [32]. However, the exact role of such integrins in H_2S-induded angiogenesis remains to be elucidated.

To explore the probability that some proangiogenic factors might be released from endothelial cells in response to H_2S treatment and consequently induce angiogenesis *in vitro*, we measured the levels of VEGF in cultured endothelial cells stimulated with H_2S and observed that VEGF was not increased in these H_2S-treated cells. Thus, the

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present data do not suggest a role of VEGF in mediating the proangiogenic effect of H_2S.

H_2S-induced protective effect against severe metabolic inhibition in isolated rat ventricular myocytes has been reported to be mediated by NO production [33]. NO has also been shown to be proangiogenic [27] In the present study, we did not find significant change in NO metabolite levels. Thus, the present data do not suggest a role of NO in H_2S-induced angiogenesis. In addition, NO has been reported to activate K_{Ca} channels either directly or indirectly by the cGMP pathway [4]. We found here that exogenous H_2S had no effect on cGMP and cAMP levels in endothelial cells. These data do not suggest a role of cGMP and cAMP in the proangiogenic effect of H_2S.

In vascular smooth muscle cells, H_2S has been reported to increase phosphorylation of ERK and p38, and ERK activation is associated with cell apoptosis [15]. In these experiments, H_2S was administered at a rather high concentration in the form of 200–500 μmol/l NaHS. Although phosphorylation of MAPKs such as ERK or p38 has been reported to mediate the proangiogenic signals in endothelial cells [34,35], This may not be applicable to the present study, since neither ERK nor p38 was activated by H_2S at a low concentration (in the form of 10 μmol/l NaHS), at which a significant proangiogenic effect was induced.

In contrast, the side effects of H_2S treatment should be noted when this gasotransmitter is being explored to develop novel therapeutic approaches for the treatment of ischemic diseases. Cytochrome oxidase activity is decreased following exposure to ≥30 ppm (~0.9 mM) H_2S in rats [36]. Inhalation of H_2S at dosages ranging from 30 to 80 ppm (~0.9–2.4 mM) causes nasal lesions in rats [37]. Workers exposed to H_2S at concentrations of ~20 ppm (~0.6 mM) show rather diffused neurological and mental symptoms [38]. While the present study showed a proangiogenic effect of NaHS administered in mice at lower dosages of 10 and 50 μmol kg^{-1} day^{-1}.

In summary, the present study provides the first evidence of the proangiogenic effect of exogenously administered H_2S at physiologically relevant concentrations/doses. This effect is mediated by phosphorylation of Akt. The proangiogenic effect of H_2S may be explored to develop novel approaches in treating ischemic diseases.

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