의학 박사 학위논문

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지도교수 윤 병 우

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Hydrogen Sulfide Treatment Induces Angiogenesis After Cerebral Ischemia

By

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A thesis submitted in partial fulfillment of the requirement
for the degree of Doctor of Philosophy in Medicine
(Neuroscience)
In Seoul National University, Seoul, Korea

June, 2015

Doctorial committee

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Abstract

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Hydrogen sulfide (H_2S) is a potent vasodilator and regulates cardiovascular homeostasis. Furthermore, H_2S has a crucial role in ischemia-reperfusion injuries, especially of the heart, liver and kidneys. In this study, we suggest that post-treatment with hydrogen sulfide is able to recover neurological function after ischemic stroke by promoting angiogenesis. Post-treatment with H_2S augments angiogenesis at the peri-infarct area, and it significantly improves functional outcomes after 2 weeks in a rat model of transient middle cerebral artery occlusion (MCAO). H_2S promotes the phosphorylation of AKT and extracellular signal-regulated kinase (ERK) and increases the expression of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1). H_2S-treated rats showed more newly synthesized endothelial cells in the ischemic lesion (2.31-fold, $p<0.01$). H_2S-treated astrocytes increased VEGF and Ang-1 expression, and the inhibition of phosphatidylinositide 3-kinase (PI3K)/AKT signaling by LY294002 significantly reduced H_2S-induced VEGF and Ang-1 expression in astrocytes. Finally, H_2S stimulated endothelial cells migration (3.92-fold increase of wound healing assay) and tube formation (3.69-fold increase, $p<0.001$) through PI3K/AKT signaling. In conclusion, post-treatment with H_2S

promotes angiogenesis and thereby contributes to improvement of functional outcome after cerebral ischemia. Our findings strongly suggest that H_2S may be of value in regenerative recovery after stroke.

Key words: Ischemic reperfusion injury, Hydrogen sulfide, Angiogenesis

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List of Figures

Contents

Introduction

Hydrogen sulfide (H_2S) is a gaseous signaling molecule that is endogenously produced from cysteine metabolism, and its biological roles are variable and rapidly expanding. These regulatory functions include the cardiovascular, nervous and immune systems.

H_2S protects tissue from injury through several pathways. H_2S can suppress leukocyte adherence to the vascular endothelium, leukocyte extravasation and consequent formation of edema.(1) It substitutes for oxygen in driving mitochondrial respiration, thereby attenuating oxidative-stress-related tissue injury.(2) H_2S can modulate the activity of a number of transcription factors. It inhibits nuclear factor-κB, leading to a reduction of the pro-inflammatory cytokines, and increase expression of antioxidant-response elements.(3, 4) The vascular regulatory roles of H_2S have been extensively studied, including vasodilation, vascular protection and the regulation of blood pressure.(5, 6)

Stroke is the second most common cause of death in the world, and it brings about severe, long-term disability. Ischemic stroke causes neuronal death and brain injury, and it then concomitantly entails regenerative processes, such as angiogenesis and vascular remodeling, in the area adjacent to the ischemic core. Cerebral angiogenesis is critical for cerebrovascular

changes after brain injury, and it is usually associated with astrocyte and blood vessels. Vascular endothelial growth factor (VEGF) has a crucial role in angiogenesis because it regulates endothelial growth and differentiation.(7) VEGF cooperates with angiopoietin-1(Ang-1) and establishes dynamic blood vessel structure.(8) Therefore, the expression of VEGF and Ang-1 may result in angiogenesis and promote better functional outcomes after stroke.

The effects of H_2S on cerebral ischemic stroke are controversial. The inhibition of H_2S synthesis reduced the infarct volume in a cerebral ischemic model.(9) However, recent data suggest that H_2S is produced by ischemic stress to protect myocardial injury.(10, 11) In an *in vivo* model of myocardial ischemia/reperfusion (I/R) injury, the administration of H_2S at the time of reperfusion decreased the infarct size and preserved left ventricular function, implying that H_2S has a therapeutic role in cardiovascular disease.(12-14) We therefore investigated the therapeutic possibilities of H_2S for cerebral ischemic stroke. Specifically, we present a role for H_2S in angiogenesis. Here, we show that the administration of H_2S increases VEGF and angiogenic factors and subsequently promotes angiogenesis. Our findings support the therapeutic application of H_2S in cerebral ischemia.

Materials and Methods

Animals and a transient focal cerebral ischemia-reperfusion injury model

A transient focal cerebral I/R injury model was induced using the endovascular internal carotid artery suture method of Longa *et al*. with minor modifications, as previously described.(15) After inhalation anesthesia using 3% isoflurane in 30% oxygen and 70% air, the left common carotid artery (CCA) was exposed at its bifurcation using a midline cervical incision. The external carotid artery (ECA), ICA, and CCA were ligated using a 5-0 silk suture.

The CCA was then transected, and a 5-0 nylon monofilament suture (with its tip rounded by heating) was inserted into the CCA. To occlude the origins of the MCA and proximal anterior cerebral artery, the suture was advanced into the ICA for a distance of 20 mm. The suture was secured in place using a ligature, and the wound was closed. The middle cerebral artery of Sprague-Dawley male rats weighing 250 to 300 grams (KOATECH, Seoul, Korea) was reperfused 120 minutes after occlusion. Seizure events were not observed during the experiments. Rectal temperature was maintained at 37 ± 0.5 °C with the use of a thermistor-controlled heating blanket. Free access to food and water was allowed following recovery from anesthesia. During recovery,

we measured the behavior test to confirm the induction of focal cerebral ischemia.

This study was carried out according to the National Institutes of Health Guide of the Care and Use of Laboratory Animals, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital and adhered to the guidelines set by the Canadian Council for Animal Care and ARRIVE (Animal Research: Reporting In Vivo Experiments).

Material

H_2S was administered in the form of sodium hydrosulfide (NaHS, Sigma-Aldrich Co) diluted in saline. Either normal saline or NaHS (5 mg/kg) in a final volume of 1mL was injected via the intraperitoneal (IP) route, using a 32-gauge needle, from day 3 after reperfusion to day 14. The dosage of NaHS was determined based on previous studies.(11, 16) In a rat model of chronic hind limb ischemia, the therapeutic intraperitoneal (IP) administration of the H_2S donor NaHS (at 10–200μmol/kg) was tested.(16) A higher dose of NaHS was not found to be effective. In addition, higher dosage (180μmol/kg) in cerebral ischemic reperfusion injury brought about deterioration.(11, 17) Blood pressure was measured before IP injection of NaHS, 5 minutes, 30

minutes, and 60 minutes after injection.

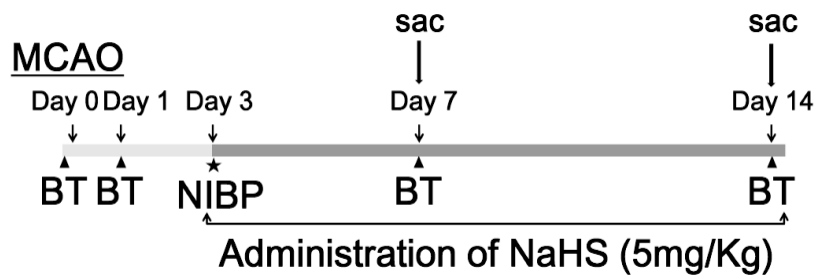


Figure 1. Scheme for post-treatment effects of H_2S in ischemic rat brain

MCAO was induced on day 0, and NaHS (5mg/kg; given by the intraperitoneal route) and BrdU were administered on days 3-14. Rats were euthanized for western blotting and immunohistochemical analysis on day 7 and for behavior tests on day 14.

BT, behavior test; NIBP, Non-invasive blood pressure; sac, the day on which the rats were euthanized; MCAO, middle cerebral artery occlusion

Cell culture and Oxygen-Glucose Deprivation

Mouse brain endothelial cells (MBECs) (bEnd.3; ATCC, CRL-2299) and astrocytes (Astrocytes Type I clone; ATCC, CRL-2541) were used for the *in vitro* experiment. The LY294002 (PI3 kinase inhibitor) was obtained from cell signaling (Boston, MA, USA), and the STAT3 inhibitor VI was from MERCK Millipore.

Astrocyte cell culture

Frozen cells were thawed by gentle agitation in a 37°C bath for approximately 2 minutes. Under strict aseptic condition, the vial contents were plated into a 75 cm^2 tissue culture flask and diluted in Dulbecco's modified eagle's medium (WELGENE Inc. Daegu, Republic of Korea) supplemented with 10% fetal bovine serum, and antibiotics (100 Units/mL penicillin, and 100 μg/mL streptomycin). We checked for microbial contamination, and also confirmed that the majority of cells attached to the bottom of the flask. The cells were incubated in a humidified incubator maintained at 37°C in a 5% CO_2 and 95% air atmosphere. The medium was changed every 3 days with a 10% fetal bovine serum media. On subculturing, the cell layer was briefly rinsed with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution, followed by addition of 1.0 to 2.0 mL of Trypsin-EDTA solution to the flask. When the cell layer began to detach, cells were aspirated by gently

pipetting. The appropriate aliquots of the cell suspension were added to new culture vessels.

Mouse brain endothelial cell culture

Frozen endothelial cells were thawed by gentle agitation in a 37°C bath for approximately 2 minutes. The contents were transferred to a centrifuge tube containing 9 mL culture medium, and span at approximately 125 x g for 5 to 7 minutes. The resuspended cell pellet was cultured using 75 cm^2 tissue culture flasks in Dulbecco's modified eagle's medium (WELGENE Inc. Daegu, Republic of Korea) supplemented with 10% fetal bovine serum, 2 mmol/L-glutamine, 100 Units/mL penicillin, and 100 μg/mL streptomycin. To avoid excessive alkalinity, the culture vessels containing the complete growth medium were placed into the incubator for at least 15 minutes to reach its normal pH (7.0 to 7.6) prior to the addition of the vial contents. After checking for microbial contamination, we also confirmed that the majority of cells were attached to the bottom of the flask. The cells were incubated in a humidified incubator maintained at 37°C in a 5% CO_2 and 95% air atmosphere. The medium was changed every 3 days with a 10% fetal bovine serum media. On subculturing, the cells were treated as described above.

Oxygen-Glucose Deprivation

The Oxygen-Glucose Deprivation (OGD) condition was performed using a glucose free media(Gibco, Grand island, NY), and was achieved by placing the cultured cells into a hypoxia chamber (VS-9000GC, Bionex, Bucheon, Korea), and flushing the chamber with 95% N_2/5% CO_2 for 1 hour, then sealing the chamber for the duration of the experiment. OGD was stopped by replacing the glucose free media with normal media and returning the cells to the normoxic incubator for 24 hours before sampling.

Real-time Polymerase Chain Reaction

MBECs and astrocytes were harvested, and total RNA was isolated from treated cells with TRIzol reagent(Invitrogen, La Jolla, CA, USA).We obtained Taqman probes for VEGF, eNOS, Ang-1, Ang-2, Tie-2 and GAPDH RNA from Applied Biosystems. Quantitative polymerase chain reaction (PCR) was performed using the TaqMan real-time PCR method on an ABI 7500 Real Time PCR System (Perkin-Elmer Applied Biosystems, Lincoln, CA, USA). The following PCR conditions were used: after the initial activation of uracyl-N-glycosylase at 50°C for 2 minutes, AmpliTaq Gold was activated at 95°C for 10 minutes; the subsequent PCR condition consisted of 45 cycles of denaturation at 95°C for 15 seconds and annealing extension at 60°C for 1 minute per cycle. During the PCR amplification, the amplified products were

measured continuously by determining the fluorescence emission. All PCR reactions were performed in triplicate, and the relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method by normalizing to GAPDH RNA.

Wound Healing and Tube formation assay

The cell migration scratch assay and tube formation assay were performed as previously described.(18) For the wound healing assay, MBECs (5×10^5 per well) were seeded in six-well plates and allowed to adhere for 24 hours. The cells were wounded with a yellow tip and washed with PBS. Fresh, full medium was added, and the cells were treated with NaHS and LY2994002 (20 μM) as indicated for 20 hours. Photographs were taken at the same position of the wound. For tube formation assay, 24-well plates were coated with Matrigel and incubated at 37°C for 1 hour. MBECs (2×10^4 cells) were plated with NaHS and LY294002, as indicated in the Results section. Tube formation was quantitated 6 hours after treatment.

Western blot analysis and immunofluorescent staining

Rat brain tissues were extracted at day 7 after I/R injuries (n=5 per group), and astrocytes and bEnd.3 cells were harvested at the indicated conditions for Western blot analysis, immunofluorescent staining and TUNEL

assay. For Western blot analysis, equal amounts of each protein sample were subjected to analysis using specific antibodies against extracellular-signal-regulated kinase (ERK; Santa Cruz Biotechnology, Santa Cruz, CA, USA), AKT (Santa Cruz Biotechnology), phospho-ERK, phospho-AKT (Cell Signaling Technology, Danvers, MA, USA), phospho-endothelial nitric oxide synthase (p-eNOS), eNOS (Abcam, Cambridge, UK), phospho-hypoxia-inducible factor1α (HIF1α), VEGF, Ang-1, Ang-2 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich, St. Louis, MO, USA). Immunofluorescent staining of brain tissue was performed using cryopreserved 40-μm coronal sections. The sections were blocked, and probed with indicated antibodies against VEGF, Ang-1(Santa Cruz Biotechnology), BrdU(5-bromo-2-deoxyuridine;Boehringer Mannheim, Indianapolis, IN, USA), CD-31(cluster of differentiation 31; Abcam, Cambridge, UK), GFAP (glial fibrillary acidic protein; Chemicon), MAP2 (microtubule-associated protein 2; Cell Signaling Technology) and NeuN (neuron-specific nuclear protein; MERCK Millipore, Billerica, MA, USA). After washing, the sections were stained with the fluorophore-conjugated secondary antibody for 30 minutes or with peroxidase substrate–diamibenzidine (DAB; Dako, Carpinteria, CA, USA) for 5 minutes. For double immunofluorescent staining, FITC-conjugated streptavidin secondary antibodies (Chemicon, MA, USA) and Alexa 488-conjugated goat anti-mouse secondary antibodies (Molecular Probes, Eugene, OR, USA) were used. Cell nuclei were visualized with 4, 6-

diamidino-2-phenylindole (DAPI) staining. Confocal laser scanning biological microscope (LSM 410 META; Carl Zeiss, Jena, Germany) was used to count the double positive cells. Six predefined sections through the peri-infarction area were used for cell quantification.

TUNEL assay

Paraffin-embedded sections were dewaxed, rehydrated, and then incubated in 20 mg/mL of proteinase K for 15 minutes. TUNEL was accomplished using a DNA fragmentation detection kit (QIA33; Oncogene, Boston, MA, USA). After the sections were immersed in 100 μl of 3% H_2O_2 for 5 minutes, they were incubated in a TdT labeling reaction mixture in a humidified chamber for 90 minutes at 37°C. Then, they were incubated in the stop buffer at 37°C for 5 minutes. Sections were washed with PBS before being incubated in a blocking buffer for 30 minutes at room temperature, colored with diaminobenzidine–H_2O_2 solution, and counterstained with methyl green.

Behavioral Testing

Neurological evaluation was performed using the forelimb placing and corner tests before middle cerebral artery occlusion (MCAO) and at 1, 7 and

14 days after MCAO by researchers who were blinded to the experimental groups (n=10). The forelimb placing test was scored using vibrissae-elicited forelimb placing.(19) Independent testing of each forelimb was induced by brushing the vibrissae ipsilateral to that forelimb on the edge of a tabletop once per trial for 10 trials. Intact animals placed the forelimb quickly onto the countertop. The percentage of successful placing responses was determined. The corner test was performed as previously described,(20) with slight modifications. Briefly, the rat was allowed to proceed into a corner, the angle of which was 30°. To exit the corner, the rat could turn to either the left or right, and this choice was recorded. This process was repeated 10 times, with at least 30 seconds between trials, and the percentage of right turns was calculated. Only turns involving full rearing along either wall were included. Rats were not picked up immediately after each turn, so that they did not develop an aversion for their pre-potent turning response.

Infarction volume

Following a cardiac perfusion-fixation with 4% paraformaldehyde, brains were cut into 40 μm-thick coronal sections on a freezing microtome (CM 3050S, Leica, Wetzlar, *Germany*). A total of 7 brain sections were processed for Nissl staining. The infarction volume was measured using an image analyzer. The percentage of infarction was then determined by

comparing the infarction volume and the total volume of the contralateral hemisphere.

Statistical analysis

The values are presented as the mean±S.D. Data were analyzed using the unpaired Student's *t*-test and ANOVA (analysis of variance) test when appropriate. Otherwise, the non-parametric Mann–Whitney *U* test or Wilcoxon signed-rank test was used for unpaired or paired samples, respectively. A two-tailed value of $p<0.05$ was considered significant.

Results

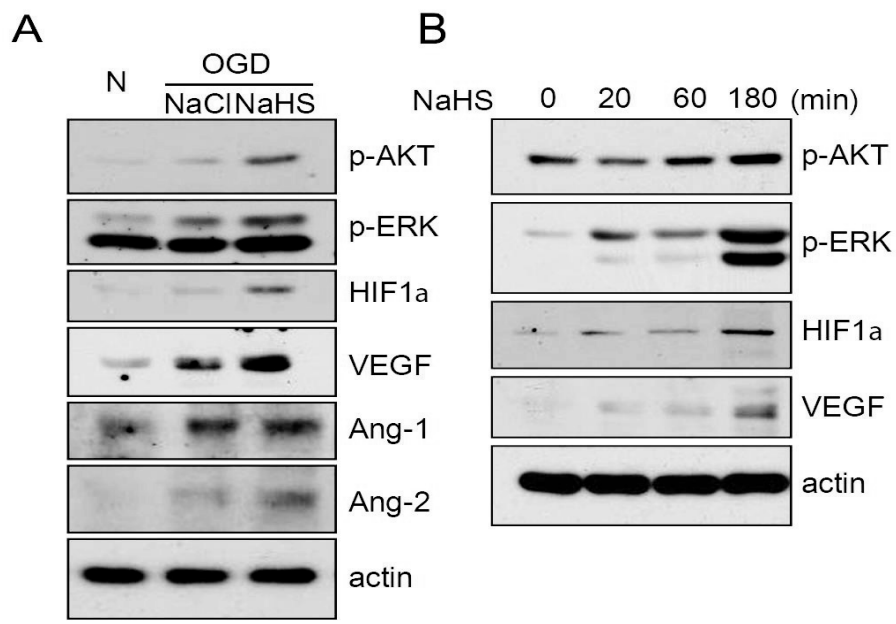
In vitro study

H_2S enhances angiogenic gene expression in astrocytes and MBECs

To elucidate H_2S effects in ischemic stroke, we first assessed an *in vitro* oxygen glucose deprivation (OGD) model in the astrocytes. Astrocytes form contacts to blood vessels and secrete crucial angiogenic factors that promote the proliferation of endothelial cells. The induction of vestibular endothelial growth factor (VEGF), Angiopoietin (Ang)-1and Ang-2 was observed after OGD in astrocytes , and quantitative analysis showed 2.5-, 1.48-, 2.65-fold increases, respectively(Figure 2A). OGD with 5 μM of NaHS significantly upregulated a 4.13-, 1.59-, 4.61-fold increase in VEGF, Ang-1 and Ang-2, respectively, compared with OGD with NaCl ($*p*<0.001; Figure 2A). The real-time PCR data revealed that astrocytes exhibited a notable induction of VEGF, Ang-1 and Ang-2 expression (**p*<0.05, ***p*<0.01, n.s., not significant; Figure 2C). AKT and ERK phosphorylation were increased after OGD, and HIF1α was also increased. Interestingly, 5 μM of NaHS enhanced AKT and ERK phosphorylation as well as the level of HIF1α

protein, compared with NaCl- treated cells (Figure 2A).

In MBECs, H_2S treatment significantly increased the levels of p-AKT, p-ERK, HIF1α and VEGF in a time-dependent manner up to 180 minutes ($ $p<0.001$, ** $p<0.01$, n.s., not significant; Figure 2B). In real-time PCR data, MBECs also showed the induction of VEGF, Ang-1 and Ang-2 expression, but not as much as was exhibited by the astrocytes (* $p<0.05$, n.s., not significant; Figure 2D). These data suggest that treatment with H_2S induced VEGF and Ang-1, which promotes vascular regeneration.



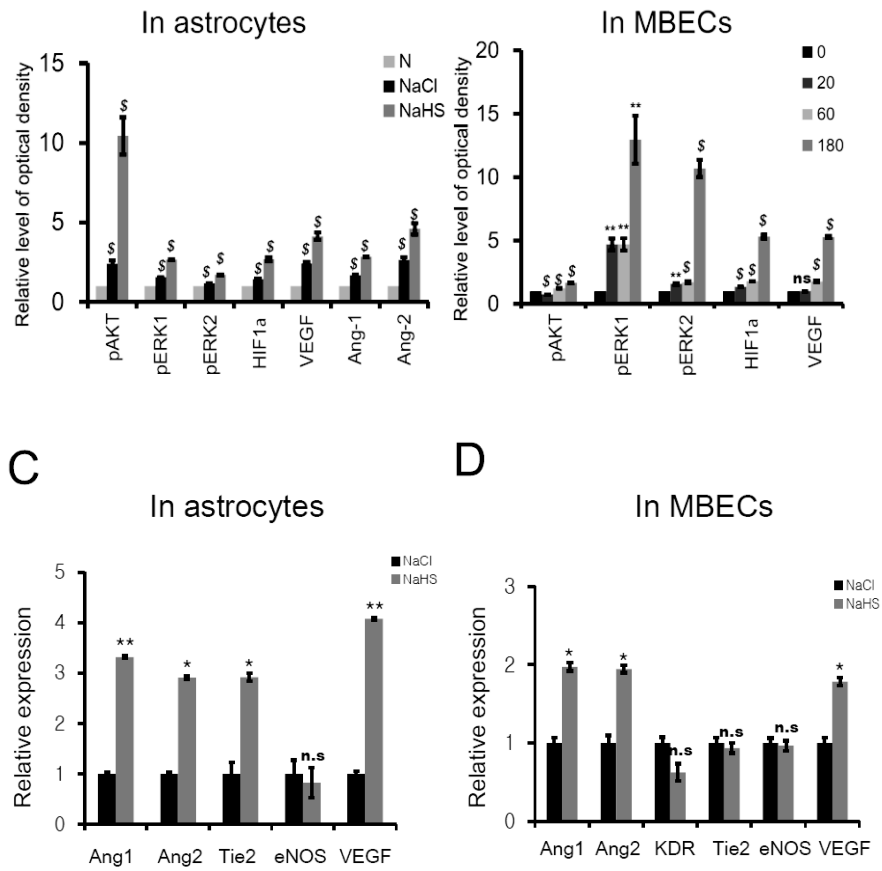


Figure 2. H_2S regulates angiogenic gene expression and proteins in astrocytes and MBECs

(A) Changes with or without 5 μM NaHS in p-AKT, p-ERK, HIF1, VEGF, Ang-1 and Ang-2 after 2 hrs of OGD in astrocytes. Actin was used as a loading control. The expression level was quantified (n=5/group, $p<0.001 versus control). (B) MBECs were stimulated with 5 μM NaHS for the indicated times, and changes in the crucial proteins were analyzed by Western blotting. Quantification of the expression level of

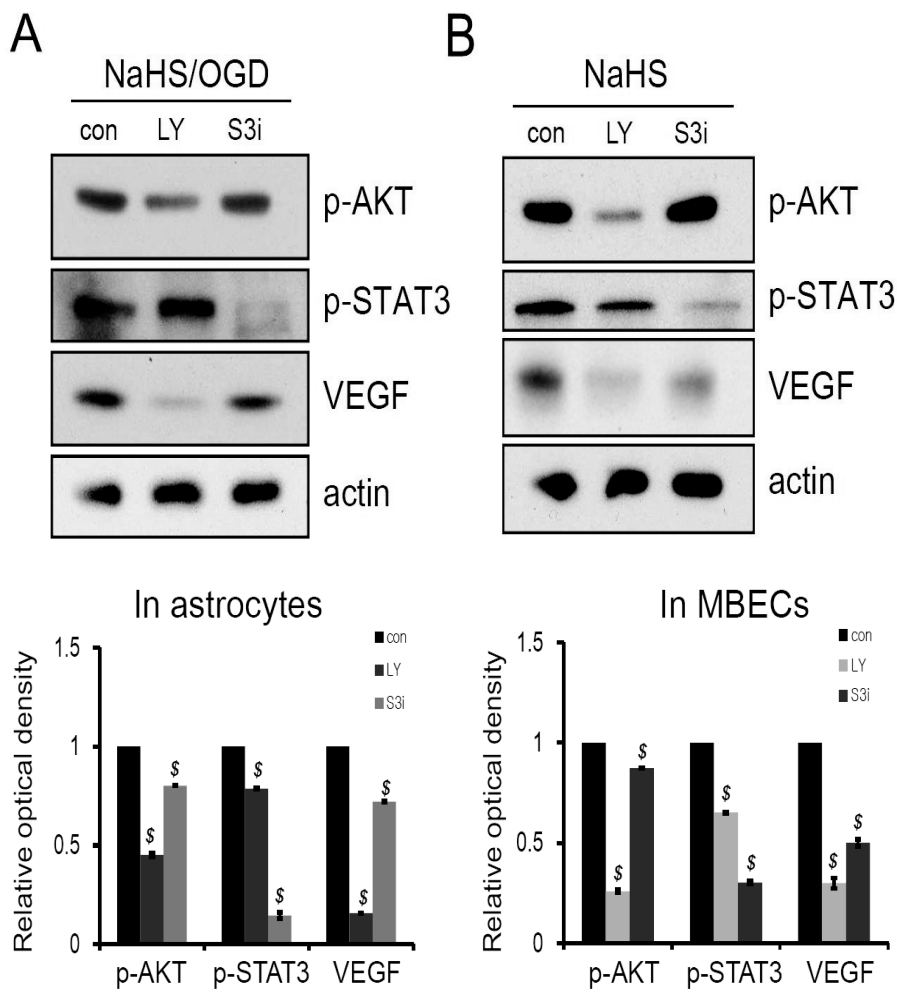
p-AKT, p-ERK, HIF1, and VEGF (n=5/group, **p<0.01, $p<0.001, *n.s.*, not significant versus control). (C, D) Gene expression in astrocytes (C) and MBECs (D) treated with or without NaHS for 3 hrs. Ang-1, Ang-2, Tie-2, eNOS, VEGF, and KDR gene expression was measured by real-time polymerase chain reaction (n=3/group, *p<0.05, **p<0.01, *n.s.*, not significant versus control).

OGD, oxygen glucose deprivation; MBEC, mouse brain endothelial cell; ERK, extracellular signal-regulated kinase; HIF, hypoxic induced factor; VEGF, vascular endothelial growth factor; Ang, angiopoietin; KDR, kinase insert domain receptor, also known as vascular endothelial growth receptor -2(VEGFR-2); eNOS, endothelial nitric oxide synthase

PI3K-AKT signaling mediates H_2S-induced VEGF expression and angiogenesis in vitro

To elucidate the mechanism underlying H_2S-induced VEGF expression, we treated H_2S with or without phosphatidylinositide-3-kinase (PI3K) or signal transducer and activator of transcription (STAT)-3 inhibitor in astrocytes and MBECs. Astrocytes were treated with 5 μM of NaHS for 2 hours of OGD stimuli. The increase in the VEGF levels was significantly blocked by the specific PI3-kinase inhibitor LY294002; in contrast, the STAT3 inhibitor was not able to change the VEGF protein level ($*p*<0.001;

Figure 3A) or the RNA expression (**p<0.01, n.s., not significant; Figure 3C). MBECs were treated with 5 μM of NaHS for 6 hours with either LY294002 or STAT3 inhibitor VI. The VEGF level was significantly blocked by LY294002 but not STAT3 inhibitor VI. Consistently, the real-time PCR data showed that VEGF induction was inhibited by LY294002 but not STAT3 inhibitor in MBECs ($$p$<0.001, *$p$<0.05, n.s., not significant; Figures 3B and 3D).



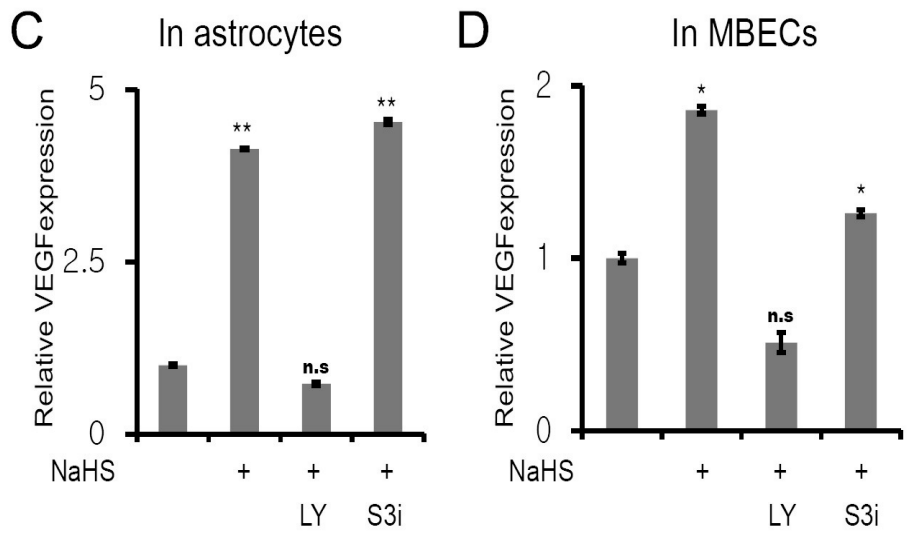


Figure 3. PI3K-AKT signaling regulates H_2S-induced VEGF expression

(A) Astrocytes were pretreated with 1.5 μM LY294002 (LY), 10 μM STAT3 inhibitor VI (S3i), or DMSO (con), which was followed by 2 hrs of OGD with 5 μM NaHS and 24 hrs of reoxygenation. Whole cell extracts were subjected to Western blot analysis with specific antibodies as indicated. Actin was used as a loading control. Quantification of the expression level of p-AKT, p-STAT3 and VEGF (n=5/group, $p<0.001). (B) MBECs were treated with 1.5 μM LY294002 (LY), 10 μM STAT3 inhibitor VI (S3i), or DMSO (con) for 6 hrs in cultured media, including 5 μM NaHS. Quantification of the expression level of p-AKT, p-STAT3 and VEGF (n=5/group,

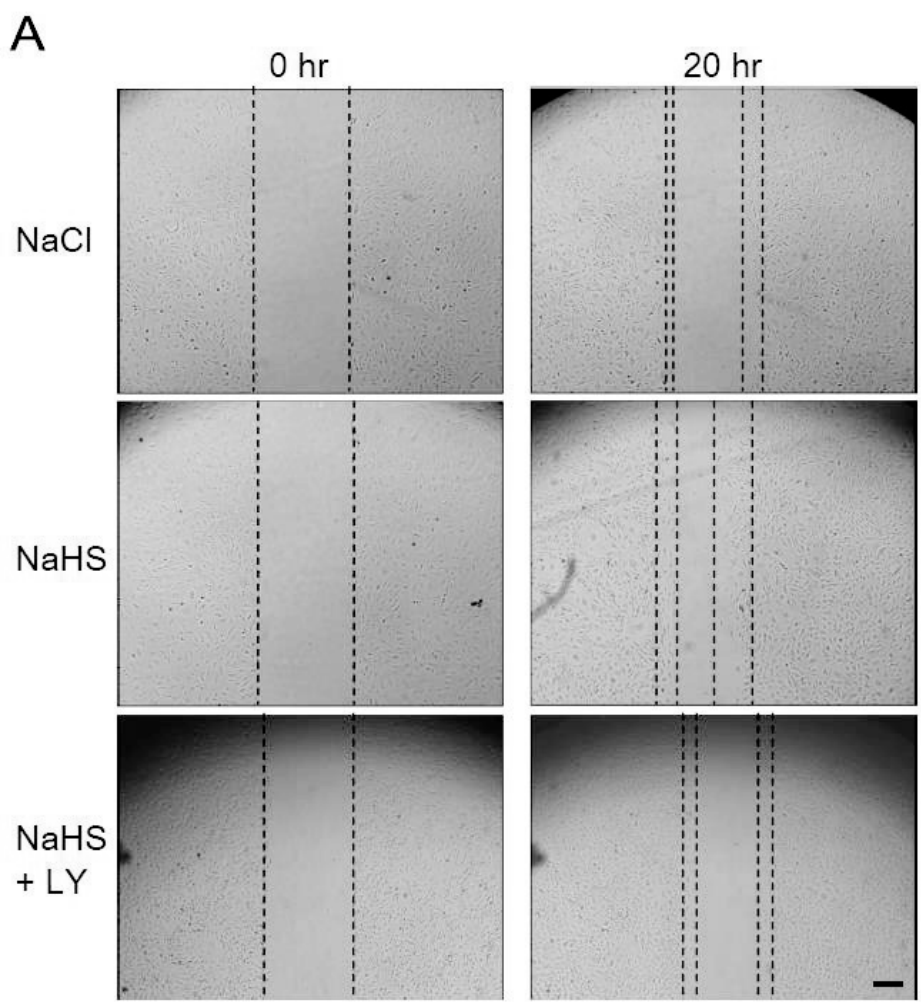
$p<0.001*vs* control). (C) VEGF mRNA expression in astrocytes incubated with 1.5 μM LY294002 (LY), 10 μM STAT3 inhibitor VI (S3i), and 5 μM NaHS for 3 hrs as described (**p<0.01, *n.s.*, not significant versus control). (D) VEGF mRNA expression in MBECs incubated with 1.5 μM LY294002 (LY), 10 μM STAT3 inhibitor VI (S3i), and 5 μM NaHS for 3 hrs, as described (*p<0.05, *n.s.*, not significant versus control).

OGD, oxygen glucose deprivation; PI3K, phosphatidylinositide 3-kinase; VEGF, vascular endothelial growth factor; STAT, signal transducer and activator of transcription; DMSO, dimethyl sulfoxide

H_2S-induced endothelial cell migration and tube formation

During the angiogenesis, endothelial cells have to migrate and then reorganize individual cells into a three-dimensional tube-like structure to form vessel lumen. Therefore, we investigated the effects of H_2S on endothelial cell migration using the scratch wound healing assay and the tube formation assay. 5 μM of NaHS accelerated the wound healing of MBECs 3.92-fold compared with NaCl-treated cells (***$p<0.001$, n.s., not significant; Figures 4A and 4C). The wound healing effect of H_2S treatment was not detected by treatment with LY294002, suggesting that H_2S promotes endothelial cell migration through

PI3K/AKT cascade signal transduction. The effect of H_2S on tube formation was examined after 20 hours of culture. The NaHS (5 μM) treatment showed a 3.69-fold increase in microvessel tube formation compared with the NaCl treatment, and LY294002 (1.5 μM) treatment blocked the formation of the tube-like structure (***p<0.001, **p<0.01; Figures 4B and 4C).



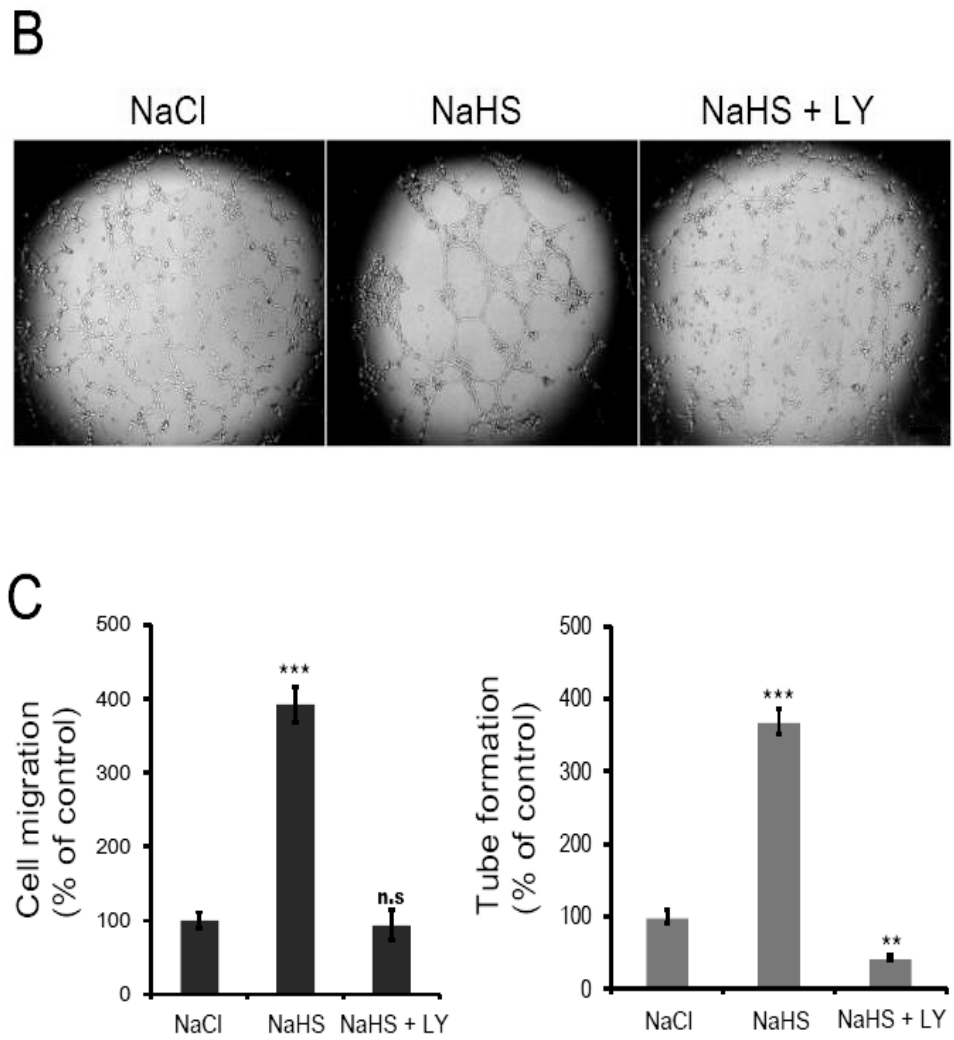


Figure 4. H_2S promotes cell migration and increases capillary-like tube formation in MBECs

(A) Representative images of the denuded zone, with filling after 20 hrs in control wells. Treatment with 5 μM NaHS increases the number of cells entering zone. Scale bar=100 μm. (B) Treatment with 5 μM

NaHS increased capillary-like tube formation, and the inhibition of PI3K significantly decreased H_2S-induced tube formation *in vitro*. The bar indicates 100 μm. (C) Quantitative analysis of cell migration (*Left*) and capillary-like tube formation (*Right*). N=5/group, ***p<0.001, **p<0.01, *n.s.*, not significant versus NaCl control

LY, 1.5 μM LY294002; PI3K, phosphatidylinositide 3-kinase

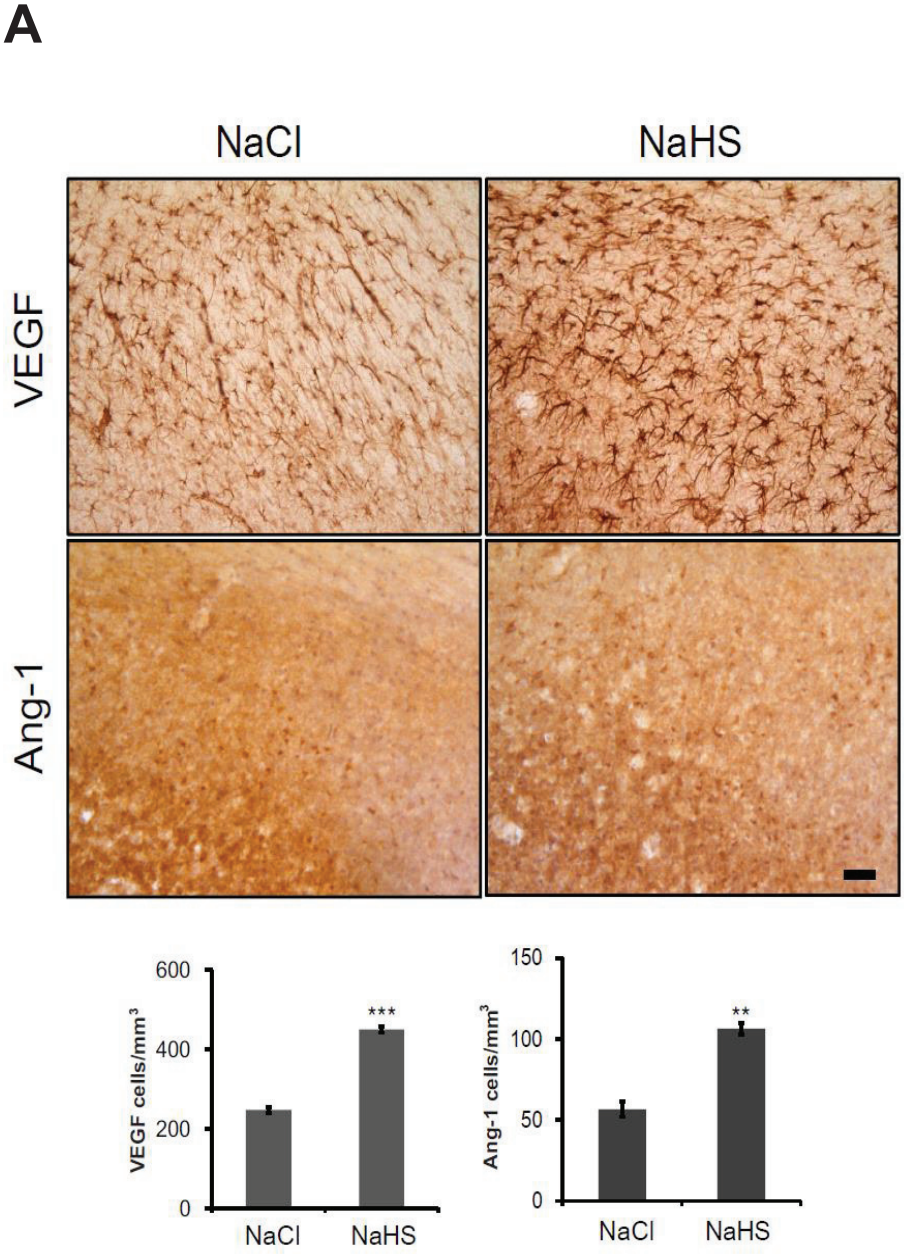
In vivo study

H_2S treatment induces VEGF and angiogenic factor after ischemic stroke through AKT and ERK phosphorylation

Because VEGF and Ang-1 are important angiogenic factors, we next confirmed their expression in the ischemic brain with or without the administration of H_2S in a rat cerebral ischemic reperfusion model. H_2S treatment significantly increased the VEGF levels (3.9-fold, ### $p<0.001$) at the ischemic boundary regions compared to non-treated control rats (Figures 5B and 5C). Ang-1, a family member of endothelial growth factors, was highly expressed in H_2S-treated ischemic lesions (1.65-fold, #$p<0.05$; Figures 5B and 5C). Consistently, H_2S treatment showed a 1.82-fold increase of VEGF+ cells and a 1.87-fold increase of Ang-1+ cells (**$p<0.01$, ***$p<0.001$; Figure 5A). In addition, stroke itself induced the BrdU-positive cells, and treatment with H_2S increased the number of BrdU-positive cells, indicating newly proliferating cells (Figure 6B). Considering together, these data indicate that treatment with H_2S induced VEGF and Ang-1, which promotes vascular regeneration.

To examine the signaling pathways through which H_2S induces angiogenesis after cerebral ischemia in vivo study, we measured the

phosphorylation of AKT and ERK, and the level of HIF1α using Western blot analysis. AKT phosphorylation was minimally detected in the contralateral hemisphere, and a 4-fold increase in AKT phosphorylation was detected in the ischemic lesion (***p<0.001; Figures 5B and 5C). Notably, the ischemic lesion of H_2S treated rats showed a significantly increased phosphorylation of AKT (1.6-fold increase, ##p<0.01) compared to the control group, and even the contralateral hemisphere showed AKT phosphorylation (Figures 5B and 5C). Consistently, the densities of p-ERK1 and p-ERK2 in the ischemic lesion were respectively 1.27- and 1.32-fold higher (###p<0.001 and #p<0.05) in the H_2S-treated group compared with the control group (Figures 5B and 5C). HIF1α was highly expressed in the ischemic lesion of H_2S-treated rats (3.93-fold increase, ##p<0.01; Figures 5B and 5C). In addition, the densities of phosphorylated-endothelial nitric oxide synthase (p-eNOS) in the ischemic lesion were respectively 1.82- and 2.45-fold higher (###p<0.001 and #p<0.05) in the H_2S-treated group compared with the control group (Figures 5D).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NaCl
NaHS
VEGF
Ang-1
VEGF cells/mm³
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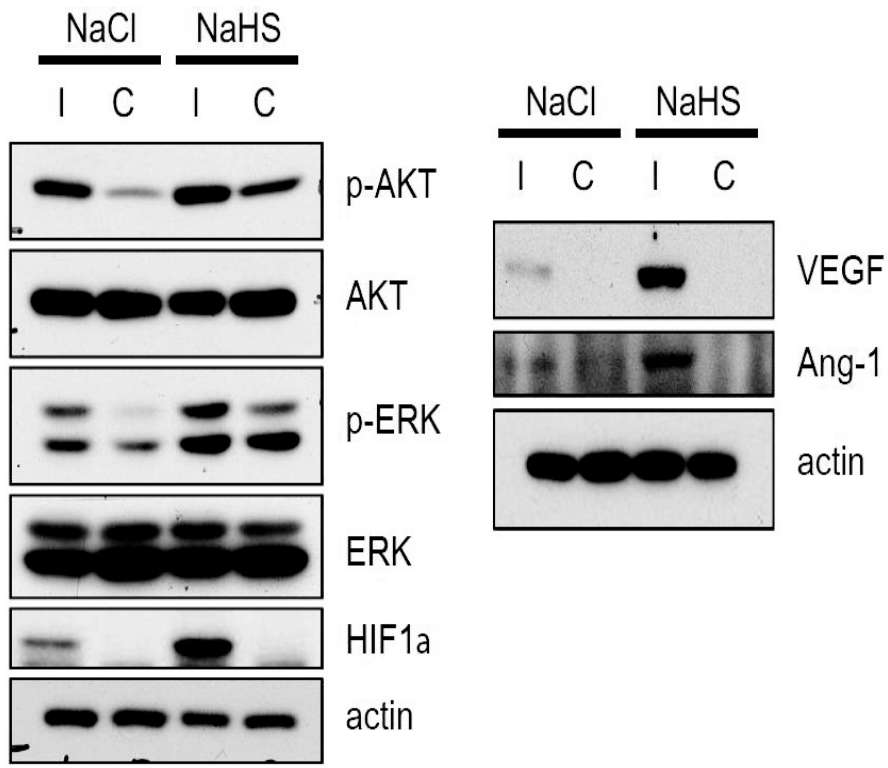
400
200
0
***
NaCl
NaHS
Ang-1 cells/mm³
150
100
50
0
**
NaCl
NaHS

B


NaCl
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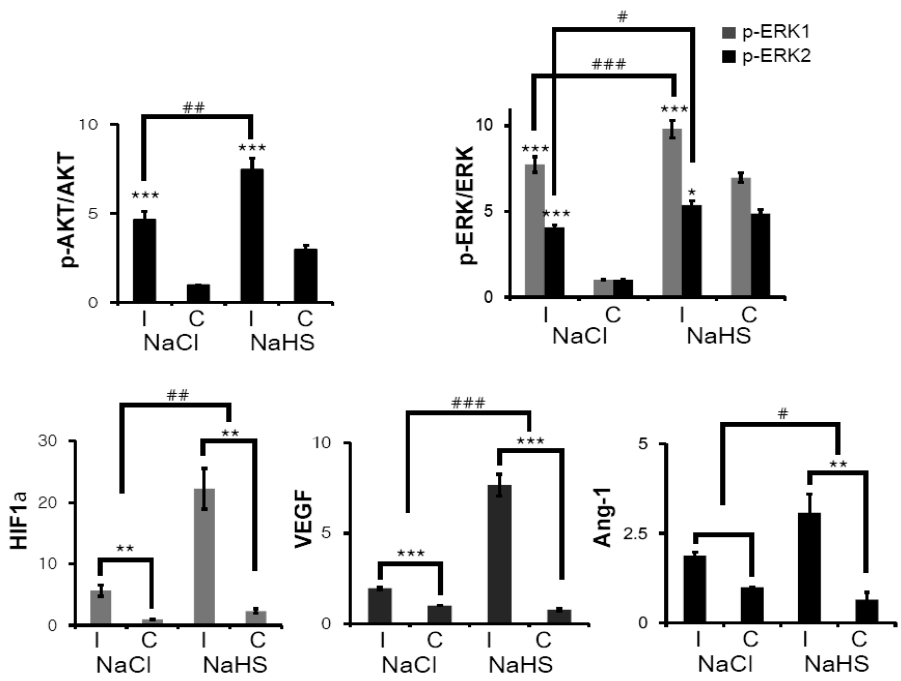


D

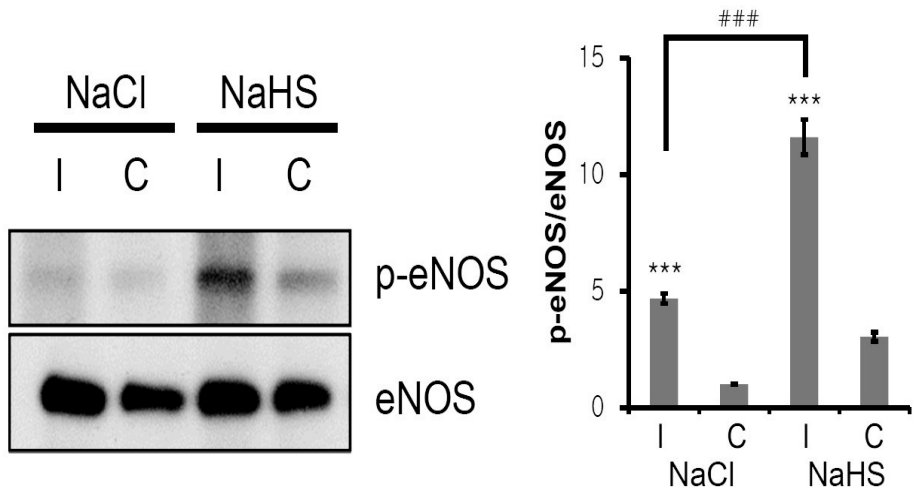


Figure 5. H_2S increases VEGF and Ang-1 expression and induces p-AKT, p-ERK, HIF1, and p-eNOS in the ischemic brain

(A) Immunohistochemical analysis of VEGF and Ang-1 in the ischemic hemisphere 1 week after MCAO from 5mg/kg NaCl- or NaHS-treated rats (n=8/group). Representative images show NaCl-treated (Left), NaHS-treated (Right), VEGF immunoreactive (*Upper*), or Ang-1 immunoreactive (*Bottom*) rats in the ischemic lesion. The bar indicates 50 μm. Quantification shows that the NaHS-treated group exhibited a higher number of VEGF+ cells (***p<0.001) and Ang-1+ cells (**p<0.01) than did the NaCl-treated group (*Bottom Left*; VEGF, *Bottom Right*; Ang-1). (B) Representative immunoblots of ischemic brain tissue; Brain tissue

samples were isolated from ischemic lesions (I) and contralateral hemispheres. (C, D) Brain lysates were analyzed by Western blotting using the indicated antibodies and were then reprobed with actin as a loading control. (C) Western blot analysis probed with VEGF and Ang-1 antibody: Quantification of the expression levels of p-AKT/total AKT and p-ERK/total ERK, HIF1/actin, VEGF/actin, and Ang-1/actin (n=5/group, *p<0.05, **p<0.01, ***p<0.001 ischemic versus contralateral hemisphere, #p<0.05, ##p<0.01, ###p<0.001 NaHS-versus NaCl-treated ischemic lesion). (D) Quantification of the expression levels of p-eNOS/total eNOS (n=5/group, ***p<0.001 ischemic versus contralateral hemisphere, ###p<0.001 NaHS-versus NaCl-treated ischemic lesion)

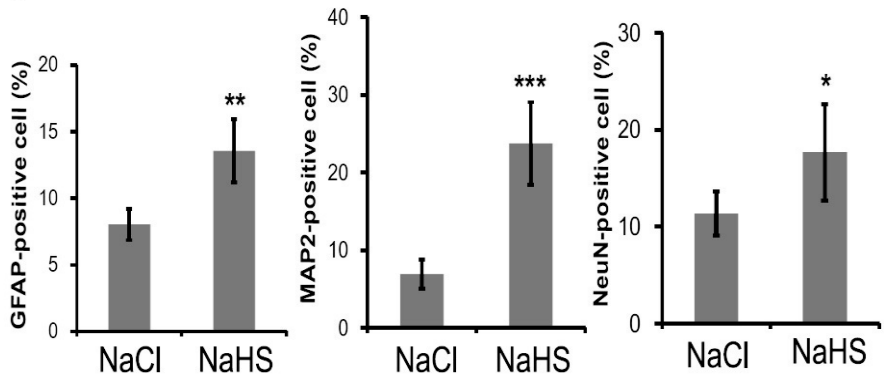
VEGF, vascular endothelial growth factor; Ang, angiopoietin; p-AKT, Phosphorylated AKT; p-ERK, Phosphorylated-ERK; HIF, hypoxic induced factor; eNOS, endothelial nitric oxide synthase, also known as nitric oxide synthase 3; p-eNOS, Phosphorylated -eNOS

Post-treatment with H_2S significantly enhances endothelial cell synthesis

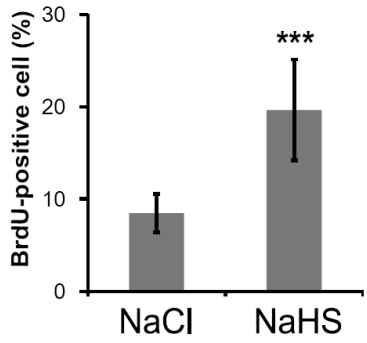
Treatment with H_2S significantly increased the percentage of GFAP+ (8.03±1.17% versus 13.55±2.36%, ***p*<0.01), MAP2+ (6.93±1.89% versus 23.74±5.31%, ****p*<0.001), and NeuN+ (11.35±2.25% versus 17.67±4.95%,

*p <0.05) cells in the ischemic lesion compared with the percentage in the control group (Figure 6A). In addition, H_2S treatment increased the number of BrdU-positive cells (8.47±2.08% versus 19.65± 5.4%, ***p <0.001) in the ischemic brain compared with the control (Figure 6B). However, H_2S treatment did not significantly increase the number of BrdU-MAP2 or BrdU-NeuN double-positive cells. Instead, the percentage of TUNEL-positive cells was significantly smaller in the H_2S-treated group (102.2±19.3% versus 77.85 ±4.31%, ** p<0.01; Figure 6C). Therefore, we investigated whether the hypothesized neurological recovery ability of H_2S comes from vascular regeneration. Importantly, Figure 6D indicates that BrdU and CD31 double-positive cells, which were newly synthesized endothelial cells, were significantly (3.2 ± 0.8 versus 7.4 ± 0.7 cells/mm^2, ***p<0.001) increased in the ipsilateral hemisphere of H_2S-treated rats, compared with the ipsilateral hemisphere of control rats. Our data indicated that H_2S promotes vasculogenesis after stroke.

A


GFAP-positive cell (%)
20
15
10
5
0
**
NaCl
NaHS
MAP2-positive cell (%)
40
30
20
10
0
***
NaCl
NaHS
NeuN-positive cell (%)
30
20
10
0
*
NaCl
NaHS

B


BrdU-positive cell (%)
30
20
10
0
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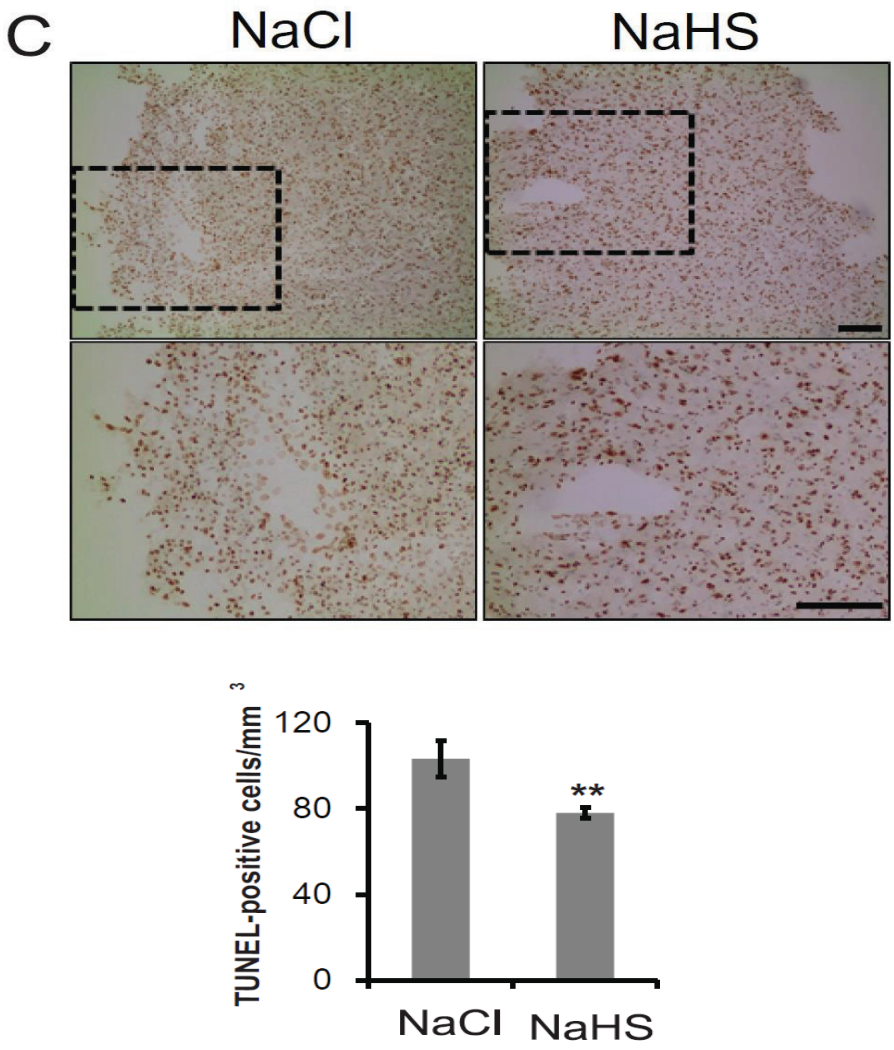
NaCl
NaHS


C
NaCl
NaHS
TUNEL-positive cells/mm 3
120
80
40
0
**
NaCl
NaHS

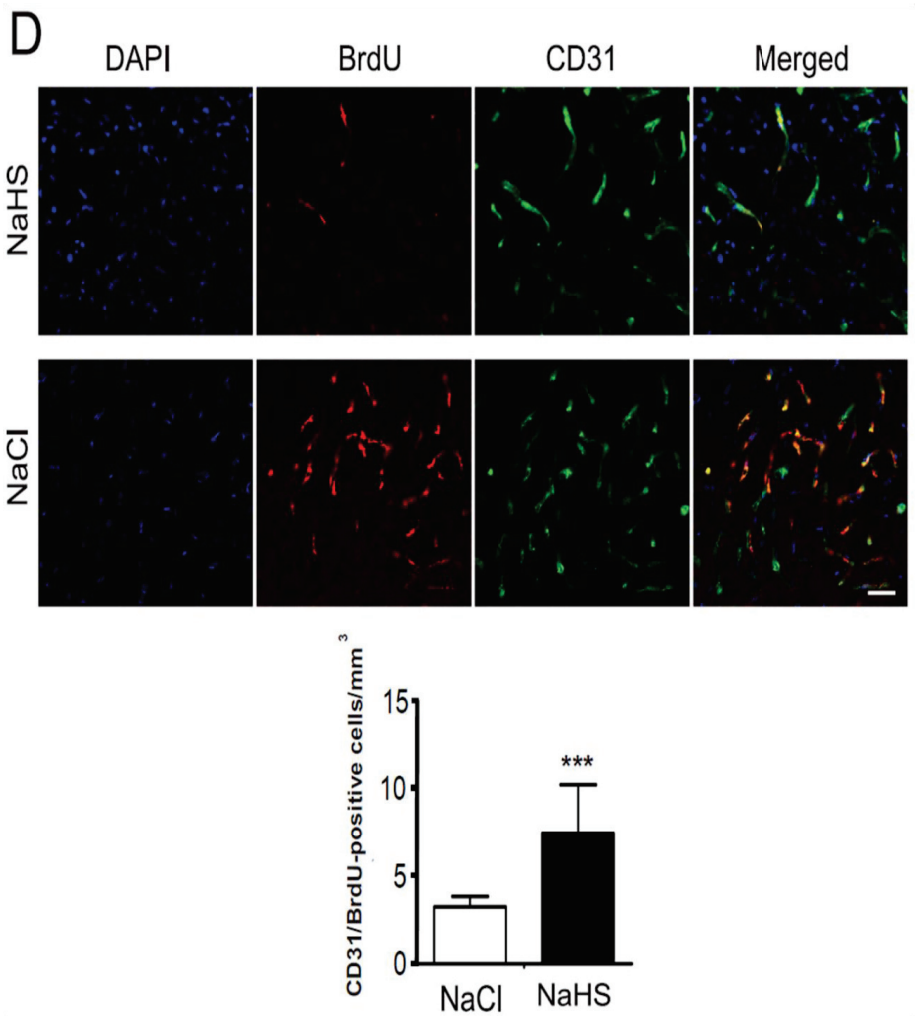


Figure 6. Effects of H_2S post-treatment on the cell populations in the ischemic brain of rat cerebral ischemic/reperfusion model

(A) Treatment with H_2S significantly increases the number of GFAP-, MAP2-, and NeuN-positive cells in the ipsilateral hemisphere. n=10/group, *p<0.05, **p<0.01, ***p<0.001 versus NaCl (B) Percent of BrdU-

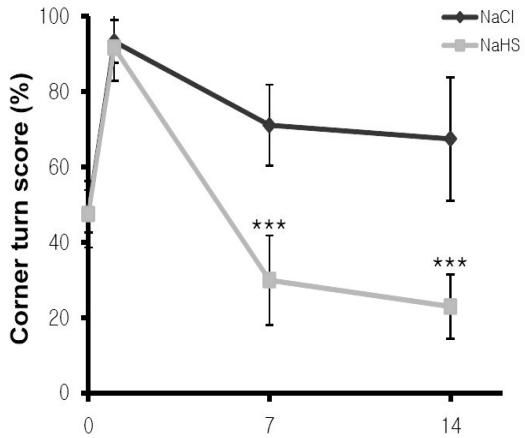
positive cells n=15/group, ***p<0.001 versus NaCl (C) TUNEL immunostaining of peri-infarct area from rats administered NaHS or NaCl; The pooled data show significantly fewer TUNEL-positive cells according to ischemia-reperfusion injury model in NaHS-administered rats than in controls. n=5/group, **p<0.01 versus NaCl (D) H_2S-induced angiogenesis after ischemia-reperfusion injury; Double immunostaining shows CD31 (*green*) and BrdU (*red*) at the peri-infarct area from NaCl- or NaHS-treated ischemic brain. Nuclei were stained with DAPI (*blue*). The bar indicates 50μm. Quantitative analysis shows that the number of CD31 and BrdU double-labeled cells per $2\times10^5\mu m^2$ were higher in the NaHS group (***p<0.001 versus NaCl treated ischemic lesion, *Bottom*)

Post-treatment of H_2S improves neurological outcome

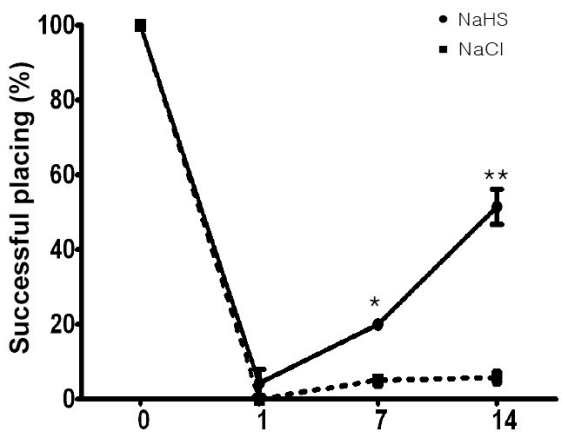
To investigate the therapeutic possibility of H_2S for the treatment of cerebral ischemic stroke, we administered daily either NaHS (5mg/kg) or NaCl from the third day after the reperfusion. Interestingly, treatment with NaHS significantly improved neurological outcomes on the corner turn test (****p*<0.001; Figure 7A) and the limb placing test (**p*<0.05, ** *p*<0.01; Figure 7B) compared with control rats. In contrast, the infarction volume was not significantly reduced in NaHS-treated rats compared with control group

rats (Figure 7C). After the administration of NaHS, the mean blood pressure at 30 minutes was decreased in the NaHS- treated group (*p<0.05; Figure 7D). However, it was not significantly different in the blood pressure at 60 minutes between NaHS– and NaCl-treated groups (Figure 7D). Therefore, we hypothesized that H_2S affects regeneration after ischemic brain injury and that it improves neurological function.

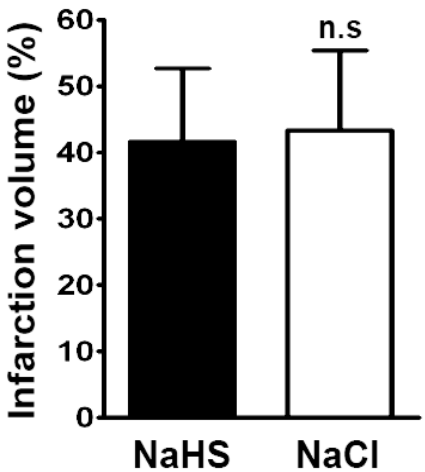
A


NaCl
NaHS
Corner turn score (%)
100
80
60
40
20
0
0
7
14
***
***

B


NaHS
NaCl
Successful placing (%)
100
80
60
40
20
0
0
1
7
14
*
**

C


Infarction volume (%)
60
50
40
30
20
10
0
n.s
NaHS
NaCl

D

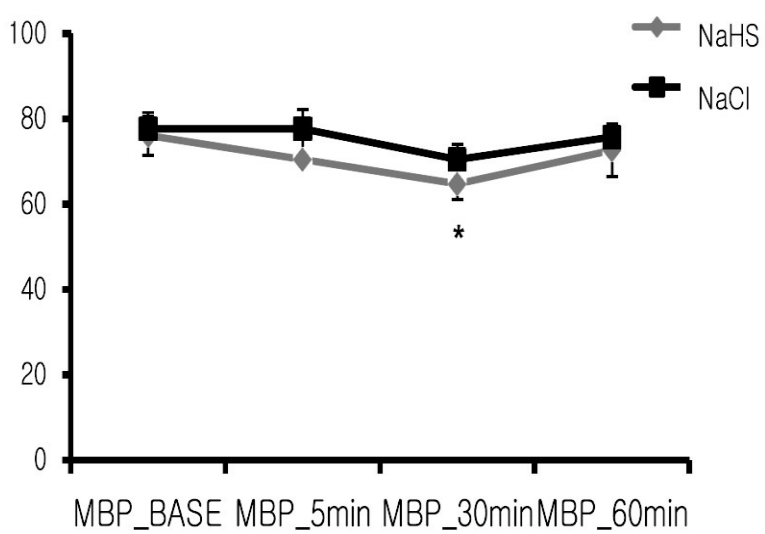

100
80
60
40
20
0
NaHS
NaCl
*
MBP_BASE
MBP_5min
MBP_30min
MBP_60min

Figure 7. Post-treatment with H_2S improves functional outcomes

(A, B) The NaHS-treated group showed better functional outcomes in the corner turn test on days 7 and 14 after MCAO (n=10/group, ***p<0.001 versus NaCl) (A) and in the limb placing test on days 7 and 14 (n=10/group, *p<0.05, **p<0.01 versus NaCl) (B). (C) Percentage of infarction area relative to the contralateral hemisphere. Infarct volumes were estimated on day 7 after MCAO (n=7/group) from 12 serial sections per animal (*n.s*., not significant). (D) The administration of NaHS did not influence on the blood pressure significantly at 60 mins after injection (*p<0.05 versus NaCl).

MCAO, middle cerebral artery occlusion

Discussion

In this study, we investigated whether H_2S plays a role in regeneration to explore potential therapeutic possibilities. Through *in vivo* and *in vitro* analysis, we demonstrated that post-treatment with H_2S enhances angiogenesis in ischemic stroke via a cooperative interaction between astrocytes and endothelial cells.

In ischemic stroke, H_2S may enhance the NMDA receptor mediated excitotoxicity of glutamate and increase the ischemic damage in the global ischemic model. (9) In addition, reducing the H_2S concentration may reduce the blood brain barrier disruption, resulting in decreased infarct volume. (21) However, another studies showed that the administration of H_2S had neuroprotective effect against cerebral I/R injuries to withstand oxidative stress.(10, 11) A recent study pointed out that these controversial results were due to the various dosages of H_2S donors, infusion methods, infusion velocities, and different animal models such as global ischemia or focal I/R injury models.(17) With regard to regeneration, the previous studies consistently reported the angiogenic potential of H_2S, which enhances wound healing. In a model of cutaneous burn injury, the topical administration of a H_2S saturated physiological solution significantly enhanced the closure of the wound.(22) H_2S promoted collateral vessel formation and regional blood flow

after femoral artery occlusion in the rat hind limb ischemia model.(16) Interestingly, H_2S donors at higher doses failed to promote angiogenesis. The bell-shaped biological dose–response has been frequently noted with H_2S.(23) Consistent with the effects of H_2S in hind limb ischemia, the pro-angiogenic effect of H_2S supplementation in the ischemic rat heart improved cardiac function assessed by electrocardiography at 42 days after permanent ligation of the left anterior descending artery. These alterations were associated with a significant increase in capillary density in the myocardium.(24)

Cerebral blood flow is important for the maintenance of neural function.(25) Stroke patients who had an increase in cerebral blood vessel density, for instance, showed better progress and longer survival times.(26) We noticed more neural cells and less apoptotic cells in the H_2S-treated group, and these differences are related to the neuroprotective effect, not to the neurogenesis or astrocytogenesis (Figures 6A and 6C). In this study, we hypothesized that H_2S promotes angiogenesis, improves cerebral blood flow in the ischemic lesion, and is eventually beneficial for neural function. Figures 6B and 6D support our hypothesis, showing that newly synthesized endothelial cells were increased upon H_2S treatment. Considering the physiologic vasodilator effect of H_2S, H_2S administration may also increase microcirculation and collateral blood flow in the penumbra, thereby enhancing neuronal survival.(5) In addition, the enhancement of angiogenic factors may have a role as neurotrophic factors.(27)

Very early after the onset of the focal perfusion deficit, release of excitotoxic material can damage neurons and glia lethally. The excitotoxicity triggers a number of events that can further contribute to the demise of tissue including inflammation, and apoptosis.(28) The neuroprotective effect of H_2S in cerebral ischemia has been explained by decreasing the oxidative stress in the early phase of cerebral ischemia. (2, 10, 11) To exclude the anti-oxidative effect of H_2S, we administered H_2S three days after MCAO (Figure 1), and we maintained daily administration for 11 days. In Figures 7A and 7B, we noticed that the administration of H_2S significantly improved functional recovery without reducing infarct volume (Figure 7C) or changing blood pressure (Figure 7D).

Interestingly, the VEGF protein level was markedly increased in the ischemic hemisphere after ischemic-reperfusion injury (Figures 5B and 5C). Because VEGF represents a master mediator that initiates the process of angiogenesis through the recruitment and proliferation of endothelial cells, the induction of VEGF by H_2S is crucial for reinforcing stroke-induced angiogenesis. In a previous study, the administration of recombinant VEGF to the ischemic rats enhanced angiogenesis in the ischemic penumbra and improved neurological recovery.(27, 29, 30) Ang-1 is a family member of endothelial growth factors and a ligand for the endothelial specific receptor tyrosine kinase. Tie-2, Ang-1 and Ang-2 have been found to regulate the maturation of new blood vessels from proliferated endothelial cells.(7, 8)

Consistently, we observed that H_2S treatment increased Ang-1 expression in the ischemic brain after western blotting (Figures 5B and 5C). We demonstrated that H_2S affects VEGF and Ang-1gene expression *in vitro* using real-time PCR in astrocytes and MBECs (Figures 2C and 2D).

It is notable that increases in the VEGF protein level follow AKT and ERK phosphorylation in ischemic brain tissue. H_2S treatment activates AKT and ERK phosphorylation in astrocytes and MBECs (Figures 3A and 3B). Thus, our data suggest that H_2S-induced angiogenesis may be mediated through AKT and/or ERK. The activation of PI3K/AKT signaling has been reported to promote VEGF production through the regulation of HIF1 stability.(31-33) Indeed, HIF1 was increased in ischemic brain tissue (Figure 5B). Conversely, STAT3 regulates VEGF expression, thereby contributing to HIF1expression/activity and to oncogenic angiogenesis.(34) Interestingly, LY294002 treatment and the inhibition of PI3K/AKT signaling decreased the H_2S-mediated expression of VEGF; in contrast, the STAT3 inhibitor did not affect the H_2S-mediated induction of VEGF (Figure 3). Our data suggest that PI3K/AKT signaling regulates the H_2S-mediated increase of VEGF expression. Astrocytes are able to actively secrete a variety of chemicals for neurovascular function. Figure 5A implies that VEGF induced by H_2S treatment seems to localize in astrocytes. Indeed, the *in vitro* data revealed that astrocytes exhibited significantly increased VEGF and Ang-1 gene expression in the H_2S stimuli (Figure 2C); in contrast, MBECs showed only

mildly increased expression (Figure 2D). Because MAP2-positive and NeuN-positive cells are significantly increased in the ischemic lesion of H_2S treated rats (Figure 6A), we could not exclude a possibility that H_2S also increase phosphorylation of AKT in neurons. In fact, the previous studies have explained that the activation of AKT promotes cell survival and growth, and increases the ability of neurons to survive after brain injury (35-37). Therefore PI3K/AKT signaling should be further characterized.

AKT has a multipotent role that can impact diseases of the central nervous system, including stroke.(38-40) AKT phosphorylation has been reported to mediate endothelial cell proliferation and migration *in vitro* and to promote vascularization *in vivo*.(41-44) H_2S treatment phosphorylated AKT in a time-dependent manner in MBECs (Figure 2B), and it increased endothelial cell migration and tube formation (Figures 4A and 4B). The inhibition of PI3K/AKT cascade did not show H_2S-induced angiogenic effects in MBECs (Figures 4A and 4B). Previous studies have demonstrated that AKT dependent mechanism mediates the phosphorylation of eNOS at S1177 (45, 46). Furthermore, H_2S regulates cardioprotection through eNOS phosphorylation at the S1177 (47). In this study, H_2S treatment showed increase of p-eNOS in ischemic lesion (Figure 5D). In Figure 8, we propose a model for H_2S effects in ischemic stroke. H_2S administration provokes astrocytes to secrete angiogenic factors, which influence endothelial cells to facilitate angiogenesis, and this cascade significantly improves neurological function after ischemic

stroke. PI3K/AKT signaling is crucial for the H_2S-mediated angiogenic effect in ischemic stroke.

An appreciation of the physiological and pathological importance of H_2S has followed attempts to develop novel therapeutics that aimed to deliver hydrogen sulfide.(48) Drug development has been focused on the anti-inflammatory effect of H_2S.(49) The main target for these drugs was pain and inflammation, which reduced gastrointestinal ulceration or arthritis.(50-52) A clinical trial of Na_2S for critical-care applications, involving the regulation of the metabolic rate for the reduction of myocardial injury after myocardial infarction had been stopped.(5) In contrast, the second study of H_2S donors targeting oxidative stress has been designed to evaluate the ability to reduce heart failure. (13, 14) Drug development to magnify the angiogenic ability of H_2S may be considered as a new therapeutic application in cerebral ischemic stroke.

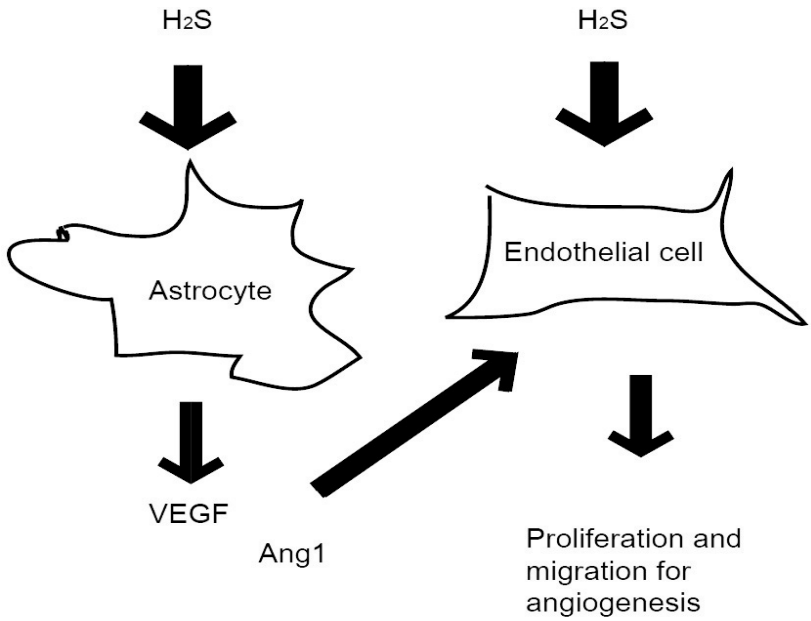


Figure 8. Proposed regulatory model for H_2S-promoted angiogenesis

H_2S stimulates astrocytes to express VEGF, Ang-1 and Ang-2. H_2S and those proteins cooperatively regulate the proliferation and migration of endothelial cells.

VEGF, vascular endothelial growth factor; Ang, angiopoietin

In this study, there are several caveats. First, neurogenesis and synaptogenesis in the long term was not evaluated. Neurogenesis in the dorsal dendate gyrus and subventricular zone was apparent at 28 days, and it takes time to migrate to the peri-infarcted area in a sequence.(53) Neurogenesis was

evaluated at 7 days after I/R injury in the present study. That may explain why neurogenesis was not prominent, even though we demonstrated that H_2S induced angiogenesis successfully. Angiogenesis reestablished functional microvasculature in the ischemic border zone, creating a microenvironment hospitable for neuronal plasticity including neurogenesis and synaptogenesis, which can lead to functional recovery.(54) In future studies, the angiogenic effects of H_2S should be examined over a longer period to demonstrate neurogenesis and synaptogenesis. It may show strong evidence for the potentials of H_2S as a therapeutic agent.

Second, we did not examine the sophisticated, cooperative mechanism between endothelial cells and astrocytes. The main manufacturer of VEGF affected by H_2S was astrocytes in this study. Angiogenic factors including VEGF bind to specific receptors located on brain endothelial cells.(53-55) This sprouting of endothelial cells leads to tube formation, branching, pruning and recruiting of supporting cells, which form mature vessels.(56) We did not examine how astrocytes and endothelial cells synergized with each other throughout angiogenesis in this experiment. That topic may be an intriguing issue to investigate the specific effects of H_2S on individual components of the neurovascular unit in a follow-up study.

Conclusion

The strategies of neurorestorative treatment aim to extend the therapeutic window for stroke recovery, and increase the diversity in therapeutic options in ischemic stroke. In the post-acute phase of ischemia, enriching the potential of the brain for neuroplasticity may affect subsequent recovery.(53) Angiogenesis is one of the pivotal neurorestorative events, along with neurogenesis, and synaptic plasticity after stroke.(54, 55) We showed that H_2S induced angiogenesis, which resulted in improved the neurological outcome. These findings support the possibility of H_2S as a therapeutic agent in the post ischemic period. In future studies, H_2S may be shown to have clinical importance in the treatment of ischemic stroke to mitigate its morbidity and mortality.

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초록

배경: 황화수소는 강력한 혈관확장제이자 심혈관계평형을 유지하는 물질이다. 나아가 황화수소는 특히 심장, 간, 신장 등의 기관에서 폐색-재관류 모델에서 중요한 역할을 하는 것 알려져 있다. 이 연구에서는 뇌허혈-재관류 백서모델에서 손상 이후 황화수소치료가 혈관신생을 증진시킴으로써, 기능회복에 기여한다는 가설을 고찰하였다.

실험 재료 및 방법: 백서를 이용한 뇌경색 모델에서 중대뇌동맥을 폐색하여 뇌허혈을 일으키고 두시간후 혈관폐색을 제거하여 뇌허혈-재관류 모델을 제작하였다. 모델 제작 직후 2주에 걸쳐 황화수소 5mg/kg(총 주입부피: 1mL)를 복강 내에 주입하였고, 대조군에는 생리식염수 1mL을 복강 내에 주입하였다. 모델제작 직후부터 2주에 걸쳐 신경학적 결손의 회복 정도를 측정하여 두 군간의 차이를 비교하였다. VEGF, Ang-1, Ang-2 등의 발현 유무를 Western blot과 RT-PCR을 통해 확인하였다. 황화수소가 혈관신생작용에 관여하는 기전을 살펴보기 위해 *in vitro* model에서 PI3K 억제제를 사용하였을 때 혈관신생작용에 차이가 있는지 확인하였다.

결과: 황화수소 주입군이 대조군에 비해 2주후 신경학적 결손의 호전이 통계적으로 유의하였다. 하지만 뇌경색의 부피 차이는 뚜렷하지 않았으며, 신생혈관생성의 증가를 위한 혈관내피세포수가 대조군에 비해 황화수소를 주입한 군에서 뚜렷하게 증가되어 있고 신생혈관 발현이 증가되어 있음을 확인하였다. 이에 대한 기전으로 황화수소가 PI3K/AKT 회로를 통해 AKT 인산화를 증진시키고 이로 인해 VEGF, Ang-1등 혈관신생촉진인자의 발현이 증가하여 혈관신생

작용이 촉진됨을 본 연구에서 확인하였다. 또한, *in vitro* model에서 VEGF를 주로 발현하는 것은 astrocyte임을 확인하였다.
결론: 황화수소가 뇌허혈-재관류 손상에서 혈관 신생 작용 증진을 통해 손상 이후 신경학적 기능회복에 기여하는 것으로 보인다.

주요어: 황화수소, 뇌허혈-재관류 뇌손상, 혈관신생

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